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Dong-Hee Lee
Stephen W. Schaffer
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Taurine 10

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Preface

The twentieth International Taurine Meeting was held from May 23 to 27, 2016, at the Plaza Hotel in downtown Seoul, South Korea. The focus of the meeting was "Taurine and Brain Function," which is a promising area of recent taurine research. In accordance with the aim of the meeting, important information was presented and discussed on the pathology and potential treatment of various CNS diseases, as well as the function of taurine as a neuroregulator, cytoprotective agent, and important nutrient. Presentations in other sessions discussed the role of the beta-amino acid, taurine, in the regulation of energy metabolism, gene expression, cell signaling, inflammation, apoptosis, blood pressure regulation, exercise physiology, cellular development, and alcohol toxicity. Enthusiastic and provocative discussions of the new findings and their meaning were initiated by the participants, which included scientists from 15 countries.

Taurine is a simple compound but plays a fundamental role within the cell. Although it is considered a semi-essential nutrient in man, there is little doubt that taurine is essential for normal cellular function. It has been 46 years since the initial taurine meeting was organized by Dr. Ryan Huxtable in Tucson, Arizona. During that time, much has been learned about the physiological functions of taurine and the mechanisms underlying those actions. In addition, taurine exerts numerous nutritional and pharmacological actions, many of which are mediated by undetermined mechanisms. As the field moves from the benchtop to the bedside, these mechanisms assume clinical relevance. Also important are potential interactions between taurine and specific cellular processes, as well as untoward actions of taurine. We look forward to future research addressing these clinically relevant questions.

The twentieth International Taurine meeting also played a pivotal role in the future development of the taurine field, as participants approved the creation of an International Taurine Society, which was given marching orders to facilitate future taurine meetings, promote scientific collaborations, advance the clinical importance of taurine, and create a taurine journal.

The organizers of the meeting would like to express their sincere thanks to the participants for ensuring a successful meeting. We would also like to acknowledge Red Bull GmbH of Vienna, Austria, the Seoul Metropolitan Government and Dong A Pharmaceutical Company of Seoul, South Korea, for their generous support.

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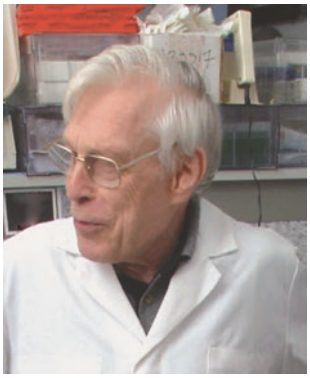
Mobile, AL — Stephen W. Schaffer
New York, NY — Eunkyue Park
Seoul, South Korea — Ha Won Kim

Eulogy for Dr. Rebel



Gérard Rebel, Ph.D., Directeur de Recherche at the CNRS, died on June 21, 2015.

Born on February 1935 in Selestat-France, Dr. Rebel received his doctoral degrees from the University of Strasbourg, France. Gerard began his career first as a technician at the laboratory of Professor P. Mandel (1957–1958), then as a research fellow at the Department of Biochemistry at the University/Medical School of Strasbourg and at the Center of Neurochemistry CNRS Strasbourg. In October 1959, he did his military service in the army, spent first at the Department of Bacteriology at the Military Hospital in Lyon, thereafter serving during the war in Algeria in the operating theater until 1962. He received his Ph.D. in 1967 and rose through the ranks at the CNRS to become Director of Research in 1979. In parallel, he was also Associate Professor at the Faculty of Medicine in Strasbourg, teaching biochemistry to medical students (1964–1984). Starting in 1980, he also provided teaching in tissue culture technologies in continuing education for the Departement d'Education Permanente de l'Université Louis Pasteur and consulting for industry.

The main early themes addressed in his research concerned the study of the structure and metabolism of lipids, mainly of glycolipids, first in bacteria and later in various tissues of mammals and of their derived cultures. He addressed brain lipid metabolism and its modulation, with studies covering the full panel of lipid species

expressed during brain development in animal models and in brain cell cultures (FA, gangliosides, sulfatides phospholipids, *etc*.). He showed among others induction at the transcriptional level of enzymes responsible for catabolism of glycolipids caused by HEPES buffer used in tissue culture media. This naturally led him to take an interest in achieving physiologically more appropriate models addressing homeostasis and to study taurine metabolism and uptake in cultured brain cells. He demonstrated the coexistence and properties of two taurine uptake systems in cultured glial and in glioma cells, together with the differential regulation of taurine transport by protein kinase C in astrocytic and neuronal cells. In 1994, having moved to the Institut des Cancers de l'Appareil Digestif (IRCAD) (Professor Marescaux, director, Strasbourg), his interest focused on the characterization of taurine uptake systems in multidrug-resistant human colon cancer cells and oral epidermoid carcinoma cells in relation to ABCB1 transporter expression. Characterization of two saturable taurine uptake systems together with a taurine diffusion component was achieved in several multidrug-resistant cell lines and in their chemosensitive counterparts. With emphasis given to physioxia, studies were devoted to the antioxidant properties of taurine under conditions reproducing tumor oxygenation status and to its effect on the action of antineoplastic drugs in MDR and non-MDR cancer cells.

All his life was devoted to fundamental science and improving tissue culture practices. He was the pioneer of culture technologies of brain cells and visionary for the need to practice cell culture under physiological oxygen pressure (physioxia). He always encouraged everyone to leave the beaten track of conventional science. He shared his experience through numerous seminars, books, and teaching.

He was member of the European Society for Cell Culture, of the French Society of Pharmacology and Toxicology in Cultured Cells, and of the French Society of Biochemistry and Molecular Biology. He was a member of the Editorial Board of Anticancer Research and a member of the International Scientific Advisory Board of the International Institute of Anticancer Research.

He was an absolutely unconditional supporter of the "Current Contents" booklet that he used to read from beginning to end. He carried it everywhere and would consult it wherever he was. He knew all of its content, better than an Internet search engine. Rebel's "questions" at neuroscience and oncology meetings were a well-known happening for the old timers and a surprise for the young scientists whose ideas were challenged by the discrete man with a French accent.

Gerard was born in a family of three brothers and was always attracted by science. His personal life was challenged by deep events. His marriage to his first wife Michèle in 1962 was blessed with three children Viviane, Thierry, and Anne. A few years later, in 1980 he had the pain of losing his wife, and his son followed the fate of his mother in 1991.

He met his second wife Isabelle during her doctoral training and married her in 1998. They worked in synergy at the IRCAD for their common passion devoted to cell biology and cancer research until his effective retirement in 2010. He was suffering for years from a painful chronic disease to which he finally succumbed.

He was also a man of great goodness, a philosopher at times, of great erudition—he had read all the founding religious books, knew world history in great detail, and

was able to exchange ideas on many spiritual planes. As said by other great scientists, he was convinced that “God does not play dice with the universe” but also by the well-known reply “Who are we to tell God what to do?”. He always meant that rigor and humility should guide the reflection of scientific thought. His knowledge was accompanied by a simplicity that was rare in today’s world. His kindness manifested in many ways; he was always ready to help others tactfully and discretely.

Stephen W. Schaffer
Mobile, USA

Eulogy for Dr. Chesney

Dr. Chesney was a pioneer researcher in the field of taurine and taurine transporter studies. He has discovered that taurine transporter is a cell membrane surface protein using genetic mice models and published his findings in the *Journal of Clinical Investigation* 40 years ago. Since then, his lab has made great contributions to the field by characterization of taurine transporter gene regulation and functions. Dr. Chesney was an amazing mentor. He touched my hearts and minds and enabled me to become much more than I ever would have.

Dr. Chesney also applied his knowledge of taurine and vitamin D to support the addition of taurine to human infant formulas and to help treat polar bear cubs in zoos around the country. He discovered that distortion of the polar bear cub bones, or rickets, was being caused because the cows' milk they were given did not contain the same amounts of taurine and vitamin D found in polar bear milk. Polar bear cubs in zoos get extra taurine today, thanks to Dr. Chesney.

Serving as chair for more than 23 years of the Department of Pediatrics at the University of Tennessee Health Science Center (UTHSC) and Le Bonheur Children's Hospital, Russell W. Chesney, M.D., worked tirelessly to advance the field of pediatric nephrology.

Among his contributions as a researcher and clinician-scientist in pediatric nephrology are the first use of captopril in children to treat high blood pressure and diabetic kidney problems; the first use of calcitriol to treat children with renal osteodystrophy, a bone disease that occurs when kidneys do not maintain proper calcium and phosphorus levels in the blood; and a decade long NIH-sponsored collaborative study to look at the use of antibiotics to prevent urinary tract infections in children with vesicoureteral reflux, a condition that occurs when urine from the bladder flows backward into the kidneys.

As amazing of a scientist as he was, he was an even better mentor and friend. Not a single discovery goes by in our lab without me missing him immensely to discuss and get his take on it. I miss you, Dr. Chesney. We all in the international taurine community miss you!

With deepest respect,

Xiaobin Han, M.D., Ph.D.

University of Tennessee Health Science Center

Eulogy for Dr. Sturman

IN MEMORY OF

JOHN A. STURMAN—August 10, 1941–November 29, 2016

Dr. John Sturman worked at NYS Institute for Basic Research in Developmental Disabilities from July 1, 1967, until his retirement on August 18, 1997. Dr. Sturman was diagnosed with chronic myeloid leukemia (CMI) in 1996 at age 54. At that time he was considered too old for a bone marrow transplant, the only curative treatment for CML. His successful transplant and recovery helped open the door to transplants for older patients.

John studied taurine deficiency using a feline animal model, which led to the discovery that taurine should be included in infant formula. His research is focused on understanding the role of taurine in reproduction as well as brain and eye development. Due to low cysteine dioxygenase and cysteine sulfinic acid decarboxylase, taurine is an essential amino acid in the cat and a conditional essential amino acid in nonhuman primates.

John was one of the initial leaders for establishing International Taurine Meeting (ITM). His dedication to taurine research and ITM led to the development of International Taurine Society in 2016 at the Seoul Meeting. John's passion toward taurine research will not be forgotten.

Georgia Schuller-Levis

Eunkyue Park

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Part I
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Is Taurine a Biomarker in Autistic Spectrum Disorder?

Eunkyue Park, Ira Cohen, Maripaz Gonzalez, Mario R. Castellano, Michael Flory, Edmund C. Jenkins, W. Ted Brown, and Georgia Schuller-Levis

Abstract Taurine is a sulfur-containing amino acid which is not incorporated into protein. However, taurine has various critical physiological functions including development of the eye and brain, reproduction, osmoregulation, and immune functions including anti-inflammatory as well as anti-oxidant activity. The causes of autistic spectrum disorder (ASD) are not clear but a high heritability implicates an important role for genetic factors. Reports also implicate oxidative stress and inflammation in the etiology of ASD. Thus, taurine, a well-known antioxidant and regulator of inflammation, was investigated here using the sera from both girls and boys with ASD as well as their siblings and parents. Previous reports regarding taurine serum concentrations in ASD from various laboratories have been controversial. To address the potential role of taurine in ASD, we collected sera from 66 children with ASD (males: 45; females: 21, age 1.5–11.5 years, average age 5.2 ± 1.6) as well as their unaffected siblings (brothers: 24; sisters: 32, age 1.5–17 years, average age 7.0 ± 2.0) as controls of the children with ASD along with parents (fathers: 49; mothers: 54, age 28–45 years). The sera from normal adult controls (males: 47; females: 51, age 28–48 years) were used as controls for the parents.

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Taurine concentrations in all sera samples were measured using high performance liquid chromatography (HPLC) using a phenylisothiocyanate labeling technique. Taurine concentrations from female and male children with ASD were 123.8 ± 15.2 and 145.8 ± 8.1 μM, respectively, and those from their unaffected brothers and sisters were 142.6 ± 10.4 and 150.8 ± 8.4 μM, respectively. There was no significant difference in taurine concentration between autistic children and their unaffected siblings. Taurine concentrations in children with ASD were also not significantly different from their parents (mothers: 139.6 ± 7.7 μM, fathers: 147.4 ± 7.5 μM). No significant difference was observed between adult controls and parents of ASD children (control females: 164.8 ± 4.8 μM, control males: 163.0 ± 7.0 μM). However, 21 out of 66 children with ASD had low taurine concentrations (<106 μM). Since taurine has anti-oxidant activity, children with ASD with low taurine concentrations will be examined for abnormal mitochondrial function. Our data imply that taurine may be a valid biomarker in a subgroup of ASD.

Keywords Taurine • ASD • Mitochondrial dysfunction • Antioxidant

Abbreviations

ASD	Autistic spectrum disorder
PITC	Phenylisothiocyanate
HPLC	High performance liquid chromatography

1 Introduction

Autistic spectrum disorder (ASD) is a neuro-development disorder affecting an estimated 1 in 68 children in the US (CDC 2014). ASD is characterized by impairments in social reciprocity and communication and by abnormal repetitive behaviors, movement patterns and sensory interests (American Psychiatric Association 2013). Co-morbid conditions commonly associated with ASD include gastrointestinal disease, epilepsy and dysbiosis (White 2003), autoimmune disease and intellectual disability (Sweeten et al. 2003; Bolte and Poustka 2002). The etiology of ASD is unclear, but genetic factors are important. Current heritability estimates for ASD are on the order of 50–55% in several studies (Sandin et al. 2014; Gaugler et al. 2014). Further, the risk for siblings of a child with ASD is more than 10 times higher than for siblings of a non-ASD child (Ozonoff et al. 2011; Chawarska et al. 2014).

In order to understand the underlying pathogenesis of ASD, several research groups have conducted evaluations of potential candidate causative genes (Nakagawa and Chiba 2016; Bourgeron 2016; Geschwind and State 2015) but no single gene has been found to be causative for ASD. It has been recently postulated that the increasing rates of diagnosed ASD and the less than 100% monozygotic concordance of ASD support a more inclusive reframing of ASD as a multi-system disorder with

both genetic influences and environmental contributions (Sealey et al. 2016). Environmental factors including inflammation due to various infections (Patterson 2011), oxidative stress and biohazardous chemicals including pesticides and plastics have being considered as potential contributors to ASD (Chauhan and Chauhan 2006; Nakagawa and Chiba 2016). Recent studies also suggest that innate immunity may have a pivotal role in the pathogenesis of ASD and future therapies could involve immune modulation (Vargas et al. 2005; Onore et al. 2012). Hallmarks of ASD include cerebellar migration abnormalities and Purkinje cell loss (Hampson and Blatt 2015; Wegiel et al. 2015). TH1/TH2 dysregulation, an increase in CNS chemokines, increased NO, HLA association, increased microglial activation, and astrocytosis demonstrate the role of immunity in ASD (Vargas et al. 2005; Ashwood et al. 2006).

Taurine, an amino acid containing sulfur, is a unique abundant amino acid in various tissues, especially the brain and leukocytes, and it modulates the innate immune response via inhibition of various cytokine production in human and rodents (Park et al. 1993, 1995, 1998, 2002; Schuller-Levis and Park 2006). Taurine plays an important role in several essential biological processes such as development of the eye and brain, reproduction, immune function including anti-inflammatory activity, anti-oxidant activity, membrane stabilization and osmoregulation (Schuller-Levis and Park 2003, 2006). Supplementation of taurine has been shown to prevent oxidant-induced damage (Schuller-Levis et al. 1995, 2009) while deficiency of taurine in a cat leads to immune activation in the CNS with Purkinje cell loss, microglial activation and astrocytosis, similar to that observed in the ASD brain (Sturman 1993; Vargas et al. 2005; Hampson and Blatt 2015).

Therefore, to examine whether taurine may be involved in the pathogenesis of ASD, we determined taurine concentrations in sera from children with ASD in comparison with unaffected siblings and their parents who in turn were compared to adult controls.

2 Materials and Methods

2.1 Reagents

Chemicals used in this study were purchased from Sigma Chemicals (St. Louise, MO) if not otherwise noted. Column for HPLC was obtained from Waters corporation (Milford, MA).

2.2 Sample Collection

Our investigation involving human subjects has been approved by the Institutional Review Board (IRB) of the New York State Institute for Basic Research in Developmental Disabilities in accordance with the NIH Guide and Federal Wide Assurance FWA00006105. Blood samples were collected from children with ASD

and their siblings as well as their parents in families from the same population located in the NYC area to limit differential influences of external environmental factors, e.g., air pollution and water contaminations. Variety of diet, differences in child care, maternal and children's diets, exposures to customs and preferences associated with households, and other non-specific environmental factors, were not controlled because relevant conditions associated with the development of ASD are not yet established. The diagnosis of ASD was carried out using various methods including the Autism Diagnostic Interview (ADI), the Autism Diagnosis Observation Schedule (ADOS) and the PDD Behavior Inventory (PDDBI) (Lord et al. 1994; Lord et al. 2000; Cohen 2003; Cohen et al. 2003, 2016) with all cases verified based on DSM-IV or DSM-IV-TR criteria. Sixty six children with ASD were recruited for this study including 21 girls and 45 boys (age range: 1.5–11.5 years). Blood samples were collected from 66 children with ASD, 55 unaffected siblings (age range: 1.3–17.4 years), 102 parents (age range 27–45 years) and 99 adult controls for parents (age range: 25–48 years). A summary of subjects used in this study and diagnosis of subjects using various diagnostic methods for ASD is shown in Table 1. Also shown is the severity of adaptive functioning based on the Vineland Adaptive Behavior Scales.

Diagnostic status was assessed, in most cases, using the ADOS or ADI-R or both, in addition to the PDDBI. Briefly, the ADOS is filled out based on observations made during the assessment. The ADOS protocol involves a series of tasks presented by a trained person. The sophistication of the task varies according to the language level of the person assessed. The reactions of the probands are coded, and a diagnostic algorithm is then employed to arrive at a diagnosis of autism, ASD, or neither of these. The examination takes about 30–45 min to complete, depending on the module used. Sensitivity and specificity for ASD vs. other diagnoses are quite good for all modules. The ADI–Revised (ADI-R) (Lord et al. 1994), a briefer version of the ADI, is a parent interview. The ADI-R takes about 2 h to administer and can be used to classify children as young as 2 years of age if their mental age is greater than 18 months. Sensitivity and specificity are excellent (96% and 92%, respectively). The PDDBI is a rating scale filled out by parents and teachers that is designed to assess response to intervention in children with PDD. It consists of subscales that measure both maladaptive and adaptive behaviors and also provides a summary "autism score" reflective of the severity of the condition. The scale has been shown to have very good internal consistency as well as developmental and construct validity and recently, very good diagnostic validity (Cohen et al. 2016).

Ninety nine sera from healthy adult controls (female; 51 and male; 47) were provided from Staten Island University Hospital, Staten Island, NY. Blood samples were collected with IRB approval in Staten Island University Hospital in accordance with the NIH Guide and Federal Wide Assurance FWA00006105.

Table 1 Demographic and clinical characterization of children with autistic spectrum disorder

1. *Demographic characteristics*					
		Autism	Sibling	Parents	Controls of parents
Sex					
	Female	21	32	54	51
	Male	45	24	49	47
Total		66	55	102	98
Age					
	Range	1.5–11.5 years	1.3–17.4 years	27–45 years	27–48 years
	Average	5.2 ± 1.6[a]	7.0 ± 2.0		
Ethnic					
	Hispanic	7			
	Causian	52			
	Causian/	3			
	Hispanic				
	Asian	4			
2. *Clinical characteristics*					
Vineland[b]					
	Typical	9			
	Border	3			
	Mild	31			
	Moderate	17			
	Severe	6			
ADI	Broad	2			
	spectrum				
	Autism	31			
	N/A[d]	7			
	ND[e]	26			
ADOS	Autism	52			
	Spectrum	2			
	N/A	2			
	ND	10			
PDDBI-autism score[c]					
	Average	50.6 ± 1.5			
	N/A	5			
Diagnosis	Autism	53			
	Rett-like	1			
	PDDNOS	11			
	Residual[f]	1			

(continued)

Table 1 (continued)

[a]Data presented as mean ± SE
[b]VINELAND LEVELS
TYPICAL > = 85
BORDERLINE > = 70
MILD > = 55
MODERATE > = 40
SEVERE > = 25
PROFOUND < 25
[c]PDDBI-autism score: The PDDBI is a standardized assessment for children with autism and its AutismT-score reflects problems with repetitive behaviors, social behaviors and language. The higher the score, the greater the severityThe mean (SD) for ASD is 50(10)
[d]N/A means not done because the child is too young or the language level is less than 2 years. Or if an early case, training on ADOS was not yet done or the PDDBI had not yet been developed as an assessment tool
[e]ND means no test because the diagnosis relies on observations and the ADOS and/or the PDDBI
[f]Residual means person once had typical autism but has shown marked improvement

2.3 Taurine Determination

The taurine concentrations were determined using HPLC (Waters, Milford, MA) (Park et al. 2014). Briefly, sera were mixed with 5% TCA and centrifuged for removal of proteins. After samples were dried using a Speedvac (Savant, Holbrook, NY), they were derivatized using phenylisothiocyanate (PITC) and separated using a C18 column with a gradient of acetate buffer containing 2.5% acetonitril (pH 6.5) and 45% acetonitril solution containing 15% methanol at 45 ° C. The flow rate was 1 mL/min. Taurine concentrations were determined by comparison to a standard.

2.4 Statistical Analysis

Taurine concentrations of male vs. female probands with ASD, male vs. female adult controls, and parents vs. unrelated adult controls were compared in unmatched t-tests, with Welch's correction for unequal variances applied in the male-female proband comparison. Taurine levels of probands vs. siblings and probands vs. parents were compared in matched t-tests. A two-way ANOVA with an interaction term was used to test for any interaction of sex and relationship status in determining taurine concentrations of adult controls. These sex effects were examined because the literature suggests that females with ASD are more severely affected than males with ASD (Cohen et al. 2016). All comparisons were performed in Stata 13.1 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP).

3 Results and Discussion

To determine the possible role of taurine and its association with ASD, the concentrations of taurine were measured in sera from children with ASD, their families and age-matched adult controls. Demographical and clinical characteristics of children

with ASD are summarized in Table 1. Most of the subjects in this study were Caucasian (79%). Blood samples were collected in a limited area less differentially influenced by geographical conditions including water or air pollution. The ratio of boys to girls (2 to 1) in ASD subjects in this study is different from the normal ratio in ASD (4 to 1). The majority of children in this study are diagnosed as having mild and moderate adaptive impairment by the Vineland (73%). Among the children in this study 80% and 17% of probands were diagnosed as ASD and PDD-NOS, respectively.

Taurine concentrations from female and male children with ASD were 123.8 ± 15.2 and 145.8 ± 8.1 μM, respectively, and those from their unaffected brothers and sisters were 142.6 ± 10.4 and 150.8 ± 8.4 μM, respectively (Figs. 1 and 2). There was no significant difference in taurine concentration between autistic children and their unaffected siblings. Taurine concentrations in children with ASD were also not significantly different from their parents (mothers: 139.6 ± 7.7 μM, fathers: 147.4 ± 7.5 μM). No significant difference was observed between adult controls and parents of ASD children (control females: 164.8 ± 4.8 μM, control males: 163.0 ± 7.0 μM).

We found that taurine concentrations (<106 μM) from 21 (10 females and 11 males) out of 66 children with ASD were remarkably lower, compared to the aver-

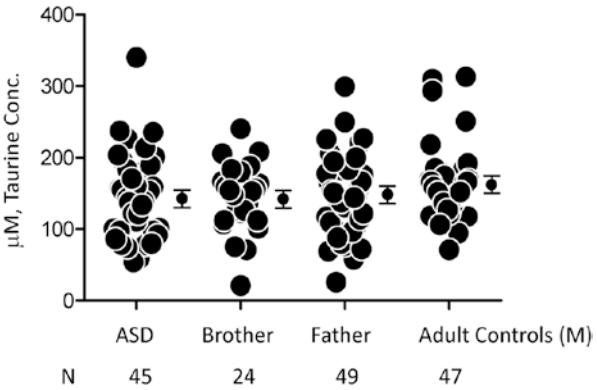

Fig. 1 Taurine concentrations in female children with autism, their sisters and mothers as well as adult female controls. Data were expressed as mean ± SE. N stands for number of subjects in each group. There is no significant difference between groups

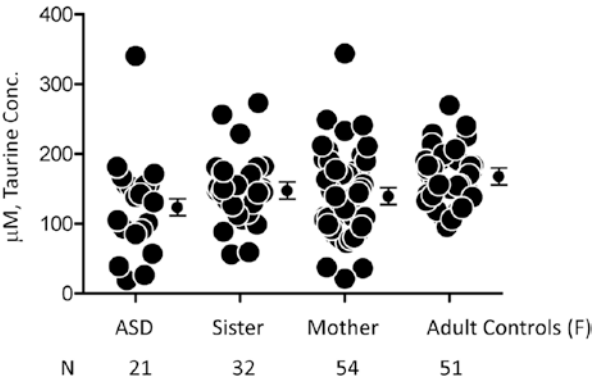

Fig. 2 Taurine concentrations in male children with autism, their brothers and fathers as well as adult male controls. Data were expressed as mean ± SE. N stands for number of subjects in each group. There is no significant difference between groups

age taurine concentrations of controls (Table 2) and this effect was more evident in females with ASD, with 48% having low concentrations vs. 24% in males with ASD. Taurine concentrations in 9 ASD out of 21 ASD are lower than average of their families (Table 2).

Taurine concentrations in ASD children have been determined in several laboratories. However, the reports are controversial, with some studies showed higher taurine concentrations in autistic groups (Moreno et al. 1992; Moreno-Fuenmayer et al. 1996; Tu et al. 2012), no change (Aldred et al. 2003; Arnold et al. 2003) or lower (Geier et al. 2009; Ghabizadeh 2013), compared to controls. Geier et al. studied transsulfuration biomarkers including plasma cysteine, reduced glutathione (GSH),

Table 2 Summary of low taurine concentrations in children with ASD and their family

ASD	Diagnosis*	sex	Taurine	M^	F	S	B1	B2	AV**
1	Mild	F	19.1	362.0	25.7	56.0	21.2		34.8
2	Mild	F	26.5	177.2	205.7		166.8		183.2***
3	Mild	F	101.0	105.5	138.8	98.8			114.4
4	Mild	F	93.3	72.6	104.5		111.1		96.1
5	Mild	F	57.0	108.5	96.7				102.6
6	Mild	F	105.1	108.2	109.6	90.9			102.9
7	Mild	F	90.9	108.2	109.6	105.1			107.6
8	Mild	F	85.0	121.1					121.1
9	Mild	F	39.3	96.3	194.5				145.4
10	Severe	F	91.7	77.9	119.9		91.7		96.5
11	Typical	M	101.8	174.8	87.2		156.6	112.4	139.5
12	Border	M	100.9	77.7	69.1	59.1	71.3		69.3
13	Mild	M	54.3	86.4	72.3				79.4
14	Mild	M	97.6	92.1	102.1	88.6			94.3
15	Moderate	M	79.8	98.9	166.6				132.8
16	Moderate	M	97.5	109.7	80.0	152.0			114.0
17	Typical	M	58.7	79.8	57.8		189.6	154.6	120.5
18	Mild	M	74.3		88.2	110.0			98.9
19	Mild	M	78.6	77.9	119.9		78.6		92.1
20	Typical	M	78.1	192.5	77.2		75.6		115.1
21	Moderate	M	86.6	80.4	142.6		237.4		153.5

*Diagnosis was done using the Vineland.
** AV means average taurine concentrations in families with ASD children.
***Taurine concentrations in ASD families are higher than in ASD (gray).
^ M, F, S, B1 and B2 stand for mother, father, sister, brother 1 and brother 2, respectively.

oxidized glutathione (GSSG), total sulfate and free sulfate as well as plasma taurine. They found all biomarkers including taurine in children with ASD were decreased compared to controls. Two groups (Aldred et al. 2003; Arnold et al. 2003) investigated not only taurine but also other amino acids. Aldred et al. demonstrated that taurine levels in children with ASD were not changed compared to controls while glutamic acid, phenylalanine, asparagine, tyrosine, alanine and lysine were increased. The Arnold group studied autistic children with an unrestricted diet vs. gluten/casein restricted diet. Taurine was not changed in either group but isoleucine, leucine, tyrosine and lysine were decreased only in the restricted diet group. Our findings from the largest group of subjects (n = 66) demonstrated that mean taurine concentrations in children with ASD were not significantly different from unaffected siblings as controls as well as from their parents and adult controls, confirming data from Aldred and the Arnold groups. Using metabolomic technology and ^{1}H-^{13}C NMR, urinary taurine was measured in children with ASD (Mavel et al. 2013; Ming et al. 2012; Yap et al. 2010). The reports from Mavel et al. and Ming et al. demonstrated that taurine from children with ASD was increased in urinary samples, compared to controls. Correlation of taurine between sera and urine in ASD may provide a possible biomarker of ASD.

Despite the fact that there were no overall group mean difference, the fact that a significant number of children had low taurine levels is interesting. Since children with ASD have food selectivity based on taste, texture preferences and/or perseverative behaviors, a balance of nutrition has been required to maintain health (Arnold et al. 2003). One explanation of low taurine concentrations may be due to food preference in the family's or children's diet. If these families prefer vegan diets (no taurine concentrations), they will have low taurine concentrations because humans require dietary taurine due to low levels of cysteine dioxygenase and cysteine sulfinic acid decarboxylase (Huxtable 1999). A different sex ratio was also observed in the low taurine group. The ratio of males to females was 1:1 in the low taurine concentration group, while the normal ASD male to female ratio was 4:1 (Table 2). These results may imply that low taurine levels could be involved in increased seizure risk since females with ASD tend to be more severely affected including increased seizure activity (Cohen et al. 2016). Junyent et al. demonstrated that mice with taurine supplementation were protected from kainic acid-induced seizure (Junyent et al. 2009).

Mitochondrial dysfunction has been reported in ASD from various laboratories (Giulivi et al. 2011; Oliveira et al. 2005; Frye and Rossignol 2011; Rossignol and Frye 2012). Impaired mitochondrial function is frequently caused by an abnormality in the mitochondrial respiratory chain, the final common pathway for aerobic metabolism. Giulivi et al. demonstrated that children with ASD were more likely to have mitochondrial dysfunction including respiratory chain reactions, mtDNA deletion and mt DNA overreplication, than typically developing children. Data from a population-based study (Oliveira et al. 2005) indicated that a defect in oxidative phosphorylation might be one of the most common medical conditions associated

with ASD. A meta-analysis of studies of mitochondrial dysfunction in autism disorders by Rossignol and Fyre showed that the prevalence of mitochondrial disease in the ASD population was much higher (5%) than found in the general population (~0.01%). Taurine has important actions within mitochondria although the antioxidant activities of taurine has been largely attributed to events outside the mitochondria (Schuller-Levis and Park 2006). Parvez et al. (2008) found that taurine pretreatment significantly reduced mitochondrial superoxide generation and lipid peroxidation in tamoxifen-treated animals. Taurine appears to be linked to a conjugation reaction between taurine and the wobble uridine of $tRNA^{Leu(UUR)}$, forming the product 5-taurinomethyluridin-$tTNA^{Leu(UUR)}$. This translational conjugation reaction is important because it substantially strengthens the interaction between the UUG codon of leucine mRNA and the AAU anticodon of $tRNA^{Leu(UUR).}$ Thus, the decoding of UUG in the presence of conjugation–free $tRNA^{Leu(UUR)}$ is severely diminished, resulting in reduced expression of UUG dependent mitochondria encoded protein. The most UUG dependent proteins are subunits of electron transport chain complex I. Therefore, in the taurine deficiency, the activity of complex I is dramatically reduced, forming superoxide production (Shimada et al. 2014). Taurine treatment reversed this oxidative stress in taurine deficient cardiomyocytes and fibroblases (Jong et al. 2012). A recent study demonstrated that taurine reversed β-alanine toxicity in the mitochondria (Shetewy et al. 2016). β-alanine reduces cellular levels of taurine which are required for normal respiratory chain reaction; cellular taurine depletion is known to reduce respiratory function and elevate mitochondrial superoxide generation. Due to the antioxidant activities of taurine through respiratory chain reactions, children with lower taurine compared to average taurine of their family are a subgroup of ASD to examine for abnormal mitochondrial function (Table 2).

Although treatment for ASD is limited, a few promising drugs have been investigated in various laboratories (Erickson et al. 2011; Kern et al. 2011; Singh et al. 2014; Naviaux et al. 2014). Acamprosate containing taurine, an FDA approved drug, for maintenance of abstinence from alcohol in adults, is a novel agent with multiple mechanisms of action. This study has a limitation due to a lack of controls and the number of subjects (n = 6), although results showed improvement of language understanding, social language and eye contact (Erickson et al. 2011). Sulforaphane, an isothiocyanate derived from broccoli, significantly improved social interaction, abnormal behaviors and verbal communication in ASD (Singh et al. 2014). A single dose of suramin (20 mg/kg), an antipurinergic agent, in mice also demonstrated that autism-like behaviors, novelty preference and metabolism were improved, indicating purine metabolism is a master regulator of behavior and metabolism in maternal immune activation model mice (Naviaux et al. 2014). Recently Wink et al. demonstrated that a randomized placebo-controlled pilot study of N-acetylcysteine (2016), an antioxidant, in youth with ASD showed an effect of increasing glutathione production but no significant impact on social impairment. We hypothesize that abnormalities of the innate immune system and oxidative stress play an important role in the pathogenesis of ASD and that taurine and/or it

metabolites can ameliorate the development of ASD when delivered at the appropriate time and dosage. Dietary nontoxic taurine supplementation as a therapeutic regimen for ASD at various stages of development including pregnancy and infancy is a long-term goal.

4 Conclusion

Mean taurine concentrations in sera from 66 children with ASD (male: 45 and female: 21) were not significantly different from unaffected siblings and their parents, whose taurine concentration were also not significantly different from adult controls. Low taurine concentrations (<106 μM) in sera from 21 children with ASD were not characteristic of the taurine concentrations in their families. More females with ASD had low taurine concentrations than males, Abnormality of mitochondrial function may be considered in this subgroup of children due to the antioxidant activity of taurine. Therefore, taurine may be a valid biomarker in a subgroup of ASD.

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Functions of Maternally-Derived Taurine in Fetal and Neonatal Brain Development

Shiro Tochitani

Abstract Taurine (2-aminoethanesulfonic acid) is a sulfur-containing organic acid, which has various physiological functions, including membrane stabilization, cell-volume regulation, mitochondrial protein translocation, anti-oxidative activity, neuroprotection against neurotoxicity and modulation of intracellular calcium levels. Taurine also activates $GABA_A$ receptors and glycine receptors. Mammalian fetuses and infants are dependent on taurine delivered from their mothers via either the placenta or their mother's milk. Taurine is a molecule that links mother-fetus or mother-infant bonding.

This review describes the functions of taurine and the mechanisms of action of taurine in fetal and brain development. Taurine is involved in regulating the proliferation of neural progenitors, migration of newly-generated neurons, and the synapse formation of neurons after migration during fetal and neonatal development. In this review, we also discuss the environmental factors that might influence the functional roles of taurine in neural development.

Keywords Taurine • Brain development • Placental transfer • Mother-infant relationship • Environmental factor • Obesity • Obstetric complication

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Abbreviations

CNS	Central nervous system
GABA	γ-aminobutyric acid
NMDA	N-methyl-D-aspartate
TauT	Taurine transporter
VZ	Ventricular zone

1 Introduction

Taurine (2-aminoethanesulfonic acid) is a sulfur-containing organic acid with various physiological functions, including membrane stabilization, cell-volume regulation, mitochondrial protein translocation, anti-oxidant effects, neuroprotection against L-glutamate-induced neurotoxicity and modulating intracellular calcium levels (Wu and Prentice 2010; Lambert et al. 2014). Taurine is structurally similar to the neurotransmitter γ-aminobutyric acid (GABA) and glycine and interacts with both $GABA_A$ receptors and glycine receptors to induce chloride currents in neuronal cells (Malminen and Kontro 1986; Kontro and Oja 1987a, b; Linne et al. 1996; Ye et al. 1997; Yoshida et al. 2004). Of note, taurine is one of the most abundant neurotransmitters in the developing mouse cortex (Benítez-Diaz et al. 2003).

Mammalian fetuses and infants are dependent on taurine given by their mothers via either the placenta or mother's milk. Taurine deficiency causes retinal degeneration and growth retardation in infancy (Hayes et al. 1975; Hayes et al. 1980; Sturman 1988). Therefore, taurine is an intriguing molecule that links the mother-fetus or mother-infant bonding.

This review provides an overview of the functions of taurine in brain development and the signaling mechanisms involved in the development of neural cells by taurine.

2 The Function of Taurine in Brain Development

2.1 Taurine in the Developing Brain

Taurine is one of the most abundant free amino acids in the developing central nervous system (CNS: Huxtable 1989; Benítez-Diaz et al. 2003). In many species, taurine concentrations in the fetal or neonatal brain are higher than in the adult brain. The concentration of taurine in the Rhesus monkey occipital lobe increases in the prenatal days before birth, while the concentration decreases gradually after birth, reaching the level of adult brains at 200 days after birth (Sturman and Gaull 1975).

The taurine concentration in fetal mouse brain and newborn mouse brain is greater than that of an adult mouse brain (Sturman et al. 1977c). In rat brain, the taurine concentration increases during the last part of gestation (Sturman et al. 1977c).

In mammals, taurine required for embryonic development is received from the mother via the placenta, while neonates obtain taurine from the mother's milk (Sturman et al. 1977a, b; Sturman 1982). Taurine is a major constituent of the free amino acid pool of milk in a number of species, second in concentration to glutamate (Sturman et al. 1977c). The concentration of taurine in rat milk is high for the first few days after birth, and then decreases rapidly thereafter and by 1 week after birth it maintains an approximately constant value (Sturman et al. 1977c). Like breast milk, amniotic fluid is rich in taurine, which is found in greater quantities in the amniotic fluid than in the maternal serum (Underwood et al. 2005). Although the functional role of taurine in the amniotic fluid has not been clarified, its importance during embryonic development has been implied.

2.2 The Function of Taurine in Mammalian Brain Development

During the development of the CNS, cortical neurons are generated from neural progenitors located in the ventricular zone (VZ; Götz and Huttner 2005). After terminal mitosis, neurons differentiated from neural progenitors migrate to specific areas. CNS development involves coordinated cellular events including the proliferation and differentiation of neural progenitors, migration and outgrowth of axons and dendrites, the formation of synapses, myelination, and programmed cell death of neurons differentiated from neural progenitors. In cats, taurine levels can be diminished merely by a reduction of dietary taurine (Hayes et al. 1975). Kittens born from dams exposed to taurine-deficient conditions exhibited a number of CNS abnormalities such as continued mitosis of the cells in the cerebellar external granule cell layer at postnatal week 8 (Sturman et al. 1985, 1986). Furthermore, the developing visual cortices of the kittens contained neural stem cells aggregated in the VZ, having failed to migrate and differentiate (Palackal et al. 1986). These observations suggest that taurine has pivotal roles in the optimal proliferation, development, and maturation of brain cells. Shivaraj et al. examined this possibility using neural stem cell cultures, primary neuronal cultures, and organotypic brain slice cultures (Shivaraj et al. 2012). Their results have shown that taurine increases cellular proliferation in the dentate gyrus of the developing hippocampus by way of ERK1/2 signaling pathways without having obvious influences on neural differentiation (Shivaraj et al. 2012). They also observed that taurine supplementation of the primarily cultured neurons enhanced the expression of Synapsin I and PSD95, factors associated with synaptic development, suggesting that taurine stimulated synaptic formation. In the developing cortex, neurons derived from the VZ migrate radially through the intermediate zone into the cortical plate to differentiate finally into glutamatergic neurons (Tan

et al. 1998). Furukawa et al. first reported that the continuous blockade of $GABA_A$ receptors accelerated the radial migration of newly generated neurons, suggesting GABAA receptors negatively regulated radial migration (Heck et al. 2007; Furukawa et al. 2014). Because they did not observe any change in the radial migration of neurons in GAD67-GFP knock-in mice ($GAD67^{GFP/GFP}$), which lack GABA synthesis and contains lower levels of GABA than wild type ($GAD67^{+/+}$) and heterozygous mice ($GAD67^{+/GFP}$), they tested whether taurine functions as an agonist for $GABA_A$ receptors (Tamamaki et al. 2003; Furukawa et al. 2014). Taurine-evoked currents detected in migrating neurons were abolished by SR95531, a $GABA_A$ receptor antagonist. In the cortices of taurine-deficient mouse embryos, whose mothers received an intraperitoneal injection of D-cysteine sulfinic acid, a taurine synthesis inhibitor, the tonic currents by $GABA_A$ receptors were significantly reduced, and radial migration was enhanced. This indicated that taurine-evoked currents in the migrating neurons were dependent on the activation of $GABA_A$ receptors by taurine (Furukawa et al. 2014). They also reported that taurine uptake was blocked by a taurine transporter (TauT) inhibitor, 2-(guanidine)ethanesulfonic acid, while taurine release from the migrating cells was inhibited by a volume-sensitive anion channel blocker, 4-(2-butyl-6,7-dichlor-2-cyclopentylindan-1-on-5-yl) oxobutyric acid (Furukawa et al. 2014). Furthermore, nonsynaptically-released taurine was reported to activate glycine receptors in the developing cortex (Flint et al. 1998; Yoshida et al. 2004). Glycine receptors α2 subunits are expressed in newly generated interneurons (Avila et al. 2013b). Glycine promotes interneuronal migration via glycine receptors, although taurine promotes interneuron cell migration independent of the activation of glycine receptors (Avila et al. 2013a, b).

2.3 The Functional Role of Taurine in Human Brain Development

Although few studies have reported taurine concentrations in human fetal and neonatal brain or the function of taurine in human brain development, it has been reported that taurine concentrations in the fetal human brain at 5 and 8 months gestation were greater than that of adult human brains (Okumura et al. 1960; Sturman et al. 1977c). Human milk collected no earlier than 7 days after the start of lactation contained 26–34 μmoles/100 mL of taurine (Sturman et al. 1977c). An epidemiological study showed that low plasma neonatal taurine in preterm babies was associated with lower scores based on the Bayley mental development index at 18 months old and lower scales based on the Wechsler Intelligence Scale for Children-revised (WISC-R) arithmetic test at 7 years old compared to the scores and the scales of preterm babies with normal plasma concentration of taurine, suggesting that nutritional conditions with low taurine level during the neonatal period of preterm babies adversely affects later neurodevelopment (Wharton et al. 2004). Therefore, the advantage of breast milk for the healthy neurodevelopment of offspring is partly due to taurine (Wharton et al. 2004).

3 Environmental Factors Perturbing the Functional Role of Taurine in Brain Development of Offspring: Association with the Risks of Psychiatric Disorders

The concentration of taurine in the milk from taurine-deprived feline mothers was reduced to 5.9% of that in milk from taurine-supplemented dams (Sturman et al. 1985, 1986). Apart from cats, the size of the intracellular taurine pool of most animal species remains constant even with significant reductions in dietary taurine content (Ito et al. 2008); however, severe malnutrition during pregnancy might cause fetal reduced taurine conditions in mothers, which may result in inhibition of the healthy development of fetal brains. Consistent with this, exposure to famine in early gestation increased the risks for adult schizophrenia in offspring (Susser et al. 1998; St. Clair et al. 2005; Xu et al. 2009; Schmitt et al. 2014). These findings suggest taurine might be involved in the etiological mechanisms related to psychiatric disorders in offspring exposed to severe malnutrition during gestation.

Taurine activates $GABA_A$ receptors and glycine receptors (Linne et al. 1996; Ye et al. 1997; Flint et al. 1998). Taurine-evoked currents in migrating neurons are mediated by $GABA_A$ receptors (Furukawa et al. 2014). In addition, $GABA_A$ receptors and glycine receptors are the targets of ethanol (Harris et al. 2008). The most relevant effects of ethanol on neural cells are the inhibition of N-methyl-D-aspartate-type glutamate receptor function and its enhancement of $GABA_A$ and glycine receptors (Harris et al. 2008). Ethanol functions as a positive allosteric modulator of $GABA_A$ and glycine receptors (Mihic et al. 1997; Harris et al. 2008). These studies suggest that alcohol drinking during pregnancy can perturb the interaction between taurine and its receptors. Indeed, ethanol is a teratogenic substance. Ethanol consumption during pregnancy can lead to CNS malformations such as microcephaly, lissencephaly, cortical lamination defects, and cortical ectopias (Riley and McGee 2005). With regard to the biological bases of teratogenesis induced by ethanol, it has been shown that embryonic exposure to ethanol disturbs the regulation of mitotic spindle orientation of neural progenitors in the developing mouse neocortex by way of $GABA_A$ receptors (Tochitani et al. 2010). Some anti-epileptic and anesthetic drugs also act on $GABA_A$ receptors, and fetal exposure to these drugs can cause brain malformations (Rho et al. 1996; Manent et al. 2007; Tochitani et al. 2010). These studies indicate that fetal exposure to $GABA_A$ receptor-acting drugs can disturb the interactions between taurine and its receptors in the developing brain.

As mentioned, mammalian fetuses are dependent on taurine transported via the placenta (Sturman 1988) by way of the actions of TauT amongst other factors (Heller-Stilb et al. 2002; Ito et al. 2008). Maternal obesity increases the risks of poor pregnancy outcome such as stillbirth, pre-eclampsia, fetal growth restriction, and fetal outgrowth, which are associated with dysfunctional syncytiotrophoblasts, the outer transporting epithelium of the human placenta. Notably, the appropriate turnover of syncytiotrophoblasts is dependent on the activity of TauT (Desforges et al. 2013). Recently, it was reported that placental TauT activity was significantly lower in obese woman than woman of ideal weight. However, there was no apparent

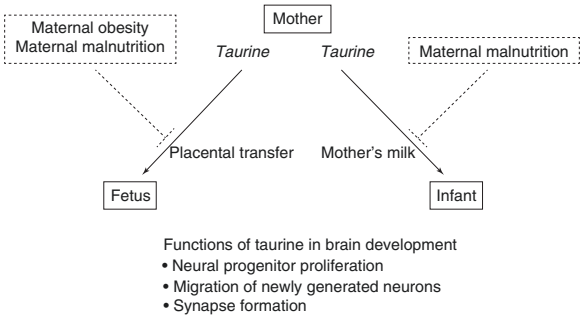

Fig. 1 Taurine transfer from mother to fetus/infant. Environmental factors such as maternal obesity and maternal malnutritional condition can adversely influence the transfer of taurine from mothers to fetuses/infants. Taurine is involved in neural progenitor proliferation, neuronal migration and synapse formation

difference in TauT expression between placentas of ideal weight and obese subjects (Ditchfield et al. 2015). This suggests that placental TauT activity is reduced in maternal obesity, which leads to lower syncytiotrophoblast taurine concentrations, perturbation of placental development, and a reduction of the driving force for taurine efflux from the placenta to the fetus, thus increasing the risk of poor pregnancy outcome (Brett et al. 2014; Ditchfield et al. 2015). Obstetric complications are potent risk factors for severe psychiatric disorders (Brown 2011; Schmitt et al. 2014). Maternal obesity has a long-term impact on offspring behavior and physiology (Rivera et al. 2015). Many studies have reported that maternal obesity increases the risks for various mental health disorders including autism spectrum disorder, attention deficit hyperactivity disorder, anxiety/depression, schizophrenia, food addiction, anorexia, and cognitive impairments (Rivera et al. 2015). These findings imply that a low activity of transplacental delivery of taurine from the mother to the fetus may underlie, at least in part, the etiological basis of psychiatric disorders caused by maternal obesity and/or obstetric complications (Fig. 1).

4 Conclusion

This review highlights recent research demonstrating the functional roles of taurine in brain development and the mechanisms involved. Taurine is the β-amino acid, which is most abundant in the developing brain and is involved in the regulation of neural progenitor proliferation, migration of newly-generated neurons, and synapse formation of neurons after migration during fetal and neonatal development. Some of its functions in neural cells are based on its agonist effect at $GABA_A$ receptors and glycine receptors. In mammals, fetuses receive taurine from their mothers by way of the placenta, whereas neonates obtain taurine from their mother's milk. Environmental factors such as maternal malnutrition and maternal obesity might have adverse effects on the amount of taurine delivered to fetuses/neonates.

Epidemiological studies demonstrate that maternal malnutrition and maternal obesity can be potent risk factors for various psychiatric disorders. However, there has been no published study demonstrating a direct link between alterations in taurine delivery to fetuses and neonates and the etiological mechanisms of psychiatric disorders, indicating a need for further research in this area.

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Taurine Supplementation Reduces Renal Nerve Activity in Male Rats in which Renal Nerve Activity was Increased by a High Sugar Diet

Sasipa Rakmanee, Supaporn Kulthinee, J. Michael Wyss, and Sanya Roysommuti

Abstract This study tests the hypothesis that taurine supplementation reduces sugar-induced increases in renal sympathetic nerve activity related to renin release in adult male rats. After weaning, male rats were fed normal rat chow and drank water containing 5% glucose (CG) or water alone (CW) throughout the experiment. At 6–7 weeks of age, each group was supplemented with or without 3% taurine in drinking water until the end of experiment. At 7–8 weeks of age, blood chemistry and renal nerve activity were measured in anesthetized rats. Body weights slightly and significantly increased in CG compared to CW groups but were not significantly affected by taurine supplementation. Plasma electrolytes except bicarbonate, plasma creatinine, and blood urea nitrogen were not significantly different among the four groups. Mean arterial pressure significantly increased in both taurine treated groups compared to CW, while heart rates were not significantly different among the four groups. Further, all groups displayed similar renal nerve firing frequencies at rest and renal nerve responses to sodium nitroprusside and phenylephrine infusion. However, compared to CW group, CG significantly increased the power density of renin release-related frequency component, decreased that of sodium excretion-related frequency component, and decreased that of renal blood flow-related frequency component. Taurine supplementation completely abolished the effect of high sugar intake on renal sympathetic activity patterns. These data indicate that in adult male rats, high sugar intake alters the pattern but not firing frequency of sympathetic nerve activity to control renal function, and this effect can be improved by taurine supplementation.

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Keywords Arterial pressure • High sugar intake • Renal nerve • Renin-angiotensin system • Taurine

Abbreviations

BSRA-PE	Baroreflex sensitivity control of renal nerve activity measured by phenylephrine infusion
BSRA-SNP	Baroreflex sensitivity control of renal nerve activity measured by sodium nitroprusside infusion
BUN	Blood urea nitrogen
CG + T	Control with high sugar intake plus taurine
CG	Control with high sugar intake
Cr	Plasma creatinine
CW + T	Control with water intake plus taurine
CW	Control with water intake alone
RVLM	Rostral ventrolateral medulla

1 Introduction

Carbohydrates are the main source of energy for humans and animals; however, regular consumption of high carbohydrate-low protein diets has been reported to underlie hypertension (Jayalath et al. 2015; Klein and Kiat 2015; Xi et al. 2015). In animal models, high sugar diets, particularly fructose and glucose, induce hypertension by increasing sympathetic nerve activity and/or renin-angiotensin system action (Tran et al. 2009). Our previous experiments indicate that male rats treated with 5% glucose in drinking water for 4 weeks after weaning display renal dysfunction but not glucose intolerance or hypertension Further, these adverse effects are abolished by short-term inhibition of the renin-angiotensin system (Roysommuti et al. 2002). This high glucose intake does not affect resting autonomic nerve activity and baroreflex-mediated renal nerve activity in male and female rats (Roysommuti et al. 2009; Thaeomor et al. 2010, 2013). However, other studies suggest that total nerve activity to an individual organ, particularly the kidney, may not be very informative, because recorded total nerve activity contains several different groups of axon, each of which has a slightly different function (DiBona 2005a, b).

Within the kidney, renal nerve fibers innervate three main renal structures; renin releasing cells, renal tubules, and renal vessels, and each fiber type responds differentially to different stimulus types and strengths (DiBona 2005a, b; Lupa et al. 2005). Under normal conditions, efferent renal nerve activity is relatively low with both firing frequency and firing synchronization differentially influencing renal function. Different types of stimuli and stimulation patterns differently affect renal function. For instance, renin release is activated at a very low frequency spectrum, while renal tubular sodium reabsorption and renal blood flow respond are elicited at higher frequency spectrum (DiBona 2005b). While direct renal nerve activity recording at the

different innervation sites is difficult practically, power spectral analysis of renal sympathetic nerve activity can differentiate frequency components, e.g., 0.5–1.5 Hz (renin release), 1.0–2.5 Hz (sodium excretion), and 2.0–5.0 Hz (renal blood flow). Several lines of evidence report that the renal nerve firing pattern rather than firing rate most greatly affects renal function; i.e., different patterns of renal nerve activity alter different renal parameters despite similar renal nerve firing rate (DiBona 2005b).

Epidemiological studies report an inverse relationship between the incidence of cardiovascular diseases and consumption of high taurine diets (Yamori et al. 2010). Low taurine intake is also related to hyperlipidemia and diabetes mellitus, and animal studies support this inverse relationship. High taurine diets decrease the rate of age-related organ damage, especially damage to the heart, blood vessels, brain, and kidneys (Huxtable 1992; Sturman 1993). Taurine reduces or delays diabetes mellitus and prevents sugar-induced hypertension (Roysommuti and Wyss 2014). Further, the taurine supplementation or diets high in taurine are reported to improve sugar-induced hypertension by inhibition of renin-angiotensin system (Nandhini et al. 2004) and/or sympathetic nervous system in adult animals (Anuradha and Balakrishnan 1999). However, the effect of taurine supplementation on renal nerve activity and renin release following high glucose intake has not been reported. The present study tests the hypothesis that taurine supplementation reduces renal nerve activity in adult male rats in which renal nerve activity was increased by a high sugar diet.

2 Methods

2.1 Animal Preparation

Sprague-Dawley rats were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. They were then treated at the Animal Unit of Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. Female Sprague-Dawley rats were fed normal rat chow (C.P. Mice Feed 082) and tap water from conception to weaning. Male offspring were fed the normal rat chow with either 5% glucose in tap water (CG group) or tap water alone (CW group) throughout the experiment. At 6–7 weeks of age, a half of rats in each group were supplemented with 3% taurine in tap water (CW + T and CG + T groups). Blood chemistry and cardiovascular parameters were studied at 7–8 weeks of age in anesthetized rats.

All experimental procedures were approved by the Khon Kaen University Animal Care and Use Committee (AEKKU 9/2557) and were conducted in accordance with the National Institutes of Health guidelines.

2.2 Experimental Protocol

At 7–8 weeks of age, male rats were anesthetized with Nembutal (50 mg/kg, i.p.) and implanted with femoral arterial and venous catheters and were then allowed to recover in individual cages. Three days later, the male rats were anesthetized with Nembutal

and tracheostomized, and arterial pressure was continuously recorded. After laparotomy, right renal sympathetic nerve was then exposed and its activity was continuously recorded using stainless steel electrodes (12 MΩ, 0.01 Taper, Model 5727; A-M System, Sequim, WA, USA) connected to a DAM-80 amplifier (DAM 80; World Precision Instruments, Sarasota, FL, USA) and the BIOPAC Systems (BIOPAC Systems, Goleta, CA, USA). Multiunit recording of renal nerve activity was conducted only on nerve units that responded to changes in arterial pressure following sodium nitroprusside or phenylephrine infusion. Body temperature was servo-control at 37 ± 0.5 °C by a rectal probe connected to a temperature regulator controlling an overhead heating lamp. At the end of the experiment, blood (volumes of about 1.0 mL) were collected from abdominal aorta for non-fasting plasma sodium, plasma potassium, plasma bicarbonate, plasma chloride, plasma creatinine, and blood urea nitrogen. Finally, all animals were euthanatized by Nembutal overdose and thoracotomy.

2.3 Data Analyses

Mean arterial pressure, heart rate, and renal nerve frequencies and patterns were analyzed by using the Acknowledge software (BIOPAC Systems). The baroreflex sensitivity control of renal nerve activity was calculated as a change in renal nerve firing rate to a change in mean arterial pressure. Both hypertensive and hypotensive baroreflex sensitivity values were analyzed.

The power spectral densities of renal sympathetic nerve activity between frequencies 0.5–1.5 Hz stand for renin release, 1.0–2.5 Hz for sodium excretion, and 2.0–5.0 Hz for renal blood flow were analyzed by using fast Fourier transformation (Hanning window, pad with zeros, remove mean, remove trend, and linear magnitude) (Acknowledge software; BIOPAC Systems). Each power spectral density was normalized to the percent of total power spectral density (summation of the three power spectral densities). In addition, the graph showing the pattern of the power spectral density of renal sympathetic nerve activity in each group was averaged from 7–12 rats by using Acknowledge software (BIOPAC Systems). The standard error of mean for this pattern could not be interpreted in the present study.

Plasma sodium, plasma potassium, plasma bicarbonate, plasma chloride, plasma creatinine, and blood urea nitrogen were measured by the Srinagarind Hospital Laboratory Unit (Faculty of Medicine, Khon Kaen University).

2.4 Statistical Analysis

All data are expressed as mean ± SEM. Statistical comparisons among the four groups were performed by using one-way ANOVA followed by the *post hoc* Duncan's Multiple Range test (StatMost32 version 3.6, Dataxiom, CA, USA). The Wilcoxon

signed-rank test was also used to find the difference between groups in renal nerve frequencies and power spectral densities. The significant criterion is $p < 0.05$.

3 Results

3.1 General Characteristics

At 7–8 weeks of age, body weights slightly increased in CG compared to CW ($P < 0.05$), but not in CW + T and CG + T groups (CW, 243 ± 3 g; CW + T, 246 ± 1 g; CG, 253 ± 3 g; CG + T, 250 ± 2 g), but heart weights were not significantly different among the four groups (data not shown). In addition, plasma sodium, plasma potassium, plasma chloride, plasma creatinine, and blood urea nitrogen were not significantly different among the four groups, while plasma bicarbonate significantly decreased in CG compared to CW + T and CG + T, but not CW groups (Table 1).

3.2 Hemodynamic Parameters

High sugar intake did not significantly increase mean arterial pressure in CG compared to CW. Further, taurine supplementation slightly and significantly increased mean arterial pressures in CW + T and CG + T compared to CW, but CW + T, CG, and CG + T were not significantly different from each other (Fig. 1 upper). In addition, heart rates (Fig. 1 lower) and baroreflex sensitivity control of renal nerve activity induced by both phenylephrine (BSRA-PE) and sodium nitroprusside infusion (BSRA-SNP) (Fig. 2) were not significantly different among the four groups.

Table 1 General blood chemistry in experimental groups

Parameters	Na^+ (mEq/L)	K^+ (mEq/L)	HCO_3^- (mEq/L)	Cl^- (mEq/L)	Cr (mg/dL)	BUN (mg/dL)
CW (n = 15)	138.4 ± 1.0	4.8 ± 0.3	21.3 ± 1.7	101.6 ± 1.1	0.23 ± 0.02	26.5 ± 1.6
CW + T (n = 12)	139.4 ± 1.3	4.3 ± 0.1	24.2 ± 0.6	100.6 ± 1.5	0.21 ± 0.01	21.5 ± 1.0
CG (n = 12)	141.4 ± 1.5	4.3 ± 0.2	$18.2 \pm 1.2^{\beta}$	101.1 ± 1.0	0.24 ± 0.02	21.9 ± 1.2
CG + T (n = 9)	139.6 ± 1.8	4.4 ± 0.1	$24.0 \pm 1.5^{\delta}$	99.1 ± 1.3	0.22 ± 0.02	20.1 ± 1.6

Values are mean ± SEM; *BUN* blood urea nitrogen, *Cr* plasma creatinine, *CW* control with water intake alone, *CW + T* control with water intake plus taurine, *CG* control with high sugar intake, *CG + T* control with high sugar intake plus taurine; $^{\beta,\delta}P < 0.05$ compared to CW + T and CG, respectively

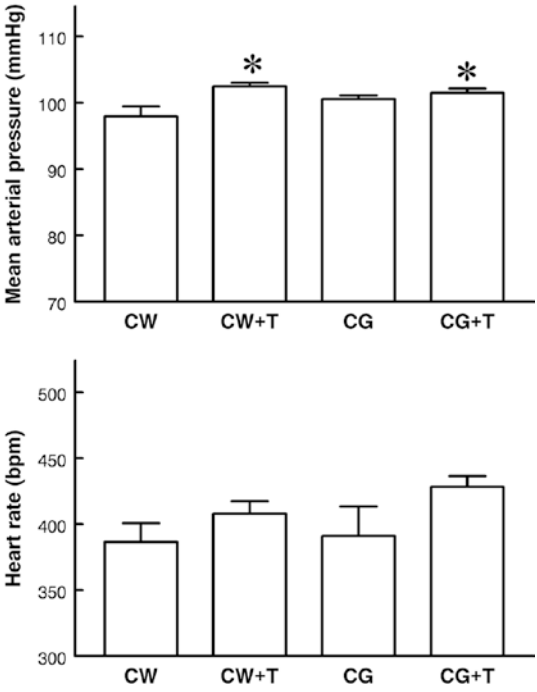

Fig. 1 Mean arterial pressures (*upper*) and heart rates (*lower*) in anesthetized male rats (*CW* control with water intake alone, *CW + T* control with water intake plus taurine, *CG* control with high sugar intake, *CG + T* control with high sugar intake plus taurine; $^{*}P < 0.05$ compared to CW)

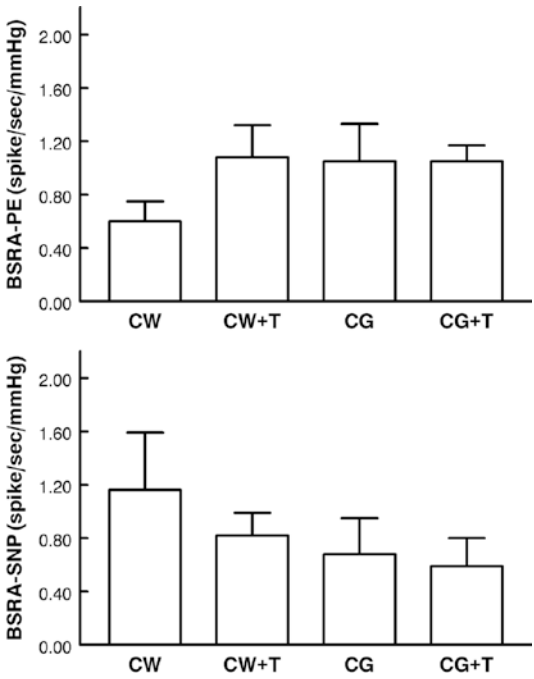

Fig. 2 Baroreflex sensitivity control of renal nerve activity measured by phenylephrine infusion (BSRA-PE; *upper*) and by sodium nitroprusside infusion (BSRA-SNP; *lower*) in anesthetized male rats (*CW* control with water intake alone, *CW + T* control with water intake plus taurine, *CG* control with high sugar intake, *CG + T* control with high sugar intake plus taurine). No significant differences were observed among the four groups

3.3 Renal Sympathetic Nerve Frequencies and Patterns

Multiunit recording of renal nerve activity indicates that high sugar intake since weaning with or without taurine supplementation did not significantly affect renal sympathetic nerve firing rates; i.e., the four groups displayed similar renal nerve firing frequencies (Table 1).

Power spectral analysis of renal nerve activity indicates that the renal sympathetic nerve activity pattern of CG group differed from the other three groups (Fig. 3). Further, the power spectral density of the renin release-related frequency component (0.5–1.5 Hz) significantly increased, that of sodium excretion-related frequency components (1.0–2.5 Hz) significantly decreased and that of renal blood flow-related frequency components significantly decreased in CG compared to CW groups (Table 2). Although taurine supplementation did not significantly affect these power spectral densities in CW + T compared to CW groups, it restored the renal nerve activity pattern of CG to CW levels (Fig. 3).

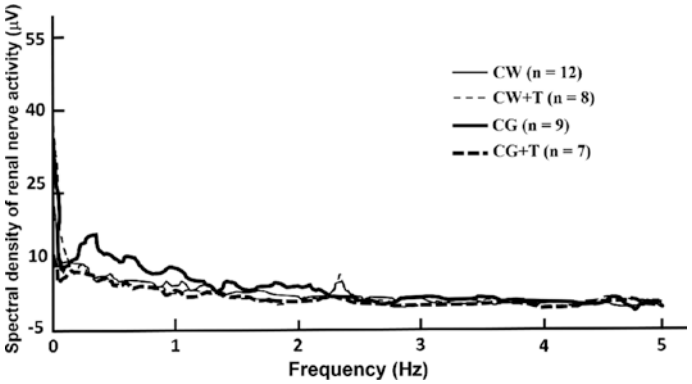

Fig. 3 Patterns of renal nerve activity in anesthetized male rats (*CW* control with water intake alone, *CW + T* control with water intake plus taurine, *CG* control with high sugar intake, *CG + T* control with high sugar intake plus taurine). Each graph was averaged from 7–12 rats by the Acknowledge software (BIOPAC Systems, Goleta, CA, USA)

Table 2 Renal nerve firing rates and power spectral densities of renal nerve activity at the frequencies related to renin release (0.5–1.5 Hz), sodium excretion (1.0–2.5 Hz), and renal blood flow (2.0–5.0 Hz) in anesthetized male rats

		Power spectral densities (%)		
Treatment	Firing rates (spike/sec)	0.5–1.5 Hz	1.0–2.5 Hz	2.0–5.0 Hz
CW (n = 12)	100.9 ± 1.2	41.7 ± 5.3	33.4 ± 2.1	24.9 ± 3.6
CW + T (n = 8)	100.2 ± 1.0	42.4 ± 5.5	33.1 ± 2.0	24.6 ± 3.9
CG (n = 9)	98.0 ± 1.3	51.8 ± 3.0*	29.3 ± 1.7*	18.9 ± 1.4*
CG + T (n = 7)	100.4 ± 1.0	37.4 ± 3.1	32.3 ± 1.0	30.3 ± 3.7

Values are mean ± SEM; *CW* control with water intake alone; *CW + T* control with water intake plus taurine, *CG* control with high sugar intake, *CG + T* control with high sugar intake plus taurine; *$P < 0.05$ compared to CW (Wilcoxon signed-rank test)

4 Discussion

High sugar intake from weaning onward decreases water and sodium excretion, due to an increase in renal tubular water and sodium reabsorption and despite a sharp rise in glomerular filtration rate in adult male rats (Roysommuti et al. 2002). Neither renal vascular resistance nor blood flow are affected by this high sugar intake. The high sugar intake does not alter sympathetic or parasympathetic nerve activity (estimated by arterial pressure variability in both male and female rats) (Roysommuti et al. 2009; Thaeomor et al. 2013). In the present study, multiunit recording of renal nerve firing frequency did not indicate that high sugar intake altered sympathetic nerve activity, but the spectral power analysis of renal sympathetic nerve activity strongly suggests that the renal nerve activity selectively increases in nerve fibers innervating the juxtaglomerular cell, i.e., the renin releasing cell. This finding confirms the likely, major role of renin-angiotensin system overactivity in high sugar-induced renal dysfunction. In addition, the present data indicate that a short-term taurine supplementation can counteract the adverse effects of high sugar intake on renal nerve activity pattern.

The systemic renin-angiotensin system is mainly activated by renin release from juxtaglomerular cells. Low renal perfusion pressure and increased renal sympathetic nerve activity are the main factors that increase renal renin secretion (Friis et al. 2013). Renal innervation is quite complex. Although some nerve axons of renal sympathetic nerve may innervate several structures of the kidney, a group of nerve axons or nerve unit to juxtaglomerular cells are mostly distinct from those destined for the renal tubule or renal vasculature (DiBona 2005b). Thus, the central sympathetic pathway can specifically regulate the renal function. Hypotension, hypercapnia, hypoxia, and pain can stimulate renal sympathetic nerve activity but at different nerve units (DiBona 2005b).

Renal nerves are composed of afferent and efferent fibers. While the afferent fibers involve renal ischemic and injury responses, the efferent fibers are sympathetic nerve fibers supplying three different functions of the kidney (Johns et al. 2011). Although the firing rate of the renal nerves may not increase in some forms of hypertension, renal denervation can prevent or decrease hypertension in animals and humans (Papademetriou et al. 2014; Schlaich et al. 2013). This change may be due to the fact that both firing rates and patterns of renal nerve activity contribute importantly to renal function. This study indicates that the pattern rather than the firing rate of renal nerve activity, specifically affects renin secretion, sodium excretion, and renal blood flow, at least in high sugar-fed rats.

It is well-known that dietary sugar - induced hypertension can be prevented or improved by taurine supplementation or diets high in taurine, by inhibition of sympathetic nerve activity and renin-angiotensin system (Roysommuti and Wyss 2014). Renal sympathetic activity is controlled by a complex of central sympathetic pathways above spinal cord, particularly residing in five cell groups in brain stem and hypothalamus. These include medullary raphe nuclei, rostral ventrolateral medulla (RVLM), ventromedial medulla, pontine A5 noradrenergic cells, and paraventricular

nucleus (Schramm et al. 1993). Further, most sympathetic renal premotor neurons are located in the RVLM and the caudal pons (Ding et al. 1993). In the present experiments, high sugar intake did not significantly affect fasting and non-fasting blood sugar in either male or female rats (Roysommuti et al. 2009; 2013) and plasma insulin levels in female rats (Roysommuti et al. 2013). It is likely that periodic (postprandial) rather than sustained hyperglycemia/hyperinsulinemia plays an important role in high sugar intake-increased renin release.

Hyperinsulinemia appears to increase renal sympathetic nerve activity and renin release by acting at the RVLM (Bardgett et al. 2010) and the hypothalamus (Cassaglia et al. 2011, 2016), while taurine is reported to decrease sympathetic nerve activity by acting at both hypothalamus (Fujita et al. 1986; Fujita and Sato 1988) and RVLM (Amano and Kubo 1993). The main neurons in RVLM is noradrenergic (Ding et al. 1993) and sympatholytic activity of taurine also involves hypothalamic noradrenergic pathway activity (Fujita et al. 1986). Thus, taurine supplementation may counteract the effect of high sugar/hyperinsulinemia on renal nerve activity and renin release by altering the noradrenergic pathways in the hypothalamus and the RVLM. However, the detailed mechanisms how these brain areas specifically regulate the renal nerve activity to the specific parts within the kidney has not been conclusively reported (Nishi et al. 2015).

In the present study, high sugar intake did not affect arterial pressure and most of plasma electrolytes except bicarbonate in CG compared to CW groups, suggesting that baroreceptor and probably chemical-mediated renal sympathetic responses is unlikely. Normal levels of plasma creatinine and blood urea nitrogen, as well as plasma electrolytes indicate no severe renal dysfunction after a high sugar diet. These changes are similar to our previous reports (Roysommuti et al. 2009; Thaeomor et al. 2010). Further, the slight increase in mean arterial pressure in taurine supplemented groups compared to CW group, irrespective of high sugar intake, might be due to the effect of taurine on gastrointestinal sodium and water absorption (Suwanich et al. 2013). However, this arterial pressure difference (about 4 mmHg) is too small to mediate baroreflex bradycardia or decreased renal sympathetic nerve activity in both taurine-treated groups compared to CW and CG groups. This is supported by the data that both heart rates and baroreflex controls of renal nerve activity were not significantly different among the four groups.

5 Conclusion

High sugar intake affects neural, cardiovascular, endocrine, and renal functions leading to several disorders particularly hypertension and kidney disease. These adverse effects of high sugar intake have been studied in both animals and humans. Lines of evidence indicate that renal nerve activity patterns rather than firing rates specifically explain the effects of renal sympathetic nerve on renal renin release, sodium excretion, and renal blood flow. The present data indicate that a high sugar

diet alters renal sympathetic nerve activity pattern, specifically increasing renin release. In addition, this adverse effect of high sugar intake since weaning can be restored to normal levels by a short-term taurine supplementation, at least in young adult, male Sprague-Dawley rats.

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Taurine Recovery of Learning Deficits Induced by Developmental Pb^{2+} Exposure

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Abstract Lead (Pb^{2+}) is a historically well-documented environmental neurotoxin that produces developmental cognitive learning and memory impairments. These early neurodevelopmental impairments cause increased brain excitability via disruption of Ca^{2+} mediated signaling during critical periods of synaptogenesis inducing competition with $I_{ca}{}^{2+}$ through $NMDA_R$s resulting in altered brain development and functioning across the lifespan. Interestingly, Pb^{2+} has been shown to decrease GABA transport and uptake, decrease spontaneous and depolarization-evoked GABA neurotransmission and lower the expression of glutamic acid decarboxylase (GAD); thereby, limiting excitatory GABAergic influences that

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regulate early developmental brain excitability and reducing inhibition across mature GABAergic networks. Taurine has been shown to regulate brain excitability in the mature brain through $GABA_{AR}$ mediated inhibition, thereby attenuating improper brain excitability. Mechanistically, taurine is developmentally a potent neuromodulator that acts as a $GABA_{AR}$ agonist and more recently has been reported as a partial agonist for $NMDA_R$s through glycine sites. We investigated the effects of developmental Pb^{2+} exposure on the rat's mature inhibitory cognitive control abilities pharmacologically through anxiety and emotional learning-related behaviors and whether taurine could recover Pb^{2+} induced neurodevelopmental behavioral deficits later in life. Results showed that Pb^{2+} increased anxiety symptoms in the open field and hole board test, increased sensitivity to context fear training with cognitive deficits in both acquisition and extinction learning while producing learning deficits and inabilities in acquiring inhibitory learned associations through the acoustic startle response and pre-pulse inhibition (ASR-PPI) test. Interestingly, taurine recovered Pb^{2+} developmentally induced behavioral deficits in the open field and hole board test evidenced by decreased freezing and increased exploration behaviors and facilitated inhibitory dependent ASR-PPI learning to levels higher than controls. In contrast, Baclofen, a $GABA_{BR}$ agonist, dose dependently showed no interaction with Pb^{2+} effects on ASR-PPI learning. Thus, taurine may work as an important neuromodulator at both $GABA_{AR}$s and $NMDA_R$s glycine sites, thereby increasing inhibition, enhancing Ca^{2+}-mediated signaling, and decreasing the altered brain excitability, which impedes learning and memory from early Pb^{2+} exposure. Taken together our data suggests that $GABA_{AR}$ dependent inhibitory learning is altered by early Pb^{2+} exposure and taurine was able to recover these Pb^{2+} induced deficits through neuromodulation of $GABA_{AR}$s and potentially $NMDA_R$s later in life. These findings may pave the way for further exploration of taurine as a pharmacotherapy for neurodevelopmental lead poisoning in both animal and clinical models.

Keywords Lead (Pb^{2+}) • GABA • Taurine • Neurodevelopment • Recovery • Behavior

Abbreviations

ACF	Auditory context cued fear conditioning
ASR	Acoustic startle response
CFC	Context fear test
HB	Hole board test
OF	Open field
Pb^{2+}	Lead
PPI	Pre-pulse inhibition
Tau	Taurine

1 Introduction

To date, the majority of the research conducted on developmental lead poisoning (Pb^{2+}) has been directed towards low levels of environmental lead exposure and its effects on learning and memory with focus on the glutamatergic system. Specifically, the Ca^{2+}/Pb^{2+} competition through the *N-methyl-D-aspartate receptor* ($NMDA_R$) in animal models has been the focus of investigation (Toscano and Guilarte 2005) and far less attention has been directed towards the GABAergic system (Neuwirth 2014). Lasley and Gilbert (2000) observed that Pb^{2+}-depressed long-term potentiation (LTP) in the hippocampus and suggested a cellular level learning deficit that correlated with cognitive learning impairments observed at the behavioral level of animals developmentally exposed to Pb^{2+}. Notably, GABAergic interneurons provide critical synchronization of distinct neuronal rhythms between the hippocampus and other association neural networks, such as the frontal cortex, which is essential for learning and memory (Bragin et al. 1995; Buzaki and Chrobak 1995; Soltesz and Deschenes 1993). Early brain excitability is restricted to GABAergic regulation as NMDARs are expressed, but lack functionality until the potassium chloride co-transporter (KCC2) reverses the intracellular chloride gradient; resulting in the shift from early GABA-excitation-to-mature GABA-inhibition (Ben-Ari 2002). Moreover, $NMDA_R$s, *γ-amino butyric acid receptors* ($GABA_{AR}$s), and *voltage sensitive calcium channels* (VSCCs) collectively drive early brain excitation via the regulation of tightly controlled Ca^{2+} oscillations through giant depolarizing potentials (GDPs). Thus, GDPs synchronize neural activity across brain structures differentially (Yan 2013) and as a function of GABAergic system maturation in normal and disease models (Neuwirth 2014; Yan 2013); whereby they shape the timing and tonic phases of neural excitation-inhibition balancing (Ben-Ari 2002).

Disruption to such early brain excitation-to-inhibition balancing is a contributing risk factor for the onset of epileptogenesis and other developmental neuropathologies (Ben-Ari et al. 2012). To further elucidate Pb^{2+} neurotoxicity and the relationship between glutamine, glutamate, and GABA with respect to seizure susceptibility, Strużyñska and Sulkowski, (2004) showed Pb^{2+} treatment diminished GABA transport, decreased uptake and depolarization evoked release, lowered the expression of *glutamate decarboxylase* (GAD), the GABA synthesizing enzyme, and over expression of the GABA transport protein GAT-1. Thus, Pb^{2+} induces brain excitability from both Glutamatergic and GABAergic mechanisms. To date little to no glutamatergic therapies have evidenced benefit or recovery of symptoms that would be a potentially viable pharmacotherapy. In contrast, taurine, a potent neuromodulator that acts as a $GABA_{AR}$ agonist, has been consistently shown to offer neuroprotection, reduces brain excitability, anxiety, and learning deficits (Neuwirth et al. 2013, 2015) and may be a viable alternative pharmacotherapy through GABAergic neuromodulation for treating developmentally Pb^{2+}-induced neuropathologies. In addition, taurine has recently been shown to be a partial $NMDA_R$ agonist through glycine binding sites, which may positively influence learning and memory (Chan et al. 2014).

Further, taurine regulates brain excitability by increasing GABA neurotransmission in animal models of epilepsy, ameliorating brain excitability through increased inhibition (El Idrissi et al. 2003); in particular, through upregulation of GAD and interaction with the $GABA_{AR}$ β2/β3 subunits (L'Amoreaux et al. 2010). Furthermore, taurine is suggested to be neuroprotective in its ability to forestall the natural aging process associated with the GABAergic systems when challenged by neurodegeneration and other related neurodevelopmental disorders (El Idrissi et al. 2013). However, taurine may also play a similar role in protecting the GABAergic system from early exposure to neurotoxins and protect against neurodevelopmental disabilities through re-establishing $GABA_{AR}$, $NMDA_R$, and Ca^{2+} synchronized GDP's during early brain insults.

Here we evaluated the effects of developmental exposure to Pb^{2+} on anxiety, freezing, exploration, and learning and memory behaviors regulated in part by the GABAergic system and whether acute taurine administration, consistent with our previous work using autistic-like rodent models with reduced $GABA_{AR}$ neural systems, would recover these neurodevelopmental induced Pb^{2+} neurobehavioral aberrations in this later life neurotoxicology rodent model.

2 Methods

2.1 Subjects

In accordance with The College of Staten Island (CUNY) IACUC approval guidelines, Long-Evans Norwegian hooded male and female rats (Taconic, N.J.) were paired for breeding and their male F1 generation were used for future experimentation. Rat litters were culled to 8–10 pups in order to control for maternal social influences on neurodevelopment and behavior. Control rats were fed a regular Purina rat chow (Dyets Inc. # 61212) [containing 970 gm/kg Purina RMH 1000 chow, 30 gm/kg maltose dextrin], while the Pb^{2+} rats were fed a diet containing 1.5 g/kg lead acetate (Dyets Inc. # 612113) [containing 968.4 gm/kg Purina RMH 1000 chow, 30 gm/kg maltose dextrin, 1.5 gm/kg lead acetate, and 0.1 gm/kg yellow dye] (956 ppm) *ad libitium* from pairing throughout gestation and continued through weaning (*i.e.,* life-long exposure model). Prior to behavioral testing all rats were handled for 10 min per day for 1 week.

At postnatal day (PND) 22 independent sets of rats (N = 176) were randomly assigned to a treatment condition (Control n = 56, Control + Taurine n = 24, Pb^{2+} n = 69, Pb^{2+}+Taurine n = 27) in the following behavioral assays: Open Field (OF), Hole Board test (HB), Context Fear Conditioning (CFC) and Auditory Cued Fear Conditioning test (ACFC), and the Acoustic Startle Response (ASR) with pre-pulse inhibition (PPI) test to ensure experience dependent effects were restricted to early developmental Pb^{2+} exposure. Rats were administered either taurine, a $GABA_{AR}$ agonist, 43 mg/kg i.p. injection or Baclofen, a $GABA_{BR}$ agonist, in a dose response of 3 mg/kg, 6 mg/kg, or 10 mg/kg i.p. injections 15 min prior to behavioral testing to assess and compare taurine's and Baclofen's influences on GABAergic behavioral

regulation in reducing anxiety, hyper-activity, emotional irritability, hyper-excitability, stress and increasing inhibitory learning and memory outcomes consistent with our previous reports of recovering similar behavioral phenotypes in the Fragile X mouse model (Neuwirth et al. 2013, 2015; El Idrissi et al. 2003, 2009, 2010; El Idrissi and L'Amoreaux 2008; El Idrissi 2008).

2.2 Blood Lead Level Analyses

Following all behavioral testing, at the point of animal sacrifice transcardial blood samples were collected in *ethylenediamine-tetraacetic acid* (EDTA) coated S-Monovette® syringes (Sarstedt, Germany) to prevent coagulation, then mixed, and immediately frozen on dry ice then stored at −80 °C. Samples were then sent out for BLL determination to Magellan Diagnostics (North Bellirica, MA). BLLs were determined using atomic absorption spectrophotometry (AAS) with a sensitivity detection level of ±1 μg/dL.

2.3 Open Field

At PND 22 naivë set of Control ($n = 24$), Control + Taurine ($n = 10$), Pb^{2+} ($n = 24$), and Pb^{2+}+Taurine ($n = 10$) rats were examined during 10 min of locomotor exploration in the open field test (OF) (376 mm H × 914 mm W × 615 mm L) in an illuminated room (300 Lux). Locomotor variables including time mobile (s) in zone and latency of first exit from zone (s) were recorded and post analyzed using the Anymaze® video tracking software transmitted via a ceiling mounted SONY-Handy Camera and transmitted to a standard HP 1.6 GHz computer.

2.4 Contextual Fear Conditioning

At PND 24–30 behaviorally naivë rats (Control $n = 10$ and Pb^{2+} $n = 9$) were transferred to the testing room and remained in their home cage for 60 min in order to acclimate in the dark with red ambient light (3 Lux). Post acclimation, rats were gently placed into the context fear conditioning chamber (Med Associates) (215.9 mm H × 260.35 mm W × 254 mm L). The testing paradigm was modified from (Neuwirth et al. 2013, 2015) as follows: (a) **Day 1 acquisition phase:** 120 s baseline, 10s later sound was emitted for a 30s duration, after 10s of the sound a light was illuminated for 10s and during the last 2 s of the sound a 0.5 mA foot shock through a floor grid was given for 5 s in duration as the conditioned aversive stimulus. During baseline and post foot shock the rat's latency to break 3 infrared beams were measured every 10 s for 60 s followed by a 70 s inter-trial-interval. Three trials were presented during day 1 which was considered the learning

acquisition phase. (b) **Day 2 retention phase:** The exact same testing procedures were administered as in day 1 except that no foot shocks were delivered.

2.5 Auditory Cued Conditioning

Another set of naivë animals were used as in the CFC test (Controls $n = 9$ and Pb^{2+} $n = 10$) and test conditions were identical to the CFC test; except that on Day 3 rats were placed in an altered context. The chamber was the same, but was divided diagonally with a black plexiglass (349.25 mm) and the floor coated with a black rubber mat. On the opposite inaccessible side of the plexiglass, a Petri dish was filled with a vanilla extract to motivate the rat to explore towards to novel odor in the altered context chamber. Motion was recorded for a single trial with a baseline measure of 180 s followed by the onset of the learned auditory cue used in the CFC training two days prior to assess ACFC learning and memory. However, the tone lasted 180 s.

2.6 Hole Board Test

At PND 30–40 another naivë set of Control ($n = 7$), Control + Taurine ($n = 7$), Pb^{2+} ($n = 10$), and Pb^{2+}+Taurine ($n = 10$) rats were examined during 10 min of anxiety fear escape testing in the Hole Board test (HB) (610 mm H × 610 mm W × 610 mm L) in an illuminated room (300 Lux) on Day 1. On Day 2 rats were tested in the same chamber and lighting at the same time of day, but 4 novel olfactory gradients (i.e. vanilla, orange, lemon, and almond) were placed in petri dishes below the four corners of the HB test with equal odor gradients to manipulate the prior day's exposure of fear responding (*i.e.,* prevent habituation due to the potential environmental assimilation within the same context) and instead promote exploratory behavior. Locomotor dependent variables included time freezing (s) and time exploring in zone (s) to overcome recurrent fear were recorded and post analyzed using the Anymaze® video tracking software transmitted via a ceiling mounted SONY-Handy Camera transmitted to a standard HP 1.6 GHz computer.

2.7 Acoustic Startle Response Habituation

At PND 30–46 behaviorally naivë rats (Control and Pb^{2+} $n = 5$ per group) were transferred to the testing room and remained in their home cage for 60 min in order to acclimate in the dark with red ambient light (3 Lux). Post acclimation rats were gently placed into the ASR chamber MED-ASR-Pro1 (Med Associates Inc., VA) for the length of the testing session (*i.e.,* 30 min). All subjects were administered a one block design consisting of 20 trials presenting an auditory stimulus of 115 decibels (dB) with an inter-trial-interval (ITI) of 15 ms to assess startle habituation in a non-associative test condition.

2.8 Acoustic Startle Response and Pre-pulse Inhibition

At PND 30–46 behaviorally naivë rats (Control $n = 11$, Control + Taurine $n = 7$, Pb^{2+} $n = 11$, Pb^{2+}+Taurine $n = 7$) were handled and acclimated as in the startle response habituation test. However, subjects were administered a three block design to assess PPI learning to test associative learning and memory modified from (El Idrissi et al. 2012; Neuwirth 2008). Block one consisted of 4 trials with a startle stimulus of 115 dB. Block two contained a random matrix of 16 trials with unpredictable pre-pulses of 75 dB, 85 dB, 95 dB, and 105 dB with an ITI of 15 ms. Block three was identical to block one. Data were driven via the motion force sensor transducer platform and transmitted to a standard desktop computer to analyze the data with the supplied Med Associated Software. Peak amplitudes of each motion force response were measured and compared as the percent difference of experimental rat's startle sensori-motor magnitude from control rats and their Pre-vs. Post-startle response following PPI respectively.

2.9 Data Analysis

Data were recorded as digital video clips using an analog-digital converter. The movies were analyzed using AnyMaze software. Animal tracking was based on contrast relative to background. Different zones were labeled and indicated on the monitor. Two tracking points were specified, one on the animal's head and the other on the center of the animal. An excel spreadsheet was generated containing all the parameters specified.

2.10 Statistical Analyses

All data were analyzed in *Statistica* V. 12.7 (Statsoft, Inc. Tulsa, OK). Factorial *ANOVAs* were used to assess *Treatment*, *Taurine,* and *Treatment* X *Taurine* interaction effects. Significance levels were set at $\alpha = 0.05\%$ with a 95% ± SEM. Significant differences were determined by equal and unequal Tukey's HSD post hoc comparisons test.

3 Results

3.1 Pb^{2+} Exposure Induced Anxiety Behaviors that Were Recovered by Acute Administration of Taurine

Developmental Pb^{2+} treatment resulted in BLL's ranging from 34–42 µg/dL ($M = 38.67$ µg/dL, $SD = 3.27$) ($p < 0.001^{***}$) at the time of testing, while controls were Pb^{2+} negative. The open field test revealed preliminary anxiety-like behaviors

relative to an animal's assimilation to a novel arena. We assessed the effects of Pb^{2+} on anxiety-like behaviors associated with this test by measuring the latency to exit the center zone and time mobile within the center zone as an index of anxiety behaviors. Pb^{2+} treatment induced levels of anxiety similar to controls. However, taurine treatment to both control and Pb^{2+} rats (*i.e.,* 43 mg/kg i.p.) delayed initial center zone exit latency (Fig. 1a) ($p < 0.1^{**}$) and increased time spent mobile exploring the center zone (Fig. 1b) ($p < 0.001^{***}$) rather than thigmotaxis. This indicates that Pb^{2+} treated animals respond to pharmacological actions of taurine similar to controls and showed behavioral recovery through decreased anxiety-like and increased exploratory behaviors. The OF test for center zone exploration latency revealed a *Tau Treatment* effect ($F_{(1)} = 8.97$, $p < 0.001^{**}$) (Fig. 1a). In the OF the time mobile in the center zone showed Pb^{2+} effect ($F_{(1)} = 4.08$, $p < 0.05^{*}$) and a *Tau Treatment* effect ($F_{(1)} = 10.7$, $p < 0.001^{***}$) (Fig. 1b).

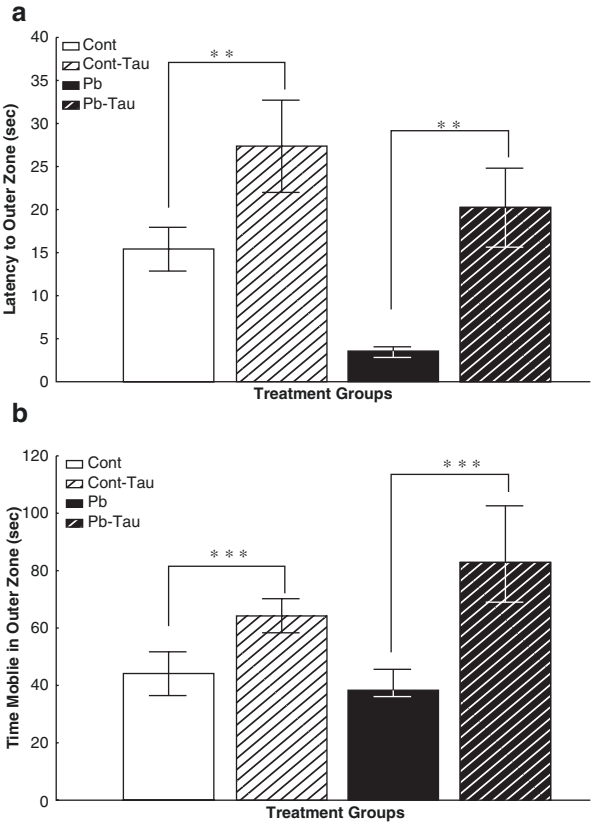

Fig. 1 Pb^{2+} treated rats exhibit similar anxiety behaviors as Control rats, both of which are recovered by taurine administration. Pb^{2+} treated rats presented with similar locomotor activity and preliminary anxiety behaviors as Control rats in the open field. Specifically, taurine increased the latency to leave the center zone (**a**) (**$p < 0.01^{**}$**) and time mobile exploring the center zone (**b**) (**$p < 0.001^{***}$**) indicating that Pb^{2+} treated animals have similar anxiety levels as controls and were recovered by taurine administration. Data are presented as ±SEM

3.2 *Pb^{2+} Induces Deficits in Hole Board Test Escape and Exploration Behaviors Which Are Recovered by Taurine*

To further evaluate the effects of Pb^{2+} on anxiety, we subjected rats to a 2-day hole board test paradigm to assess anxiety as measured by escape behaviors in Day 1 followed by habituation and exploratory behaviors in Day 2 when the apparatus was baited with four novel odorants. Data revealed that Pb^{2+} increased freezing behaviors on Day 1 ($p < 0.01^{**}$) and reduced exploration behaviors on Day 2 ($p < 0.001^{***}$) when compared to controls. Interestingly, taurine administration showed little effects on controls during both days of testing. In Pb^{2+} rat's taurine reduced freezing and increased exploration behaviors ($p < 0.01^{**}$) at levels comparable to controls (Fig. 2) The behavioral engagement time comparisons between tests showed a *Condition* effect ($F_{(1)} = 221.88$, $p < 0.001^{***}$), a *Pb^{2+}* effect ($F_{(1)} = 6.23$, $p < 0.01^{**}$), a *Condition* **X** *Pb^{2+}* interaction ($F_{(1,1)} = 44.73$, $p < 0.001^{***}$), a *Condition* **X** *Taurine* interaction ($F_{(1,1)} = 10.83$, $p < 0.001^{***}$) and a *Condition* **X** *Pb^{2+}* **X** *Taurine* interaction ($F_{(1,1,1)} = 12.51$, $p < 0.001^{***}$) (Fig. 2).

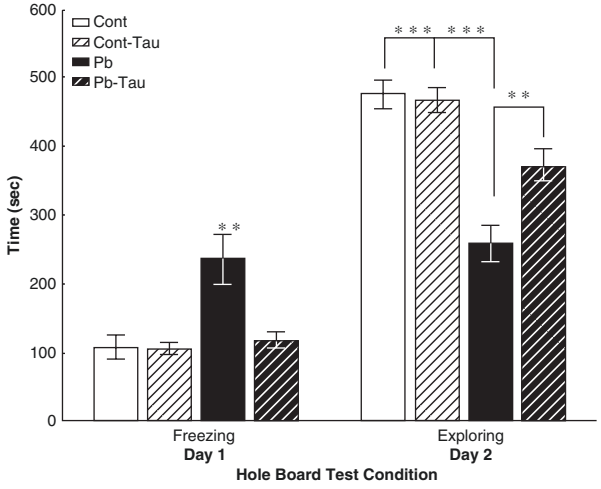

Fig. 2 Pb^{2+} treated rat's evidence increased anxiety and decreased exploratory behaviors on the hole board test when compared to Control rats, and taurine recovers these Pb^{2+} induced neurobehavioral deficits. Pb^{2+} treated rats exhibited increased freezing behaviors on Day 1 (**$p < 0.001^{**}$**) and decreased exploratory behaviors on Day 2 (**$p < 0.001^{***}$**). Notably, in Pb^{2+} treated rat's taurine administration reduced freezing and increased exploratory behaviors comparable to Control rat (**$p < 0.001^{***}$**). Data are presented as ±SEM

3.3 Pb^{2+} Induces Deficits in Context Fear and Auditory Cued Fear Conditioning Learning

We next investigated the effects of Pb^{2+} on contextual fear and auditory cued fear conditioning as a robust measure of emotional learning and memory. Pb^{2+} showed no differences in contextual acquisition trials (i.e. A-T1-4) but had selective deficits in contextual extinction learning trials (i.e. E-T1-3) (Fig. 3a) ($p < 0.05^{*}$ E-T1-2).

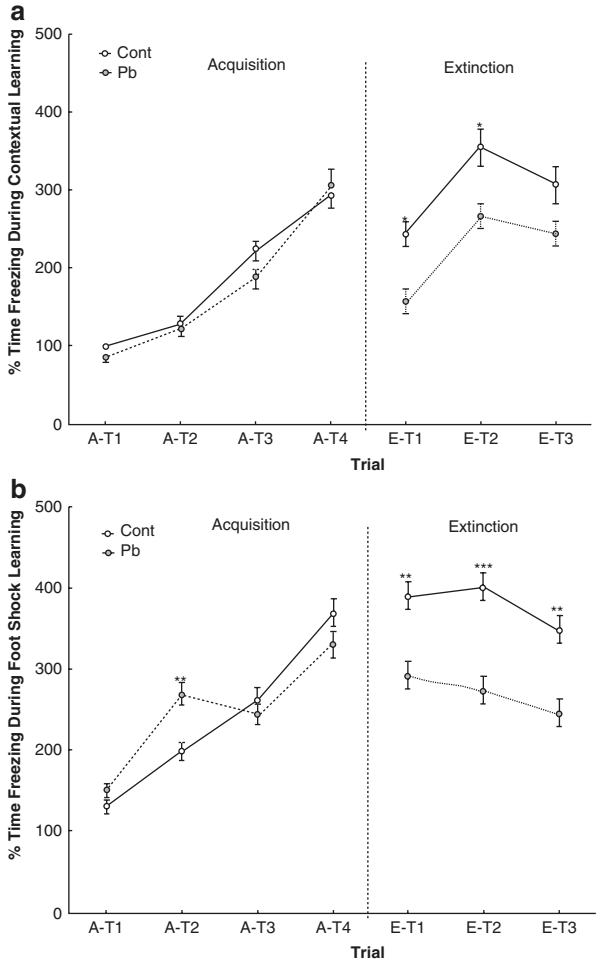

Fig. 3 Pb^{2+} treated rat's exhibit the ability to learn context fear associations, but have deficits in consolidating these memories for later recall evidenced as a reduced behavioral response to learned stimuli 24-h during extinction testing. Pb^{2+} treated rat's exhibit memory retention problems in associating the test context with foot shock administration (**$p < 0.05^{*}$**, **$p < 0.01^{**}$**, **$p < 0.001^{***}$**) (**a**). In addition, Pb^{2+} treated rats were more sensitive to foot shocks during the initial stages of fear acquisition (**$p < 0.01^{**}$**) (**b**). This suggests that Pb^{2+} treated rats respond negatively to stressors and which may impair their spatial information processing during context dependent learning resulting in emotional memory deficits. Data are presented as ±SEM

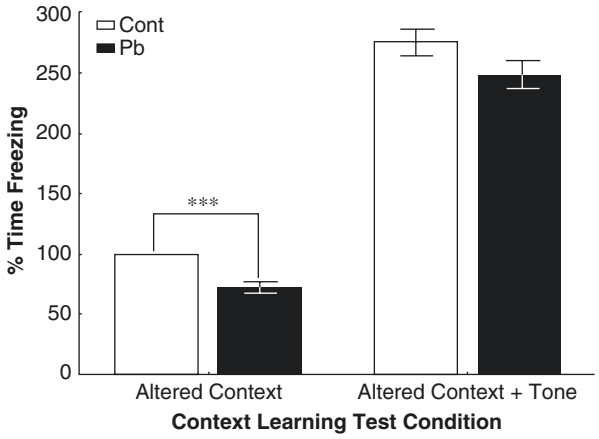

Fig. 4 Pb^{2+} treated rats exhibit context specific learning deficits in the auditory cued and context conditioning test. Pb^{2+} increased locomotor activity and hyperexcitability when place in a novel context (**$p < 0.001^{***}$**) when compared to controls. Notably, there were no treatment effects observed for auditory cued conditioning. Data are presented as ±SEM

Following the aversive foot shock Pb^{2+} rats were initially more sensitive to learn the fear association (Fig. 3b) during acquisition trials ($p < 0.01^{**}$ A-T2) but showed significantly lower memory retention 24 h following training ($p < 0.01^{**}$ E-T1 & 3; $p < 0.001^{***}$ E-T2) when compared with controls. In the context extinction condition revealed a Pb^{2+} effect ($F_{(1)} = 21.74$, $p < 0.001^{***}$) (Fig. 3a). In the aversive extinction condition showed during acquisition trials a *Trial* **X** Pb^{2+} interaction ($F_{(1,3)} = 5.14$, $p < 0.001^{**}$), and in the extinction trials a Pb^{2+} effect ($F_{(1)} = 48.89$, $p < 0.001^{***}$) (Fig. 3b). This suggests that Pb^{2+} induces learning deficits relating to spatial cue context dependent information processing. In order to further assess these findings, we evaluated whether or not Pb^{2+} rats would either show similar behavioral problems that would generalize into novel contextual exposures such as in an altered context when compared to controls. In the ACFC test Pb^{2+} showed increased hyperactivity in the altered context when compared to controls (Fig. 4) ($p < 0.001^{***}$) but were not different than controls when the prior learned tone was presented in the altered context. In the ACFC test revealed a *Condition* effect ($F_{(1)} = 335.41$, $p < 0.001^{***}$) and a Pb^2 Effect ($F_{(1)} = 22.76$, $p < 0.001^{***}$). This indicates that Pb^{2+} rats may by hyper excitable and new environments induce an exaggerated stress response which is further exacerbated by aversive stimuli thereby affecting emotional learning and memory. However, in an environment absent of the floor grid Pb^{2+} rats are better able to retain memory of the tone associated with the prior learned foot shock.

3.4 Pb^{2+} Induced Auditory Hypersensitivity to Startle Stimuli and Pre-pulse Conditioning Deficits Which Were Recovered by Taurine

In order to assess whether or not learning deficits in the context fear and auditory cued conditioning tests were selective to context dependent spatial information processing, we directed our focus on assessing auditory learning deficits induced by Pb^{2+}. To approach this problem, we subjected rats to the acoustic startle

non-associative habituation learning test. This test allowed for us to parse auditory sensory motor gating through habituation of repeated exposures of sensory stimuli in the absence of any learning associations. Data revealed that Pb^{2+} rats were initially hypersensitive to auditory non-associative startle stimuli (Fig. 5a) ($p < 0.001^{***}$) and eventually reached levels comparable to controls. The startle non-associative habituation test showed a *Trial* effects ($F_{(19)} = 2.47$, $p < 0.001^{***}$) and a Pb^{2+} effect ($F_{(1)} = 64.52$, $p < 0.001^{***}$) (Fig. 5a). Following this another set of naivë animals were exposed to a startle and pre-pulse conditioning test to evaluate the roles of auditory inhibitory learning and the effects on taurine ($GABA_{AR}$ agonist) verses Baclofen ($GABA_{BR}$ agonist) to determine which GABAergic receptors are mediated Pb^{2+}induced hypersensitivity and learning deficits. Pb^{2+} treated rats showed significantly less inhibitory learning when compared to controls (Fig. 5b) ($p < 0.05^{*}$ vs. all other treatments $p < 0.001^{***}$). The startle PPI pre- and post-test comparisons revealed a *Condition* effect ($F_{(1)} = 130.39$, $p < 0.001^{***}$), a *Taurine* effect ($F_{(1)} = 23.28$, $p < 0.001^{***}$), a *Condition* **X** *Taurine* interaction ($F_{(1,1)} = 6.0$, $p < 0.01^{**}$), a Pb^{2+} **X** *Taurine* interaction ($F_{(1,1)} = 8{,}50$, $p < 0.01^{**}$) and a *Condition* **X** Pb^{2+} **X** *Taurine* interaction ($F_{(1,1,1)} = 11.4$, $p < 0.001$***) (Fig. 5b).

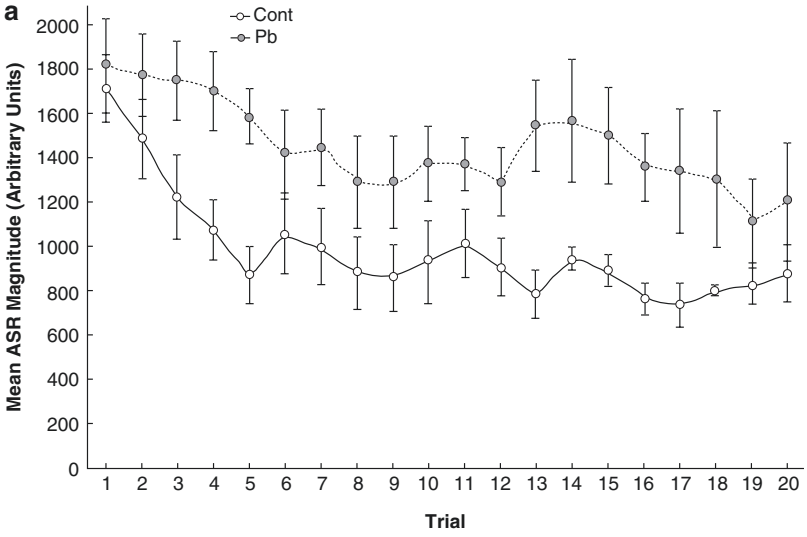

Fig. 5 Pb^{2+} treated rats exhibit auditory hypersensitivity, reduced non-associative startle habituation, and reduced startle pre-pulse inhibition which is recovered by taurine treatment. Pb^{2+} treated rats tested with non-associative (*i.e.,* startle stimuli not paired with any other stimuli) startle stimuli evinced an auditory hyper sensitivity profile when compared to Control rats (**a**) (**$p < 0.001^{***}$**). Pb^{2+} treated rats also showed reduced inhibitory PPI learning, while taurine selectively recovered and enhanced PPI learning in Pb^{2+} rats only (**b**) (**$p < 0.001^{***}$**). Data are presented as ±SEM

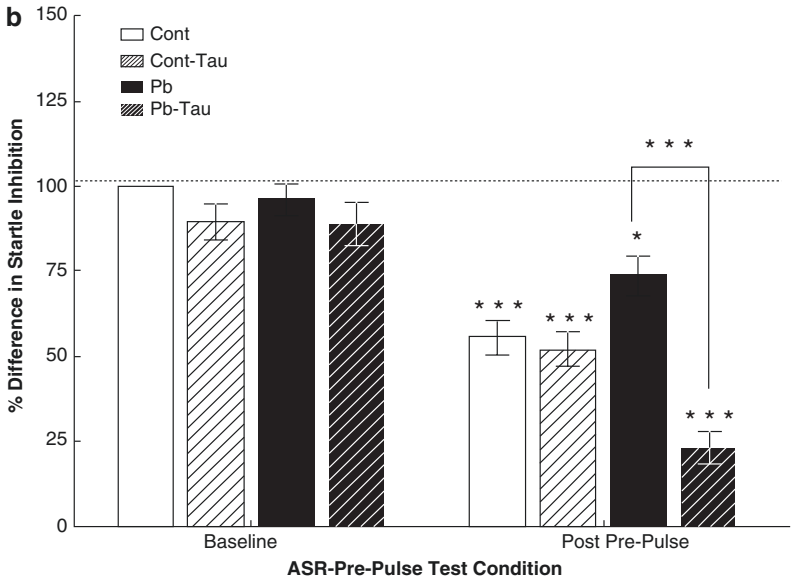

Fig. 5 (continued)

Taurine showed little contribution to PPI inhibition learning for controls. Notably, taurine significantly enhanced PPI learning in Pb^{2+} treated rats (Fig. 5b) ($p < 0.001^{***}$). There were no Pb^{2+}+Baclofen or Control + Baclofen treatment differences observed. However, there was only a *Condition Effect* ($F_{(1)} = 121.72$, $p < 0.001^{***}$) (Fig. 6).

4 Discussion

Taken together these results suggest that Pb^{2+} exposure during early brain development disrupts GABAergic mediated neurobehaviors (i.e., tonic and cortical inhibition) that persist into adulthood. Here, we identified that early developmental Pb^{2+} exposure induced anxiety-like behaviors in the open field and hole board test which persisted into later life. Taurine administration recovered these Pb^{2+} induced behavioral deficits. Interestingly, we identified that the 2-day hole board test was very sensitive in revealing anxiety in the Pb^{2+} rats. The first day of the hole board test induced elevated anxiety responses in the Pb^{2+} rats. However, when re-exposed to the same testing apparatus the next day, rats typically would habituate and show a reduction in anxiety behaviors. However, 24 h later in the hole board test we presented rats with novel odorant stimuli to decrease their anxiety behaviors and to increase their exploratory behaviors. Interestingly, these Pb^{2+} rats remained fearful of the hole board test irrespective of habituation and prior acclimation to the test. This suggests that Pb^{2+} rats have hypersensitivity and increased stress responses to

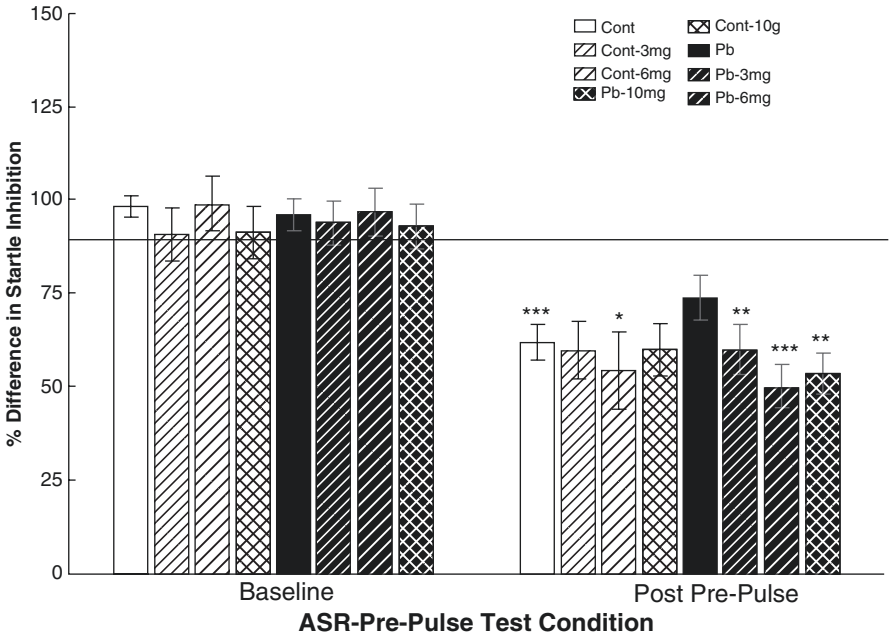

Fig. 6 Pb^{2+} treated rats exhibit startle PPI learning deficits which are minimally recovered by Baclofen administration. Pb^{2+} treated rats showed reduced inhibitory learning and Baclofen dose dependently did not significantly improve or recover their behavioral inhibition irrespective of treatment. Thus, Baclofen a $GABA_{BR}$ agonist, is insensitive in addressingPb^{2+} treated rat's deficits in PPI learning. Notably, all post-test conditions were significantly different from baseline ($\mathbf{p < 0.05}^{*}$, $\mathbf{p < 0.01}^{**}$, $\mathbf{p < 0.001}^{***}$). Data are presented as ±SEM

novel test conditions that may impair contextual spatial learning. To evaluate the contribution of elevated neophobic (*i.e.,* fear of novelty) context specific learning deficits we utilized a context fear test. Here Pb^{2+} rats showed initial hypersensitivity to foot shock during acquisition trials with deficits in context dependent spatial learning. This suggests that Pb^{2+} increases stress responses that may impair the ability of rats to attend to contextual spatial cues; thereby, reducing memory association, consolidation, and later retention.

We next assessed whether Pb^{2+} learning deficits were specific to context information processing or compounded by auditory processing issues as well. In the altered context auditory cued conditioning experiment Pb^{2+} rats were initially hyperactive in the novel environment and seeking out ways to escape the arena. When the auditory tone was presented the Pb^{2+} rats responded similar to controls. Taken together, these findings suggest that Pb^{2+} rats respond with elevated stress to novel environments, but were able to recall the learned auditory cue in an altered context. We then decided to use an acoustic startle response test to assess hypersensitivity to auditory stimuli. Pb^{2+} rats showed an initial hypersensitivity to 20 trials of a 115 dB startle stimulus that eventually returned to control levels indicating delays in inhibition and non-associative learning outcomes as a function of Pb^{2+} developmental neurotoxicity.

These experiments were controlled whereby no stimulus-paired associations occurred with the 115 dB startle stimuli, thus restricting interpretation to auditory sensory processing deficits induced by Pb^{2+}. In the startle PPI experiments, taurine induced inhibition recovered the startle response in Pb^{2+} rats evidenced by PPI returning to control levels and further enhanced the amount of baseline PPI levels when compared to controls. In addition, Baclofen, the $GABA_{BR}$ agonist, had no positive effect dose dependently on control and Pb^{2+} rats. Suggesting that developmental Pb^{2+} neurotoxicity and persistent Pb^{2+} exposure induced behavioral learning and memory deficits that could be recovered by $GABA_{AR}$ pharmacotherapy via taurine treatment.

Taken together, Pb^{2+} induces anxiety-like behaviors, increased freezing, decreased exploration, and produced context specific deficits in spatial information processing, auditory hypersensitivity, and increased stress responses to foot shock and novel environments, which can be treated and recovered through the $GABA_{AR}$ agonist taurine. Notably, Baclofen a $GABA_{BR}$ agonist did not recover behavioral deficits induced by Pb^{2+}. We suggest that early developmental Pb^{2+} exposure, in part, may alter the early GABA-shift and later alter the functioning of the mature $GABA_{AR}$ and its expression levels in the brain resulting in aberrant increased brain excitability. More research in this area of GABAergic brain development under the influence of Pb^{2+} and taurine interactions are required to further elucidate specifically how Pb^{2+} alters GABAergic neurons, and in turn, how taurine recovers behaviors in this developmental neuropathological disease model. In addition, the need for more sensitive and multiple testing paradigms are required in order to obtain a "full picture" of how exactly environmental toxins such as lead may contribute to different sensory processing disorders that will certainly impact a wide-range of learning and memory symptoms. Thus, our findings suggest that animal researchers must be cautious and adequately show convincing systematic evidence in deducing what behavioral pharmacological pathways may be involved in their animal model of human disability.

5 Conclusion

In summary, this study shows that early developmental Pb^{2+} exposure can induce deficits in the proper establishment of GABAergic mediated networks that are, in part, responsible for regulating anxiety-like behaviors, auditory hyper excitability, emotional learning and memory disruption later in life which can be recovered by acute taurine administration. This suggests that taurine may be neuroprotective against Pb^{2+} induced GABAergic aberrant neural signaling, whereby enhancing inhibition to reduce neuronal excitability via $GABA_{AR}$s and learning enhancement as a partial agonist at $NMDA_R$ glycine sites. Moreover, here we showed selective pharmacological action of taurine with $GABA_{AR}$s and an insensitivity to Baclofen with $GABA_{BR}$s, suggesting a potential therapeutic role of $GABA_{AR}$ drugs such as taurine administration in treating Pb^{2+} toxicity in animal and clinical interventions.

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Comparison of Urinary Excretion of Taurine Between Elderly with Dementia and Normal Elderly

Ranran Gao, Mi Ae Bae, Kyung Ja Chang, and Sung Hoon Kim

Abstract The purpose of this study was to investigate the differences in dietary intake, serum level and urinary excretion of taurine between the elderly with dementia and the normal elderly. Subjects with dementia were 22 (8 men, 14 women) and normal were 26 (2 men, 24 women). The general characteristics, anthropometric data were considered together. The blood and urine samples were obtained from the elderly in the morning fasting state. Taurine concentrations in serum and urinary excretion were determined using high performance liquid chromatography (HPLC). Dietary intake data were collected using questionnaires, and analyzed by Computer Aided Nutritional analysis program (CAN-pro 4.0). Statistical analyses were carried out using SPSS 20.0. There were no significant differences in age and BMI (body mass index) between the elderly with dementia and the normal elderly, however, blood total cholesterol, LDL cholesterol and HDL cholesterol levels of the elderly with dementia were relatively higher than the normal elderly. The elderly men with dementia took more lipid, riboflavin higher than the normal elderly men ($P < 0.05$). The elderly women with dementia took more nutrients except vitamin D, vitamin B_{12} and taurine than the normal elderly ($P < 0.001$). There were slight differences in serum taurine level between the two groups. However, urinary excretion of taurine in the elderly with dementia was significantly higher than the normal elderly (41.2%, $P < 0.05$).

Keywords Taurine concentration • Blood • Urine • Dementia • The elderly

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1 Introduction

Dementia is not just a simple disease but a type of complicated syndrome, which may lead to many problems on the respect of memory, thinking, and communication. According to the report of The Global Impact of Dementia, the amount of people living with dementia in the world in 2013 was 44.33 million, and would reach 135.46 million in 2050 (Prince et al. 2013). According to previous studies, the growth rate of the illness would be more than 3 times. It is obvious that not only the elderly with dementia would have low-quality lives, but also the family caregivers would face with great difficulties from the health care and social pressure (Wortmann 2012).

There are many etiological factors in dementia such as brain cell death, stroke, head injury and brain tumor, *etc*. Recently, the administration of taurine contributes to recovering learning and memory of the rats with dementia (Kim et al. 2014). Taurine exists in body tissues (for instance, skeletal and cardiac muscles) of every human being, especially, with a high concentration in the brain. Also, it is well known that taurine has physiological functions such as antioxidant and anti-inflammatory (Javed et al. 2013), promoting skeletal muscle cell's growth and development (Zielinska et al. 2012), and accelerating brain nervous conduction of premature infants (Tyson et al. 1989).

There are many methods reported to measure the concentration of taurine (Anzano et al. 1978; Fekkes et al. 2000; Inoue et al. 2003; Park et al. 2001; Qu et al. 1999). Fluorescamine is one of the best reagents in the derivatization of taurine (Kelly et al. 2000). The formation of taurine-fluorescamine fluorophore is shown in Fig. 1.

Recently, many researchers measured the concentration and content of taurine in their studies. However, the study about the relationship between concentration of taurine and dementia is very rare. Therefore, the purpose of this study was to investigate the differences in dietary intake, serum taurine level and, urinary excretion of taurine between the elderly with dementia and the normal elderly.

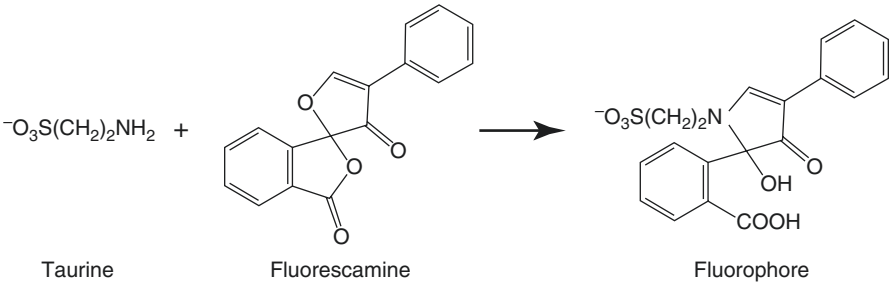

Fig. 1 Formation of taurine-fluorescamine fluorophore

2 Methods

2.1 Subjects

Forty-eight urine samples and forty-eight serum samples were collected from 22 elderly with dementia (8 men, 14 women) using 3 dementia daytime care centers and 26 normal elderly (2 men, 24 women) living in Incheon, Korea. The study protocol was approved by the IRB (Institutional Review Board) of Inha University. (150604-1A).

2.2 Data Collection

It is well known that the elderly with dementia cannot answer the dietary questionnaires properly. So their family caregivers finished the questionnaires instead of them in July, 2015. The items of the questionnaires included general characteristics (gender, age, height, and body weight) and dietary intakes. Height and body weight were measured with stadiometer and digital weight scale. Dietary intakes (all foods and beverages) of the subjects were surveyed for the previous 24 h. Blood samples (after 12 h of overnight fasting) were collected with the agreement of elderly themselves or their family caregivers. The urine and serum samples were stored at −20°C before taurine concentration measurement.

2.3 Chemicals

Taurine (99%) was from Dong-A Pharmaceutical Company. Boric acid, super-purity acetonitrile, and fluorescamine (98%) were from Sigma-Aldrich. HPLC grade acetonitrile, methanol, and tetrahydrofuran were from J.T. Baker.

2.4 Sample Preparation

One hundred microliters of serum or urine samples were mixed with 150 μL of super-purity acetonitrile and then centrifuged at 3000 rpm for 15 min to obtain supernatants. Borate buffer (50 μL), which was prepared from aqueous disodium tetraborate solution (100 mM) adjusted to pH 9.2 with 10 mM boric acid, were added to the supernatants to give approximately pH 9 solution. Then 50 μL of fluorescamine reagent in acetonitrile (5 mM) was added to each solution, immediately vortex mixed (McMahon et al. 1996). The resulting sample solutions (20 μL) were analyzed on the HPLC system within 40 min.

2.5 Experiment

Blood lipid levels (total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglyceride) were determined using Lipido Pro (Impopia, Korea). The taurine concentrations were measured using HPLC system (Agilent Technologies 1200 series HPLC) and Waters C18 reverse-phase column (250 mm × 4.6 mm *i.d.*) at 20 °C. The mobile phase was composed of tetrahydrofuran-acetonitrile-phosphate buffer (pH 3.5) (4:22:74, v/v/v). The flow rate of the elution is 1 mL/min. UV/VIS detection was carried out at 382 nm, which is the maximum absorbance wavelength for the taurine-fluorescamine derivative.

2.6 Statistic Analysis

The significance was determined by Student t-test and Mann-Whitney-test. Each value was expressed as mean ± SE (standard error). Difference was considered statistically significant when the calculated *P* value was less than 0.05.

3 Results and Discussion

3.1 General Characteristics

General characteristics of the elderly were presented in Table 1. The percentage of women (79.2%) was higher than men (20.8%). Although age distribution of the subjects was from 60 to 90 years old, more than 50% was over 80 years old.

Table 1 General characteristics

Variables	Total (n = 48)	Dementia (n = 22)	Normal (n = 26)
Gender			
Men	10(20.8)	8(36.4)*	2(7.7)
Women	38(79.2)	14(63.6)	24(92.3)
Age			
60 ~ 69	7(14.6)	5(25.0)	2(7.7)
70 ~ 79	14(29.2)	5(25.0)	9(34.6)
80≤	25(52.1)	10(50.0)	15(57.6)
BMI[a]			
Under weight	1(2.1)	0(0)	1(3.8)
Normal	27(56.3)	12(60.0)	15(57.7)
Overweight≤	18(37.5)	8(40.0)	10(38.5)

Data were expressed as N (%).

*$P < 0.05$(by student t-test and x^2-test).

[a]BMI (body mass index) was calculated as body weight (kg) divided by height squared (m^2) BMI < 18.5(under weight), 18.5 ≤ BMI < 23(normal), BMI ≥ 23(overweight)

The BMI level of subjects over 50% was normal, and there was no significant difference between the two groups.

3.2 Blood Lipid Level

The relationship between serum lipids and dementia in the elderly is not clear (Lesser et al. 2001). However, it has been reported, that many risk factors can increase the risk of dementia. The risk factors include age, high total cholesterol level in midlife, cardiovascular related disease, low-density lipoprotein cholesterol (LDL cholesterol) level, high serum apolipoprotein level, and high lipoprotein levels, etc. High total cholesterol level is one of the predominent risk factor leading to dementia (Panza et al. 2006; Wakutani et al. 2002; Zarrouk et al. 2015). So, the blood lipids (total cholesterol, HDL cholesterol, LDL cholesterol, and triglyceride) were measured in this study. According to Table 2, the total cholesterol level of elderly with dementia is higher than the normal elderly, which is similar to the previous study (Zarrouk et al. 2015). Cholesterol is the main lipid ingredient of myelin and neuronal membranes. Myelin breakdown as a risk factor to dementia's disease might degrade cognitive processing speed. Abnormality of cholesterol homeostasis may have impact on maintenance of myelin (Bartzokis et al. 2007).

According to Table 2, LDL cholesterol of the elderly with dementia is higher than that of normal elderly. However, HDL cholesterol level is different from that of Bartzokis G's study where the HDL cholesterol level has no difference between elderly with dementia and normal elderly (Bartzokis et al. 2007). LDL cholesterol of 22 elderly people with dementia is significantly higher than that of 23 normal elderly people. There was no significant difference in total cholesterol and HDL cholesterol between elderly with dementia and normal elderly (Moroney et al. 1999).

Table 2 Blood lipid levels of the subjects

Blood lipid (mg/dL)	Dementia	Normal
Total cholesterol	191.7 ± 7.0***	141.4 ± 5.6
HDL cholesterol	46.0 ± 2.4**	31.8 ± 2.7
LDL cholesterol	114.1 ± 4.9**	70.1 ± 6.6
Triglyceride	140.0 ± 20.0	223.4 ± 29.4

Data were expressed as mean ± SE
***P<0.001, **P<0.01 (by student t-test and Mann-Whitney-test)

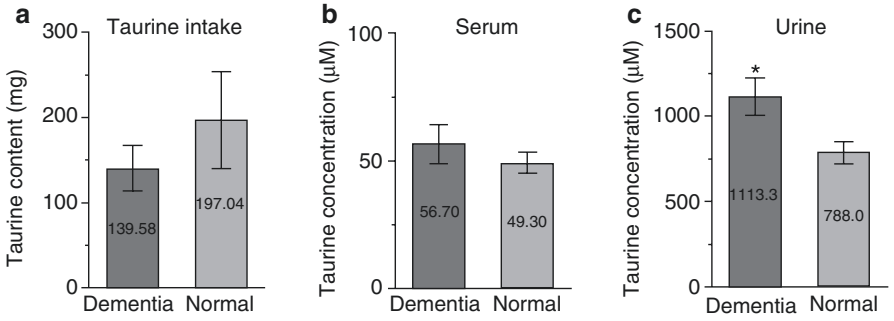

Fig. 2 Comparison between taurine contents in the dietary intake, urine, and serum. Mean values were marked in the middle of the bar. $^{*}P < 0.05$ by Student t-test and Mann-Whitney-test

3.3 Comparison Between the Elderly with Dementia and the Normal Elderly in Taurine Contents of Dietary Intake, Urine, and Serum Samples

According to Fig. 2, the normal elderly took more taurine than the elderly with dementia. The taurine concentration in serum in the elderly with dementia was 56.7 ± 7.57 μM, however, that was just 49.30 ± 4.06 μM in the normal elderly. The results were similar to those previously reported (Inoue et al. 2003; Kataoka et al. 1984; Trautwein and Hayes 1991). There was a slight difference in serum taurine level between the elderly with dementia and the normal elderly. However, urinary excretion of taurine in the elderly with dementia was approximately 40% higher than that of the normal elderly ($P < 0.05$). Healthy people have a wide range in urine taurine concentration which varies from 8 to 1500 μM (Fekkes et al. 2000; Mou et al. 2002; Qu et al. 1999). The results of taurine concentration in this study were similar to the previous study. The result of this study showed that urinary excretion of taurine in the elderly with dementia was much higher than that of the normal elderly.

3.4 Dietary Nutrients Intake Data Including Taurine

Supply of nutrients is vitally important for neuronal health of the brain (Bourre 2006). To a certain extent, nutritious food intake may prevent or delay the onset of neurodegenerative diseases such as dementia (Joseph et al. 2009). According to the following study, nutritional supplements were applied to a group of elderly with dementia living in nursing-homes and later it was found that morbidity and mortality of this group were reduced after few year follow-up (Gil et al. 2002). In this study, dietary nutrients intake was surveyed in men and women groups which took

Table 3 Dietary nutrients intake data including taurine

Nutrients (unit)	Men		Women	
	Dementia	Normal	Dementia	Normal
Energy (kcal)	2031.9 ± 154.2	1644.4 ± 40.3	1939.4 ± 113.3***	941.9 ± 72.3
Protein (g)	95.7 ± 8.5	40.2 ± 28.5	81.3 ± 5.4***	30.4 ± 2.7
Lipid (g)	58.5 ± 6.3*	16.7 ± 16.0	50.0 ± 5.1***	13.9 ± 1.4
Vitamin A (μg RE)	1065.8 ± 193.1	537.8 ± 493.4	807.0 ± 96.5**	544.5 ± 99.3
Vitamin D (μg)	4.6 ± 1.4	1.0 ± 1.0	1.8 ± 0.5	0.8 ± 0.2
Riboflavin (mg)	1.6 ± 0.2*	0.7 ± 0.4	1.2 ± 0.9***	0.6 ± 0.1
Vitamin B_6 (mg)	1.9 ± 0.2	1.1 ± 0.7	1.7 ± 0.1***	0.8 ± 0.1
Folate (μg)	672.9 ± 87.9	513.1 ± 328.4	546.3 ± 41.8***	301.6 ± 32.2
Vitamin B_{12} (μg)	10.2 ± 2.4	6.6 ± 6.0	8.3 ± 2.4	5.1 ± 1.0
Calcium (mg)	956.9 ± 102.8	409.5 ± 300.6	735.4 ± 79.5***	303.5 ± 41.5
Phosphorus (mg)	1603.9 ± 124.2	769.0 ± 570.8	1355.9 ± 98.1***	553.2 ± 48.9
Taurine (mg)	116.6 ± 39.2	467.0 ± 434.2	151.1 ± 35.7	203.9 ± 58.7

Data were expressed as mean ± SE
***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ Student t-test and Mann-Whitney-test

different amount of energy. The intakes of lipid and riboflavin of elderly men with dementia were higher than the normal elderly ($P < 0.05$). In the previous study, high fat and high cholesterol diet raised the content of β-peptide in mice's brain, and excessive β-peptide precipitation in the brain would be a risk factor to dementia (Refolo et al. 2000). In a test of 815 elderly persons as objects studied for about 4 years, it was reported that higher intakes of saturated and trans-unsaturated fats increased the risk of dementia (Morris et al. 2003). In this study, women with dementia, all nutrients except vitamin D, vitamin B_{12} and taurine were higher than those of the normal elderly women ($P < 0.01$). The taurine intake of the normal elderly was higher than that of the elderly with dementia. However, there was no statistically difference between the elderly with dementia and normal elderly in taurine intake (Table 3).

4 Conclusion

According to the results of this study, taurine excretion has a significant relationship with dementia, also, it is found that dementia has no relationship with gender, BMI level and age from 60 to 90. Although the taurine contents of dietary intake of the elderly are approximately equal, the elderly with dementia have higher urinary excretion of taurine. It is convincing that the higher urinary excretion of taurine in the elderly can be an evidence or imminent sign of dementia. This specific evaluation method based on this study might be useful in the diagnosis or in the prevention of dementia.

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Past Taurine Intake Has a Positive Effect on Present Cognitive Function in the Elderly

Mi Ae Bae, Ranran Gao, Sung Hoon Kim, and Kyung Ja Chang

Abstract This study investigated the associations between dietary history of past taurine intake and cognitive function in the elderly. Subjects of this study were 40 elderly persons with dementia (men 14, women 26) and 37 normal elderly persons (men 5, women 32). Data were collected using questionnaires by investigator-based interview to the elderly and family caregivers. We examined their general characteristics, anthropometric data, cognitive function, and taurine index. Cognitive function was measured using MMSE-DS and higher score means better cognitive function. As dietary history of past taurine intake, taurine index was evaluated by scoring the intake frequency of 41 kinds of taurine-containing foods. Part correlation analysis (sex, age, and school educational period correction) was used to analyze associations between taurine index and cognitive function. The analysis of all data was carried out by the SPSS 20.0 program for windows. The age, height, weight, and BMI of elderly with dementia showed no statistical significance compared to normal elderly. The elderly with dementia had significantly higher school education period (7.4 years) than the normal elderly (4.8 years) ($p < 0.01$). Nevertheless, the average total score of cognitive function (MMSE-DS) of the elderly with dementia (18.1 points) was significantly lower than score of the normal elderly (21.7 points) ($p < 0.05$). The average taurine index of the elderly with dementia (104.7 points) was significantly lower than average taurine index of the normal elderly (123.7 points) ($p < 0.01$). There were positive correlations between total taurine index and total score of cognitive function in all the elderly subjects ($p < 0.05$). In particular, as taurine index was higher, there were significantly higher scores of cognitive function such as 'time orientation' and 'judgement and abstract thinking' ($p < 0.01$). In conclusion, these results suggest that past taurine intake may have a positive effect on present cognitive function in the elderly.

Keywords Taurine • Dementia • Dietary history • Cognitive function • The elderly

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Abbreviations

BMI	Body Mass Index
MMSE-DS	Mini-Mental State Examination-Dementia Screening

1 Introduction

The life expectancy is increasing by economic growth, improved living levels, and developments of medical technology. Therefore, the ratio of the elderly population has increased rapidly in Korea. The elderly has various age-related chronic diseases such as diabetes, hypertension, dementia, and dyslipidemia (Kim et al. 2015). Dementia which has been one of the serious social problems is a typical chronic disease of the elderly and it is degenerative brain disorder that developed steadily over several years (Cooper 2014).

Currently there are over 67 million dementia patients in Korea and the number is increasing gradually every year (Central dementia center 2016). The cause and pathogenesis of Alzheimer's disease, accounting for 60 ~ 70% of dementia were not clearly identified (Kim and Han 2012). However, it is known fact that a protein called beta-amyloid had detrimental effect on brain cells (Braak and Braak 1991; Morris et al. 2004).

According to previous studies, there have been reported the risk factors of dementia such as age, sex, educational level, genetic factors, marital status, diabetes, and meal factors (Suh et al. 2000; Won and Kim 2003). Also the poor nutritional status of the B vitamins including folic acid was related to lower cognitive function in the elderly (Dangour et al. 2010; Duthie et al. 2002; Kim et al. 2011; Wang et al. 2001). Although many researchers are actively studying the risk factors that may cause dementia, there is no radical prevention and treatment for Alzheimer's disease yet.

Recently it was reported that taurine may recover learning and memory in dementia in animal experiments (Kim et al. 2014). Taurine is the free sulphur-containing amino acid that is distributed at high concentrations in human body (Huxtable 1992). Taurine plays a significant role in biological function such as brain function and it has been known that there are neuroprotective effects for the dementia of various types (Chen et al. 2001; Wanga et al. 2007). Although recent study related to taurine have been reported in animal experiments, it was result that supported specific association between taurine and dementia (Kim et al. 2014). However, it is not a study of people with dementia, and studies of the elderly with dementia are lacking.

Therefore, we tried to investigate the associations between taurine intake and cognitive function through present taurine intake as well as dietary history of past taurine intake in the elderly with or without dementia.

2 Methods

2.1 Subjects

In this study, the subjects were 40 elderly persons with dementia using in three dementia daytime care centers (men 14, women 26) and 37 normal elderly persons using in one welfare center (men 5, women 32) in Incheon, a Metropolitan City of Korea.

2.2 Data Collection

Data were collected using questionnaires by investigator-based interview to the elderly and family caregivers in July, 2015. We examined the general characteristics, anthropometric data, cognitive function, taurine intake, and taurine index of the subjects. In the section of general characteristics, the subjects were asked about age, school education period. For anthropometry, height and body weight measured using a stadiometer and a digital scale, respectively. Body weight divided by height squared was calculated as BMI. Cognitive function was measured using MMSE-DS. It is most widely used, easy, and simple dementia screening questionnaire in the world and higher score means better cognitive function (Han et al. 2010).

The plan of study was got approval by the Institutional Review Board (IRB) of the Inha University (150604-1A) and after receiving an explanation of the study purpose, written consent was obtained from participants.

2.3 Taurine Intake and Taurine Index

Assessment of present taurine intake was conducted through the 24-h recall method and analyzed using computer program (The Korean Nutrition Society, CAN-pro 4.0, Korea).

As dietary history of past taurine intake, taurine index was evaluated by scoring the intake frequency of 41 kinds of taurine-containing foods. The foods of 41 kinds of taurine-containing were selected with reference to taurine content in the previous study (Kim et al. 1999). Taurine was mainly contained in seafood.

The foods of 41 kinds are divided by 3 groups according to taurine content; group with high content of taurine (HTG, $\geq$ 500 mg/100 g), group with intermediate content of taurine (ITG, 100 ~ 499.9 mg/100 g), and group with low content of taurine (LTG, <100 mg/100 g), and scores of the taurine-containing food group were scored 1 ~ 3 points.

There are 17 kinds of foods in the HTG group; octopi, short arm octopus, cockle, mussel, little neck clam, blue crab, abalone, oyster, dried anchovy, dried shrimp, and taurine-containing drinks. In the ITG group, 13 foods are included; cuttlefish, octopus, mackerel, saury, spanish mackerel, flatfish, atka mackerel, frozen pollock,

anglerfish, skate, shrimp, capsosiphon, and laver. In the LTG group, 11 foods are included; boiled rice, rice with beans, multi-grain rice, pork, beef, chicken, duck, egg, green vegetables, fruits, cutlassfish, croaker, eel, seaweed, green laver, sea tangle, and dried calamari.

Frequency of each food intake was asked according to a 5-point Likert scale and it was scored 0 ~ 4 points as follows; never eat (0 point), rarely eat (1 point), 2 ~ 3 times per a month (2 points), 2 ~ 3 times per a week (3 points), everyday (4 points).

Therefore, taurine index was calculated by multiplying the score of the taurine-containing food group and the score of the taurine-containing food intake frequency, and range of taurine index were 0 ~ 12 points.

Taurine index (point) = score of the taurine-containing food group × score of the taurine-containing food intake frequency.

2.4 Statistical Analysis

We analyzed all data statistically with the SPSS 20.0 program for windows. Statistical significance was decided by Student t-test, and correlations of taurine intake, taurine index, and cognitive function were decided by part correlation analysis (sex, age, and school educational period correction). Significantly differences of results were considered at $p < 0.05$.

3 Results and Discussion

3.1 General Characteristics and Anthropometric Data

Table 1 shows general characteristics and anthropometric data of the subjects. Although we tried to conduct case-control study (sex, age, and BMI—matched), the number of the subjects was not correctly sex, age, and BMI matched due to difficulties of subject collection.

Table 1 General characteristics and anthropometric data of the subjects

	Men		Women	
Variables (units)	Dementia	Normal	Dementia	Normal
Age (year)	78.2 ± 1.8	78.2 ± 3.6	80.3 ± 1.5	79.4 ± 1.2
Height (cm)	160.7 ± 2.0	161.6 ± 2.4	147.1 ± 1.1	148.1 ± 1.5
Weight (kg)	62.1 ± 2.7	63.1 ± 1.9	52.3 ± 1.4	54.6 ± 1.5
BMI (kg/m^2)	24.0 ± 0.9	24.2 ± 0.8	24.2 ± 0.6	24.9 ± 0.6
Education period (year)	9.0 ± 0.7	7.8 ± 1.2	6.6 ± 0.8*	4.3 ± 0.7

Mean ± SE, $^*p < 0.05$

The age, height, weight, and BMI of elderly with dementia showed no statistical significance compared to normal elderly. However, according to the 6th Korea National Health and Nutrition Examination Survey (KNHNE), the average height, weight, and BMI of men ≥70 years old were 164.3 cm, 62.2 kg, and 23.0 kg/m^2 and those of women ≥70 years old were 149.6 cm, 53.6 kg, and 23.9 kg/m^2, respectively. The average height and weight of our subjects were lower or similar to those of the KNHNE, and the BMI of our subjects was higher compared to the same age group in Korea.

There was no significant difference in school education period between elderly men with dementia and normal men elderly. However, school education period of the elderly women with dementia was significantly longer than that of the normal women elderly (6.6 years vs. 4.3 years) ($p < 0.05$). Although it was not shown directly in Table 1, The elderly with dementia had significantly higher school education period (7.4 years) than the normal elderly (4.8 years) ($p < 0.01$).

Many previous studies reported association with the educational level and prevalence of dementia. According to the previous studies, the people with low educational level may have higher prevalence of dementia (Kim et al. 1998; Kim and Han 2012; Van Oijen et al. 2007), and the prevalence of Alzheimer's disease correlated with the level of education, and it was highest in the illiterate group defined as 1 year of education or less (Zhou et al. 2006).

Because the people with low educational level may have low income, medical benefits, and nutrition intakes compared to people with higher educational level, they seems to have higher prevalence of Alzheimer's disease as well as many chronic diseases.

Nevertheless, the result in this study showed a difference compared to the previous studies in which people with low educational level may have higher prevalence of dementia.

3.2 Cognitive Function

The average total score of cognitive function (MMSE-DS) of the elderly with dementia (18.1 points) was significantly lower than score of the normal elderly (21.7 points) ($p < 0.05$). In particular, scores of 'time orientation', 'memory', and 'judgement and abstract thinking' of the elderly with dementia were significantly lower than that of the normal elderly ($p < 0.01$) (Table 2).

According to previous studies related to cognitive function (evaluated by MMSE, MMSE-K, and K-MMSE), the average scores of cognitive function of the elderly with dementia living in care facilities were 9.13 ~ 15.41 points (Kim 2006, 2009; Kim and Jung 2013; Lee 2008; Yu et al. 2015). In particular, score of 'time-place orientation' was the lowest and that of 'language' was the relatively higher score (Kim and Jung 2013). The average score of cognitive function of the

Table 2 Cognitive function of the subjects

MMSE-DS (point)	Dementia	Normal
Average total	18.1 ± 0.8**	21.7 ± 0.8
Time orientation	2.1 ± 0.3**	4.0 ± 0.2
Place orientation	3.1 ± 0.3	3.6 ± 0.2
Memory	3.5 ± 0.2**	4.7 ± 0.2
Attention	2.1 ± 0.3	2.2 ± 0.3
Language	2.9 ± 0.1**	2.5 ± 0.1
Ability to execute	2.4 ± 0.1	2.5 ± 0.1
Visuospatial construction	0.6 ± 0.1*	0.3 ± 0.1
Judgement and abstract thinking	1.6 ± 0.1**	1.9 ± 0.1

Mean ± SE, $^{*}p < 0.05$, $^{**}p < 0.01$

elderly with dementia in this study was 18.1 points. It was higher score compared to previous studies. It may be considered that the subjects of previous studies have more severe dementia of the care facility than the dementia daytime care center in the study.

3.3 Taurine Intake and Taurine Index

Present taurine intake and taurine index as dietary history of past taurine intake of the subjects are shown in Table 3. There was no significant difference in present taurine intake between elderly with dementia and normal elderly. Taurine intakes of the elderly with dementia and the normal elderly in this study were 236.2 mg/day and 231.1 mg/day, respectively. Taurine intake references were not in the 2015 Dietary Reference Intakes for Koreans (KDRI) and KNHNE. However, taurine dietary intakes of two groups were higher compared with that in previous studies (85.5 ~ 216 mg) (Kim et al. 2003; Park et al. 2001; Yim et al. 2004).

The average total of taurine index of the elderly with dementia (105.3 points) showed statistical significance, being lower compared to the normal elderly (124.2 points) ($p < 0.01$). These results could have significant implications in terms that reflect the frequency of taurine intake in the past, and as taurine index was higher, there were significantly higher scores of cognitive function.

As for taurine index of each food, croaker, seaweed, sea tangle, dried calamari, atka mackerel, frozen pollock, anglerfish, shrimp, capsosiphon, cockle, mussel, little neck clam, blue crab, oyster, and dried shrimp in the elderly with dementia were significantly lower than that of normal elderly ($p < 0.05$). In particular, the intake of shellfish such as cockle, mussel, and little neck clam in the elderly with dementia was significantly lower than the normal elderly ($p < 0.01$).

Table 3 Present taurine intake and taurine index of the subjects

Variables (units)	Dementia	Normal
Taurine intake (mg/day)[a]	236.2 ± 37.5	231.1 ± 51.5
Taurine index (point)[b]		
Average total	105.3 ± 4.8**	124.2 ± 4.2
Foods included in the HTG		
Octopi	3.6 ± 0.4	3.5 ± 0.4
Short arm octopus	3.4 ± 0.4	4.1 ± 0.4
Cockle	3.5 ± 0.3**	4.8 ± 0.3
Mussel	3.5 ± 0.3***	5.3 ± 0.2
Little neck clam	3.8 ± 0.3**	5.4 ± 0.3
Blue crab	4.1 ± 0.3*	5.0 ± 0.3
Abalone	3.5 ± 0.3	4.4 ± 0.3
Oyster	3.8 ± 0.3*	4.9 ± 0.3
Dried anchovy	5.7 ± 0.4	6.4 ± 0.4
Dried shrimp	3.7 ± 0.4**	5.4 ± 0.3
Taurine-containing drink	0.3 ± 0.1	0.2 ± 0.1
Foods included in the ITG		
Cuttlefish	2.8 ± 0.3	3.0 ± 0.4
Octopus	2.0 ± 0.2	2.1 ± 0.3
Mackerel	3.1 ± 0.3	2.9 ± 0.3
Saury	2.8 ± 0.2	3.2 ± 0.2
Spanish mackerel	2.9 ± 0.2	3.3 ± 0.2
Flatfish	2.7 ± 0.2	3.2 ± 0.3
Atka mackerel	2.2 ± 0.2*	2.9 ± 0.3
Frozen pollock	2.6 ± 0.3**	3.6 ± 0.2
Anglerfish	2.2 ± 0.2*	2.8 ± 0.2
Skate	2.0 ± 0.2	2.4 ± 0.3
Shimp	2.7 ± 0.2**	3.4 ± 0.2
Capsosiphon	2.4 ± 0.1**	3.1 ± 0.2
Laver	5.0 ± 0.3	5.4 ± 0.3
Foods included in the LTG		
Boiled rice	1.7 ± 0.2	2.2 ± 0.2
Rice with beans	1.9 ± 0.1	1.8 ± 0.1
Multi-grain rice	3.3 ± 0.1	3.1 ± 0.1
Pork	1.9 ± 0.1	1.8 ± 0.1
Beef	2.1 ± 0.1	1.7 ± 0.1
Chicken	1.8 ± 0.1	1.7 ± 0.1
Duck	1.6 ± 0.1	1.6 ± 0.1
Egg	2.1 ± 0.1	2.0 ± 0.1
Green vegetables	2.4 ± 0.1	2.4 ± 0.1
Fruits	2.4 ± 0.2	2.5 ± 0.2
Cutlassfish	1.7 ± 0.1	1.8 ± 0.1
Croaker	1.7 ± 0.1*	2.0 ± 0.1

(continued)

Table 3 (continued)

Variables (units)	Dementia	Normal
Eel	1.0 ± 0.1	1.2 ± 0.1
Seaweed	1.9 ± 0.1*	2.3 ± 0.1
Green laver	1.4 ± 0.1	1.6 ± 0.1
Sea tangle	1.3 ± 0.1**	1.8 ± 0.1
Dried calamari	0.7 ± 0.1*	1.4 ± 0.1

Mean ± SE, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

[a]Present taurine intake (by 24-h recall and analysis using CAN-pro 4.0)

[b]Dietary history of past taurine intake (score of the taurine-containing food group × score of the taurine-containing food intake frequency)

According to previous study, the elderly who ate seafood (including fish) more than once a week had lower risk of developing dementia, and the elderly who do not eat fish have the risk of Alzheimer's disease 5 times higher than the elderly who eat fish more than once a day (Barberger-Gateau et al. 2002). Recently it was reported that older adults without dementia who eat one or more servings of seafood per week have less cognitive decline than those who eat less than one serving of seafood per week (Shinto 2016). According to Luchsinger and Mayeux (2004), Fish-related fat is associated with a low risk of vascular disease and it may be beneficial to prevent from cognitive decline and Alzheimer's disease. From these results, development of dementia may lower through the dietary habits of eating seafood including fish.

In addition, omega-3 fatty acids contained in fish inhibited accumulation of abnormal protein beta-amyloid in the brain and reduced the risk of dementia (Jin and Jeon 1999; Kalmijn 2000; Barberger-Gateau et al. 2007). Also there was a positive correlation between the memory and the intake of fish and shellfish (Jung et al. 2008). Therefore, it may be necessary to recommend eating fish and shellfish that contain a large amount of taurine and omega-3 fatty acids.

3.4 Part Correlations of Taurine Intake, Taurine Index, and Cognitive Function

There were no significant associations between present taurine intake and scores of cognitive function in all the elderly subjects. However, positive association was shown between taurine index which mean past taurine intake and total score of cognitive function ($p < 0.05$). In particular, as taurine index was higher, there were significantly higher scores of cognitive function such as 'time orientation' and 'judgement and abstract thinking' (Table 4) ($p < 0.01$).

Table 4 Part correlations of present taurine intake, taurine index, and cognitive function of the subjects

Cognitive function (MMSE-DS)	Taurine Intake[a]	Taurine Index[b]
	The correlation coefficient	
Total score	0.008	0.250*
Time orientation	0.017	0.376**
Place orientation	−0.020	0.195
Memory	0.109	0.216
Attention	−0.104	0.085
Language	−0.131	−0.155
Ability to execute	0.045	0.044
Visuospatial construction	0.153	−0.203
Judgement and abstract thinking	0.086	0.311**

Mean ± SE, $^{*}p < 0.05$, $^{**}p < 0.01$

[a]Present taurine intake (by 24-h recall and analysis using CAN-pro 4.0)

[b]Dietary history of past taurine intake (score of the taurine-containing food group × score of the taurine-containing food intake frequency)

4 Conclusion

These results suggest that past taurine intake may have a positive effect on present cognitive function in the elderly. Therefore, it may be necessary for prevent dementia to eat enough taurine-rich seafood or to supplement taurine as health foods continuously.

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Dietary Taurine Supplementation in School Meals Has Positive Effect on School Attitude Assessment in Korean High School Students

So Hyun Park, Su Ji Park, and Kyung Ja Chang

Abstract This study was conducted to evaluate the effect of supplementation with taurine-rich foods on school attitude assessment (SAA) in high school students. A total of 134 subjects were divided into a taurine-rich food supplemented (TS) group (68 subjects) and control group (66 subjects). For the TS group, school dinners supplemented with taurine-rich foods were provided for 5 days and average dietary amount of taurine supplementation was 466.2 mg/school dinner. Control group ate dinner at home or at restaurant *ad libitum*. The school attitude assessment survey-revised and 24-h recall method were used for SAA and dietary assessment, respectively. There were no significant differences in scores of dietary attitudes between the TS and control groups by gender. Average dietary taurine intake of the TS group (649.8 mg/day in males, 634.5 mg/day in females) was significantly higher compared to the control group (392.4 mg/day in males, 334.4 mg/day in females) ($p < 0.01$ in males and $p < 0.001$ in females, respectively). Total SAA scores in the TS group were significantly higher compared to the control group ($p < 0.01$) for attitudes toward teachers, goal valuation, and motivation/self-regulation ($p < 0.01$). Dietary taurine intake was showed positive correlations with scores for academic self-perception ($p < 0.05$), attitudes towards teachers ($p < 0.001$), goal valuation ($p < 0.01$), motivation/self-regulation ($p < 0.05$), and total scores ($p < 0.01$). According to the results, dietary taurine supplementation in school meals for 5 days had a positive effect on SAA in high school students. Therefore, dietary taurine supplementation in school and home meals may be necessary for improving SAA of high school students.

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Keywords Taurine supplementation • School meals • School attitude assessment • Nutrient intakes • High school

Abbreviations

BMI Body mass index
SAA School attitude assessment
TS Taurine-rich food supplemented

1 Introduction

It has been reported that student achievement is higher when school attitudes are positive including attitudes towards school and teachers, academic self-conception, motivation, and self-regulation (Ablard and Lipschultz 1998; Ak and Sayil 2006; d'Ailly 2003; Powers et al. 2005; Trautwein et al. 2006). Previous studies reported that low physical fatigue and psychological well-being against stress, depression, etc. have a positive effect on school attitudes (Kim and Choi 2013; Kim et al. 2006).

Taurine may play an important role in physical and psychological well-being (Stapleton et al. 1997). Dietary taurine intake is associated with physical effects such as reduction of exercise-induced fatigue and improvement of exercise performance ability in rats (Dawson et al. 2002; Yatabe et al. 2009). Dietary taurine intake also was shown to have significant effects on endurance exercise performance and serum fatigue parameters in athletes (Lee et al. 2003). Dietary taurine intake was shown to be associated with a psychological anxiolytic-like effect in mice in an elevated plus-maze (Chen et al. 2004). Further, dietary taurine intake was shown to be negatively correlated with stress frequency and total scores of life stress in Korean female college students (Sung and Chang 2009). Although there have been previous studies on the relationship between dietary taurine intake and psychological well-being in students (Park et al. 2010, 2013), there has been no study on the relationship between dietary taurine supplementation and school attitudes in students. Therefore, this study was conducted to evaluate the effect of supplementation with taurine-rich foods on school attitude assessment (SAA) in high school students.

2 Methods

2.1 Subjects and Study Design

The subjects were 134 high school students (71 male and 63 female) residing in the Incheon area. The subjects were divided into the taurine-rich food supplemented (TS) group and control group. The TS group consisted of 35 male and 33 female

high school students who ate both lunch and dinner at school. The control group consisted of 36 male and 30 female high school students who ate only school lunches. This study was carried out using a self-administered questionnaire in order to examine general characteristics, anthropometric data, dietary intake, and SAA. After supplementation of school dinners with taurine-rich foods for 5 days in the TS group, part of the questionnaire was used to assess changes in dietary intakes and SAA of the TS and control groups.

2.2 Supplementation with Taurine-Rich Foods

Major source of dietary taurine is animal protein and it was reported that taurine intake of approximately 40–400 mg per day through food intake was possible (Rana and Sanders 1986). Average taurine intake of Korean high school students was 219 ± 16.9 mg per day (Park et al. 2001). To provide the maximum daily intake of taurine in one meal for TS group, school dinners were supplemented with taurine-rich foods (Kim et al. 1999; Park et al. 1998). Taurine-rich foods used in school dinners were squid, webfoot octopus, dried shrimps, short neck clam, and mussels. As shown in Table 1, school dinner menus were provided for 5 days. The average dietary taurine amounts before and after supplementation with taurine-rich foods in school dinners were 216.7 mg and 466.2 mg, respectively.

Table 1 School dinner menu supplemented with taurine-rich foods for 5 days

Day	Conventional menus	Dishes supplemented with taurine-rich foods	Dietary taurine (mg)	
			Before[b]	After[c]
1st	Boiled rice, **Bean sprout soup**[a], Stir-fried sausage, **Stir-fried boiled fish paste**, Kimchi	Spicy seafood stew, Boiled sausage quail eggs	148.7	348.7
2nd	Multi-grain Rice, **Egg drop soup**, **Stir-fried pork**, Seasoned acorn jelly salad, Kimchi	Dried pollack with egg drop soup, Stir-fried pork with webfoot octopus	522.3	785.7
3rd	Multi-grain Rice, **Banquet noodles**, **Braised meatballs**, Seasoned spinach, Kimchi	Noodle soup with seafood, Braised fish meatballs	164.2	382.2
4th	**Vegetarian Bibimbap**, **Egg drop soup**, Fried chicken, Kimchi	Seafood fried rice, Soybean paste stew with clams	159.4	425.8
5th	Boiled rice, **Spicy chicken soup**, **Braised meatballs**, **Fruit salad**, Kimchi	Spicy chicken soup and dried shrimps, Braised fish meatballs, Caesar salad	88.7	388.7
Total			216.7	466.2

[a]Bold was changed a dish
[b]Before: Menu prepared without taurine-rich foods
[c]After: Menu prepared with taurine-rich foods

2.3 General Characteristic and Anthropometric Data

General characteristics of subjects, including age, person preparing meals, and experience of taurine-containing drink intake, were examined. The subjects' height and body weight values were presented as self-reported estimates, and BMI was calculated as the weight in kg divided by the height in meters squared (kg/m^2).

2.4 Dietary Taurine and Nutrient Intake Assessment

The 24-h recall method was used for assessment of dietary intake. Dietary taurine and nutrient intakes were estimated using the computer-aided nutrition program (CAN-pro 4.0, The Korean Nutrition Society Korea), which contains a taurine content database for 17 food groups (Kim et al. 1999; Park et al. 1998).

2.5 Measurement of SAA Scores

SAA scores were measured using SAAS-R (School Attitude Assessment Survey-Revised), which is comprised of 29 items of five sub-scales including academic self-perception (seven items), attitudes toward teachers (seven items), attitudes towards school (five items), goal valuation (four items), and motivation/self-regulation (six items). Each item response ranged from 1 (strongly disagree) to 5 (strongly agree) (Ahn and Kim 2009; McCoach and Siegle 2003b).

2.6 Statistical Analysis

Statistical analysis was performed using SPSS 20.0 for Windows. Frequency, percentage, and the mean and standard error (SE) were calculated for all variables and analyzed by paired Student *t*-test. Since pre-test scores of total and sub-scales in SAA showed significant differences between TS and control groups, pre-test scores were entered as covariate variables to perform ANCOVA. The correlation between dietary taurine intake and SAA were analyzed using Pearson's correlation coefficient.

3 Results and Discussion

3.1 General Characteristics and Anthropometric Data

General characteristics and anthropometric data of the subjects are shown in Table 2. The average age of the subjects was 17.6 years (range 17–18 years), and the person preparing meals was the mother in more than 70% of the subjects. There was no significant difference in experience of taurine-containing drink intake between TS and control groups by gender. Experience rates of taurine-containing drink intake of the TS and control groups of male were 40.0% and 44.4%, and those of female were

Table 2 General characteristic of the subjects

Variables		Male (n = 71)		Female (n = 63)	
		TS (n = 35)	Control (n = 36)	TS (n = 33)	Control (n = 30)
Age (years)		17.7 ± 0.1[a]	17.7 ± 0.1[NS]	17.5 ± 0.1	17.6 ± 0.1
Person who prepares meals	Mother	28(80.0)[b]	26(72.2)	29(87.9)	21(70.0)
	Father	2(5.7)	4(11.1)	1(3.0)	0(0.0)
	Grandmother	3(8.6)	4(11.1)	1(3.0)	2(6.7)
	Brother/sister	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	Myself	2(5.7)	2(5.6)	2(6.1)	6(20.0)
	Others	0(0.0)	0(0.0)	0(0.0)	1(3.3)
Experience of taurine-containing drinks intake	Yes	14(40.0)	16(44.4)	16(48.5)	13(43.3)
	No	21(60.0)	20(55.6)	17(51.5)	17(56.7)
Height (cm)		173.5 ± 0.9	171.9 ± 1.0	160.2 ± 0.7	163.3 ± 1.0
Body weight (kg)		66.8 ± 2.3	64.1 ± 2.3	56.1 ± 1.6	55.2 ± 1.3
BMI (kg/m^2)		22.2 ± 0.7	21.6 ± 0.7	21.2 ± 0.5	20.7 ± 0.5

[a]Mean ± SE
[b]n (%)
[NS]Not Significant

48.5% and 43.3%, respectively. Taurine-containing drink intake of the subjects was lower than that of high school students (71.3%) in Gyeoungbuk, Korea (Lee et al. 2014). In males, average height, body weight, and BMI of the TS and control groups were 173.5 cm, 66.8 kg, and 22.2 kg/m^2 and 171.9 cm, 64.1 kg, and 21.6 kg/m^2, whereas those of females were 160.2 cm, 56.1 kg, and 21.2 kg/m^2 and 163.3 cm, 55.2 kg, and 20.7 kg/m^2, respectively. According to the 2015 standard of Korean nutrient intake based on age, the average height, weight, and BMI of male and female students aged 15–18 years were 173.3 cm, 63.1 kg, and 21.0 kg/m^2 and 160.9 cm, 53.1 kg, and 20.5 kg/m^2, respectively (Ministry of Health and Welfare and The Korean Nutrition society 2015). The average height, body weight, and BMI of our subjects were similar to those of the same age group of Korean high school students by gender.

3.2 Dietary Taurine and Nutrient Intakes

The average dietary taurine and nutrient intakes are shown in Table 3. After supplementation with taurine-rich foods in school dinners, there was a significant difference in average dietary taurine intake between the TS and control groups by gender. In male subjects, taurine intake was significantly higher in the TS group (649.8 mg/day) than the control group (392.4 mg/day) ($p < 0.01$). In female subjects, average dietary taurine intake was significantly higher in the TS group (634.5 mg/day) compared to the control group (334.4 mg/day) ($p < 0.001$). Average dietary taurine intake in subjects was higher than that in high school students (219 mg/day) in Seoul, Korea (Park et al. 2001).

Table 3 Changes in nutrient intakes by supplementation with taurine-rich foods

Variables (per day)	Male (n = 71)				Female (n = 63)			
	Before		After		Before		After	
	TS	Control	TS	Control	TS	Control	TS	Control
Taurine (mg)	189.8	269.0	649.8**	392.4	167.3	242.9	634.3***	343.9
Energy (kcal)	1662.5	1658.9	2142.6	1858.4	1474.5	1444.3	1925.8	1740.8
Carbohydrate (g)	241.0	260.0	314.7	272.6	213.8	230.8	281.9	250.3
Total protein (g)	87.8*	70.2	53.2*	49.4	74.2	60.0	96.9	108.3
Total fat (g)	36.3	35.9	94.7	76.7	34.3	30.7	51.5	52.7
Vitamin A (μg RAE)	466.6	567.4	683.0	596.8	542.7	571.5	672.4	572.7
Vitamin B_1 (mg)	1.4	1.3	2.4*	1.9	1.2	1.2	2.3	2.0
Vitamin B_2 (mg)	1.5*	1.2	1.9	1.1	1.4	1.1	1.2	1.3
Vitamin B_6 (mg)	1.5*	2.1	2.3**	1.7	1.6*	2.1	2.2	1.9
Folic acid (μg)	302.9	368.6	408.6	353.2	279.5	333.6	385.7	325.1
Vitamin C (mg)	39.4*	54.9	64.8	62.1	36.0	57.3	69.4	56.8
Vitamin D (μg)	0.9***	6.4	5.8	4.1	4.1	6.6	4.8	5.4
Vitamin E (mg)	8.1	9.1	9.6	10.5	7.4	7.7	11.2	12.6
Vitamin K (mg)	94.8	89.4	113.0	104.9	72.6	74.6	117.4	96.8
Calcium (mg)	250.9*	358.2	620.4	566.1	305.6	355.0	612.3	663.8
Iron (mg)	14.6	12.7	18.6*	13.7	12.1	11.7	16.2	14.0
Sodium (mg)	2549.8	3284.1	3417.9	2968.1	2465.9	2752.3	3353.6	2916.4

*$p < 0.5$; **$p < 0.01$; ***$p < 0.001$ (different between TS and control groups by Student *t*-test)

After supplementation with taurine-rich foods in school dinners, average intakes of vitamin B_1 ($p < 0.05$), vitamin B_6 ($p < 0.01$), iron ($p < 0.05$) in male subjects in the TS group were significantly higher than those in the control group. In the study of Park et al. (2001) it showed a positive correlation between the consumption of taurine and other nutrient intakes (calorie, carbohydrate, protein, lipids, Vitamin A, B_1, C, iron, sodium). Therefore, consumption of foods with high taurine content seems to increase the intake of other nutrients.

3.3 SAA Scores

SAA scores of subjects are shown in Table 4. Average total SAA score of the TS group (107.0 points) was significantly higher compared to the control group (100.0 points) ($p < 0.01$). Especially, scores for attitudes towards teachers ($p < 0.01$), goal

Table 4 Changes in SAA scores by supplementation with taurine-rich foods

	Male (n = 71)				Female (n = 63)				Total (n = 134)			
	TS (n = 35)		Control (n = 36)		TS (n = 33)		Control (n = 30)		TS (n = 68)		Control (n = 66)	
Variables	Pre-test	Post-test	Pre-test	Post-test	Pre-test	Post-test	Pre-test	Post-test	Pre-test	Post-test	Pre-test	Post-test
Academic self-perceptions	20.7 ± 0.8 (3.0 ± 0.6)	23.9 ± 0.9*[a] (3.4 ± 0.7)[b]	18.2 ± 0.8 (2.6 ± 0.7)	20.8 ± 0.6 (3.0 ± 0.5)	19.1 ± 0.9 (2.7 ± 0.7)	21.1 ± 0.7 (3.0 ± 0.6)	19.4 ± 0.6 (2.8 ± 0.5)	21.1 ± 0.7 (3.0 ± 0.5)	19.9 ± 0.6 (2.8 ± 0.7)	22.5 ± 0.6 (3.2 ± 0.7)	18.7 ± 0.5 (2.7 ± 0.6)	20.9 ± 0.4 (3.0 ± 0.5)
Attitudes towards teachers	24.3 ± 0.6 (3.5 ± 0.5)	27.1 ± 0.7** (3.9 ± 0.6)	23.6 ± 0.6 (3.4 ± 0.5)	24.4 ± 0.6 (3.5 ± 0.5)	24.2 ± 0.7 (3.5 ± 0.5)	25.8 ± 0.7 (3.7 ± 0.6)	25.3 ± 0.8 (3.6 ± 0.6)	24.5 ± 0.9 (3.5 ± 0.7)	24.3 ± 0.5 (3.5 ± 0.5)	26.5 ± 0.5** (3.8 ± 0.6)	24.3 ± 0.5 (3.5 ± 0.6)	24.4 ± 0.5 (3.5 ± 0.6)
Attitudes towards school	17.6 ± 0.6 (3.5 ± 0.7)	18.9 ± 0.7 (3.8 ± 0.9)	17.5 ± 0.7 (3.5 ± 0.8)	17.8 ± 0.6 (3.6 ± 0.7)	18.6 ± 0.6 (3.7 ± 0.7)	18.9 ± 0.6 (3.8 ± 0.6)	18.2 ± 0.7 (3.6 ± 0.8)	17.6 ± 0.7 (3.5 ± 0.7)	18.1 ± 0.4 (3.6 ± 0.7)	18.9 ± 0.5 (3.8 ± 0.8)	17.8 ± 0.5 (3.5 ± 0.8)	17.7 ± 0.4 (3.5 ± 0.7)
Goal valuation	15.4 ± 0.4 (3.8 ± 0.7)	16.5 ± 0.5* (4.1 ± 0.7)	13.8 ± 0.6 (3.5 ± 0.9)	14.7 ± 0.4 (3.7 ± 0.6)	16.8 ± 0.4 (4.2 ± 0.6)	17.4 ± 0.4 (4.4 ± 0.6)	14.4 ± 0.6 (3.6 ± 0.8)	15.5 ± 0.5 (3.9 ± 0.7)	16.0 ± 0.3 (4.0 ± 0.7)	16.7 ± 0.3** (4.2 ± 0.6)	14.1 ± 0.4 (3.5 ± 0.8)	15.3 ± 0.3 (3.8 ± 0.7)
Motivation/ self-regulation	20.9 ± 0.7 (3.5 ± 0.7)	22.7 ± 0.6* (3.8 ± 0.7)	18.4 ± 0.8 (3.1 ± 0.8)	20.3 ± 0.6 (3.3 ± 0.6)	22.0 ± 0.7 (3.7 ± 0.7)	23.3 ± 0.5 (3.9 ± 0.5)	21.4 ± 0.6 (3.6 ± 0.6)	22.1 ± 0.7 (3.7 ± 0.7)	21.4 ± 0.5 (3.6 ± 0.7)	22.9 ± 0.4** (3.9 ± 0.6)	19.7 ± 0.5 (3.3 ± 0.7)	21.2 ± 0.4 (3.5 ± 0.6)
Total	98.9 ± 2.3 (3.4 ± 0.5)	107.6 ± 2.2** (3.8 ± 0.6)	97.5 ± 1.8 (3.2 ± 0.5)	99.3 ± 2.1 (3.4 ± 0.4)	100.7 ± 2.5 (3.5 ± 0.5)	106.5 ± 2.1 (3.7 ± 0.4)	98.6 ± 2.3 (3.4 ± 0.4)	100.8 ± 2.6 (3.5 ± 0.5)	99.8 ± 1.7 (3.4 ± 0.5)	107.0 ± 1.5** (3.7 ± 0.5)	94.7 ± 1.8 (3.3 ± 0.5)	100.0 ± 1.6 (3.4 ± 0.4)

[a]Mean ± SE = sum of sub-scales/n, *$p < 0.5$; **$p < 0.01$ (different between TS and control groups by ANCOVA)

[b](Mean ± SE) = [a]Mean ± SE/items number of sub-scales

valuation ($p < 0.01$), and motivation/self-regulation ($p < 0.01$) of the TS group were significantly higher than those of the control group. Scores for academic self-perception ($p < 0.05$), attitudes towards teachers ($p < 0.01$), goal valuation ($p < 0.05$), motivation/self-regulation ($p < 0.05$), and total SAA ($p < 0.01$) in male subjects of the TS group were significantly higher than average scores. In female subjects, however, there was no significant difference in scores for total SAA and sub-scales between the TS and control groups. It was reported that the SAA scores of middle school students were lower than those of the TS group but similar to those of the control group in our study (Kim and Ahn 2010). In the previous study on SAA between achievers and underachievers groups using SAAS-R in American high school students, achievers group showed significantly higher scores in attitudes toward teachers, motivation/self-regulation, and goal valuation compared to underachievers groups (McCoach and Siegle 2003a). These previous results were same as our result that the TS group obtained significantly higher scores in factors of SAA than those of the control group.

When adolescents have been psychologically stable, they were reported to have high academic achievement. In the results of these previous studies using SAAS-R (Castejon et al. 2016; Dedrick et al. 2015), students with high academic achievement were even higher in scores of SAAS-R. In addition, taurine has been reported neuroprotective effect (Wang et al. 2007). Therefore, the increase of taurine intake might be considered to give the psychological stability and to bring positive effects in academic achievement in the high school students.

3.4 Correlation Between Dietary Taurine Intake and SAA Scores

Table 5 shows the correlation between dietary taurine intake and SAA scores. There was a significant positive correlation between dietary taurine intake and SAA scores such as academic self-perception ($p < 0.05$), attitude toward teachers ($p < 0.05$), goal valuation ($p < 0.01$), and motivation/self-regulation ($p < 0.01$), and total scores ($p < 0.01$). There was a significant correlation between dietary taurine intake and scores for attitudes towards teachers, and total SAA in male subjects and score for goal valuation in female subjects of the TS group ($p < 0.05$).

Table 5 Correlation coefficient between dietary taurine intake and SAA score

Variables	Academic self-perception	Attitudes towards teachers	Attitudes towards school	Goal valuation	Motivation/ self-regulation	Total score
Male	0.249	0.257*	0.152	0.231	0.213	0.281*
Female	0.076	0.245	0.217	0.326*	0.197	0.272
Total	0.193*a	0.242*	0.170	0.250**	0.202*	0.275**

a*$p < 0.05$; **$p < 0.01$ (by Pearson's correlation coefficient)

4 Conclusion

Our study evaluated the effect of supplementation with taurine-rich foods on SAA in Korean high school students. In the TS group, average intakes of taurine, Fe, and vitamin B_1 in male subjects and taurine intake in female subjects over 5 days were significantly higher compared to the control group. The average scores for total SAA, academic self-perception, attitudes towards teachers, goal valuation, and motivation/self-regulation of the TS group were significantly higher compared to the control group. There was a significant correlation between dietary taurine intake and SAA scores. These results show that dietary taurine supplementation in school meals for 5 days had a positive effect on SAA in high school students. Therefore, it may be necessary to supplement school meals with taurine-rich foods for improvement of SAA in high school students. Further study should be conducted on the effect of longer supplementation with taurine-rich foods on SAA.

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Significance of Taurine in the Brain

Simo S. Oja and Pirjo Saransaari

Abstract Two main functions of taurine in the brain are here discussed: the role of taurine in cell volume regulation and the neuromodulatory actions of taurine liberated by depolarization. Taurine takes part in cell volume regulation with other small-molecular compounds. Extracellular taurine inhibits neuronal firing through GABA and glycine receptors. However, the existence of specific taurine receptors is still not excluded.

Keywords Brain • Development • Neuromodulation • Cell volume regulation Release

1 Introduction

There is a lot of taurine in the brain. The taurine concentration is particularly high in the developing rodent brain, higher than those of all other amino acids (Oja and Saransaari 2007). In some other species, e.g. in the guinea pig, its concentrations are smaller (Oja et al. 1968). The deficiency of taurine delays in kittens the normal migration of cells from the cerebellar granule cells and evokes disorganization of tapetal cell layer in the eye (Imaki et al. 1986). In human newborns, vision development is disturbed when they have been fed totally with parenteral nutrition not containing taurine (Ament et al. 1986). The concentration of taurine diminishes during the maturation of the central nervous system (Oja and Saransaari 2007). Taurine possesses at least two main functions in the brain. It participates in cell volume regulation (Pasantes-Morales and Schousboe 1997) and also attenuates electrical activity and hyperpolarizes neurons by increasing the membrane conductance of chloride ions (Saransaari and Oja 2008).

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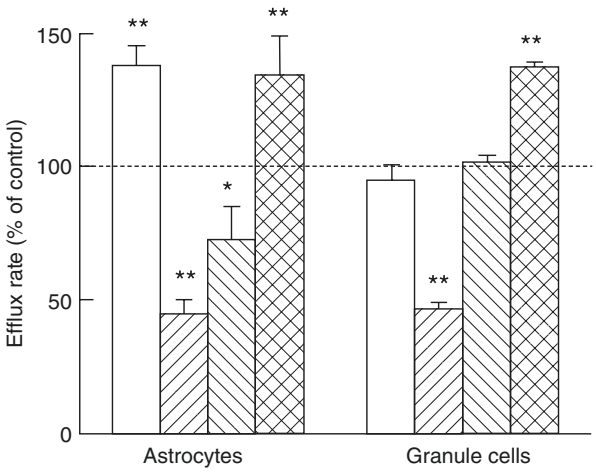

Fig. 1 Release of [^{3}H]taurine from cerebellar astrocytes and granule cells when 50 mM Na^+ in medium is replaced by equimolar K^+ (*open bar*), 50 mM K^+ is added to the medium (*right-hand striped bar*), in medium in which the ionic product 13 mM Cl^- × 50 mM K^+ is constant (*left-hand striped bar*), and when 50 mM Na^+ is omitted from the medium (*cross-striped bar*). The cells were exposed to each condition for 20 min. Mean values with SEMs of four independent experiments. Significance of differences from the control (100%) $^*P < 0.05$, $^{**}P < 0.01$

2 Cell Volume Regulation

The role of taurine in cell volume regulation in marine animals has long been known. Later, it was also shown to have an osmoregulatory role in the mammalian brain (Walz and Allen 1987), and to be released from cultured glial cells and neurons by hypoosmotic media (Pasantes-Morales and Schousboe 1988; Schousboe and Pasantes-Morales 1989). Indeed, the replacement of 50 mM sodium ions by potassium in the culture medium evokes taurine release from cerebellar astrocytes. In vivo hypoosmotic media also evoke taurine release into the extracellular spaces (Solís et al. 1988). However, in hyperosmotic media the release is blocked, even inhibited, from both astrocytes and granule cells when 50 mM potassium is added without reduction of sodium (Fig. 1). The omission of 50 mM sodium from medium without other changes significantly foments taurine release. The mechanisms of taurine partification in cell volume regulation have not yet been thoroughly characterized. They are apparently complex (Pasantes-Morales and Franco 2002). It thus has a role in cell volume regulation, together with other osmoregulatory compounds (Kimelberg et al. 1990). However, this is not its only function.

3 Taurine Transport

A depolarization concentration of potassium ions has frequently been shown to evoke taurine release from different kinds of brain preparations (Saransaari and Oja 2008). The release from brain slices in incubation media of different ionic

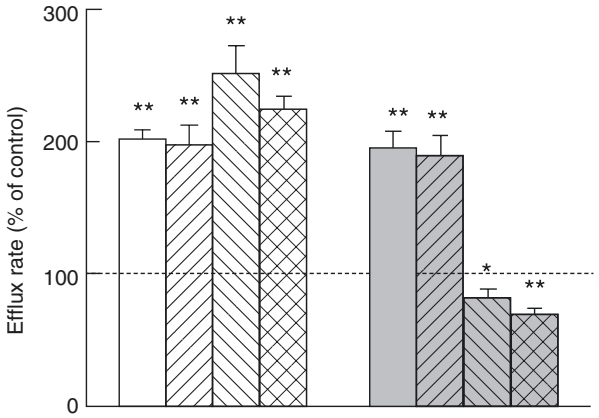

Fig. 2 Release of [^{3}H]taurine from cerebral cortex slices under different experimental conditions: when 50 mM Na^+ in medium is replaced by equimolar K^+ (*open bar*), 50 mM K^+ is added to the medium (*right-hand striped bar*), 50 mM K^+ and 50 mM sucrose are added to the medium (*left-had striped bar*), 50 mM Na^+ is omitted from the medium (*cross-striped bar*), in medium supplemented by 1.0 mM N-methyl-D-aspartate (*gray open bar*) or 1.0 mM kainate (*gray right-hand striped bar*), and in media in which 50 mM sucrose (*gray left-hand striped bar*) or 100 mM sucrose are added (*gray cross-striped bar*). The slices were superfused with each medium for 20 min. Mean values with SEMs of 5–12 independent experiments. Significance of differences from the control (100%) $^*P < 0.05$, $^{**}P < 0.01$

compositions is also fomented under conditions in which there occurs intracellular shrinking (Oja and Saransaari 1992). The evoked release is similar both when 50 mM sodium ions are replaced by equimolar potassium and when 50 mM potassium ions are simply added to the medium rendering it thus hyperosmotic (Fig. 2). The addition of 50 mM sucrose even slightly potentiates the evoked release. The omission of 50 mM sodium ions also increases taurine release in brain slices (Oja and Saransaari 1992). Hyperosmotic media supplemented by 50 mM or 100 mM sucrose inhibit the release (Fig. 2). Taurine release evoked by the agonists of ionotropic glutamate receptors, N-methyl-D-aspartate and kainate, is comparable to the release evoked by depolarizing concentration of potassium ions (Fig. 2). The evoked release of taurine is apparently somewhat complex, since it is affected by both protein kinase C and adenosine (Saransaari and Oja 2003). The N-methyl-D-aspartate-induced taurine release seems to be mediated via the nitric oxide cascade (Scheller et al. 2000). Nitric oxide stimulates the formation of cyclic guanosine monophosphate and thus enhances the release of neurotransmitters and taurine (Guevara-Guzman et al. 1994; Saransaari and Oja 2002).

The analyses of taurine influx have indicated that it comprises three components, two saturable ones, one high-affinity and the other low-affinity, together with non-saturable penetration. In brain slices in the presence of depolarizing potassium ion concentration the saturable components disappear, as well as in hypoosmotic media (Oja and Saransaari 1996). Since intracellular taurine in brain slices is first released into the extracellular spaces, the changes in influx must also modify the amount of taurine finally liberated into the incubation media.

4 Role of Extracellular Taurine

In the rat brain extracellular spaces the concentration of taurine is normally markedly lower than the intracellular concentration, being 20–25 μM or less (Jacobson and Hamberger 1985; Molchanova et al. 2004), The intra-extracellular concentration gradients have been assayed to be more than 2000 for the known neurotransmitters GABA and glutamate and less than 100 for non-neurotransmitter amino acids. The intra-extracellular concentration gradient about 400 for taurine is maintained by rather effective transport mechanisms at brain cell membranes (Oja and Saransaari 2007). It thus has an intermediate position between established neurotransmitters and non-neuroactive compounds (Lerma et al. 1986).

Taurine has been shown to displace GABA binding from the GABA-benzodiazepine receptor complex (Malminen and Kontro 1987). Taurine and GABA thus act at Cl^- channels governed by the same receptor (El Idrissi 2006). Due to the competition of taurine with GABA it inhibits GABA-induced Cl^- fluxes into synaptic membrane microsacs at low concentrations. However, at higher concentrations, apparently due to inhibitory properties of its own, taurine enhances the fluxes (Oja et al. 1990). The increase of chloride ion permeability induces hyperpolarization and reduces neuronal firing. The interactions of taurine with glycinergic transmitter systems have also been demonstrated. It displaces glycine from its strychnine-sensitive binding sites in the mouse brain stem (Kontro and Oja 1987a) and acts at glycine receptors in *Xenopus* oocytes injected with mouse brain messenger RNA (Horikoshi et al. 1988). In some cases taurine acts simultaneously at both $GABA_A$ and glycine receptors. For example, it blocks the ammonia-induced accumulation of cyclic guanosine monophosphate in the rat striatum by interacting with both GABA and glycine receptors (Hilgier et al. 2005). In certain brain preparations both taurine and glycine activate the Cl^- conductance and apparently act at the same recognition site.

Very minor modifications in cloned glycine receptors could convert them to respond to taurine with a high efficacy (Saransaari and Oja 2008). For instance, the glycine receptor α1 subunit of the zebrafish in which there is valine in position 111 instead of isoleucine has exhibited a high sensitivity for taurine (David-Watine et al. 1999). The existence of specific taurine receptors has also been speculated. They have not been verified, mainly because no strictly specific taurine antagonists are known. Taurine effects are stronger in the developing than in the adult nervous system (Kontro and Oja 1987b). Could the receptors which are affected by taurine in the developing brain be structurally different from those in the adult brain?

5 Conclusions

The importance of taurine in the brain is apparent. It contributes to the regulation of the volumes of neurons and astrocytes. It reduces the excitability of neurons and in this manner counterbalances overactivation of excitatory elements. In the developing brain taurine is particularly active. It is an essential amino acid during development.

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Neuroprotection by Taurine on HBCD-Induced Apoptosis in PC12 Cells

Yachen Li, Shuangyue Li, Xizhe Xie, Hang Xiu, Xiaohui Liu, Jing Shao, and Xiuli Zhang

Abstract Hexabromocyclododecane (HBCD) is a widely used brominated flame retardant (BFR). Because of their presence in human issues, including brain tissue, concern has been raised on their possible neurotoxicity. Presently, we explored the neuroprotection of taurine against HBCD-induced apoptotic damages in PC12 cells. Cells were pre-treated with taurine before HBCD exposure and the viability was assayed via the methyl-thiazolyl-tetrazolium (MTT) method. Apoptotic features were observed with Hoechst 33342 staining. Apoptotic ratio was measured using flow cytometry with Annexin V-FITC coupled propidium iodide (PI) double staining. The changes in the levels of Bcl-2 and Bax proteins were quantitated by the western blot. The activity of caspase-3 was tested and the results revealed that presence of HBCD decreased cell survival and led to apoptosis in the tested cells. Further, exposure of HBCD reduced protein expression of Bcl-2, increased expression in Bax protein and activity of caspase-3. Taurine attenuated HBCD-induced cell viability loss and cell apoptosis. Moreover, taurine significantly prevented from reducing Bcl-2 protein expression and elevating Bax protein expression and caspase-3 activity induced by HBCD. These results demonstrated that taurine can alleviate HBCD-induced apoptosis by altering Bcl-2 expression and Bax protein and Caspase-3 activity in PC12.

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Keywords HBCD • Taurine • Apoptosis • PC12 cell

Abbreviations

HBCD Hexabromocyclododecane

1 Introduction

HBCD is used as a flame retardant added to thermal insulation materials, electronic products, textiles and wall coverings. Because it is not chemically bound to the polymer product, HBCD can leach into the environment, contaminating indoor dust and foodstuff (Abdallah et al. 2008; Abdallah Mohamed et al. 2008; Asante et al. 2013; Barghi et al. 2016). Now, HBCD is widely used as a substitute of polybrominated diphenyl ethers, thus it has been a widespread environmental contaminant (Al-Odaini et al. 2015; Eljarrat et al. 2009) and its environmental and health problems raises concern (Aylward and Hays 2011; Covaci et al. 2006).

Toxicological studies have found that exposure to HBCD has toxic effects. The toxic effects of HBCD mainly involved in endocrine disturbances (Eggesbø et al. 2011; Kim and Oh 2014; Li et al. 2014; van der Ven et al. 2006), hepatotoxicity (Cantón et al. 2008; Germer et al. 2006), reproductive developmental toxicity (Ema et al. 2008), immunotoxicity (Almughamsi and Whalen 2016; Anisuzzaman and Whalen 2016; Hachisuka et al. 2010) and neurotoxicity (Lilienthal et al. 2009; Saegusa et al. 2012). Apoptosis had been found play a significant role in HBCD-mediated toxic effects. An et al. (2014) investigated the toxic effect of HBCD on HepG2 cells and found that HBCD led to elevated cell apoptosis ratio. Deng et al. (2009) also shown that HBCD could induce significant apoptosis in zebrafish embryos. Our previous study also found that HBCD induced apoptosis in PC12 cells. However, few studies have focused on how to take precautions against the harm of HBCD.

Taurine is a sulphur-containing non protein amino acid involved in a wide range of physiological processes (Lambert et al. 2015). Studies have reported that taurine exerts neuroprotective effects against various disorders and injuries (Akande et al. 2014; Alkholifi and Albers 2015; Kearns and Dawson 2000; Khan et al. 2000; Zhu et al. 2016). Recent studies showed that taurine involved in apoptosis regulation (Gao et al. 2011; Taranukhin et al. 2010). Whether taurine has protective effects on HBCD-induced apoptosis?

In this report, we explored the protective effects of taurine against apoptosis elicted by HBCD and the potential molecular mechanisms in PC12 cells.

2 Methods

2.1 Cell Culture

PC12 cells were aquired through the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and grown in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator at 37 °C with 5% CO_2.

2.2 Cell Viability

PC12 cells were seeded for 24 h in 96-well plate (1×10^4 cells/well), and then treated with different concentrations of taurine (1 mM, 3 mM and 9 mM) for 30 min before treated with 10 μM HBCD for 24 h (Sigma-Aldrich, St. Louis, MO). The viability was assayed by the MTT method. Briefly, 10 μL MTT (diluted in PBS at 5 mg MTT/mL) was aliquoted to each sample for 3 h at 37 °C. The media were discarded carefully and insoluble formazan components were dissolved in 100 μL DMSO. The absorbance (570 nm) was taken on an ELISA plate reader. The viability was presented as the percentage relative to the value in control culture.

2.3 Hoechst 33342 Staining

PC12 cells were cultured for 24 h in 24-well plates, and preexposed to 0, 1, 3 and 9 mM taurine and 10 μM HBCD as described above in Sect. 2.2. After treatment, cells were applied with 10 μg/mL of Hochest 33342 in PBS (Sigma, St. Louis, MO) and incubated in dark for 20 min followed by two PBS washes. The nuclear morphology was scrutinized with a converted fluorescent microscope (Olympus, Japan).

2.4 Apoptosis and Necrosis by Flow Cytometry

PC12 cells were evenly planted in six-well plates (5×10^5 cells/well) and treated with 0, 1, 3 and 9 mM taurine and 10 μM HBCD as described above in Sect. 2.2. The apoptotic cells and those undergoing necrosis were checked via staining by Annexin V coupled with propidium iodide (PI) by the Annexin V-FITC/PI cell apoptosis detection kit (Beyotime, China). Briefly, the cells were trypsinized and

then gathered by centrifuging at 1000 rpm of 5 min. Following 2 washes with PBS, cells were then resuspended in binding buffer and kept in dark for 10 min in the presence of annexin V-FITC + PI. Fluorescence analysis was performed by the BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

2.5 Western Blotting

PC12 cells were grown in six-well plates (5×10^5 cells/well) and exposed to 0, 1, 3 and 9 mM taurine and 10 μM HBCD as described above in Sect. 2.2. After treatment, PC12 cells were harvested and homogenized in the RIPA Tissue Protein Extraction Reagent of ice-cold (Beyotime, China) adding 1% proteinase inhibitor, then incubated for 1 h at 4 °C. The homogenate was centrifugated at 4 °C at 12,000 g for 15 min and then the supernatant was kept under −80 °C. The protein level of in the supernatant was assayed by the BCA method (Beyotime Institute of Biotechnology, China). An aliquote of 50 μg protein was resolved on the 10% SDS-PAGE Gels and transferred onto polyvinylidence fluoride (PVDF) membrane (Millipore, France) and subsequently immunoblotted with antibodies of rabbit against rat Bcl-2 and polyclonal Bax antibody (1:500) (Cell Signaling, USA). The membrane was then immunodetected using the enhanced ECL chemoluminescence kit (Cell Signaling Technology, USA). The densitometric data of protein bands were quantified using the UVP BioSpectrum Multispectral Imaging System (Ultra-Violet Products Ltd. Upland, CA, USA).

2.6 Caspase-3 Activity

The activity of caspase-3 was measured with the commercial Assay Kit (Beyotime, China). Briefly, PC12 cells were planted at 1×10^6 cells per ml. After treatment as described above in Sect. 2.2, cells were harvested and lyses, and the lysates were mixed with both Ac-DEVD-pNA, the caspase-3 substrate, and reaction buffer, followed by 2 h incubation at 37 °C. The variations in absorbance (405 nm) was taken on an ELISA reader. The activities of caspase-3 were presented as a ratio of treatment group and control group.

2.7 Statistic Analysis

All our data were shown as mean ± S.D. based on six independent measurements, with each conducted in 3 replicates. Statistical analysis was performed by the Student's t-test and one-way ANOVA. The p value of <0.05 was determined statistically significant.

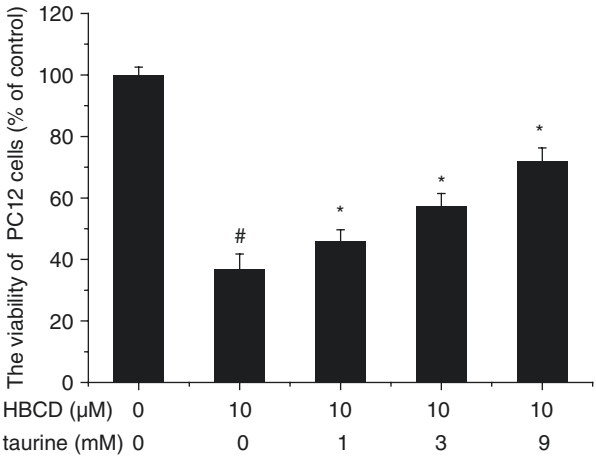

Fig. 1 The protective potential of taurine against HBCD on the PC12 viability. PC12 cells were treated with taurine for 30 min (0, 1, 3, and 9 mM) before 24 h exposure to 10 μM HBCD. The viability of PC12 was then measured with the MTT assay. Data were displayed as mean ± S.D. using the results averaged from three replicate investigations, $^{*}p < 0.05$ (of the control), $^{**}p < 0.05$ (of the HBCD exposure alone)

3 Results

3.1 Effect of Taurine on the Viability in PC12 Cells

As displayed in Fig. 1, PC12 were treated with 10 μM HBCD for 24 h, and rate of cell survival was about 36.84 ± 4.91%. Pretreated with 1, 3, 9 mM taurine before exposure to HBCD, the survival of PC12 cells was significant increased to 46.11 ± 3.56%, 57.46 ± 3.98% and 72.02 ± 4.27% in a dose-dependency, respectively.

3.2 Effect of Taurine on HBCD-Elicited Apoptosis

The potential of taurine against HBCD-stimulated apoptosis was tested by the Hoechst staining (33342) together with Annexin-FITC + PI double staining flow cytometry. According to Fig. 2, 24 h exposure of PC12 cells to 10 μM HBCD showed a classic characteristic of apoptosis, condensed chromatin and bright blue nuclei or even fragmented nucle, while the nuclei with signs of apoptosis were not found in the control. The cells containing condensed nuclei was significantly decreased in the dose-dependency, when PC12 cells treated with 0, 1, 3, and 9 mM taurine before HBCD exposure.

PC12 cells stained with FITC-conjugated annexin V + PI were detected with flow cytometric analysis. The data showed that the most of control cells negatively stained both annexin V-FITC coupled with PI (Fig. 3). The proportion of cells positively stained with annexin V significantly increased after 24 h exposure of PC12 cells to HBCD, however, cells engaged in apoptotic process were lowered upon

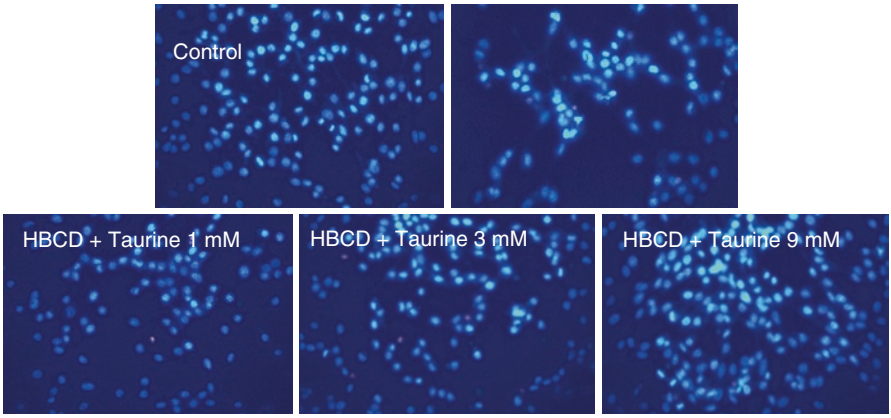

Fig. 2 Effects of taurine on nuclei morphological change of PC12 cells exposed to HBCD. The PC12 were treated with taurine for 30 min at 0, 1, 3, and 9 mM before exposure to 10 μM HBCD for 24 h. Cellular nuclei morphology was observed under fluorescence microscope after Hoechst 33342 staining (400×)

preexposure to taurine. These results present evidence that taurine can prevent PC12 cells from HBCD-induced apoptosis.

3.3 Effect of Taurine on the Proteins of Bcl-2 and Bax

The protective effect of taurine on the proteins of Bcl-2 and Bax were examined by western blotting in PC12 cells. Fig. 4a revealed that compare with the control group the protein expression in Bcl-2 was considerably reduced and the level of Bax protein was remarkably increased (Fig. 4b) in 10 μM HBCD exposure group. PC12 cells pretreated with 1, 3, and 9 mM taurine, however, a significant reduction in the protein level of Bcl-2 (Fig. 4a) and an elevation in the protein expression of Bax (Fig. 4b) were prevented in does-dependent manner. These data strongly suggest that taurine can regulate HBCD-induced apoptosis by affecting the apoptotic components of the Bcl-2 family of proteins.

3.4 Effect of Taurine on Caspase-3 Activity

Since caspase-3 is the ultimate executor of apoptosis, we explored the effects of pretreatment with taurine on caspase-3 activity in HBCD-treated PC12 cells. Fig. 5 showed that the elevation in caspase-3 activity was observed in PC12 cells when

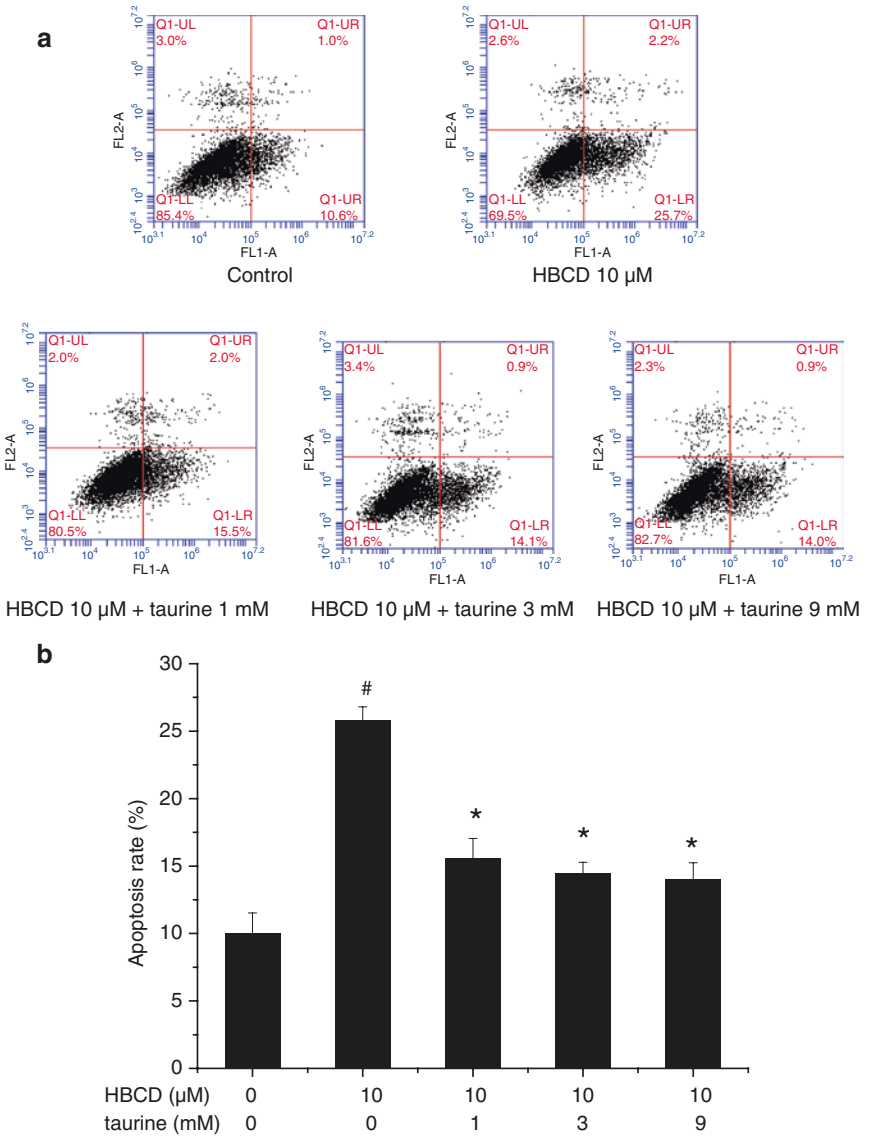

Fig. 3 Evaluation on protection of taurine against HBCD-mediated apoptosis in PC12 exposed to HBCD. Cells were pre-exposed to taurine for 30 min at 0, 1, 3, and 9 mM before exposure to 10 μM HBCD for 24 h, the percentage of apoptotic population vs. cells undergoing necrosis was examined with flow cytometry by annexin V + PI double staining. (**a**) was representative for three independent experiments. Column graphs showed the rate of apoptotic cells (**b**). Data were presented as mean ± S.D. calculated from three separately conducted assays, $^{*}p < 0.05$ (over the control) and $^{**}p < 0.05$ (over the HBCD exposure alone)

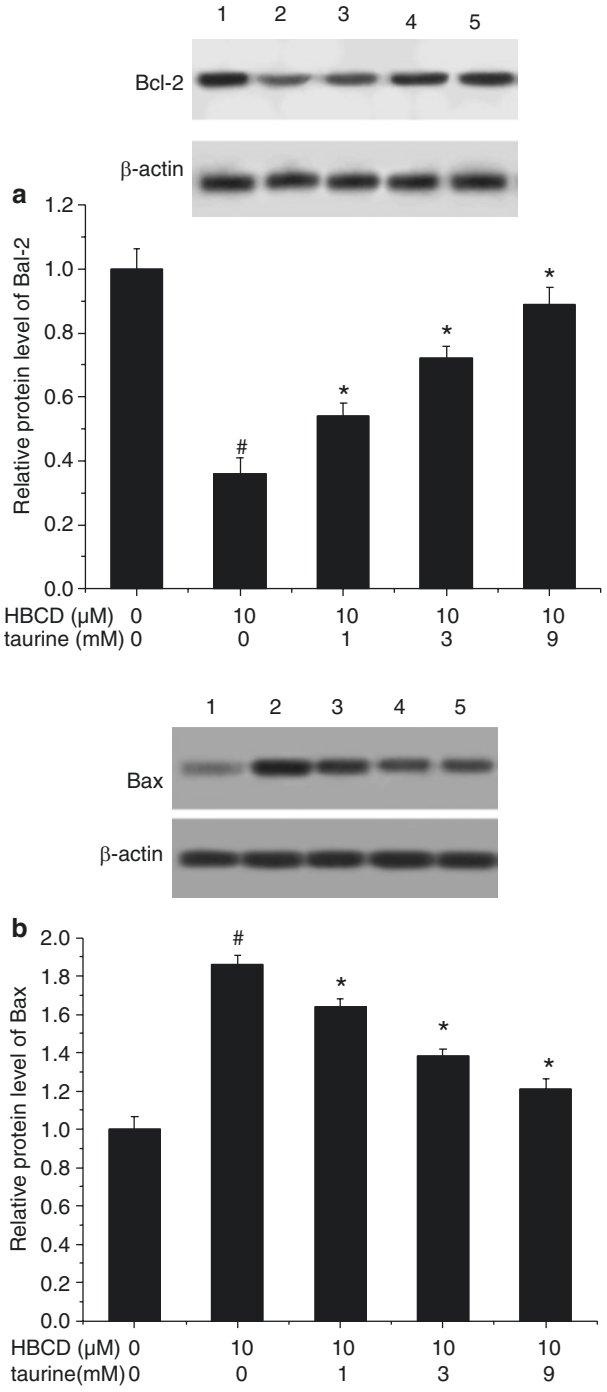

Fig. 4 Assessment of taurine on protein expression of Bcl-2 and Bax in PC12 exposed to HBCD. Cells were not treated (lane 1) or treated with taurine for 30 min at levels of 0, 1, 3, and 9 mM before exposure to 10 μM HBCD for 24 h (lane 2,3,4,5). And the expression of Bcl-2 and Bax proteins were measured using western blotting. Data were presented as mean ± S.D. calculated from three independent testings, $^{*}p < 0.05$ (over control) and $^{**}p < 0.05$ (over HBCD exposure alone)

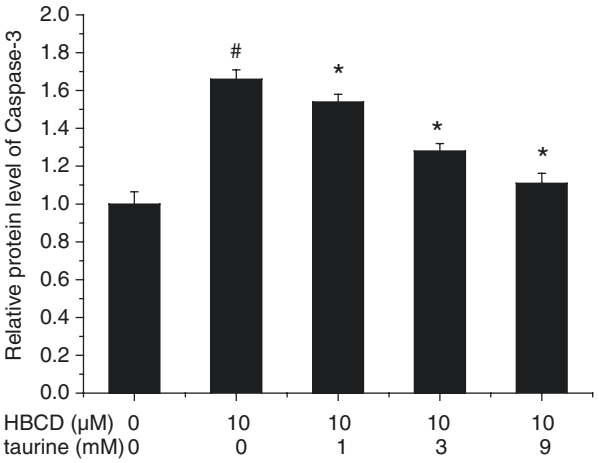

Fig. 5 Effects of taurine on caspase-3 activity in PC12 cells exposed to HBCD. Cells were preexposed to taurine (0, 1, 3, and 9 mM) for 30 min before exposure to 10 μM HBCD for 24 h. The caspase-3 activation was analyzed using the commercial kit. Data were presented as mean ± S.D. from three independently performed tests, $^{*}p < 0.05$ relative to the control and $^{**}p < 0.05$ as compared to HBCD exposure alone

exposed to HBCD, in comparison with the untreated control, while cells pretreated with taurine before HBCD exposure inhibited significantly increase in caspase-3 functionality in comparison with the HBCD-treated group.

4 Discussion

In this current research, the protective potential of taurine for PC12 cells from HBCD-induced apoptosis was investigated. Our experimental data demonstrate that HBCD can induce apoptosis in PC12 cells, pretreatment with taurine significantly inhibited HBCD-induced apoptosis in a dose-dependent manner.

There have been two proposed apoptotic pathways: the death receptor-mediated apoptosis (also called extrinsic pathway), and the mitochondria-mediated apoptosis (also known as intrinsic pathway or mitochondrial pathways) (Boatright and Salvesen 2003; Hotchkiss et al. 2009). The proteins in Bcl-2 family are the important regulators of the mitochondria-mediated apoptosis, which are involved in the positive and/or negative regulations of apoptosis (Gonzalez et al. 2008) by altering mitochondrial membrane permeability (Chipuk et al. 2006). Among the family members, Bax and Bid are pro-apoptotic members, which increase mitochondrial membrane permeability, leading to the cytochrome c leakage that mediates the stimulation in caspase cascade and a resultant apoptotic event. Bcl-2 and Bcl-XL proteins are the components against apoptosis, which functions to block the increase in membrane permiability of mitochondria and inhibit the apoptotic event evoked by a variety of chemical stimulations (McDonnell et al. 1996; Miller et al. 1997). Another kind of important cell apoptosis regula-

tors is a family of highly conserved, cystein-dependent aspartate-directed proteases called caspases.

Among them, Caspase-3 takes an essential function in regulating the process of apoptosis (Green and Kroemer 2004). As the ultimate executor in apoptotic death it is activated many protein including bcl-2 family proteins (Brauchi et al. 2006; Lakhani et al. 2006). Deng et al. (2009) demonstrated that HBCD exposure led to apoptotic process in embryo cells by supressing Bcl-2 gene expression and uprising the activity of caspase-3 in zebrafish embryos. Al-Mousa and Michelangeli (2012) also found that HBCD induced cell apoptotic death via the activation of caspases in SH-SY5Y cells. In accordance with their studies, our results also presented that HBCD induced PC12 cells apoptotic death was associated with reduced expression in Bcl-2 protein and lifted expression in Bax protein, and caspase-3 activation. Moreover, taurine was significantly effective to prevent the reduction in the level of Bcl-2 protein and the elevation in the level of Bax protein, and to suppress the activation of caspase-3.

5 Conclusion

In conclusion, the present studies show that HBCDs could significantly induce apoptosis in PC12 cells, taurine render protection against HBCDs-induced apoptosis via suppressing the caspase-3 activation, and preventing Bcl-2 protein decrease and Bax protein increase induced by HBCD. Taken together with our previous study results that taurine blocked the ROS generation and MMP collapse induced by HBCDs, suggest that taurine attenuates HBCDs-induced apoptosis in PC12 cells through prevent ROS overproduction, drop in MMP and the consequent triggering of apoptosis genes.

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Taurine Alleviate Hexabromocyclododecane-Induced Cytotoxicity in PC12 Cells via Inhibiting Oxidative Stress

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Abstract Hexabromocyclododecane (HBCD) is a widely used brominated flame retardant. Its adverse effects on brain had been observed. Taurine, a sulfur amino acid, take part in many brain physiological functions and exhibits protective effects on a variety of detrimental situations. In this paper, we explored the protections of taurine on cytotoxicity induced by HBCD in PC12 cells. PC12 cells were pretreated with taurine (1 mM, 3 mM and 9 mM) for 30 min before 10 μM HBCD treatment for 24 h. Then, the cell survival was assayed by the lactate dehydrogenase (LDH) release and trypan blue dyeing method. The formation of reactive oxygen species (ROS) and a collapse of mitochondrial membrane potential (MMP) were evaluated with a fluorescence microplate reader using the non-fluorescent probe 2′7′-dichlorofluorescin diacetate (DCFH-DA) and the fluorescent cationic dyestuff Rhodamine 123 (Rh 123), respectively. Further, the activity of many antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and the content of glutathione (GSH) were tested by kits. Our results displayed that taurine significantly decreased the cell death induced by HBCD, prevented ROS production and disruption of mitochondrial membrane potential, and reversed the decline of SOD, CAT, GPx activity and GSH content induced by HBCD. These results suggested that taurine could alleviate cytotoxicity induced by HBCD in PC12 cells through inhibition of oxidative stress.

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Keywords HBCD • Taurine • Cytotoxicity • Oxidative stress

Abbreviations

CAT	Catalase
DCFH-DA	2′7′- dichlorofluorescin diacetate
GPx	Glutathione peroxidase
GSH	Glutathione
HBCD	Hexabromocyclododecane
LDH	Lactate dehydrogenase
MMP	Mitochondrial membrane potential
Rh 123	Rhodamine 123
ROS	Reactive oxygen species
SOD	Superoxide dismutase

1 Introduction

Hexabromocyclododecane (HBCD: consisting of α-, β- and γ-HBCD isomers), widely used in electrical products, building materials and polystyrene foams, has become a ubiquitous organic contaminant detected in different species (de Wit 2002). Studied had found that lake sediments, marine mammals, sea bird eggs and even human adipose tissue, breast milk and blood had HBCD accumulation (Kohler et al. 2008; Law et al. 2006; Sellström et al. 2003; Pulkrabová et al. 2009; Eljarrat et al. 2009; Kakimoto et al. 2008). Due to the increased bioaccumulation of HBCD, researchers has paid much attention to its effects on the biotic environment and human health (Birnbaum and Bergman 2010; Roosens et al. 2010).

Some toxicological studies found that HBCD could lead neuronal cell to death because of its special neurotoxicity (Al-Mousa and Michelangeli 2012). HBCD had also been verified to be harmful to brain development, resulting in abnormal behavior and defective learning and memory (Williams and Desesso 2010). Studies by Eriksson et al. (2006) reported that neonatal exposure to HBCD had a lasting distortion of spontaneous behavior and cognitive function of adult mice. Mariussen and Fonnum (2003) had also shown that HBCDs prevented dopamine uptake from rat synaptic vesicles and synaptosomes. Reistad et al. (2006) found that cerebellar granule cells had died when exposed to HBCD for 24 h. Additionally, studied had observed that HBCD induced oxidative stress and destroyed antioxidant defense systems in the cell of fish brain (Zhang et al. 2008a). However, there are still few studies focusing on the elimination of HBCDs' harm.

Taurine (2-aminoethanesulfonic acid) is one of the major amino acids in our human body. It has spread in brain tissue, muscle, heart and blood plasma (Lourenco and Camilo 2002). It takes part in many physiological functions including cell protection, neuronal development, intracellular calcium regulation, lipid metabolism and osmoregulation (Huxtable 1992; Bouckenooghe et al. 2006). Recently, some studies have proved that taurine has neuroprotective effects in different damage model (Zhou et al. 2011; Tadros et al. 2005; Das et al. 2009; Das and Sil 2012). So whether taurine has protective effects on cytotoxicity induced by HBCD?

Our previous study found that HBCD can cause cytotoxicity and oxidative stress in PC12 cells. Our study aimed to investigate the protective effects of taurine on PC12 cells cytotoxicity induced by HBCD and its possible mechanisms.

2 Methods

2.1 Cell Culture

PC12 cells were aquired through the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and grown in DMEM including 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin in an incubator at 37 °C with 5% CO_2.

2.2 Cytotoxicity Assay

PC12 cells were cultivated for 24 h in 96-well plate (1×10^4 cells/well), and then incubated with different concentrations of taurine (1 mM, 3 mM and 9 mM) for 30 min before 10 μM HBCD exposure for 24 h (Sigma-Aldrich, St. Louis, MO). Cells death was tested by trypan blue exclusion analysis and LDH release analysis using LDH cytotoxicity detection kit (Beyotime Institute of Biotechnology, China).

Cell death rate (%) = (LDH release in Experimental group—LDH release in Control group)/(Maximum LDH release- LDH release in Control group).

2.3 Trypan Blue Dyeing

PC12 cells were treated as described above in Sect. 2.2, cell death and cellular morphology was scrutinized with a phase-contrast microscope after trypan blue staining.

2.4 Measurement of ROS

PC12 cells were seeded for 24 h in 96-well plate (1×10^4 cells/well), and then treated with different dosages of taurine (1 mM, 3 mM and 9 mM) for 30 min before 10 μM HBCD exposure for 24 h. ROS were measured by ROS assay kit with fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Beyotime Institute of Biotechnology, China). The cellular fluorescence (excitation and emission wavelength at 485 nm and 538 nm, respectively) was taken on a fluorescence microplate reader (Genios, TECAN). ROS generation was presented as the percentage relative to the value in control culture.

2.5 Measurement of MMP

Rhodamine 123 (Rh 123) was employed to estimate MMP as previous describe by Zamzami et al. (Zamzami et al. 2001). Briefly, PC12 cells were raised for 24 h in 24-well plate (5×10^4 cells/well), and then treated with different dosages of taurine (1 mM, 3 mM and 9 mM) for 30 min before 10 μM HBCD exposure for 24 h. Then, cells were washed with PBS 2 times after incubated with 10 mM Rh 123 for 30 min at 37 °C. A fluorescence microplate reader (Genios, TECAN) was used to evaluate the cellular Rh 123 fluorescence intensity (excitation wavelength of 485 nm and an emission wavelength of 530 nm). MMP was presented as the percentage relative to the value in control culture.

2.6 Measurement of SOD, CAT, GPx Activities and GSH Content

PC12 cells were grown in six-well plates (5 × 105 cells/well) and exposed to 0, 1, 3 and 9 mM taurine and 10 μM HBCD as described above in Sect. 2.2. Then, PC12 cells were harvested and homogenized. The homogenate was centrifugated at 4 °C at 10,000 g for 10 min and then the supernatant was collected for SOD, CAT, GPx activities and GSH content test by assay kit. Bovine serum albumin was employed as standard and coomasse blue protein-binding method was used for measurement protein content.

2.7 Statistic Analysis

All our data were shown as mean ± S.D. based on six independent measurements, with each conducted in 3 replicates. Statistical analysis was performed by the Student's t-test and one-way ANOVA. The p value of <0.05 was determined statistically significant.

3 Results

3.1 Effects of Taurine on the Survival of PC12 Cells Exposed to HBCD

Exposure to 10 μM HBCD for 24 h, the cell death rate was 66.23 ± 4.13% comparing with the control group. Pretreatment with 1, 3 and 9 mM taurine significantly decreased cell death rate to 52.58 ± 3.57%, 38.15 ± 3.21%, and 18.46 ± 4.29%, respectively (Fig. 1a). Similar results were observed by trypan blue dyeing method (Fig. 1b).

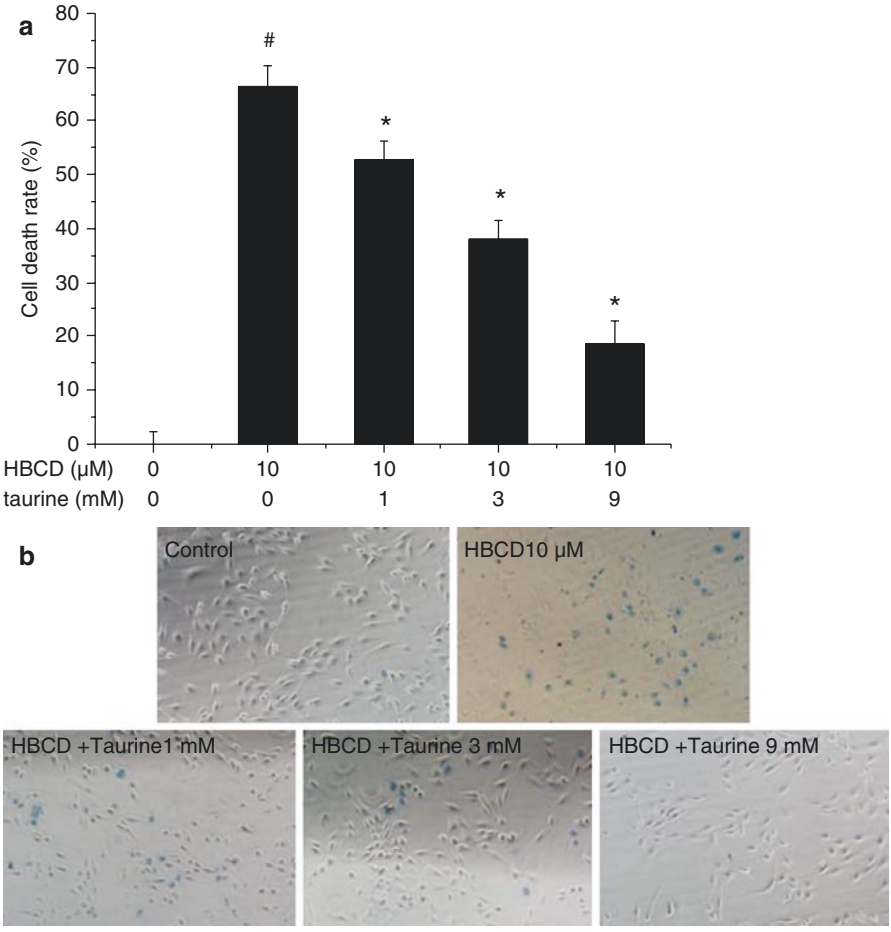

Fig. 1 The protective potential of taurine against HBCD on PC12 cell survival. PC12 cells were exposed to 0, 1, 3, and 9 mM of taurine for 30 min before 24 h treated with10 μM HBCD. Cells death was determined by LDH released assay (**a**) and observed with light microscope after trypan blue dyeing (×64) (**b**). Data were displayed as mean ± S.D. using the results averaged from three replicate investigations, $^{*}p < 0.05$ (of the control), $^{**}p < 0.05$ (of the HBCD exposure alone)

Morphological observation also demonstrated the protection of taurine against the effect of HBCD on PC12 cell survival. Visual inspection showed that normal PC12 cells were round to ovoid and having long neurite. Exposure to 10 μM HBCD, cell body shrink and lose in neurite. However, pretreatment with taurine dramatically prevented cell morphological deterioration induced by HBCD (Fig. 1b).

3.2 *Effects of Taurine on MMP and ROS Generation in PC12 Cells Exposed to HBCD*

Mitochondria play vital in normal cell function as well as the regulation of cell death. Mitochondrial depolarization that is MMP decrease could elevate mitochondrial ROS formation. To explore the impact of taurine on HBCD-induced collapse in PC12 cells MMP, the fluorescent cationic dyestuff Rh 123 was employed to test changes in MMP. HBCD- caused a collapse in MMP was inhibited by pretreatment with taurine (Fig. 2a). In addition, PC12 cells exposure to HBCD markedly raised

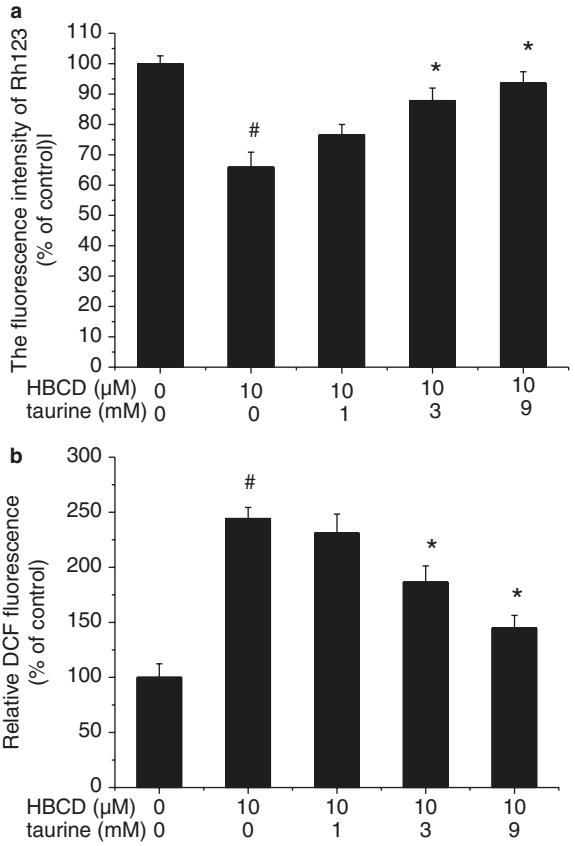

Fig. 2 Effects of taurine on MMP collapse and ROS generation in PC12 cells exposed HBCD. PC12 cells were exposed to taurine for 30 min (0, 1, 3, and 9 mM) before 24 h treated with 10 μM HBCD. Mitochondrial membrane potentials (**a**) and ROS (**b**) are displayed as percentage of DCF and Rh123 fluorescence intensity figure of control culture, respectively. Data were displayed as mean ± S.D. using the results averaged from three replicate investigations, $^{*}p < 0.05$ (of the control), $^{**}p < 0.05$ (of the HBCD exposure alone)

the formation of ROS to 244.21 ± 12.36% of control. Taurine was able to inhibite ROS formation induced by HBCD and the levels of ROS decreased to 231.05 ± 10.19%, 186.53 ± 17.31% and 144.92 ± 14.75% of control at 1, 3, and 9 mM of taurine, respectively (Fig. 2b).

3.3 Effects of Taurine on SOD, CAT, GPx Activities and GSH Content in PC12 Cells Exposed to HBCD

Antioxidant enzymes SOD, CAT, GPx and low-molecular weight antioxidant GSH play a significant protection on cell damage induced by oxidative stress in mammalian cells. In the present study, the influence of taurine on the activities of SOD, CAT, GPx and the content of GSH were evaluated in HBCD-induced PC12 cells. Data shown in Fig. 3 demonstrated that the activities of SOD, CAT and GPx were 12.22 ± 0.63 U/mg protein, 21.16 ± 1.25 U/mg protein and 60.18 ± 2.62 U/mg protein in the control group, respectively. The average GSH content was 11.32 ± 0.68 μmol/mg protein. After HBCD exposure, the cellular antioxidant system was disturbed and led to decrease the activities of SOD, CAT, GPx and the content of GSH to 6.30 ± 0.47 U/mg protein, 8.41 ± 1.17 U/mg protein, 36.25 ± 3.31 U/mg protein and 5.91 ± 0.46 μmol/mg protein (Fig.3a–d). Taurine significantly preserved the activities of SOD, CAT and GPx (Fig. 3a–c), and impeded the loss of GSH (Fig. 3d) induced by HBCD.

4 Discussion

In HBCD-induced cytotoxicity, oxidative stress has been recognized as a vital mechanism. Studies found that ROS production was positively in correlation to the release of LDH in HepG2 cytotoxicity induced by HBCD enantiomers (Zhang et al. 2008a, b). HBCD had also led to oxidative stress in zebrafish embryos (Deng et al. 2009). Due to biological function of taurine to regulate oxidative stress (Gurer et al. 2001; Aruoma et al. 1988), we deduced that taurine might prevent cells from cytotoxic damage mediated by HBCD via inhibiting oxidative stress. In our experiment, we first observed that taurine had reduced PC12 cells damage induced by HBCD by detecting LDH release and trypan blue staining method. ROS generation was assayed and the result showed that taurine may attenuate ROS production induced by HBCD. This result is similar to previous study by Xu et al. (2015).

Mitochondria play an important role on normalizing cell function and regulating cell death and intracellular ROS are primarily produced by mitochondria. Mitochondrial dysfunction can increase ROS production, reduce ATP production, release death regulatory and signaling molecules, leading to cell death

(Christophe and Nicolas 2006). MMP is one of the important parameters reflecting the function of mitochondria. Our research showed that HBCD caused a significant reduction of MMP, concomitant with an increase in ROS. Similar result has been obtained in Jurkat cells of exposure to PBDE-47 (2,2′,4,4′-Tetrabromodiphenyl ether), a brominated flame retardant (Yan et al. 2011). On the contrary, taurine could mediate recovery of the MMP of PC12 cells in our study.

Cells have own defense system to protect them from oxidative damage. SOD, CAT, GPx are antioxidant enzymes and GSH is one kind of antioxidant. They altogether used to scavenge ROS (Halliwell and Gutteridge 1990; Ibi et al. 1999). Sochor et al. (2014) investigated the influence of taurine on antioxidans status in Wistar rat found that SOD, GPx activities and GSH level increased with the increasing taurine supplementation. In the present study, our results displayed

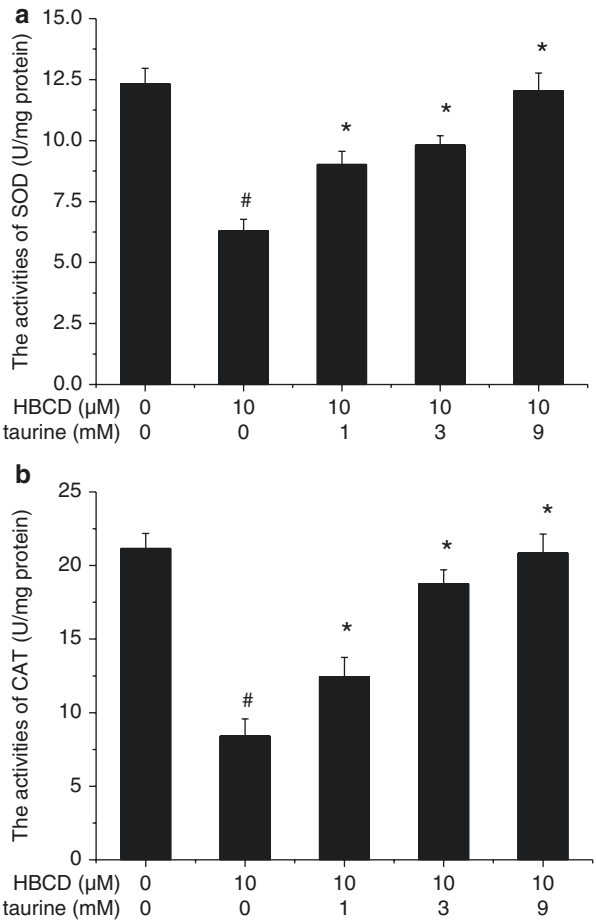

Fig. 3 Effects of taurine on the activities of SOD, CAT, GPx and the content of GSH in PC12 cells exposed to HBCD. PC12 cells were pretreated with taurine for 30 min (0, 1, 3, and 9 mM) before 24 h exposure to 10 μM HBCD. SOD, CAT, GPx activities (**a**-**c**) and GSH content (**d**) were tested by assay kit. Data were displayed as mean ± S.D. using the results averaged from three replicate investigations, $^{*}p < 0.05$ (of the control), $^{**}p < 0.05$ (of the HBCD exposure alone)

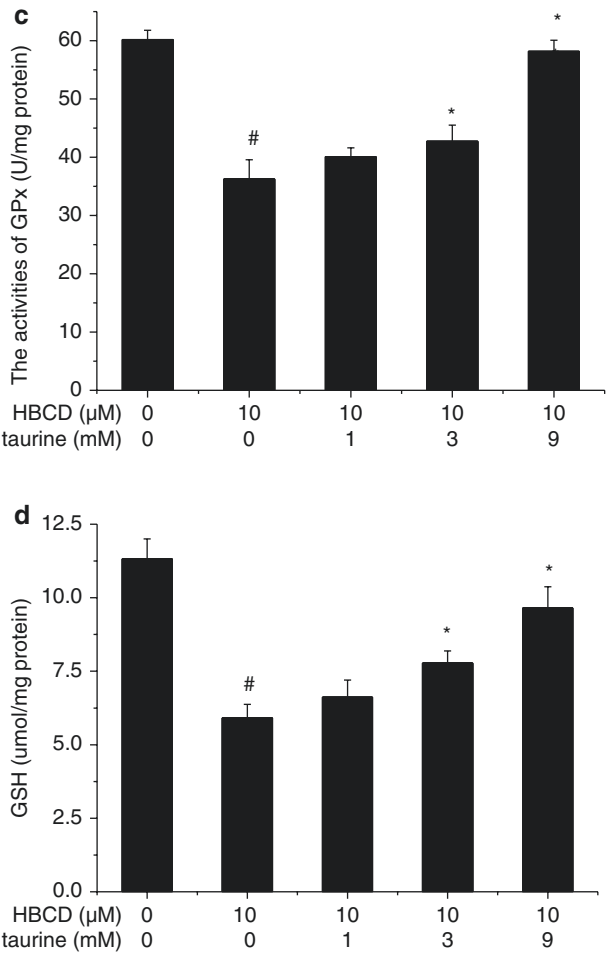

Fig. 3 (continued)

that SOD, CAT, GPx activities decreased greatly, and the GSH content also significantly reduced in PC12 cells when exposed to HBCD alone, while pretreatment with taurine can prevent the decrease of these enzymes activities and reverse effectively GSH reduction in PC12 cells exposed to HBCD in a dose-dependent manner.

5 Conclusion

In conclusion, our studies in this paper show that taurine can provide protection against HBCD-induced cytotoxicity and oxidative stress in PC12 cells. The protection of taurine may be via promoting endogenous antioxidants, prevent drop in MMP and ROS overproduction.

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Effects of Taurine on Alterations of Neurobehavior and Neurodevelopment Key Proteins Expression in Infant Rats by Exposure to Hexabromocyclododecane

Xiuli Zhang, Xiuhua Wang, Jing Zhang, Xiaohong Pan, Jing Jiang, and Yachen Li

Abstract Hexabromocyclododecanes (HBCDs) is a widely used flame retardant. Studies have found that HBCDs has toxic effects on endocrine and neural development, leading to adverse effects on behavior, learning and memory. This study aimed to investigate the protective effects of taurine on cognitive function, neurotrophic factors expression of infant rats exposured to HBCDs. Sprague-Dawley rats of 10-days old were oral gavaged of different doses (0.3, 3 and 30 mg/kg) of HBCDs and 30 mg/kg HBCDs with 300 mg/kg taurine for 60 consecutive days. Rat cognitive function was detected by the method of Morris water maze test. The protein expressions of brain derived neurotrophic factor (BDNF), nerve growth factor (NGF) and fibroblast growth factor (FGF) were assayed by Western-blotting. Results showed that rats exposed to HBCDs significantly declined rats spatial learning and memory ability by increasing the latency time of seeking the platform ($P < 0.05$), decreasing the numbers that each rat had crossed the non-exits and the time spent in the target quadrant as compared with those in control rats ($P < 0.05$). Taurine treatment significantly reversed the effects of HBCDs. Western-blotting results showed that expression of BDNF, NGF and FGF proteins in the low dose group were obviously increased compared with those in control rats ($P < 0.01$), and middle-dose and high dose groups significantly decreased. Taurine treatment increased BDNF and NGF expression as compared with high dose groups while Taurine seemed to have no effects on FGF. These result suggested that higher doses of HBCDs early exposure in the developing rats could decrease neurotrophic factors

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including BDNF, NGF, FGF, which have an impact on neural development, damage on learning and memory.

Keywords HBCD • Taurine • Neurobehavior • Neurotrophic factor

Abbreviations

HBCDs	Hexobromocyclododecanes
BDNF	Brain derived neurotrophic factor
NGF	Nerve growth factor
FGF	Fibroblast growth factor

1 Introduction

Hexobromocyclododecanes (HBCDs), primarily used as additive flame retardants in materials, are kinds of non-aromatic, brominated cyclic alkanes (Birnbaum and Staskal 2004). HBCDs can separate and spread to the environment due to their chemical character not bound to materials (de Wit 2002). HBCDs have been a ubiquitous contaminant in environmental media and biota, (Abdallah Mohamed et al. 2008; Covaci et al. 2006; Hale et al. 2006). HBCDs have high bioaccumulative character because they are extremely hydrophobic/lipophilic compounds. There are evidence that HBCDs accumulate/biomagnificate in the food chain. Researchers have found HBCDs in several species of bird eggs (Polder et al. 2008) and marine animals (Peck et al. 2008), and adipose tissue (Pulkrabova et al. 2009), even human blood (Knutsen et al. 2008), even in breast milk (Kakimoto et al. 2008; Thomsen et al. 2010). These findings have raised concerns potential toxicity effects of HBCDs particularly developmental effects on infants.

HBCDs have been found to have neurotoxic effects on brain development. Lilienthal et al. (2009) reported that exposure to HBCDs before mating and continuing mating, gestation, lactation, and after weaning in offspring affected hearing function and dopamine-dependent behavior in adult male and female offspring in Wistar rats. Saegusa et al. (2012) also found aberration neuronal migration and impaired neurogenesis in SD rats exposed to HBCDs during pregnancy. However, the machenism of behavioral impairment induced by HBCDs is still unknown and few studies have been done to investigate how to eliminate the impairment.

Previous studies found that neurotrophic factors (NTFs) including nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), fibroblast growth factor (FGF) exert a vital effect in nervous system development (Henderson 1996). They regulate synaptic structure, function, plasticity and learning and memory processes (Leal et al. 2014). Thus down-regulated NTFs expressions imply impairment neurogenesis and spatial learning and memory in various stress conditions (Kim et al. 2011; Song et al. 2006).

Taurine is a semi-essential amino acid involved in a wide range of physiological processes. Studies showed that taurine supplementation improve neuronal proliferation and synaptogenesis, indicating its effects on reinforcing synaptic plasticity and promoting learning and memory (Shivaraj et al. 2012). Taurine has also been verified to enhance the spatial cognitive function of rats damaged by sub-chronic manganese exposure (Lu et al. 2014). However, we still don't know the effects of taurine on cognitive impairment induced by HBCDs exposure to infant.

In this research, we focus on the effects of taurine on spatial learning and memory impairment of rat infant induced by HBCDs exposure and investigated the molecular mechanism of taurine by evaluating NGF, BDNF, FGF protein expressions.

2 Methods

2.1 Reagents and Drugs

HBCDs (Sigma, USA) were dissolved in peanut oil; Taurine was supplied by YUFENG Fine Chemical Company (Qingdao, China) and dissolved in 0.9% saline; Primary antibody of BDNF, FGF and NGF were purchased from cell Signaling (USA); HRP-conjugated secondary antibodies were purchased from Bioworld Technology (Nanjing, China).

2.2 Animals and Drug Administration

Sprague-Dawley (SD) rats (weighing 250–300 g) were purchased from the Center for Animal Testing of Shandong Lukang Drugs Group (Jining, Shandong, China). All these rats were kept at the room temperature (RT; 24 ± 1 °C) with relative humidity (60 ± 10%). The room was 12 light and 12 h dark cycle. When infants were born 10 days, we divided them into five groups: Normal control group (n = 9), HBCDs 0.3 mg/kg group (n = 9), HBCDs 3 mg/kg group (n = 9), HBCDs 30 mg/kg group (n = 9), Taurine group (n = 9). Except normal control group, HBCDs groups rats and taurine group rats were separately oral gavaged with HBCDs once at the dose of 0.3, 3, 30 mg/kg body weight for 60 days while control group rats were orally administrated with physiological saline at the same volume. At same time, taurine group mice were oral treated with taurine at the dose of 300 mg/kg once daily for 60 days. Control group and HBCDs group mice were treated with physiological saline. All studies were exerted according to the National Institutes of Health guidelines and were permitted by the associated Animal Care and Use Committee of Binzhou medical university. We tried to use the less number of rats according to statistical design.

2.3 Water Maze Test

The water maze was *designed as* a black circular tank whose diameter was 120 cm and depth was 50 cm. We virtually divided the tank into four quadrants and put a platform in one quadrant about 1.5 cm underwater. Each rat was faced to the tank wall and put into the pool from the rim of one quadrant. The rat was permitted to swim 60 s to find the platform. If the rat had not found the platform in 60 s, it will be recorded the latency as 60 s and put onto the platform for 30 s. Each rat had four trials daily for 5 days. The latency, time rat used to find the platform was recorded to evaluate the spatial learning performances of mice.

A probe test was performed at 6th day to evaluate memory consolidation by taking off the platform. The rat was put into tank and let it swim for 60 s. The numbers that each rat had crossed the non-exits and the time spent in the target quadrant where the platform had placed were both recorded to evaluate the spatial memory of rats.

2.4 Brain Samples Preparation

When finished water maze test, all rats were sacrificed by decapitation after one night fasted. Brains were taken carefully and quickly washed by 0.9% cold saline. The hippocampus was immediately separated from brain on a cold plate and stored at −80 °C for western blot detection.

2.5 Western Blot Assay

Hippocampus was put into lysis buffer and homogenized. The homogenate was sonicated for 10 s three times during 30 min incubation on ice. Then all the samples were centrifuged at 4 °C with 13,000 × *g* for 15 min. Bradford method was used to detect supernatant protein concentrations. Fifty micrograms of each sample was calculated and separated by electrophoresis using a 4–12% sodium dodecyl sulfate (SDS)-polyacrylamide gel. When finished, the gel was transferred onto nitrocellulose membranes. The membranes were then blocked with 5% nonfat dry milk in TBST for 1 h. Primary antibody [BDNF, NGF, FGF, β-Actin antibody; 1:1000 in TBST/5% milk] were added on the washed membranes and incubated overnight. After washing with TBST HRP-conjugated secondary antibodies (1:2000) were put onto the membranes and incubated at room temperature for 1 h. The signals were shown by enhanced chemiluminescence and quantified by ImageJ Software. β-Actin was used for inner reference.

2.6 Statistical Analysis

We used mean ± S.D. to express the data. One-way ANOVA followed by a Student's t test was chosen to analyze data. A criterion of $P < 0.05$ was considered as statistically significant.

3 Results

3.1 Morris Water Maze Test

Spatial learning and memory ability of rats is often assessed by Morris water maze. The results showed that HBCDs groups rats had significant decline of cognitive function. HBCDs groups rats took longer time to find platform during the first 5 days than the control group rats (Fig. 1; $P < 0.05$), indicating declined learning

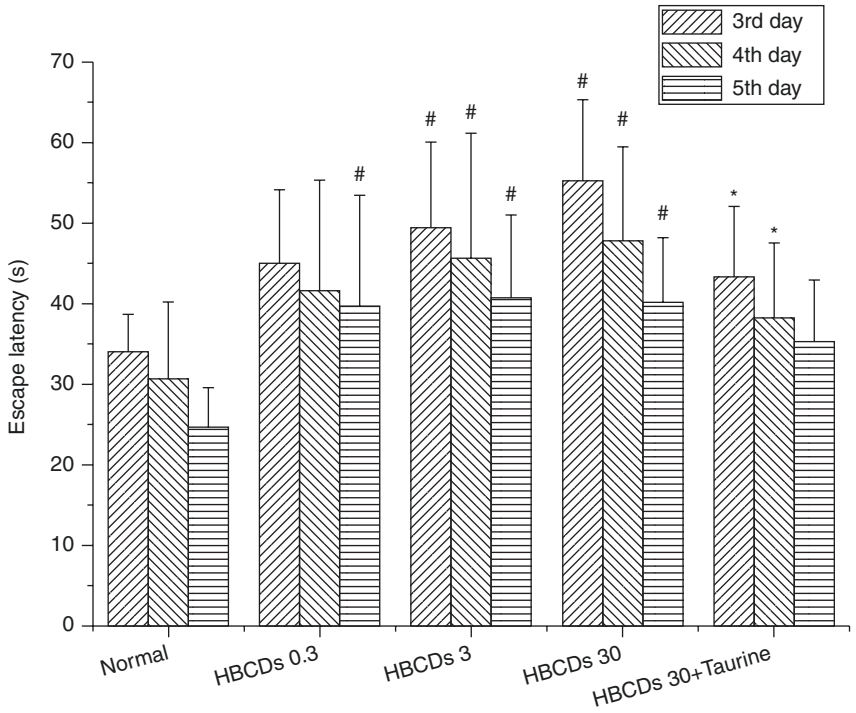

Fig. 1 Effects of taurine on the acquisition of spatial learning ability of rats detected by Morris water maze. Average latency time (days 3–5) spending to find underwater platform was extended by HBCDs ($P < 0.05$ as compared to control group) and taurine decreased the latency time ($P < 0.05$ as compared to HBCDs 30 mg/kg group)

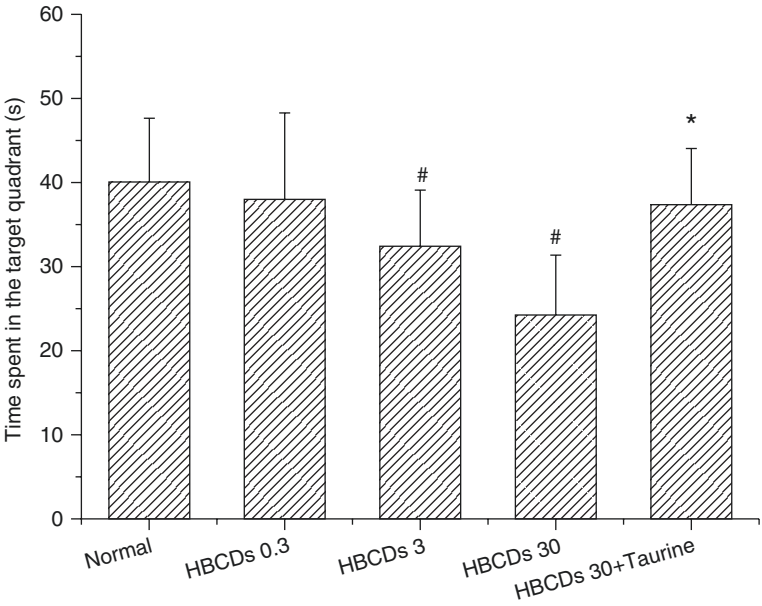

Fig. 2 Effects of taurine on the mean time staying in the target quadrant where the platform had been placed. Taurine obviously lessened high dose of HBCDs induced memory deficits ($^{\#}P < 0.01$ as compared to control group; $^{*}P < 0.01$ as compared to HBCDs 30 mg/kg group)

ability induced by HBCDs. Higher dose of HBCDs showed longer latencies to platform, indicating that HBCDs affected rats learning ability at dose dependence. Taurine (300 mg/kg day) administration obviously decreased the mean latency ($P < 0.05$) as compared with 30 mg/kg HBCDs treatment group ($P < 0.05$).

On the probe trial, HBCDs group rats seldom found the precise position of the platform, leading to short time in the target quadrant than control group (Fig. 2; $P < 0.05$). Taurine treatment increased the mean percent time staying in the target quadrant (Fig. 2; $P < 0.05$ vs. HBCDs 30 mg/kg group), indicating that taurine could enhance the damaged memory induced by HBCDs. Furthermore, the number of non-exits crossing was significantly reduced in HBCDs groups rats (Fig. 3; $P < 0.05$). Taurine administration changed the spatial navigation deficits (Fig. 3; $P < 0.05$ vs. HBCDs 30 mg/kg group). All results showed that taurine had effects on improving spatial learning and memory ability of rats exposed to HBCDs.

3.2 Effects of Taurine on BDNF Expression in Hippocampus of Infant Rats Treated with HBCDs

Studies verified that BDNF could not only promote neural stem cells differentiation into astrocytes and multi-stage nerve cells in order to maintain neuronal survival, differentiation, growth, development and repair, but also accelerate synaptic

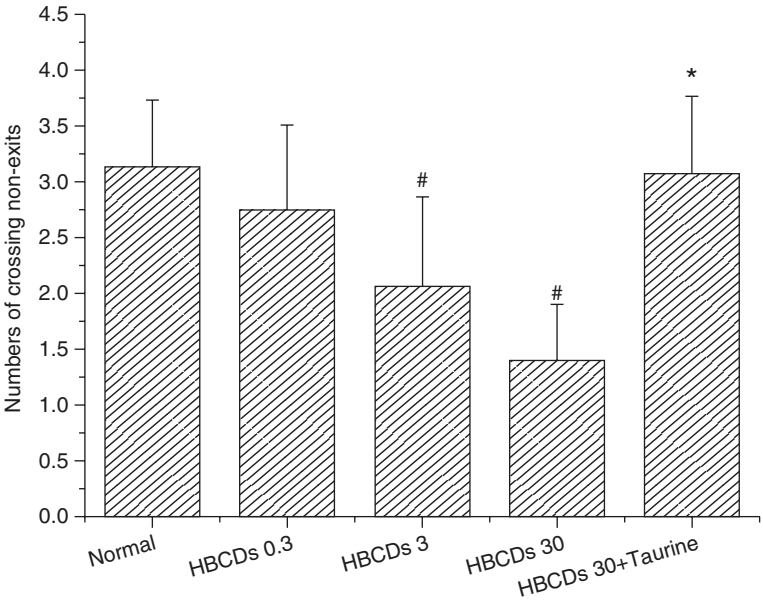

Fig. 3 Effects of taurine on the mean number of crossing non-exiting platform. The number of crossing non-exiting platform was significantly decreased in the mice with HBCDs ($^{\#}P < 0.01$ as compared to control group), taurine treatment reversed HBCDs' effect ($^{*}P < 0.05$ as compared to HBCDs 30 mg/kg group)

plasticity, neuronal regeneration after injury and promote regeneration of axons and dendrites. In Fig. 4, we can see that, compared with normal control group, BDNF expression in the rat hippocampus of lose dose HBCDs group was markedly increased ($P < 0.01$). While BDNF expression in the middle and high doses HBCDs groups were decreased when compared with the control group ($P < 0.01$). Taurine could slightly inhibit high dose HBCDs-induced BDNF expression decrease but it had not achieved significant difference. The results indicated that lose dose HBCDs exposure to infant rats for 60 days induced an increase of BDNF to protect neurons when brain met stress, but high dose of HBCDs made severe damage of hippocampus and induced dramatically decrease of BDNF. Taurine treatment seemed to have effects on increasing BDNF expression.

3.3 Effects of Taurine on NGF Expression in Hippocampus of Infant Rats Treated with HBCDs

NGF can participate in the neural development, neuron survival and damage repair of sympathetic neurons. It also has an important role on cholinergic neurons. As compared to the control group, middle and high doses of HBCDs obviously

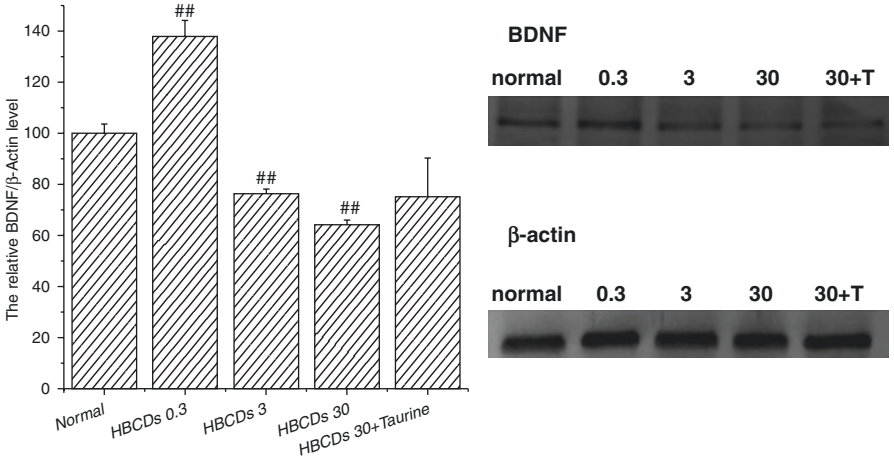

Fig. 4 Effects of taurine on BDNF expression in hippocampus of infant rats treated with HBCDs. ($^{\#\#}P < 0.01$ as compared to control group)

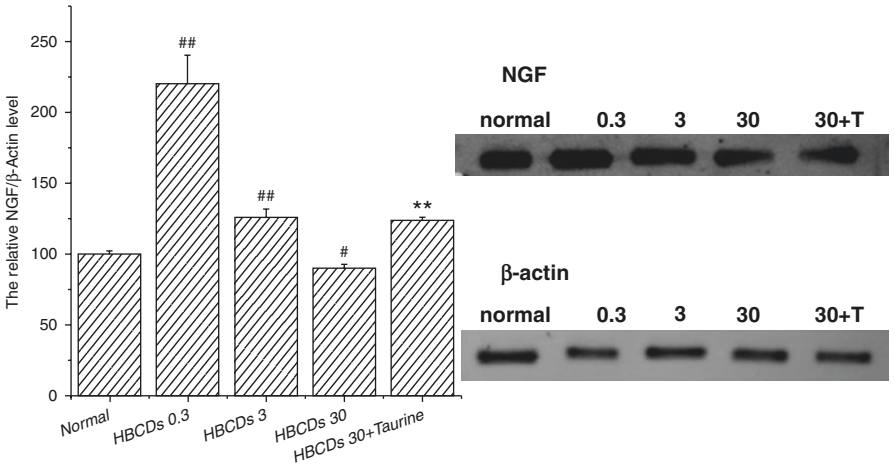

Fig. 5 Effects of taurine on NGF expression in hippocampus of infant rats treated with HBCDs. ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$ as compared to control group; $^{**}P < 0.01$ as compared to HBCDs 30 mg/kg group)

decreased NGF expression, while low dose of HBCDs interestingly increased NGF expression ($p < 0.01$; Fig. 5). Treatment with taurine could up-regulate the expression of NFG compared with the high dose HBCDs group ($p < 0.01$). These illustrated that low dose exposure HBCDs make NGF increased in order to protect nerve cells to face stress, but with the increasing concentration of HBCDs, brain hippocampal tissue got serious injury, the content of NGF declined sharply, and taurine could prevent the decline of NGF.

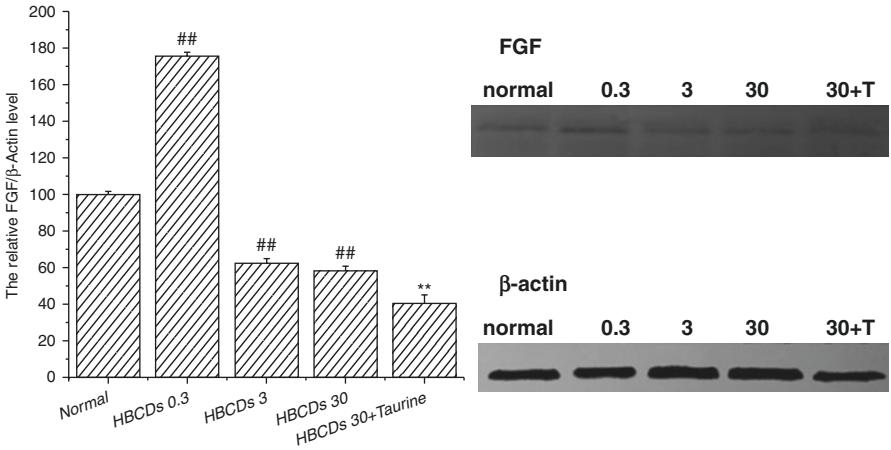

Fig. 6 Effects of taurine on FGF expression in hippocampus of infant rats treated with HBCDs. $^{\#\#}P < 0.01$ vs. control group; $^{**}P < 0.01$ vs. HBCDs 30 mg/kg group)

3.4 Effects of Taurine on FGF Expression in Hippocampus of Infant Rats Treated with HBCDs

FGF could affect neuronal growth, differentiation, development, regeneration, survival, and obviously has a regulatory role to maintain the neuronal function. As shown in Fig. 6, lose dose of HBCDs (0.3 mg/kg HBCDs) caused increase of FGF expression, but middle and high doses of HBCDs (3 and 30 mg/kg HBCDs) induced decrease of FGF expression when compared with normal control group ($P < 0.01$). Administration with taurine seemed not to improve FGF expression compared with high dose HBCDs group. The results indicated that lose dose HBCDs could induce an increase of FGF, but high dose of HBCDs induced dramatically decrease of NGF due to severe damage of hippocampus. Taurine treatment seemed to have no effect on FGF expression.

4 Discussion

Animal and in vitro studies demonstrated that HBCDs could cause neurodevelopmental toxic effects. Eriksson et al. (2006) reported that neonatal mice exposed to HBCDs may lead to developmental neurotoxic effects, which included aberrations in spontaneous behavior and cognitive function. Lilienthal et al. (2009) also reported that exposure to HBCDs before mating and continuing mating, gestation, lactation, and after weaning in offspring affected hearing function and dopamine-dependent behavior in adult male and female offspring in Wistar rats. In our experiment, we detected the protective effects of taurine on learning and memory defect in infant

rats exposed to HBCDs and its possible mechanism. Our experimental results demonstrated that infant rats exposed to HBCDs significantly declined spatial learning and memory ability. This result is coincidence with Eriksson et al.'s studies (Eriksson et al. 2006).

Taranukhin et al. (2009) found that taurine could protect immature cerebellar granullar neurons from the damage of acute alcohol treatment. Akande et al. (2014) also found that taurine alleviated cognitive impairment of male Wistar rats induced by chronic co-exposure chlorpyrifos and lead acetate. In this study we found that infant rats treatment with taurine significantly were reversed HBCDs induced spatial learning and memory ability decline. This result is similar to studies by Akande et al. (2014).

NTFs including NGF, BDNF and FGF play a crucial role in the nervous system during development and maturation (Henderson 1996). In the central nervous system of mammal, the neurotrophin brain-derived neurotrophic factor (BDNF) has been recognized as the main regulator of activity-dependent plasticity of excitatory synapses. Particularly, the neurotrophin plays a vital role on the hippocampal long-term potentiation (LTP) regulation, which is a continuous strength of excitatory synapsis recognized to be the basis of cognitive processes (Leal et al. 2015). Ibhazehiebo et al. (2011) reported that HBCD at the Low dose significantly inhibited neurite extension of granule cell aggregate induced by Tyrosine hydroxylase. BDNF prevented the inhibition of granule cell neurite extension mediated by HBCD. In this study, we found that the expression of BDNF, NGF and FGF proteins in the low dose group of HBCDs were obviously increased, and middle-dose and high dose groups of HBCDs significantly decreased. Taurine treatment increased BDNF and NGF expression in high dose groups while taurine seemed to have no effects on FGF.

5 Conclusion

These result suggested that higher doses of HBCDs early exposure in the developing rats could decrease neurotrophic factors including BDNF, NGF, FGF, which have an impact on neural development, damage on learning and memory.

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Taurine Chloramine Suppresses LPS-Induced Neuroinflammatory Responses through Nrf2-Mediated Heme Oxygenase-1 Expression in Mouse BV2 Microglial Cells

Dong-Sung Lee, Ki Han Kwon, and Sun Hee Cheong

Abstract The brain is sensitive to the inflammation and oxidative stress that can cause the aging or neurodegenerative diseases. We investigated the anti-neuroinflammatory activities of taurine chloramine (TauCl) on lipopolysaccharide (LPS)-treated mouse BV2 microglia mediated through heme oxygenase (HO)-1 expression. TauCl inhibited the protein expressions of prostaglandin E2 (PGE_2), cyclooxygenase (COX)-2, nitric oxide (NO), and inducible nitric oxide synthase (iNOS) in LPS-treated BV2 microglia. TauCl markedly inhibited interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) production. These effects were related to the suppression of the degradation and phosphorylation of inhibition of nuclear factor kappa B-α (IκB-α), translocation of nuclear factor kappa B (NF-κB) as well as DNA binding activity. In addition, TauCl induced the HO-1 expression by increasing the nuclear factor E2-related factor 2 (Nrf2) translocation to the nucleus in mouse BV2 microglia. These findings suggest that TauCl has protective effects of neurodegenerative disorders caused by neuroinflammation.

Keywords Taurine chloramine • Lipopolysaccharide • Neuroinflammatory • Heme oxygenase-1 • BV2 microglial cells

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Abbreviations

(IκB)-α	Inhibitor kappa B-α
COX-2	Cyclooxygenase-2
HO	Heme oxygenase
IL-1β	Interleukin-1β
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
Nrf2	Nuclear factor-E2-related factor 2
PGE_2	Prostaglandin E2
TauCl	Taurine chloramine
TNF-α	Necrosis factor-α

1 Introduction

Microglial cells function as the immune cells of the central nervous system (CNS), acting as primary mediators of inflammation. In response to some negative stimulus such as a free radicals and tissue or organ damages, microglial cells presume a reactive state characterized by the widening and shortening of microglial processes (Beynon and Walker 2012). Activated microglia can produce glutamate transporters and antioxidants to promote correct neuronal function. However, activated microglia are also able to generate several neurotoxic compounds including nitric oxide (NO) and several pro-inflammatory cytokines that are associated with neurological diseases and CNS disturbances (Lucin and Wyss-Coray 2009; Polazzi and Monti 2010). In CNS inflammation, microglial responses and activation can be mediated from various agents, including lipopolysaccharide (LPS) and several pro-inflammatory cytokines (Romero et al. 1996; Nakamura et al. 1999).

It has been well known that the nuclear factor-E2-related factor 2 (Nrf2) is one of a transcription factor. It can regulate the generation of several antioxidant enzymes. The Nrf2 plays a major role in the phase 2 detoxifying enzymes expression regulated by the antioxidant response element (ARE) and caused to various inducible genes activation by several responses associated with oxidation (Itoh et al. 1997). When the cells exposed to naturally occurring antioxidants containing Michael-reaction acceptors, it can disrupt the Keap1-Nrf2 complex, followed by Nrf2 translocate into the nucleus. Then it binds to ARE and stimulates transcription (Balogun et al. 2003). Heme oxygenase (HO)-1 expression is related to the Nrf2/ARE pathways and stimulated by several oxidative and inflammatory agents (Wagener et al. 2003). It has been reported that HO-1 pathway can induced the activation of some anti-inflammatory mediators such as prostaglandin J_2 (PGJ_2), tumor growth factor-β (TGF-β), and interleukin-10 (IL-10). It has been revealed that HO-1

is induced by major haloamines, such as taurine chloramine (TauCl) and taurine bromamine (TauBr), produced by leukocytes at the inflammation sites (Olszanecki and Marcinkiewicz 2004). TauCl and TauBr also decrease the generation of reactive oxygen species (ROS), NO, and proinflammatory cytokines as well as suppress the generation of pro-inflammatory eicosanoids such as prostaglandin E_2 (PGE_2) derived from cyclooxygenase (COX) *in vitro* (Park et al. 1997; Kontny et al. 2000). However, no study has addressed the molecular mechanisms of TauCl in mouse BV2 microglial cells or whether these mechanisms related to the HO-1 induction. Therefore, we investigated the protective effects against neuroinflammation of TauCl in LPS-treated mouse BV2 microglial cells as mediated by Nrf2/ARE-dependent HO-1 expression.

2 Methods

2.1 Chemical Reagents

Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco BRL Co. (Grand Island, NY, USA). Tin protoporphyrin IX (SnPP IX) was purchased from Porphyrin Products (Logan, UT, USA). Trolox, cobalt protoporphyrin IX (CoPP), TauCl, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α, PGE_2, IL-6, and IL-1β were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Primary antibodies, including COX-2, iNOS, HO-1, p65, p50, p-IκBα, IκBα, and Nrf2, and secondary antibodies were obtained from Santa Cruz Biotechnology (Heidelberg, Germany).

2.2 Cell Culture

BV2 microglial cells were obtained from Professor Hyun Park at Wonkwang University (Iksan, Korea). The cells (5×10^5 cells/mL) were maintained in DMEM medium containing streptomycin (100 mg/mL), penicillin G (100 U/mL), L-glutamine (2 mM), and 10% heat-inactivated FBS. The cells were then incubated at 37 °C in a humidified atmosphere (95% air and 5% CO_2).

2.3 Nitrite Assay

To determine the NO levels, the concentration of nitrite was assessed by the Griess reaction. The supernatant (100 μL) was mixed with Griess reagent (100 μL), and then determined the absorbance at 525 nm with an ELISA plate reader from BIO-RAD (Hercules, CA, USA).

2.4 PGE_2, TNF-α, and IL-1β Assays

BV2 microglial cells were maintained in 24-well plates, and incubated for 12 h with TauCl, and stimulated with LPS for 18 h. After collection of the medium, the TNF-α, IL-1β, and PGE_2 concentrations were analyzed by commercial ELISA kits from R&D Systems (Minneapolis, MN, USA).

2.5 Western Blotting

BV2 cells were pelleted at 200 × *g* for 3 min, followed by washing using phosphate buffered saline (PBS). Cells were lysed using 20 mM Tris-HCl buffer (pH 7.4) containing protease inhibitors (5 mg/mL pepstatin A, 5 mg/mL aprotinin, 1 mg/mL chymostatin, 0.1 mM phenylmethanesulfonyl fluoride). We determined the protein concentration using the commercial Lowry protein assay kit (P5626; Sigma). Each sample with same amount of protein was dissolved using 12% SDS-PAGE and transferred to a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane from Bio-Rad (Hercules, CA, USA). After blocking the membrane by 5% skimmed milk, it was incubated with anti-COX-2, anti-HO-1, anti-β-actin (1/1000 dilution, respectively), and anti-iNOS (1/500 dilution) primary antibodies at 4 °C overnight. The immunoreactive band was observed by a horseradish peroxidase-conjugated secondary antibody (1/1000 dilution) followed by ECL detection using a device purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA), and the bands were quantitated using Image Gauge v3.12 software from Fujifilm (Tokyo, Japan).

2.6 Preparation of Cytosolic and Nuclear Cell Fractions

Cells were homogenized (1:20, w:v) with PER-Mammalian Protein Extraction Buffer from Pierce Biotechnology (Rockford, IL, USA) including 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail from EMD Biosciences (San Diego, CA, USA). The cytosolic cell fractions were obtained by centrifugation at 15,000 × *g* for 10 min at 4 °C. The cytoplasmic and nuclear extracts were obtained using NEPER cytoplasmic and nuclear extraction reagents. Cells (3×10^6 cells in a 60-mm dish) were collected, washed with PBS buffer and then centrifuged. Cells were lysed at 4 °C by shaking for 15 min with RIPA buffer containing protease inhibitors, 0.5% sodium deoxycholate, 20 mM NaF, 50 mM Tris-HCl, 0.1% SDS, 150 mM NaCl, 1 mM Na_3VO_4, 1% NP-40, 50 mM glycerophosphate (pH 7.4), 20 mM ethylene glycol tetra-acetic acid (EGTA), and 1 mM dithiothreitol (DTT). After centrifugation at 14,800 × *g* for 15 min, the supernatant was obtained and then stored at −70 °C. Bicinchoninic acid (BCA) protein assay kit was used for the determination of protein content.

2.7 NF-κB DNA Binding Activity

Cells were treated with the TauCl for 12 h and then exposed to the LPS (1 μg/mL) for 1 h. The DNA-binding activity of NF-κB was determined using the Trans AM kit from Active Motif (Carlsbad, CA, USA). Binding buffer (Binding Buffer AM3, herring sperm DNA, and 30 μL; DTT) was added to each well. Then 20 μg of nuclear extracts (diluted to 20 μL in lysis buffer) were treated with TauCl and stimulated with LPS for 1 h. After the incubation for 1 h at room temperature, each well were washed with fresh wash buffer, then added 100 μL of diluted NF-κB antibody (1/1000 dilution in 1× antibody binding buffer), and the plates were incubated for further 1 h. After washing the wells with wash buffer, 100 μL of diluted horseradish peroxidase (HRP)-conjugated antibody (1/1000 dilution in 1× antibody binding buffer) was added to each of the 6 wells. After the incubation for 1 h, each well was added the developing solution and washed to remove the supernatant. After incubation for 5 min, the absorbance was read on a spectrophotometer at 450 nm.

2.8 Transfection

Using Lipofectamine 2000™ (Invitrogen, Carlsbad, CA, USA), we transfected the cells with HO-1 siRNA for 6 h, and then the cells were recovered with fresh media containing 10% FBS for 24 h.

2.9 RT-PCR Analysis

Total RNA (1 μg) was isolated using TRIzol from Invitrogen (Carlsbad, CA, USA) and quantified using spectrophotometer at 260 nm, and then transcribed reversely by the High Capacity RNA-to-cDNA kit (Carlsbad, CA, USA). The cDNA was amplified by the SYBR Premix Ex Taq kit (TaKaRa Bio Inc. Shiga, Japan) and a StepOnePlus Real-Time PCR system from Applied Biosystems, respectively. We performed RT-PCR analysis in 20 μL of a total volume, containing diethyl pyrocarbonate-treated water, primer (0.8 μM), and SYBR Green PCR Master Mix (10 μL). The primer sequences designed by PrimerQuest from Integrated DNA Technologies (Cambridge, MA, USA) were as follows: mHO-1, forward 5′-CTCTTGGCTGGCTTCCTT-3′ and reverse 5′-GGCTCCTTCCTCC TTTCC-3′, and mGAPDH, forward 5′-ACTTTGGTATCGTGGAAGGACT-3′ and reverse 5′-GTAGAGGCAGGGATGATGTTCT-3′. The mRNA data were analyzed using a PCR device and the StepOne software from Applied Biosystems (Carlsbad, CA, USA).

2.10 Statistic Analysis

Data are expressed as the mean ± SD of three independent experiments. Statistical analysis was conducted by GraphPad Prism software version 3.03 from GraphPad Software Inc. (San Diego, CA, USA). The differences between means were assessed by one-way analysis of variance (ANOVA) followed by Newman-Keuls *post hoc* test, and statistical significance was defined at $P < 0.05$.

3 Results

3.1 Effects of TauCl on the Generation of Pro-Inflammatory Mediators and Cytokines in LPS-Stimulated BV2 Microglia

Our results demonstrated that TauCl inhibited LPS-stimulated proinflammatory mediators including NO, PGE_2, iNOS, and COX-2. In the present study, we also confirmed that TauCl significantly and dose-dependently reduced the LPS-mediated IL-1β, IL-6, and TNF-α generation (Fig. 1).

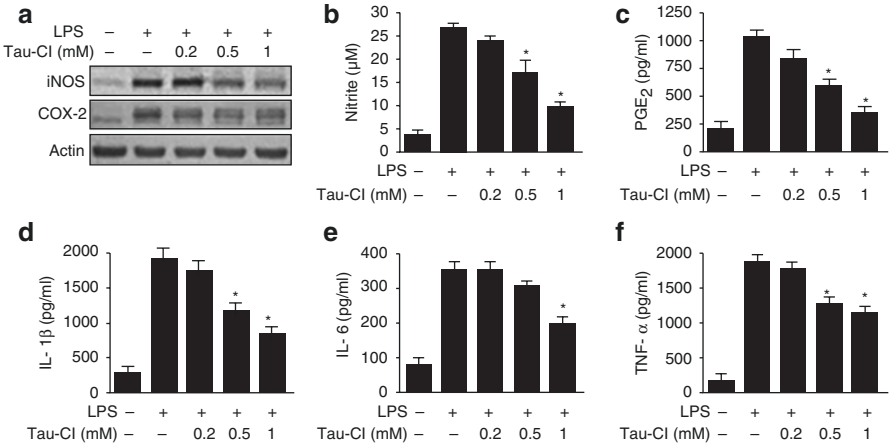

Fig. 1 Effects of TauCl on iNOS and COX-2 protein expression (**a**), nitrite (**b**), PGE2 (**c**), IL-1β (**d**), IL-6 (**e**), and TNF-α (**f**) production induced by LPS in BV2 microglial cells. Experiments were conducted in triplicate and values are expressed as means ± SD. $^{*}P < 0.05$ compared to the group treated with LPS

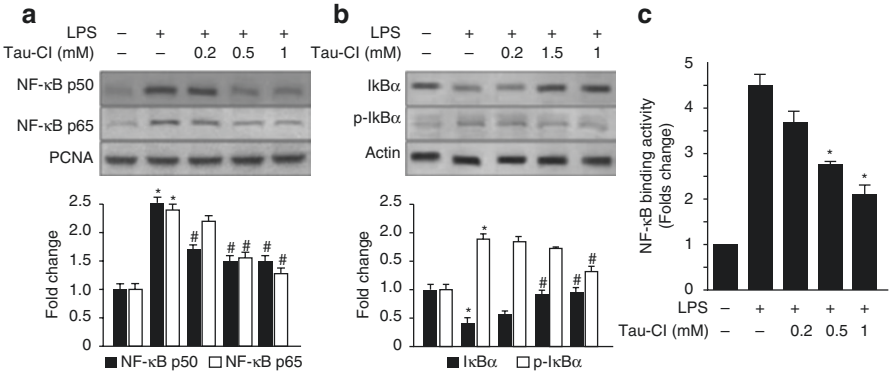

Fig. 2 Effects of TauCl on LPS-induced NF-κB activation (**a**), IκB-*α* phosphorylation, degradation of IκB-*α* (**b**), and NF-κB DNA binding activity (**c**) in BV2 microglia. Experiments were conducted in triplicate and values are expressed as means ± SD. $^{*}P < 0.05$ compared with the LPS-treated group

3.2 *Effects of TauCl on the Degradation of IκB-α, Nuclear Translocation of NF-κB, and NF-κB DNA Binding Activity in LPS-Stimulated BV2 Microglia*

NF-κB is well known as a crucial transcription factor that regulates the production of proinflammatory mediators. We therefore investigated the effects of TauCl on the NF-κB signaling pathway. As shown in Fig. 2a, TauCl significantly inhibited the translocation of p65 and p50, the two subunits of the NF-κB heterodimer, to the nucleus. In addition, TauCl inhibited the cytoplasmic phosphorylation and degradation of IκB-*α* (Fig. 2b). Furthermore, as shown in Fig. 2c, we examined the ability of NF-κB to bind DNA in the nuclear extracts from BV2 microglia induced with LPS for 1 h. LPS treatment markedly increased the amount of NF-κB that bound to DNA; however, TauCl inhibited NF-κB binding to DNA in a dose-dependent manner.

3.3 *Effects of TauCl on the Expressions of HO-1 mRNA and Protein in BV2 Microglia*

We next investigated the effects of TauCl on the HO-1 expression in BV2 microglia. The cells were treated with noncytotoxic concentrations of TauCl (0.1–1 mM) for 12 h. The results indicated that TauCl markedly increased the HO-1 mRNA (Fig. 3a) and protein expressions (Fig. 3b). The 20 μM of CoPP, a HO-1 inducer, significantly promoted HO-1 expression in BV2 microglia. However, 1 mM of TauCl treatment

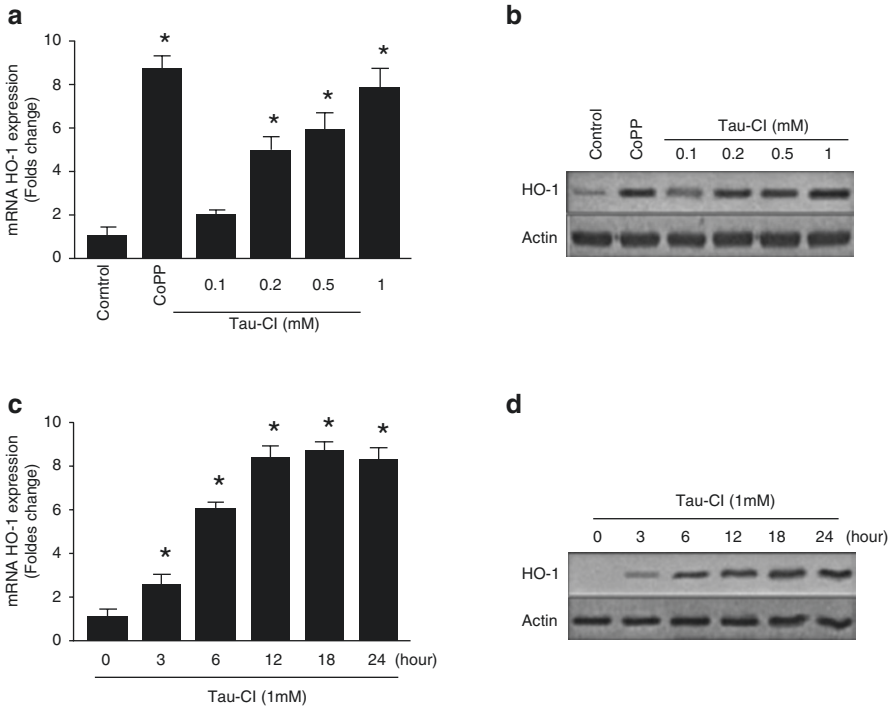

Fig. 3 The effects of TauCl on HO-1 mRNA and protein expressions. Cells were incubated for 12 h with the various concentrations of TauCl (**a**, **b**) or treated with 1 mM TauCl for 3, 6, 12, 18, or 24 h (**c**, **d**). The blots are representative of three independent experiments. $^{*}P < 0.05$ compared with the control group

significantly increased the expression levels of HO-1 mRNA as well as protein at 3 h and then peaked at around 18 h (Fig. 3c, d). These results indicated that TauCl has strong neuroprotective effect against cell death stimulated by LPS in BV2 microglia and that is partially attributed to HO-1.

3.4 The effects of TauCl on HO-1 Mediated Proinflammatory Mediator and Cytokine Production and NF-κB Binding Activity in LPS-Stimulated BV2 Microglia

To confirm that the observed anti-neuroinflammatory effects were mediated by the TauCl-induced HO-1 expression, we evaluated the effects of TauCl on proinflammatory mediators and cytokine levels using siRNA against HO-1. Our results showed that the inhibitory effects of TauCl against LPS-induced NO, PGE_2, IL-1β,

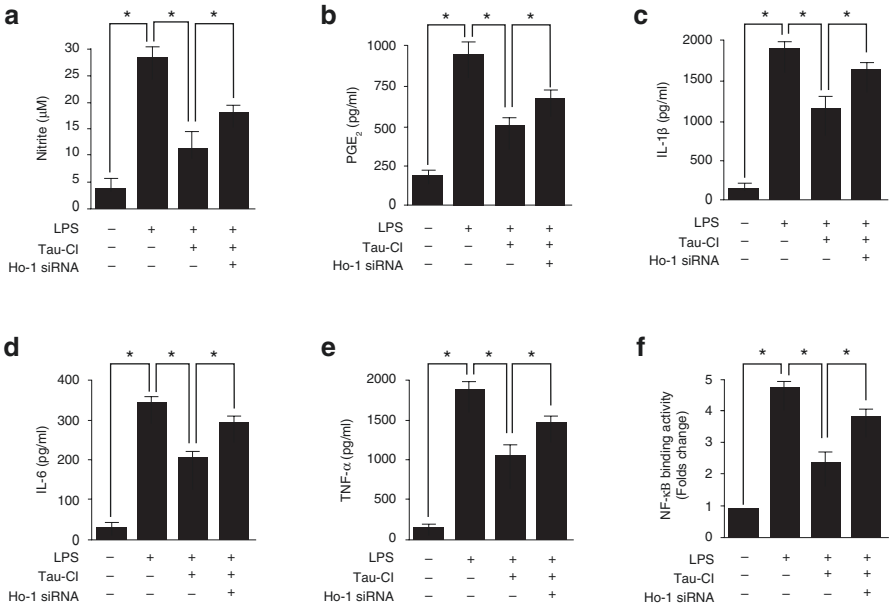

Fig. 4 The inhibitory effect of TauCl on LPS-induced proinflammatory mediator productions and NF-κB DNA binding activity mediated by HO-1 in BV2 microglia. The levels of NO (**a**), PGE_2 (**b**), IL-1β (**c**), IL-6 (**d**), TNF-α (**e**), and the degree of NF-κB binding activity in the nucleus (**f**) were determined by commercial ELISA kits. Experiments were conducted in triplicate and values are expressed as means ± SD. $^{*}P < 0.05$

IL-6, and TNF-α generation were partially reversed by HO-1 siRNA (Fig. 4a–e). Furthermore, we determined whether HO-1 upregulation by TauCl mediated the inhibition of NF-κB. NF-κB binding was observed in the nuclear extracts from BV2 microglia. However, HO-1 siRNA markedly suppressed the inhibitory property of TauCl on NF-κB DNA binding activity (Fig. 4f).

3.5 Effects of TauCl on the Nrf2 Nuclear Translocation in BV2 Microglia

The activated Nrf2 nuclear translocation regulates HO-1 mRNA and protein expression. Therefore, we determined whether TauCl treatment induced the Nrf2 nuclear translocation in BV2 microglia (Fig. 5). According to the results, the Nrf2 levels of BV2 microglia gradually increased in the nuclear fraction, whereas decreased in cytoplasmic fraction.

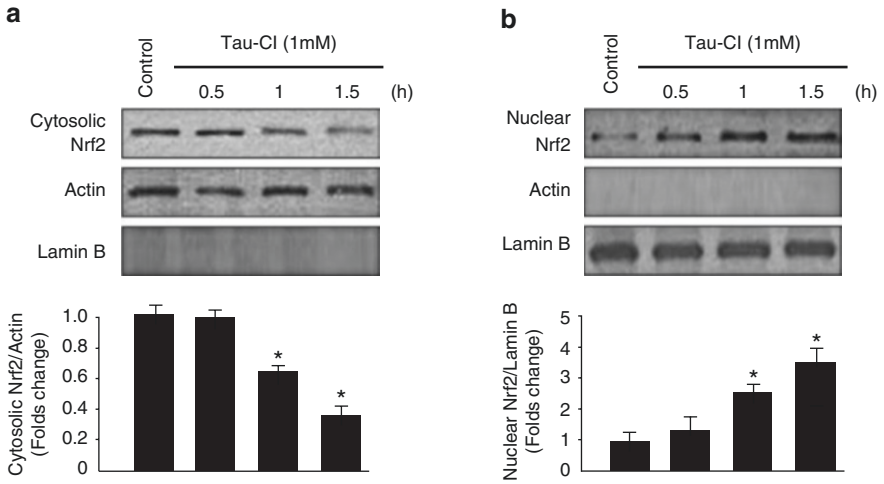

Fig. 5 The effects of TauCl on the Nrf2 nuclear translocation (**a**, **b**) in BV2 microglia. Cells were treated with 1 mM TauCl for 0.5, 1, or 1.5 h. The blots are representative of three independent experiments. $^{*}P < 0.05$ compared with the control group

4 Discussion

Brain tissues are sensitive to acute or chronic inflammation and oxidative stress, which can occur aging and neurodegenerative diseases (Hald and Lotharius 2005). In this study, we estimated the implication of HO-1 enzymatic expression by the Nrf2 nuclear translocation in the anti-neuroinflammatory property of TauCl in mouse BV2 microglial cells. The BV2 immortalized murine microglia cell line is often used as *in vitro* model, because BV2 cells maintain most of the functional characteristics for primary microglia. Especially, the endotoxin LPS is commonly used to model neuroinflammatory effects as it induces strong microglial activation through binding to toll-like receptor 4 (TLR4) (Das et al. 2015). Microglia are stimulated by brain injury and release several pro-inflammatory cytokines and inflammatory mediators including NO, TNF-α, IL-1β, and prostaglandins, which can induce or promote the inflammatory responses in the CNS (Chao et al. 1992; Meda et al. 1995). Thus, these reports demonstrate the suitability of the BV2 cell line to study anti-neuroinflammatory effects of TauCl against LPS-induced immune responses and activation *in vitro*. The transcription factor NF-κB is involved in the several genes control that encode mediators of acute-phase, immune, and inflammatory responses. Following such a response, the p50 and p65 components of the free NF-κB dimer translocate to the nucleus and bind to specific DNA sequences of proinflammatory enzyme genes, including iNOS and COX-2, resulting in their rapid transcription (O'Neill and Kaltschmidt 1997; Connelly et al. 2001). To determine the effects of TauCl on the proinflammatory mediator production and the

iNOS and COX-2 expressions in LPS-treated BV2 microglia, BV2 microglia were stimulated with LPS with or without non-cytotoxic concentrations of TauCl. The results indicated that up to 0.5 mM TauCl inhibited the expressions of iNOS and COX-2, thereby suppressing NO, PGE_2, TNF-α, IL-6, as well as IL-1β generation.

In addition, we evaluated the effects of TauCl on the activation and DNA-binding activity of NF-κB, phosphorylation of IκB-α and the nuclear translocation of the p65 and p50 NF-κB subunits in BV2 microglia. Our results indicated that the IκB-α phosphorylation and degradation, and nuclear translocation of p65 and p50 mediated by LPS were markedly reduced after pre-treatment of BV2 microglia with 0.2–1.0 mM TauCl. In the present study, we also confirmed that TauCl dose-dependently suppressed the NF-κB DNA-binding activity in BV2 microglia stimulated with LPS. These findings suggest that TauCl exerted its anti-neuroinflammatory properties by suppressing the proinflammatory enzymes expression like as iNOS and COX-2, and the secretion of proinflammatory cytokines by inhibiting the NF-κB activation in LPS-stimulated microglia. Consistent with our results, it has been reported that TauCl inhibits the DNA-binding activity of NF-κB and PGE_2 production by suppressing COX-2 gene transcription under the control of NF-κB (Liu et al. 1998; Kontny et al. 2003).

Recently, it has been reported that the anti-inflammatory effect of HO-1 is associated with the suppression of various pro-inflammatory cytokines in activated macrophages (Wiesel et al. 2000). HO-1 and its metabolites can also inhibit the expressions of iNOS and COX-2 enzymes, thereby decreasing iNOS-derived NO and COX-2-derived PGE_2 generation (Oh et al. 2006; Suh et al. 2006). Additionally, it has been known that the nuclear translocation of Nrf2 is a major up-stream event of HO-1 expression. The expression of inducible proteins, such as quinine reductase, GSH S-transferase, and HO-1 activates the Nrf2 expression (Jaiswal 2000). In the present study, we confirmed that the HO-1 expression induced by TauCl in BV2 microglia and also show that TauCl-mediated HO-1 mRNA as well as protein expression occurs in a time- and concentration-dependent manner. In addition, we showed that TauCl increased Nrf2 levels in the nuclei of BV2 microglia. In the present study, we determined whether TauCl-induced HO-1 expression was associated with inhibiting the expression of LPS-stimulated pro-inflammatory mediators. Our results demonstrate that the inhibitory effect of HO-1 via HO inhibitor HO-1 siRNA reversed the suppressive effects of TauCl in LPS-stimulated microglia. Because HO-1 siRNA blocks HO-1 activity, these results demonstrated that TauCl could partially suppress NF-κB-mediated NO, PGE_2, IL-1β, IL-6, and TNF-α generation through regulation of HO-1 expression. Several previous studies also found that TauCl is able to up-regulate the HO-1 expression and reduce the generation of IL-6 and IL-8 in several cells (Mizuno et al. 2005; Kirino et al. 2007; Orozco et al. 2007).

Based on our results, TauCl promoted HO-1 expression via the Nrf2 pathway, which suppressed NF-κB-mediated NO, PGE_2, TNF-α, IL-1β, and IL-6 production in LPS-stimulated microglia. Therefore, our findings provide evidence of a beneficial role of TauCl in the treatment of neuroinflammatory diseases.

5 Conclusion

In conclusion, we confirmed that TauCl increased the expressions of HO-1 mRNA and protein through the Nrf2 pathway. Additionally, TauCl-induced HO-1 expression suppressed NF-κB-mediated generation of proinflammatory cytokines and mediators in LPS-stimulated microglia. Thus, TauCl may represent an alternative natural therapeutic material for the treatment as well as prevention of neuroinflammatory diseases.

Acknowledgements This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2016R1D1A1B01006822).

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Taurine Chloramine Prevents Neuronal HT22 Cell Damage Through Nrf2-Related Heme Oxygenase-1

Sun Hee Cheong and Dong-Sung Lee

Abstract Oxidative cell damages are able to contribute to neuronal degeneration in several diseases of the central nervous system (CNS) including stroke as well as ischemia. Heme oxygenase (HO)-1 plays a major role in the pathogenesis of neuronal disorder. Taurine chloramine (TauCl) has been shown to possess strong neuronal activities; however, the direct effects of TauCl on neuronal cell death remain to be determined. Therefore, this study was designed to assess the neuroprotective effect of TauCl using oxidative stress-stimulated mouse hippocampal HT22 cells. TauCl showed protective effects against oxidative stress-induced neurotoxicity and inhibited the reactive oxygen species (ROS) production by inducing the heme oxygenase (HO)-1 expression in HT22 cells. TauCl upregulated HO-1 expression and it also increased the nuclear factor E2-related factor 2 (Nrf2) translocation to nuclear. Using an inhibitor of HO-1 activity, we verified that the oxidative stress-related HT22 cell death was significantly suppressed by TauCl. In addition, we found reduced TauCl-induced HO-1 expression and cytoprotection following treatment of the cells with an extracellular signal-regulated kinase (ERK) inhibitor (PD98059) or a p38 inhibitor (SB203580), but not following treatment with a SP600125 as a c-Jun NH2-terminal kinase (JNK) inhibitor. These findings suggest that TauCl improves cellular damage induced by glutamate or H_2O_2 through ERK and p38, Nrf2, and HO-1 pathways in HT22 cells.

Keywords Taurine chloramine • Oxidative stress • Heme oxygenase-1 • Nuclear factor E2-related factor 2 • Mouse hippocampal HT22 cells

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Abbreviations

CNS	Central nervous system
ERK	Extracellular signal regulated kinase
HO	Heme oxygenase
JNK	c-Jun NH2 terminal kinase
Nrf2	Nuclear factor E2-related factor 2
ROS	Reactive oxygen species
SnPP	Tin protoporphyrin
TauCl	Taurine chloramine

1 Introduction

The neuronal damages caused by overstimulated excitatory receptors, known as excitotoxicity, have been involved in many neurological disorders (Lipton and Rosenberg 1994). Glutamate is the important excitatory neurotransmitter and plays key roles in brain development and processes related to the movement control, memory, and learning in the CNS (Gasic and Hollmann 1992). However, the overstimulation of glutamate receptors has been involved in the neuronal damage observed in many neurodegenerative diseases (Difazio et al. 1992; Gasic and Hollmann 1992; Behrens et al. 2002). The neuronal HT22 cell line originated from the mouse hippocampus lacks glutamate receptors, therefore it can cause for glutamate mediated cell death (Maher and Davis 1996). Hydrogen peroxide (H_2O_2) is a major cause of free radicals and is continually produced from their metabolic activity within the body. If H_2O_2 is accumulated in the cells, it is converted to molecules which can cause cell injury irreversibly (Halliwell and Gutteridge 1993). Some enzymes such as glutathione peroxidases and catalase are able to detoxify the formed H_2O_2 in the cells. On the other hand, some neuronal cells show lower catalase levels, therefor, the burden falls on the glutathione pathway (Mavelli et al. 1982).

Taurine is generated from cysteine and is one of the most rich amino acids stored in all mammalian monocytes and tissues (Learn et al. 1990). Taurine protects activated neutrophils from inflammatory injury by detoxifying the highly oxidizing hypochlorous acid ($HOCl/OCl^-$) that is generated from H_2O_2 by the myeloperoxidase (MPO) system (Thomas et al. 1986). Taurine removes excessive $HOCl/OCl^-$ by reacting readily with it to form taurine chloramine (TauCl), which is much more stable and a weaker oxidant. TauCl also inhibits the macrophage-derived overproduction of O_2^- and NO, without causing cytotoxicity to macrophages, by inducing anti-oxidant enzymes such as peroxiredoxin (Prx)-1, thioredoxin (Trx)-1, and heme oxygenase-1 (HO-1) via activation of transcription factors including nuclear factor E2-related factor 2 (Nrf2) (Kim and Kim 2005; Jang et al. 2009).

Nrf2 is one of the basic leucine zipper protein family. Under physiological conditions, Nrf2 binds to the cytoplasmic adaptor protein, Keap1, thereby forming a complex that remains in the cytoplasm. When the cell is stimulated by certain exogenous substances, Nrf2 is phosphorylated and uncouples from Keap1. The Nrf2 protein

then translocates from the cytoplasm to the nucleus where it binds to the antioxidant responsive element sequence in the promoter region of target genes and, induces their transcription and translation. These gene products include anti-inflammatory proteins and antioxidant enzymes, which further induce the expression of HO-1, NQO1, and other enzymes related to antioxidant and detoxification (Hong et al. 2010). These antioxidant and detoxifying factors encompass many important protective mechanisms against brain injury (Miller et al. 2014; Sandberg et al. 2014).

TauCl exerts a number of biological effects such as inhibition of nitric oxide generation in lipopolysaccharide-activated macrophages (Barua et al. 2001) and inhibiting H_2O_2-induced apoptosis in macrophages (Piao et al. 2011). However, there have been very limited studies about the molecular targets of TauCl and fundamental mechanisms of it's neuroprotective activities in mouse hippocampal HT22 cells. In the present study, we investigated the protective effects of TauCl on H_2O_2- or glutamate-stimulated neurotoxicity in HT22 cells via HO-1 expression mediated Nrf2 through mitogen-activated protein kinase (MAPK) pathways.

2 Methods

2.1 Chemical Reagents

The cell culture-related reagent including Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco BRL Co (Carlsbad, CA, USA). As a HO activity inhibitor, Tin protoporphyrin IX (SnPP IX) or as a HO-1 inducer, cobalt protoporphyrin (CoPP) were purchased from Porphyrin Products. Antibodies such as HO-1, Nrf-2, actin were obtained from Santa Cruz Biotechnology (CA, USA). Taurine, small interfering RNA (siRNA), and all other chemical reagent were purchased from Sigma Chemical Co (MO, USA). Lipofectamine 2000™ was obtained from Invitrogen Life Technologies (Grand Island, NY, USA).

2.2 HT22 Cells Cultures

HT22 cells were donated from Prof. Youn-Chul Kim Wonkwang University (Iksan, Korea). The cells (5×10^6/dish) were seeded in 100 mm dishes in DMEM containing streptomycin (100 μg/mL), 10% heat-inactivated FBS, and penicillin G (100 units/mL), and then incubated at 37 °C in a humidified atmosphere (5% CO_2 and 95% air).

2.3 MTT Assay

To determine cell viability by MTT assay, HT22 cells were maintained at 2×10^4 cells/well and then treated with taurine in the absence or presence of glutamate (5 mM) or H_2O_2 (100 μM). After incubation for the indicated times, we

removed the cell culture medium from each well, and then replaced with 200 μL of fresh medium in each well. Cells were incubated with 0.5 mg/mL of MTT for 1 h, and the formed formazan were resolved in DMSO.

2.4 Measurement of ROS Generation

To measure the generation of ROS, HT22 cells were maintained at 2.5×10^4/well in 24-well plates and were stimulated with H_2O_2 (100 μM) or glutamate (5 mM). And, the cells were washed and then stained using 10 μM of 2′,7′-dichlorofluorescein diacetate (DCFDA) in the dark for 30 min. After washing the cells with PBS, the cells were extracted with 1% Triton X-100 in PBS for 10 min at 37 °C. Fluorescence was recorded at an emission wavelength of 525 nm and an excitation wavelength of 490 nm by Spectramax Gemini XS from Molecular Devices (CA, USA), respectively.

2.5 The Extraction of Cytoplasmic and Nuclear Cell

HT22 cells homogenized in PER-Mammalian Protein Extraction buffer supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail I (EMD Biosciences, San Diego, CA, USA). Cytoplasmic and nuclear fractions were separated by using NE-PER reagents (Pierce Biotechnology), respectively.

2.6 Western Blotting Analysis

Pelleted HT22 cells were obtained by centrifugation. After washing the cells with PBS, the cells were lysed in Tris-HCl buffer (20 mM, pH 7.4) supplemented with a protease inhibitor mixture containing 5 mg/mL aprotinin, 1 mg/mL chymostatin, 5 mg/mL pepstatin A, and 0.1 mM phenylmethylsulfonyl fluoride. The equal amount of each protein was dissolved using SDS-PAGE and then transferred to membrane. The membrane was blocked using skimmed milk and then incubated with primary antibodies at 4 °C overnight. The immune-reactive band was obtained by a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology).

2.7 Real-Time PCR Analysis

We isolated total RNA of the cells using TRIzol (Invitrogen, Carlsbad, CA, USA) and then quantified spectrophotometrically at 260 nm, in accordance with the manufacturer's recommendations. Total RNA (1 μg) was reverse-transcribed using the

kit of High Capacity RNA to cDNA from Applied Biosystems (CA, USA). The obtained cDNA was amplified using the TaKaRa Bio Inc kit of SYBR Premix Ex Taq and a StepOnePlus Real-Time PCR Applied Biosystems. Reaction mixture (20 μL) contained diethyl pyrocarbonate (DEPC)-treated water, 0.8 μM of primer, and 10 μL SYBR Green PCR Master Mix. The sequences of primer were designed by PrimerQuest from Integrated DNA Technologies.

HO-1 (Forward: 5′-CTCTTGGCTGGCTTCCTT-3′, Reverse: 5′-GGCTCCTTCCTCCTTTCC-3′)

GAPDH (Forward: 5′-ACTTTGG TATCGTGGAAGGACT-3′, Reverse 5′-GTAGAGGCAGGGATGATGTTCT-3′)

2.8 Statistical Analysis

All data expressed as means ± S.D. of 3 independent experiments. We used one-way analysis of variance followed by the *Newman-Keuls post hoc test*. Statistical analysis was conducted by the GraphPad Prism software, version 3.03 from GraphPad Software (CA, USA).

3 Results

3.1 Effects of TauCl, Glutamate and H_2O_2 on Cell Viability in HT22 Cells

We determined the potential cytotoxic effects of TauCl (Fig. 1a) and its effect on the viability of HT22 cells (Fig. 1b). It was revealed no cytotoxic effects at a concentration of 1 mM of TauCl using the MTT assay. By contrast, treatment with 2 mM glutamate or 50 μM H_2O_2 significantly reduced cell viability (Fig. 1c, d).

3.2 Effects of TauCl on Glutamate or H_2O_2-Induced Cytotoxicity and ROS Reduction

We examined the protective action by TauCl against glutamate or H_2O_2-induced toxicity. The viability of HT22 cells treated with glutamate or H_2O_2 only was lower than control (untreated) cells; however, pre-treatment with TauCl (0.5 and 1 mM) increased viability (Fig. 2a, c). We also studied whether TauCl affected oxidative neurotoxicity and ROS generation induced by glutamate or H_2O_2 in HT22 cells. Glutamate and H_2O_2 doubled ROS generation, whereas TauCl treatment effectively suppressed the rises in the production of ROS (Fig. 2b, d). Trolox has been

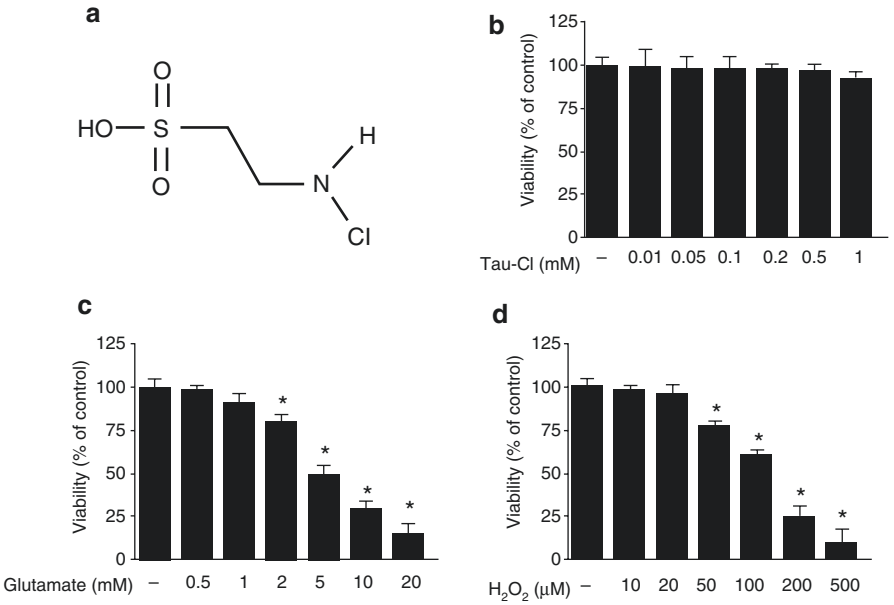

Fig. 1 The structure of taurine chloramine (TauCl) (**a**) and the effects of TauCl, glutamate and H_2O_2 on cell viability by MTT assay. HT22 cells were incubated for 48 h with various concentrations of TauCl (0.01–1.0 mM) (**b**), glutamate (0.5–20 mM) (**c**) and H_2O_2 (10–500 μM) (**d**). Data are presented as the mean values ± S.D. of three independent experiments. $^*P < 0.05$ vs. control

well-known as an anti-oxidative agent, therefore, we used Trolox as a positive control. Trolox showed a strong ROS scavenging activity as well as cytoprotective effect.

3.3 Effects of TauCl on the mRNA and Protein Expression of HO-1

To investigate the induction of HO-1 mediated by TauCl, we treated HT22 cells with TauCl (0.1–1 mM) for 12 h and then examined HO-1 levels. We used the 20 μM of CoPP, as a positive HO-1 inducer. TauCl increased the expression of HO-1 mRNA (Fig. 3a). Additionally, TauCl significantly promoted HO-1 protein expression (Fig. 3b). We also investigated whether TauCl induces the nuclear translocation of Nrf2 in HT22 cells. TauCl showed a gradual rises of Nrf2 in nuclear fractions, with a concomitant decreased in the cytoplasmic fractions (Fig. 3c, d). This result indicates that the HO-1-induced by TauCl is associated with the nuclear Nrf2 translocation in the cells.

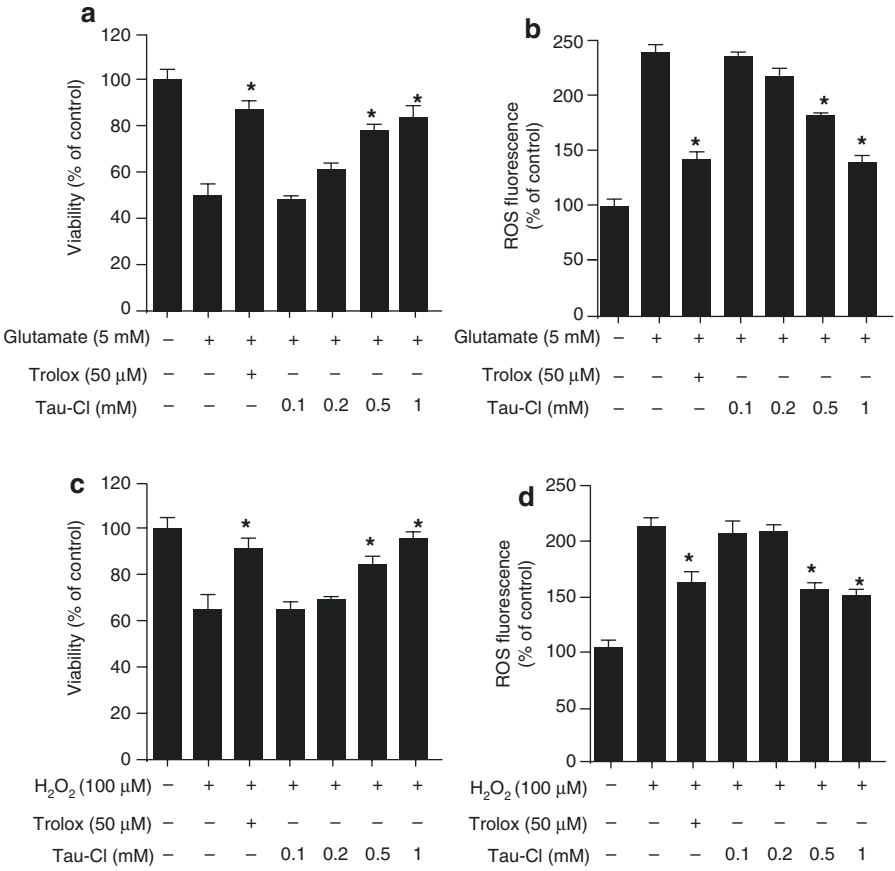

Fig. 2 The effects of taurine chloramine (TauCl) on glutamate- or H_2O_2-induced toxicity (**a**, **c**) and the production of ROS (**b**, **d**) in HT22 cells. Data are presented as the mean values ± S.D. of 3 independent experiments. $^*P < 0.05$ vs. glutamate or H_2O_2

3.4 Effects of TauCl on HT22 Cell Viability and Generation of ROS Through HO-1 Action

To confirm that the marked protective action and ROS reducing properties of pre-incubation with TauCl (Fig. 2) were correlated with TauCl-induced HO-1 expression (Fig. 3), we indicated whether the HO-1 expression induced by TauCl can be reversed by SnPP (Fig. 4). SnPP has been well known as an HO-1 inhibitor. HT22 cells pre-treated using TauCl with or without SnPP, and then exposed to glutamate or H_2O_2 for 12 h. SnPP was shown to significantly inhibit TauCl-mediated cytoprotection using the MTT assay (Fig. 4ac,). The HO-1 expression induced by TauCl was required for the suppression of glutamate or H_2O_2-stimulated ROS production

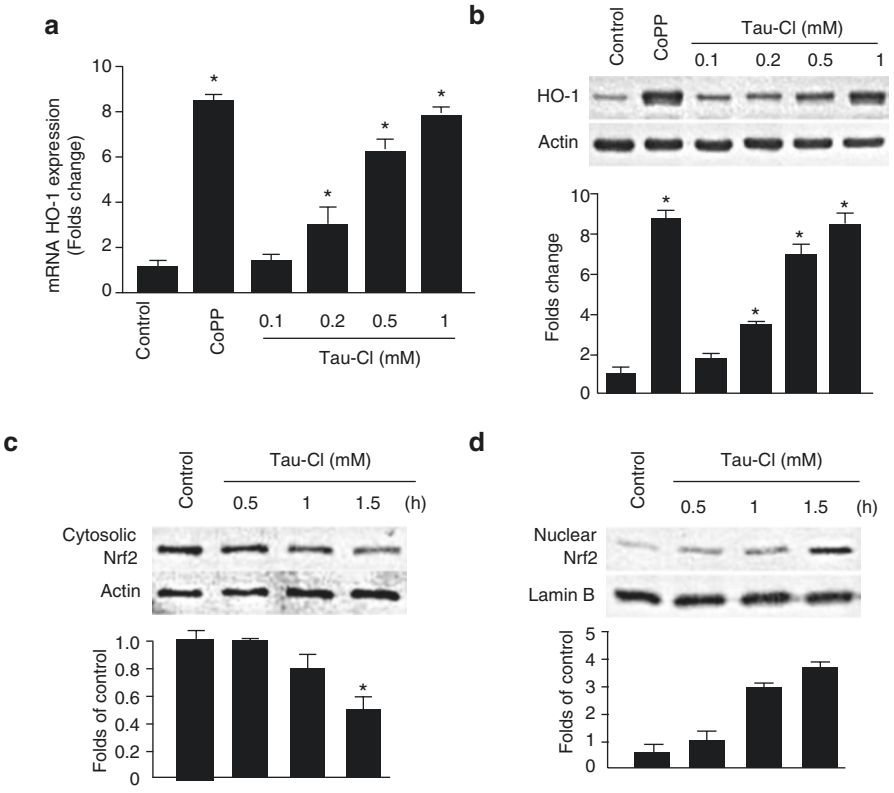

Fig. 3 Effects of taurine chloramine (TauCl) on HO-1 mRNA (**a**) and protein (**b**) expression, as well as Nrf2 nuclear translocation (**c**, **d**), in HT22 cells. Data are presented as the mean values ± S.D. of 3 independent experiments. *$P < 0.05$ vs. control

(Fig. 3b, d). The HO-1 induction mediated TauCl contributes to the cytoprotective properties in the HT22 cells.

3.5 Effects of HO-1 Induction by TauCl on the MAPK Pathway

Next, we evaluated whether the TauCl-induced HO-1 expression occurs through the MAPK pathway. Pre-treatment with a specific inhibitor of p38 (SB203580) and a specific inhibitor of ERK (PD98059) significantly attenuated TauCl-induced HO-1 expression in HT22 cells (Fig. 5a). Furthermore, the p38 inhibitor (SB203580) and ERK inhibitor (PD98059) significantly abolished TauCl-induced cytoprotection (Fig. 5b, c). We indicate in this result that the HO-1 expression induced by TauCl is correlated with the ERK and p38 MAPK pathway.

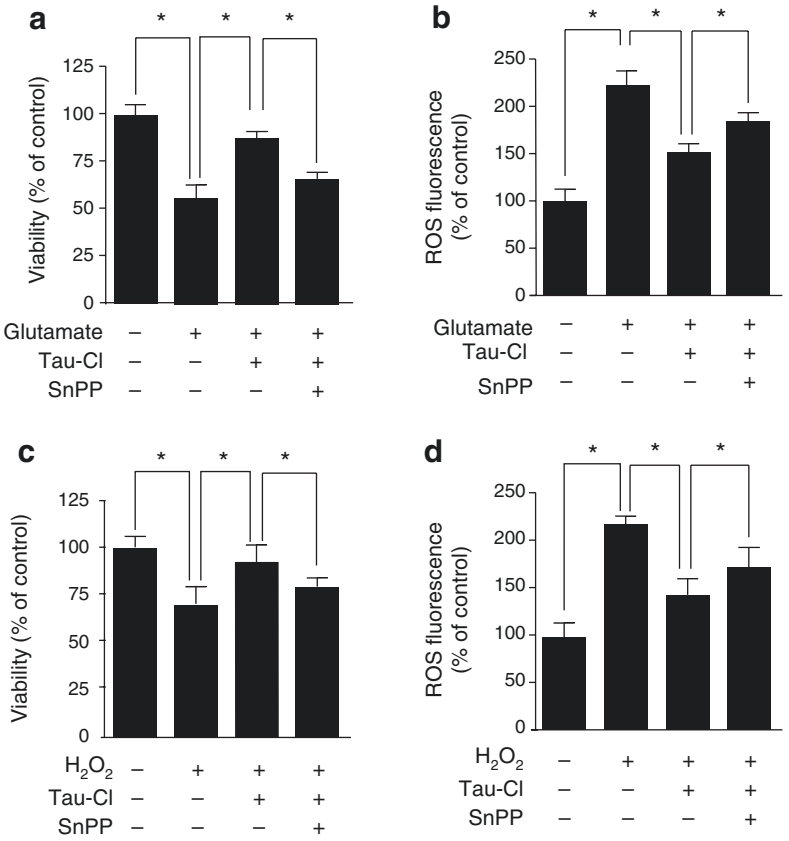

Fig. 4 Effects of Taurine chloramine (TauCl)-induced HO-1 expression on glutamate-induced neurotoxicity (**a**) and ROS production (**b**) or H_2O_2-induced neurotoxicity (**c**) and ROS production (**d**) in HT22 cells. Data are presented as the mean values ± S.D. of 3 independent experiments. $^{*}P < 0.05$

4 Discussion

Neurodegenerative diseases are disorders in which the nervous system irreversibly and progressively degenerates. Since ROS cause oxidative neurotoxicity in several neurodegenerative diseases, numerous studies have focused on the therapeutic molecules to prevent or develop the antioxidant candidates (Rahman 2007). Glutamate-stimulated neurotoxicity in the HT22 mouse hippocampal neuronal cell line has been commonly used to investigate the neurotoxicity induced by acute or chronic oxidative stress as an *in vitro* model (Coyle and Puttfarcken 1993).

Several transcription factors are known to regulate the HO-1 expression through one or more signaling pathways. Nrf2 is one of the basic transcription factor that retains in the cytoplasm. When some stimulus are exist, Nrf2 is bound to its Keap-1

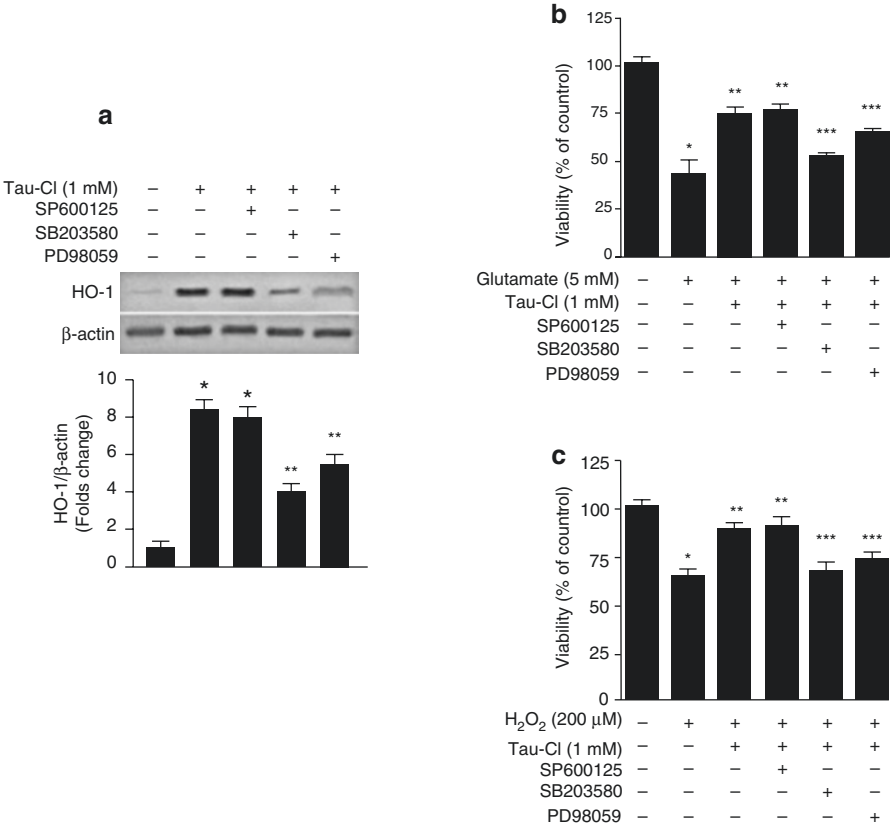

Fig. 5 Effects of TauCl-induced HO-1 expression via the MAPK pathway in HT22 cells (**a**). HT22 cells were pre-incubated with or without 10 μM SP600125 (JNK inhibitor), SB203580 (p38 inhibitor) or PD98059 (ERK inhibitor) for 1 h and then incubated with or without 1 mM TauCl for 12 h. These cells were then exposed to 5 mM glutamate (**b**) or 200 μM H_2O_2 (**c**) for 12 h. Data are presented as the mean values ± S.D. of 3 independent experiments $^{*}P < 0.05$. vs. control. $^{**}P < 0.05$ compared to the TauCl-treated group. $^{***}P < 0.05$ compared to the TauCl and SP600125, SB203580 or PD98059 treated group

protein, and then translocates to the nucleus. Once in the nucleus, it is bound to ARE sequences site which is the promoter regions of specific target genes (Qiang et al. 2004). In this study, we found that TauCl prevents glutamate- or H_2O_2-mediated cell death and ROS production by inducing the HO-1 via the Nrf2 nuclear translocation. It has been well known that Nrf2 stimulate the expression of several antioxidant proteins such as HO-1 and glutathione (GSH) (Ishii et al. 2000). HO-1 catalyzes the rate-limiting step in the degradation of free-heme separated from heme-containing protein molecules during oxidative stress. Since free-heme catalyzes the hydroxyl radicals production from H_2O_2 by the Fenton reaction, the rapid elimination of free-heme via elevated HO-1 activity appears to be essential for the survival of cells exposed to H_2O_2 in inflammatory tissues (Otterbein et al. 2000; Srisook et al. 2006). In accordance with our results, several researchers have reported that TauCl increased the both HO-1 mRNA and protein levels in macrophages, and that TauCl,

alone or in combination with LPS, increased the enzymatic activity of HO-1 (Jang et al. 2009; Kang and Kim 2013). It has also been shown that the HO-1 induction by TauCl was decreased in RAW 264.7 cells that were transfected with Nrf2 siRNA (Kim et al. 2010). Therefore, the effect of TauCl on the induction of HO-1 expression and its activity is important for the elimination of toxic hydroxyl radicals and ROS generation (Kim and Cha 2014).

MAPK signaling pathways that increase the ERK phosphorylation and p38 MAPK play a major role in inducing HO-1 expression (Elbirt et al. 1998). Our results indicate that TauCl increases cellular resistance against glutamate-stimulated oxidative damages through the ERK and p38 MAPK pathways, which participate at an early stage in the induction of HO-1. Similarly, previous studies have reported that the induction of HO-1 was dependent on JNK and p38 MAPK activation, and that the induction of HO-1 expression by arsenite depends on the activation of ERK and p38 MAPK in an *in vitro* model (Kietzmann et al. 2003; Midwinter et al. 2004). These results suggest that ERK and p38 MAPK signaling pathways are somehow involved in the phosphorylation of Nrf2 and may regulate the induction of HO-1. However, further studies are necessary to investigate the role of MAPKs in the activation of Nrf2 and the TauCl-derived upregulation of HO-1 expression.

5 Conclusion

In conclusion, we have shown that TauCl protects against glutamate- or H_2O_2-induced oxidative HT22 cell death, and that HO-1 expression through Nrf2 appears to major function in HT22 cells. Our study is the first demonstration that TauCl exerts the defense mechanism against H_2O_2- or glutamate-stimulated oxidative neurotoxicity. Therefore, these findings suggest that the HO-1 pathway can be the major biological target of TauCl to prevent several neurodegenerative disorders.

Acknowledgements This study was financially supported by Chonnam National University, 2016.

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Taurine Have Neuroprotective Activity against Oxidative Damage-Induced HT22 Cell Death through Heme Oxygenase-1 Pathway

Dong-Sung Lee and Sun Hee Cheong

Abstract Glutamate-induced oxidative neurotoxicity plays a part role in neuronal degeneration on the disorders of central nervous system (CNS). The expression of heme oxygenase (HO)-1 mediated by Inducible nuclear factor-E2-related factor 2 (Nrf2) functions as an anti-oxidants that is able to play an important role in the pathogenesis of several neuronal disorders. In the present study, taurine showed the inhibitory effect against reactive oxygen species (ROS) induction and protective effects against neurotoxicity induced by glutamate- and H_2O_2 through induction of HO-1 expression in HT22 cells. Moreover, taurine promoted the Nrf2 nuclear translocation in HT22 cells. We also verified the oxidative stress-mediated cell death of HT22 cells was significantly repressed by taurine, using tin protoporphyrin (SnPP) as an HO activity inhibitor. In addition, we found that treatment of the cells with p38 inhibitor (SB203580) suppressed taurine-induced HO-1 expression and cytoprotection, but inhibitors of c-Jun NH2 terminal kinase (JNK) (SP600125) or extracellular signal regulated kinase (ERK) (PD98059) did not. These results suggest that taurine improves the resistance against oxidative damages induced by glutamate in HT22 cells via the p38/Nrf2-dependent HO-1 expression. Our results demonstrated the potential application of taurine as a therapeutic agent for neurodegenerative diseases.

Keywords Taurine • Oxidative stress • Neuroprotection • Nuclear factor E2-related factor 2 • MAPK pathways

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Abbreviations

CNS	Central nervous system
ERK	Extracellular signal-regulated kinase
HO-1	Heme oxygenase-1
JNK	c-Jun NH2-terminal kinase
Nrf2	Nuclear factor E2-related factor 2
ROS	Reactive oxygen species
SnPP	Tin protoporphyrin

1 Introduction

Neurodegenerative disorders including Parkinson's, Huntington's, and Alzheimer's disease, are characterized by the dysfunction of the central nervous system (CNS) as well as progressive loss of neurons (Nasrallah and Wolk 2014). Chronic oxidative stress is one of the major causes of various neurodegenerative diseases, but the exact mechanism remains unknown (Cahill-Smith and Li 2014). Therefore, anti-oxidative candidates with no toxicity have been explored for the prevention of several neurodegenerative diseases (Lohle and Reichmann 2010).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is one of the transcription factors and it is essential for the anti-oxidant responsive element (ARE), which protect the oxidative stress and detoxify several carcinogens, by regulating the induction of heme oxygenase-1 (HO-1) or phase II detoxifying enzymes such as glutathione S-transferases (Pi et al. 2010). Nrf2 generally exists in the cytoplasm combined with Kelch-like ECH-associated protein (Keap1) under normal conditions. When severe oxidative stress occurs, however, Nrf2 is released from Keap1, then translocated into the nucleus where it binds to ARE to alleviate several antioxidative genes (Itoh et al. 1999). HO-1 has been known as HSP32, the HSP family. It has been reported that HO-1 and its enzymatic by-products such as carbon monoxide, biliverdin, and free iron are able to protect animal cells against oxidative stress by converting toxic heme compounds (Maines 1988). In addition, phase 2 antioxidant enzymes such as HO-1, and Nrf2/ARE signaling pathway have revealed as a therapeutic targets for neuronal protection (Zhang et al. 2013).

Taurine (2-aminoethanesulfonic acid) is well known as the predominant endogenous free amino acid in the CNS and has multiple functions in the body such as antioxidant and anti-inflammatory properties, CNS development, osmoregulation, protein stabilization, and calcium homeostasis (Frosini et al. 2003). However, there have been few studies on the therapeutic targets of taurine and their mechanism about anti-neurodegenerative properties in mouse hippocampal HT22 cells. Glutamate induces neuronal cell death through non-receptor-mediated oxidative stress as well as receptor-initiated excitotoxicity in the CNS. The HT22 cell originated from mouse hippocampal neuronal cell lacks the glutamate receptors (Chao et al. 2014). Therefore, it has been often used to study of oxidative stress-mediated neuronal cell death through high concentrations of glutamate exposure to the neuronal cells (Fukui et al. 2010; Chao et al. 2014). Taurine can inhibit several events that occur downstream of

glutamate stimulation, containing apoptotic pathways and altered enzymatic activities, but the fundamental molecular mechanisms of taurine are not fully understood yet (Ye et al. 2013). The present study aimed to investigate the protective effects of taurine on glutamate- or H_2O_2-induced oxidative neurotoxicity in HT22 cells.

2 Methods

2.1 Chemical Reagents

The cell culture-related reagent including Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco BRL Co (Carlsbad, CA, USA). Lipofectamine 2000™ was obtained from Invitrogen Life Technologies (Grand Island, NY, USA). Tin protoporphyrin IX (SnPP IX) as a HO activity inhibitor or cobalt protoporphyrin (CoPP) as a HO-1 inducer were purchased from Porphyrin Products. Antibodies such as HO-1, Nrf-2, actin were obtained from Santa Cruz Biotechnology (CA, USA). Taurine, small interfering RNA (siRNA), and all other chemical reagent were purchased from Sigma (Sigma Chemical Co., MO, USA).

2.2 HT22 Cells Cultures

HT22 cells were donated from Prof. Youn-Chul Kim at Wonkwang University (Iksan, Korea). The cells (5×10^6/dish) were seeded in 100 mm dishes in DMEM containing streptomycin (100 μg/mL), 10% heat-inactivated FBS, and penicillin G (100 units/mL), and then incubated at 37 °C in a humidified atmosphere (5% CO_2 and 95% air).

2.3 MTT Assay

To determine cell viability by MTT assay, HT22 cells were maintained at 2×10^4 cells/well and then treated with taurine in the absence or presence of glutamate (5 mM) or H_2O_2 (100 μM). After incubation for the indicated times, we removed the cell culture medium from each well, and then replaced with 200 μL of fresh medium in each well. Cells were incubated with 0.5 mg/mL of MTT for 1 h, and the formed formazan were resolved in DMSO.

2.4 Measurement of ROS Generation

To measure the generation of ROS, HT22 cells were maintained at 2.5×10^4/well in 24-well plates and were stimulated with H_2O_2 (100 μM) or glutamate (5 mM). After incubation for 8 h in the absence or presence of taurine or SnPP, the cells were washed and then stained using 10 μM of 2′,7′-dichlorofluorescein diacetate (DCFDA)

in the dark for 30 min. After washing the cells with PBS, the cells were extracted with 1% Triton X-100 in PBS for 10 min at 37 °C. Fluorescence was recorded at an emission wavelength of 525 nm and an excitation wavelength of 490 nm by Spectramax Gemini XS (Molecular Devices, Sunnyvale, CA, USA), respectively.

2.5 The Extraction of Cytoplasmic and Nuclear Cell

HT22 cells homogenized (1:20, w:v) in PER-Mammalian Protein Extraction buffer from Pierce Biotechnology (IL, USA) supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail I (EMD Biosciences, San Diego, CA, USA). Cytoplasmic and nuclear fractions were separated by using NE-PER reagents (Pierce Biotechnology), respectively.

2.6 Western Blotting Analysis

Pelleted HT22 cells were obtained by centrifugation. After washing the cells with PBS, the cells were lysed in Tris-HCl buffer (20 mM, pH 7.4) supplemented with a protease inhibitor mixture containing 5 mg/mL aprotinin, 1 mg/mL chymostatin, 5 mg/mL pepstatin A, and 0.1 mM phenylmethylsulfonyl fluoride. The equal amount of each protein was dissolved using 12% SDS-PAGE and then transferred to a Hybond-enhanced chemiluminescence nitrocellulose membrane (Bio-Rad). The membrane was blocked using 5% skimmed milk and then incubated with anti-HO-1, anti-Nrf2, or anti-actin antibodies (Santa Cruz Biotechnology) at 4 °C overnight. The immune-reactive band was obtained by a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology).

2.7 Real-Time PCR Analysis

We isolated total RNA of the cells using TRIzol (Invitrogen, Carlsbad, CA, USA) and then quantified spectrophotometrically at 260 nm, in accordance with the manufacturer's recommendations. Total RNA (1 μg) was reverse-transcribed using the kit of High Capacity RNA to cDNA from Applied Biosystems (CA, USA). The obtained cDNA was amplified using the TaKaRa Bio Inc kit of SYBR Premix Ex Taq and a StepOnePlus Real-Time PCR Applied Biosystems. Reaction mixture (20 μL) contained diethyl pyrocarbonate (DEPC)-treated water, 0.8 μM of primer, and 10 μL SYBR Green PCR Master Mix. The sequences of primer were designed by PrimerQuest from Integrated DNA Technologies.

HO-1 (Forward: 5′-CTCTTGGCTGGCTTCCTT-3′, Reverse: 5′-GGCTCCTT CCTCCTTTCC-3′)

GAPDH (Forward: 5′-ACTTTGG TATCGTGGAAGGACT-3′, Reverse 5′-GTA GAGGCAGGGATGATGTTCT-3′)

2.8 Statistical Analysis

All data expressed as means ± S.D. of three independent experiments. We used one-way analysis of variance followed by the *Newman-Keuls post hoc test*. Statistical analysis was conducted by the GraphPad Prism software, version 3.03 (GraphPad Software Inc., San Diego, CA, USA).

3 Results

3.1 Effects of Taurine, Glutamate and H_2O_2 on the Cell Viability in HT22 Cells

We evaluated the cytotoxic potential of taurine (Fig. 1a) and its effect on the cell viability (Fig. 1b) in HT22 cells. Taurine at 20 mM revealed no cytotoxicity, whereas, 2 mM of glutamate and 50 μM of H_2O_2 reduced the viability of these cells significantly (Fig. 1c, d).

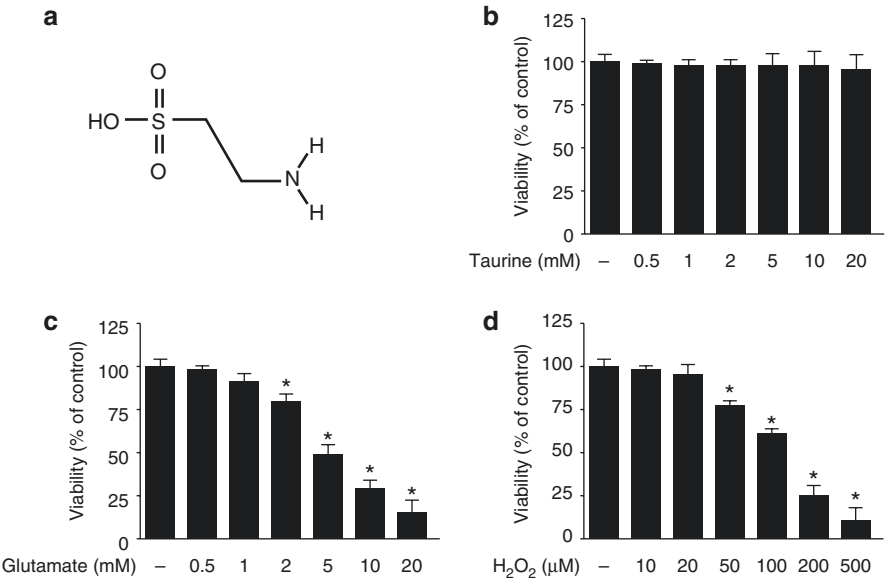

Fig. 1 The structure of taurine (**a**) and the effects of taurine, glutamate and H_2O_2 on cell viability by MTT assay. HT22 cells were incubated for 48 h with various concentrations of taurine (0.5–20 mM) (**b**), glutamate (0.5–20 mM) (**c**) and H_2O_2 (10–500 μM) (**d**). Data expressed as mean ± S.D. values of three independent experiments. $^*P < 0.05$ vs. control

3.2 *Effects of Taurine on Glutamate- or H_2O_2-Induced Cytotoxicity and ROS Generation*

We investigated the neuroprotective effects of taurine on glutamate- or H_2O_2-stimulated cytotoxicity in HT22 cells. In this study, we used Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), an anti-oxidant compound, as a positive control, and showed a strong ROS scavenging activity as well as cytoprotective effect at a concentration of 50 μM (Fig. 2). Treatment with glutamate or H_2O_2 significantly increased cell death compared to untreated cells, whereas taurine treatment at non-cytotoxic concentrations (10 and 20 mM), significantly and dose-dependently increased cell viability (Fig. 2a, c). Taurine showed potent protective effects against glutamate or H_2O_2-induced cytotoxicity. Glutamate and H_2O_2 also showed doubled ROS production, whereas taurine effectively suppressed the rises in the ROS production (Fig. 2b, d).

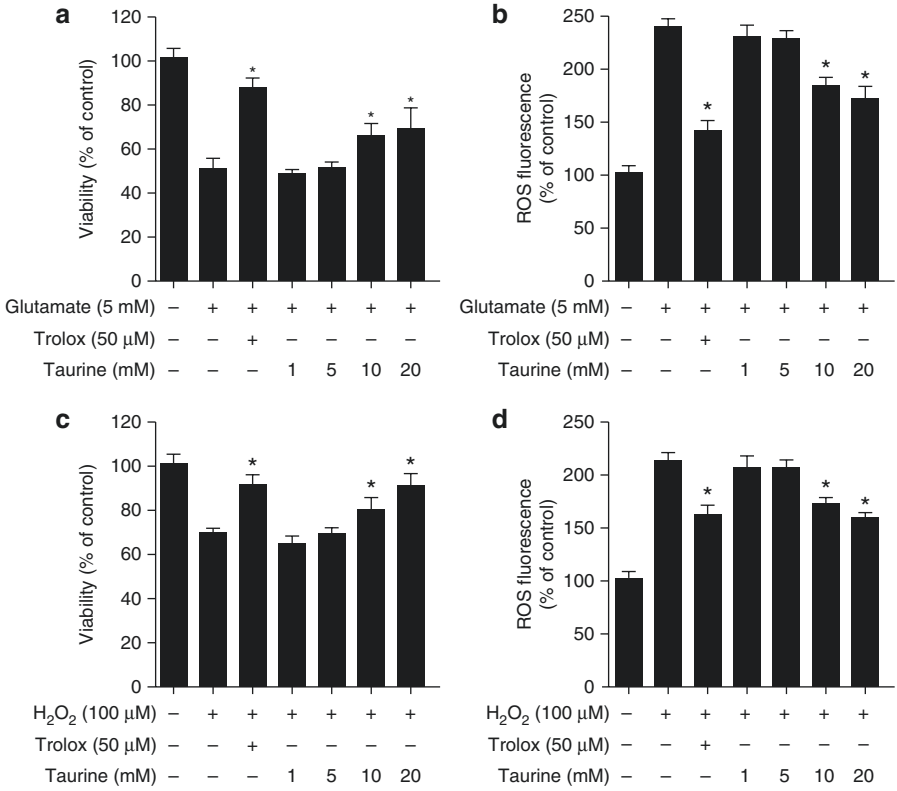

Fig. 2 The effects of taurine on glutamate or H_2O_2-stimulated neurotoxicity (**a**, **c**) and inhibition of ROS production (**b**, **d**) in HT22 cells. Data expressed as mean ± S.D. values of three independent experiments. $^*P < 0.05$ vs. glutamate or H_2O_2

3.3 *Effects of Taurine on HO-1 mRNA Expression and Nrf2-Mediated HO-1 Expression in HT22 Cells*

In the present study, taurine markedly and dose-dependently increased the expression levels of HO-1 mRNA and protein, with the maximal value at 20 mM (Fig. 3a, b). CoPP significantly induced the HO-1 expression at 20 μM. These findings indicated that the neuroprotective action of taurine against HT22 cell damage stimulated by glutamate is attributed to HO-1 expression. We treated the cells with 20 mM taurine, and then determined the Nrf2 protein level. The Nrf2 levels were time-dependently increased in the nuclear fraction, whereas gradually reduced in the cytoplasmic fractions of taurine-treated HT22 cells (Fig. 3c, d). This result indicated that HO-1 expression increased by taurine occurs through Nrf2 pathways.

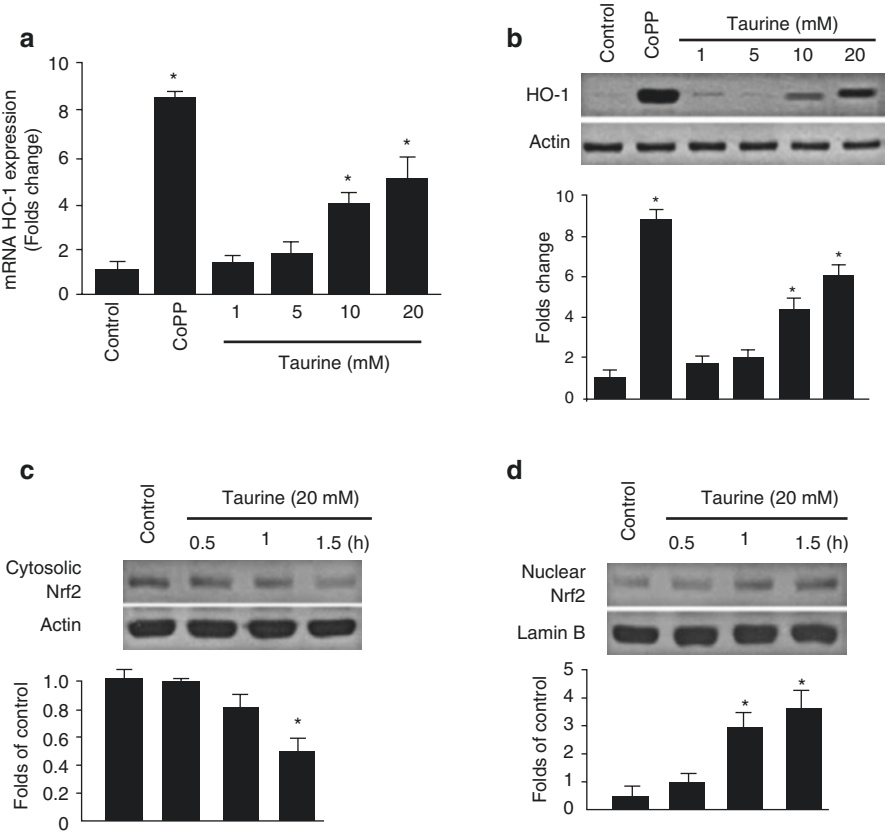

Fig. 3 Effects of taurine on HO-1 mRNA (**a**) and HO-1 protein (**b**) levels and Nrf2 translocation (**c**, **d**) in HT22 cells. Data expressed as mean ± S.D. values of 3 independent experiments. $^{*}P < 0.05$ vs. control

3.4 *Effects of Taurine on HT22 Cell Viability and ROS Reduction Through HO-1 Expression*

In this study, we also investigated whether the expression of HO-1 induced by taurine is associated with protective action on the cell death and inhibitory properties of ROS reduction. In the present study, cells were treated with 20 mM taurine for 12 h with or without in the presence or absence of SnPP, well-known as a competitive inhibitor of HO. SnPP markedly reduced the cell protective effect and ROS reduction induced by taurine (Fig. 4). These results demonstrate that taurine-induced the expression of HO-1 protein is correlated with the inhibitory effect of ROS production in HT22 cells. Additionally, SnPP partially increased the ability of taurine to inhibit the cytotoxicity and the production of ROS stimulated by glutamate or H_2O_2 (Fig. 4).

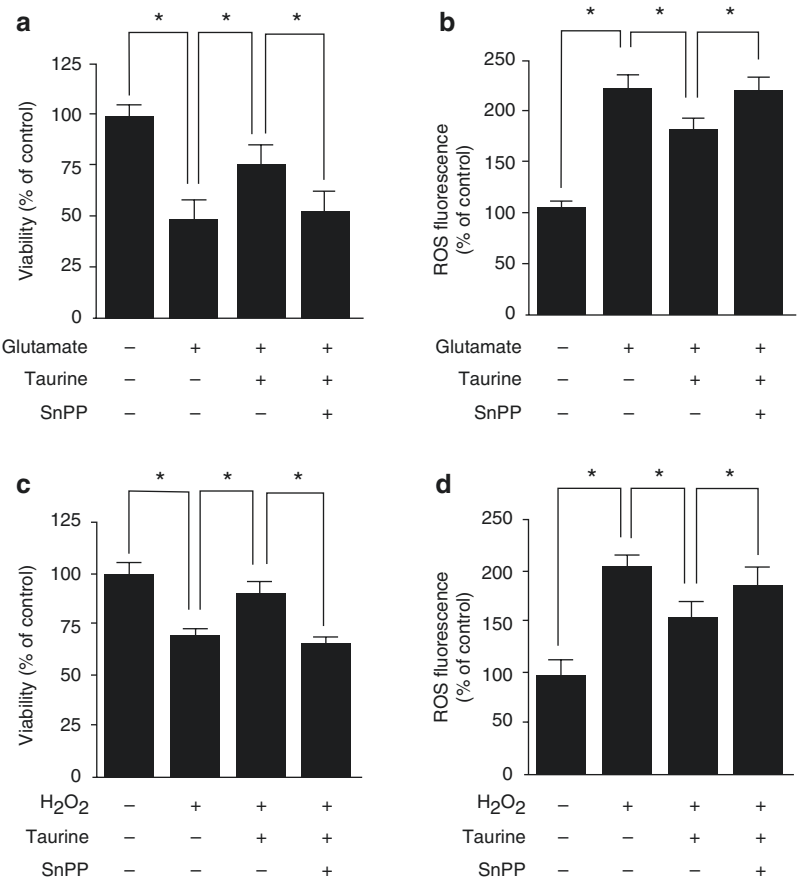

Fig. 4 Effects of Nrf2-mediated HO-1 expression induced by taurine on glutamate-stimulated neurotoxicity (**a**) and ROS production (**b**) or H_2O_2-stimulated neurotoxicity (**c**) and ROS production (**d**) in HT22 cells. Data expressed as mean ± S.D. values of three independent experiments. $^{*}P < 0.05$

3.5 *Effects of Taurine on HO-1-Related MAPK Pathway*

We determined whether taurine-mediated HO-1 expression occurs by the MAPK pathway. Figure 5a shows that pretreatment with 10 μM SB203580 (a specific inhibitor of p38) markedly reduced the taurine-induced HO-1 expression in HT22 cells. Furthermore, a p38 inhibitor (SB203580) abolished the cytoprotective effect induced by taurine, but inhibitors of extracellular signal-regulated kinase (ERK) (PD98059) or c-Jun NH_2-terminal kinase (JNK) (SP600125) did not (Fig. 5b, c). Therefore, these results suggested that taurine improves the resistance against neurotoxicity induced by glutamate or H_2O_2, via p38 MAPK-dependent HO-1 expression.

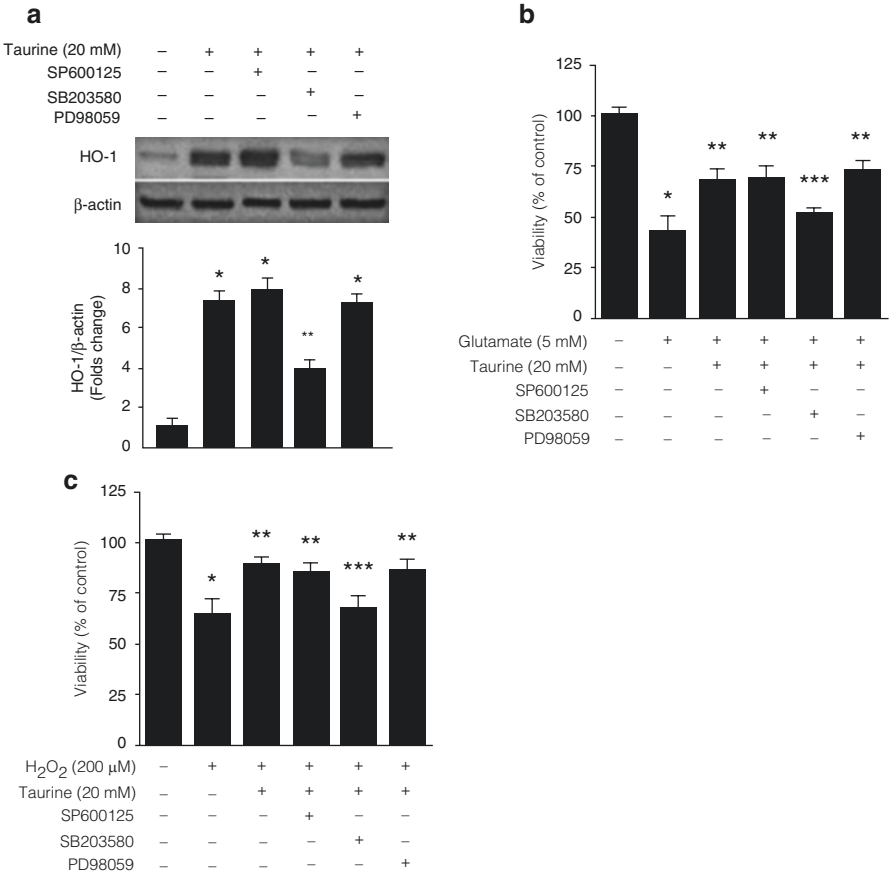

Fig. 5 Effects of HO-1 expression induced by taurine via the MAPK pathway in HT22 cells (**a**). HT22 cells were pre-incubated with or without 10 μM SP600125 (JNK inhibitor), SB203580 (p38 inhibitor) or PD98059 (ERK inhibitor) for 1 h and then incubated with or without 20 mM taurine for 12 h. These cells were then exposed to 5 mM glutamate (**b**) or 200 μM H_2O_2 (**c**) for 12 h. Data expressed as mean ± S.D. values of three independent experiments. $^{*}P < 0.05$ vs. control. $^{**}p < 0.05$ compared with the group treated with taurine. $^{***}P < 0.05$ compared with the group treated with taurine and SP600125, SB203580 or PD98059

4 Discussion

Oxidative stress is very important characteristic of many neurodegenerative processes. Several neurological injuries including stroke, Alzheimer's disease, Parkinson's disease, and Huntington's disease, have been related to the excitotoxicity and over-activation of glutamatergic transmission (Maragos et al. 1987; Greenamyre 1993). Glutamate is able to act as an inducer of oxidative stress by leading to depletion of glutathione, increasing ROS generation, and suppressing the cystine/glutamate transport system (Choi 1988; Mattson 2000). Several previous studies have reported that ER stress in neurons is also mediated by glutamate toxicity (Yu et al. 1999; Kitao et al. 2001). On the other hand, it has been reported that taurine protects the cultured retinal neurons against the neurotoxicity induced by glutamate receptor agonists and β-amyloid peptide (Aβ) (Louzada et al. 2004). Hydrogen peroxide (H_2O_2) is also a toxic product of aerobic metabolism and is produced abundantly in inflammatory tissues. A high dose of H_2O_2 is used as a disinfectant, but often leads to host cell damage (Chow et al. 2005).

Taurine (2-aminoethanesulfonic acid) is the predominant free amino acid in animal tissues including cardiac and skeletal muscles with a concentration of 20–70 mmol/kg (Chapman et al. 1993). Although taurine is not able to synthesize proteins within the body, it plays several important roles including calcium flux, membrane stabilization, bile acid conjugation as well as detoxification. Therefore, taurine deficiency can occur various chronic disease such as neurological and hepatic disorders, diabetes, obesity, cancer, and cardiovascular diseases (Hansen 2001; Balkan et al. 2002).

In this study, we examined the protective effects of taurine against glutamate- or H_2O_2-induced oxidative neurotoxicity in HT22 cells. In HT22 cells, taurine showed the inhibitory effect against glutamate- or H_2O_2-stimulated ROS generation by inducing HO-1 expression. When the cells are exposed to several oxidative stresses, they are able to induce the genes expression encoding the phase II detoxifying enzymes, which are involved in the activation of several transcription factor including Nrf2 (Copple et al. 2008). In this study, we also showed that taurine caused nuclear accumulation of Nrf2 in mouse hippocampal HT22 cells. These results suggest that taurine-mediated HO-1 expression induces by the Nrf2 signaling in mouse hippocampal HT22 cells. The HO-1 induction is an adaptive cellular defense response to several oxidative stresses that protects cells in pathophysiological conditions (Song et al. 2007). Similar to our results, some previous studies for neurodegenerative disorder have demonstrated the neuro-protective effects of Nrf2 activation-mediated HO-1 expression (Copple et al. 2008), particularly reduction of oxidative stress (De Vries et al. 2008). Agca et al. (2014) reported that taurine treatment partially overcome the HO-1 expression by activating the Nrf2 expression *in vivo* in an animal model.

In contrast, the MAPK pathway acts an important role in the induction of various pro-inflammatory cytokines and mediators by LPS (Jung et al. 2010; Kim et al. 2013). Taurine inhibited the myocardial pathophysiology induced by arsenic and

attenuated the NF-κB activation through the p38, JNK, IKK, and MAPK signaling pathways (Ghosh et al. 2009). In the present study, we evaluated whether taurine induced expression of HO-1 through the MAPK pathway. Our results indicate that HT22 cells treated with a p38 MAPK inhibitor (SB203580) exhibited significantly attenuated HO-1 expression and abolished cytoprotection mediated by taurine. In contrast, inhibitors of extracellular signal-regulated kinase (ERK) (PD98059) or c-Jun NH2-terminal kinase (JNK) (SP600125) had no such effect. These results indicated that taurine improved the resistance against glutamate- or H_2O_2-induced oxidative damages through p38 MAPK signaling pathway-Nrf2/ARE-dependent HO-1 expression in mouse hippocampal HT22 cells. Further studies to demonstrate the action of the MAPK signaling pathways in the induction of HO-1 expression are underway.

5 Conclusion

The results of the present study suggest that taurine prevents the oxidative damage induced by glutamate or H_2O_2 and it can also induce HO-1 expression through Nrf2 activation and MAPK pathways, which plays a major role for neuroprotective effect in HT22 cells. Our study is the first to show that taurine activates HO-1 signaling induced by Nrf2 and exerts an anti-oxidant effect against H_2O_2- or glutamate-induced neurotoxicity. This study suggests that the taurine might be a candidate agent for the prevention of neurodegenerative disorders through Nrf2/HO-1 signaling.

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Ethanol-Induced Taurine Elevation in the Rat Dorsal Striatum

Lisa Ulenius, Louise Adermark, Bo Söderpalm, and Mia Ericson

Abstract In the search for the primary mechanism underlying the dopamine elevating properties of ethanol we have established that raised levels of taurine in the nucleus accumbens (nAc) is pivotal. In the nAc, the release of taurine appears to be connected to osmoregulation, and neither taurine nor dopamine is increased if ethanol is administered in a hypertonic saline solution. However, even though the nAc is important for drug-reinforcement, manifestation of addiction has been postulated to recruit the more dorsal parts of the striatum (DS). How ethanol influences dopamine and taurine in the DS and their role in addiction is thus far poorly understood. By means of in vivo microdialysis in freely moving rats we concomitantly monitored extracellular levels of dopamine and taurine in the DS following administration of ethanol diluted either in an isotonic or hypertonic saline solution. In a different set of rats, placed in a voluntary ethanol consumption paradigm (intermittent access to 20% ethanol for 2 months), taurine and dopamine were monitored following an acute injection of ethanol. We found that neither administration of ethanol diluted in a hypertonic saline solution, nor 2 months of moderate ethanol consumption, influence the ethanol-induced increase of taurine in the DS. We propose that there may be regional differences in the relationship between taurine, dopamine and ethanol in the nAc and in the DS. It remains to be determined if this subregion-specificity is important for the transition from recreational drug use to a compulsive habit.

Keywords Alcohol • Taurine • Dopamine • Rat • Dorsal striatum • Microdialysis

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Abbreviations

DS	Dorsal striatum
GABA	Gamma-aminobutyric acid
GlyR	Glycine receptor
HPLC	High-performance liquid chromatography
nAc	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptor
TauT	Taurine transporter
VTA	Ventral tegmental area

1 Introduction

When trying to understand the development of addiction, a brain disease known to cause enormous suffering worldwide, much focus has been put on defining the mechanisms by which the addictive drug increase dopamine in the mesolimbic dopamine system. Ethanol, an addictive substance with a rich pharmacology, is known to modestly increase dopamine output in the nucleus accumbens (nAc), which in turn may produce positive reinforcement (Di Chiara and Imperato 1988; Wise and Rompre 1989; Spanagel 2009). The exact mechanism by which this occurs has not been determined. However, studies from our research group suggest that ethanol increases dopamine via a neuronal circuitry involving direct or indirect activation of glycine receptors (GlyR) in the nAc and indirect activation of nicotinic acetylcholine receptors (nAChR) in the ventral tegmental area (VTA), presumably through inhibition of GABAergic neurons projecting from the nAc to the VTA (Blomqvist et al. 1997; Ericson et al. 2003; Soderpalm et al. 2009). One putative candidate for mediating this effect is the endogenous GlyR agonist taurine (Olive 2002). Taurine (2-aminoethanesulfonic acid) is a semi-essential endogenous amino acid mainly produced in the liver but also in the brain (Lambert et al. 2015). Taurine-containing cells are abundant in the nAc (Madsen et al. 1987) and high expression of the taurine transporter (TauT) is found in GABAergic neurons projection to areas with dopaminergic cell bodies (Clarke et al. 1983; Liu et al. 1992). We, and others, have shown that taurine is released in response to ethanol (Dahchour et al. 1996; Ericson et al. 2011), and that taurine may increase accumbal dopamine levels via activation of GlyR (Ericson et al. 2006). In fact, research from our laboratory has shown that an increase in extracellular taurine appears to be required for the ethanol-induced dopamine release (Ericson et al. 2011). However, the exact mechanism by which ethanol produce increased levels of taurine is not known. We hypothesize that the ethanol-induced increase of taurine is related to the osmoregulatory properties of the amino acid, since administration of ethanol diluted in a hypertonic saline solution failed to produce an increase of nAc taurine (Quertemont et al. 2003; Ericson et al. 2011).

In addition to nAc, dopamine signaling is also important in the dorsal striatum (DS), a key brain region for habit formation (Packard and Knowlton 2002; Gerdeman et al. 2003; Yin et al. 2008; Chen et al. 2011). It has been suggested that during the progress of addiction the behavior turns from reward-driven to habit-driven drug-seeking behavior and that this is associated with a neuroanatomical progress from the nAc to the DS (Gerdeman et al. 2003). How ethanol influences dopamine and taurine in the DS and their role in addiction is however poorly understood.

In the present study we aimed to further explore the importance of taurine for ethanol-induced dopamine elevation with focus on the DS. By means of in vivo microdialysis in freely moving rats we concomitantly monitored extracellular levels of dopamine and taurine in the DS following administration of ethanol diluted either in an isotonic or hypertonic saline solution. In a different set of rats, with a history of voluntary ethanol consumption (intermittent access to 20% ethanol for 2 months), dopamine and taurine were monitored following an acute injection of ethanol.

2 Methods

2.1 Intermittent Ethanol Consumption

Male Wistar rats (Taconic, Ejeby, Denmark) weighing 130–250 g at arrival, had continuous access to water and was given an additional bottle of 20% alcohol three 24 h sessions per week (Sunday, Tuesday, Thursday) during 2 months (Simms et al. 2008). Water controls were housed in parallel with continuous access to one bottle of water. The bottles were weighed after every drinking session and body weight was measured once a week.

2.2 In vivo Microdialysis in Freely Moving Rats

Microdialysis probes where stereotactically implanted in the DS (A/P:+1.2, M/L:−3.4 mm to bregma, V/D:−5.0 mm relative to dura; Paxinos and Watson 2007) as previously described (Clarke et al. 2014). On the day of microdialysis experiment, the sealed inlet and outlet of the probe was cut open and connected to a microperfusion pump via a swivel, allowing the animal to move around freely. The probe was perfused with Ringer's solution at a rate of 2 μL/min. During the first set of microdialysis experiments, rats received acute treatment with vehicle or ethanol (2.5 g/kg, i.p.) diluted either in an isotonic (NaCl 0.9%) or hypertonic (NaCl 3.6%) saline solution. In the second experiment, following 2 months of intermittent ethanol consumption, rats received 2.75 g/kg ethanol i.p. Dialysate samples were collected every 20 min and analyzed for dopamine and taurine using two separate high-performance liquid chromatography (HPLC) systems as previously described (Lido et al. 2009).

2.3 Statistic Analysis

Statistical significance was determined by two-way analysis of variance (ANOVA) repeated measure (treatment group x time), followed by Tukey's multiple comparisons test. A probability value (p) less than 0.05 was considered statistically significant. All values are expressed as mean ± SEM.

3 Results

3.1 Ethanol Administrated in a Hypertonic Saline Solution does not Influence the Ethanol-Induced Release of Taurine in the Dorsal Striatum

In contrast to previous studies performed in the nAc (Quertemont et al. 2003; Ericson et al. 2011), ethanol diluted in a hypertonic saline solution (3.6%) did not prevent the ethanol-induced increase of taurine in the DS. Ethanol diluted in an isotonic saline solution (0.9%) or in a hypertonic saline solution (3.6%) significantly elevated the extracellular levels of taurine (Fig. 1a). Statistical analysis showed a group effect ($F_{(3, 23)} = 4.31$, $p = 0.0149$), a time effect ($F_{(9, 207)} = 12{,}35$, $p < 0.0001$), and an interaction between the groups over time ($F_{(27, 207)} = 1.639$, $p = 0.0297$; Fig. 1a). Post hoc analysis demonstrated that ethanol diluted in an isotonic saline solution (0.9%) increased taurine levels as compared to saline alone (Fig. 1a), as well as when diluted in a hypertonic saline solution (3.6%) (Fig. 1a). The higher concentration of saline did not modulate taurine per se.

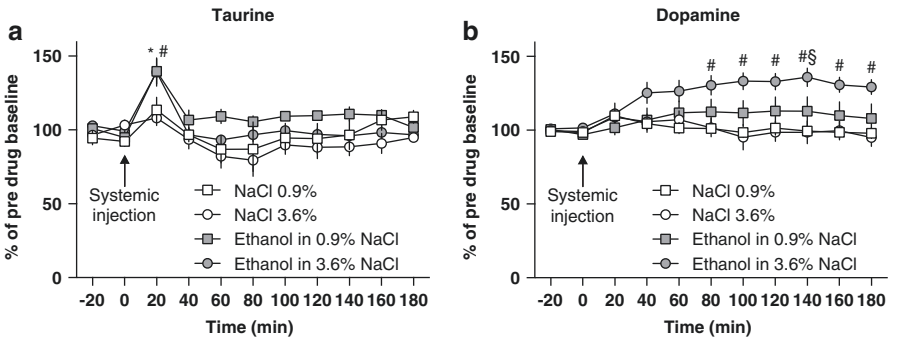

Fig. 1 Extracellular levels of (**a**) taurine and (**b**) dopamine in the DS were measured by means of microdialysis in naïve rats after administration of vehicle or ethanol (2.5 g/kg, ip) diluted either in an isotonic (NaCl 0.9%) or hypertonic (NaCl 3.6%) saline solution (n = 5–8). All data are expressed as % of baseline levels (mean ± SEM), $^{*}p < 0.05$ NaCl 0.9% to Ethanol in 0.9% NaCl; $^{\#}p < 0.05$ NaCl 3.6% to Ethanol in 3.6% NaCl $^{\S}p < 0.05$ Ethanol in 0.9% to Ethanol in 3.6% NaCl

Analyzing dopamine in the same dialysate samples revealed a group effect ($F_{(3, 25)} = 4.911$, $p = 0.0081$), a time effect ($F_{(9, 225)} = 4.846$, $p < 0.0001$) and an interaction between the groups over time ($F_{(27, 225)} = 3.639$, $p < 0.0001$; Fig. 1b). Post hoc analysis demonstrated that ethanol diluted in an isotonic saline solution (0.9%) did not affect dopamine levels as compared to saline alone (Fig. 1b). In contrast, ethanol diluted in a hypertonic saline solution (3.6%) increased dopamine levels as compared to saline alone and compared to ethanol diluted in an isotonic solution (Fig. 1b).

3.2 Two Months of Moderate Ethanol Consumption does not Influence the Ethanol-Induced Elevation of Taurine in the Dorsal Striatum

Animals had intermittent access to ethanol 3 times/week over a 2-month period resulting in a total of 25 sessions. The animals declined in ethanol intake over time (paired t-test between the average first and last three drinking sessions; $p < 0.0001$). The mean overall ethanol consumption, calculated as an average of the last five drinking sessions, was 2.22 ± 0.11 g/kg/session (Fig. 2a). In vivo microdialysis performed in animals with a history of either ethanol or water consumption (control animals) demonstrated no significant difference with respect to the relative increase in taurine (Fig. 2b) or dopamine (Fig. 2c) induced by a systemic administration of ethanol (2.75 g/kg, i.p.). Statistical analysis of extracellular taurine content after ethanol found no group effect ($F_{(1, 18)} = 0.9590$, $p = 0.3404$), but a time effect ($F_{(5, 90)} = 13.33$, $p < 0.0001$) and no interaction between the groups over time ($F_{(5, 90)} = 0.7507$, $p = 0.8577$; Fig. 2b). Statistical analysis of extracellular dopamine after ethanol found no group effect ($F_{(1, 17)} = 0.03$, $p = 0.86$), but a time effect ($F_{(5, 85)} = 12.74$, $p < 0.0001$) and no interaction between the groups over time ($F_{(5, 85)} = 0.43$, $p = 0.83$; Fig. 2c).

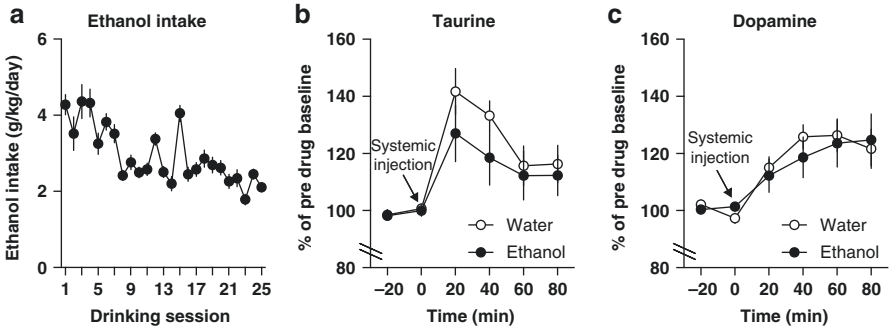

Fig. 2 (**a**) Intermittent voluntary ethanol consumption during 2 months (n = 19). (**b**) Extracellular taurine levels in the DS following an acute injection of ethanol (2.75 g/kg i.p.), in water drinking rats or rats with a previous history of ethanol consumption. (**c**) Extracellular dopamine levels in the DS following an acute injection of ethanol (2.75 g/kg i.p.), in water drinking rats or rats with a previous history of ethanol consumption (n = 7–12). All values are expressed as mean ± SEM

4 Discussion

Ethanol has for some time been known to elevate taurine in the nAc (De Witte et al. 1994; Dahchour et al. 1996), a phenomenon that appears crucial for ethanol-induced extracellular dopamine increase in the same region (Ericson et al. 2011). Since taurine is a key player in the dopamine elevating properties of ethanol in the mesolimbic dopamine system, an effect associated with addiction (Volkow et al. 2007), understanding the underlying mechanism as to why ethanol produces this increase of taurine is of major importance. As addiction is suggested to include an alteration of behavior, from reward-driven to habit-driven drug intake, exploring dorsal parts of the striatum is relevant. Whether taurine also has a major influence on ethanol-induced increases of dopamine in the dorsal striatum is poorly understood. In line with the findings of Smith and colleagues (Smith et al. 2004) we here found that systemically administrated ethanol increases extracellular taurine in the DS. In the same animals, ethanol was unable to produce increased levels of dopamine. However, in the second study ethanol-naïve control animals responded with a modest elevation of dopamine following an acute injection of ethanol. This conflicting result can also be found in the literature where there are reports of both a significant dopamine elevation following ethanol administration (Wozniak et al. 1990; Clarke et al. 2015; Jamal et al. 2016) as well as a lack thereof (Wang et al. 1997). Whether the conflicting results are due to sub regional differences within the DS or to other factors remains to be established.

Neurons and glia cells swell in response to increased external osmolarity (Allansson et al. 1999). Consequently taurine is released as part of re-equilibrating the osmotic pressure (Solis et al. 1988; Pasantes-Morales et al. 1993; Moran et al. 1994; Vitarella et al. 1994; Deleuze et al. 1998). Elevated levels of taurine are also found in ethanol-treated astrocyte cultures (Kimelberg et al. 1993) and when preventing ethanol-induced cell swelling by using the $Na^+/K^+/2Cl^-$ co-transporter blocker furosemide there was no ethanol-induced increase of taurine (Adermark et al. 2011). These findings raise the possibility that taurine is released due to an altered osmotic environment after ethanol administration. In contrast to the nAc (Ericson et al. 2011) we were not able to block the ethanol-induced taurine release in the DS by administrating ethanol in a hypertonic saline solution. This was unexpected and may question the hypothesis that ethanol produce increased levels of taurine due to osmoregulation alone. Additional studies need to explore this in depth. Furthermore, in the same samples we also found that following administration of hypertonic ethanol there was an additional increase of dopamine in the DS, an effect opposite to what was previously observed in the nAc (Ericson et al. 2011). It is possible that elevated levels of sodium or chloride might affect the balance of striatal microcircuits, thereby facilitating ethanol-induced dopamine release. The dorsal striatum and nAc were shown to exhibit distinct kinetics of dopamine release and clearance (Shu et al. 2014). We also found that GlyRs mediate ethanol-induced dopamine signaling in the nAc but not in the DS (Clarke et al. 2015). Taken together this and previous findings further support the hypothesis that ethanol-induced dopamine

elevation is differentially regulated in the DS as compared to the nAc, which could be important when considering mechanisms underlying the addictive phenotype.

The DS is proposed to be recruited during the transition from recreational to compulsive use of reinforcing drugs like ethanol (Gerdeman et al. 2003), and altered activity in the DS has been shown in addicted humans (Volkow et al. 2007). Moderate ethanol consumption over long periods of time is a risk behavior for development of addiction. It is therefore important to study possible adaptations in the dopaminergic system during these conditions. In the second experiment, we wanted to investigate possible adaptations of ethanol-induced elevations of either taurine or dopamine in the DS following 2 months of voluntary ethanol intake. We found that moderate ethanol consumption does not influence the ethanol-induced elevation of taurine or dopamine in the DS as compared to water drinking controls. This would suggest that the ethanol-induced increase of taurine in the DS is a robust phenomenon or that the amount of ethanol consumed or duration of time is to little to cause adaptations.

5 Conclusion

The findings of the present study suggest that there may be regional differences in the relationship between taurine, dopamine and ethanol in the nAc and in the DS. Whether the ethanol-induced increase of taurine is due to osmoregulatory properties of the amino acid in the nAc but not in the DS remains to be established. Furthermore, ethanol's ability to increase taurine appears to be rather robust since 2 months of modest ethanol consumption was unable to alter ethanol-induced elevation of taurine in the DS.

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Taurine Ameliorates Arsenic-Induced Apoptosis in the Hippocampus of Mice Through Intrinsic Pathway

Shuangyue Li[$], Lijun Yang[$], Yan Zhang, Cong Zhang, Jing Shao, Xiaohui Liu, Yachen Li, and Fengyuan Piao

Abstract Our group previously reported that arsenic (As) exposure induced apoptosis in hippocampus neurons. The aim of the present study was to clarify the protective capacity of taurine (Tau) on As-induced neuronal apoptosis and the related mechanism in mouse hippocampus. Mice were divided into: control group, Tau control group, As exposure group and Tau protective group, randomly. The apoptotic rate of mouse hippocampus was determined by TUNEL staining. The levels of Bcl-2 and Bax gene and protein were analyzed by real time RT-PCR and WB, respectively. Furthermore, cytochrome c (Cyt C) release, and the activity of caspase-8 and caspase-3 were also determined. The results showed that Tau treatment induced the decrease of TUNEL-positive cells, prohibited the disturbance of Bcl-2 and Bax expression, and inhibited Cyt C release and caspase-8 and caspase-3 activation significantly. The results indicated that Tau supplement markedly ameliorates As-induced apoptosis by mitochondria-related pathway in mouse hippocampus.

Keywords Arsenic • Taurine • Apoptosis • Hippocampus • Intrinsic pathway

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Abbreviations

As	Arsenic
Cyt C	Cytochrome c
Tau	Taurine

1 Introduction

Arsenic (As), a naturally toxicant existing in both organic and inorganic forms, is universal found in food, groundwater, ambient air and dust. As toxin, being inorganic and/or organic compound, is widely spread in water and environment which was resulted by the increasing pollution. Several researches indicated that inhalated- or ingested-exposure of As involved in the more and more chronic diseases which include diabetes mellitus, cardiovascular disease, peripheral vascular disease and various cancers, and one of the major targets is nerve system (Ferreccio et al. 2000, Chen and Ahsan 2004, Meliker et al. 2007, Huang et al. 2008, Das et al. 2012a, b). It was illustrated that As exposure induces dysfunctions in nervous system or severe signs of neuropathy in animal and human models (Meliker et al. 2007; Sinczuk-Walczak 2009; Zhang et al. 2013). Impaired learning ability and neural behaviors were observed in many As-intoxicated rodents at environmental relevant levels (Sinczuk-Walczak 2009; Zhang et al. 2013).

The hippocampus, one of the most important components of brain, is responsible for several vital functions, including behaviours, mental and intellectual activities, in both rodents and human. Moreover, the hippocampus is very susceptible to the toxicities of some neurotoxins such as ethanol, lead, $MnCl_2$ and CuO (Sharifi et al. 2002; Oliveira-da-Silva et al. 2009; Sinczuk-Walczak 2009; Wang et al. 2012; Zhang et al. 2013). It was reported that the gene levels of caspase-3 and 9, the effectors of apoptosis, were markedly elevated in hippocampus of As-intoxicated rats (Zhang et al. 2013). Our group recently found that As intoxication caused apoptosis in hippocampus neurons by showing the increasing number of Tunel-positivity cells (Wang et al. 2015). These researches indicated abnormal apoptosis induced by As exposure in hippocampus may involve in the neurotoxicity induced by As exposure.

Apoptosis, the basic process under both physiological and pathological condition, regulates cell death according to surrounding environmental (Namgung and Xia 2000). Disruption or over-activation of this process, the programmed cell death, may lead to the pathogenesis of nervous system. Thus, minimizing neuronal apoptosis and promoting injured neuron recovery may be the most effective and direct therapeutic approach to reduce the neurotoxic effects of As.

Taurine, a natural amino acid, exists in mammalian brain and acts as a functionally neurotransmitter or neuromodulator. It modifies protein phosphorylation, regulates calcium transport and maintains the structural integrity of membrane in nerve tissue (Zhou et al. 2011). It has been shown that Tau protected nervous system against injury, acting

as an antioxidant and apoptosis inhibitor. However, the protective capacity of Tau on As-induced apoptosis in hippocampus and its related mechanism is unknown.

In the present study, Tunel assay was used to examine the capacity of Tau on As-induced apoptosis in hippocampus. To clarify the mechanism of anti-apoptotic potential of Tau, Bax and Bcl-2 expression in hippocampus was examined by real-time PCR and Western blotting (WB). The release of cytochrome c (Cyt C) was examined by WB, and the activity of caspase-8 and caspase-3 were analyzed by commercial kits. The study aimed at illustrating the neuroprotection of Tau on As-induced apoptosis in mouse hippocampus and exploring its related mechanism.

2 Materials and Methods

2.1 Animal and Treatment

19.2 ~ 24.7 g male mice were provided by Animal Center, Dalian Medical University. During experiments, all mice were raised under 20 ~ 24 °C temperature, 55% humidity, 12 h dark-light cycle environment with an ad libitum diet and water. The 30 mice were divided into control group, As exposure group and Tau protection group randomly. As exposure group exposed to 4 mg/L As_2O_3 in double-distilled water orally; tau protection group received 4 mg/L As_2O_3 in double-distilled water orally and 150 mg/kg Tau once daily by gavage; control group only received double-distilled water. After 60-day treatment, all model mice were sacrificed and samples were collected carefully. The animal experiments were carried out according to the guidelines of the committee of Dalian Medical University.

2.2 Tunel Assay

In Situ Cell Death Detection Kit (Roche, Germany) was used to perform Tunel assay according to instructions. DAPI staining was performed to counter the number of cells. To quantify the apoptotic cells, 6 fields were randomly selected per slide under fluorescence microscope and calculated the apoptosis index (AI). AI score equals the percentage of number of Tunel-positive cells on total number of cells.

2.3 Real Time RT-PCR

Trizol® reagent (Takara, China) was used to extract RNA sample according to the instructions. Transcriptor First Strand cDNA Synthesis Kit (Roche,\USA) was used to perform RT reactions. TP800 System and SYBR Green PCR kit (Takara, Japan) were used to carry out real time RT-PCR. 95″C 5 min, followed by 95″C for 30 s, 40 cycles,

then 55″C 30 s, 72″C 30 s were used as reaction conditions. The primers for genes of interest and β-actin are as followed: Bcl-2, GACTGAGTACCTGAACCGG CATC, CTGAGCAGCGTCTTCAGAGACA; Bax, CGAATTGGCGAT GAACTGGA, CAAACATGTCAGCTGCCACAC; β-actin, GGAGAT TACTGCCCTGGCTCCTA, GACTCATCGTACTCCTGCTTGCTG.

2.4 Western Blot

Total proteins were extracted from liver tissue with lysis buffer. BCA method was used to qualify protein concentration. SDS-polyacrylamide gel electrophoresis was carried out with same gram of loading sample protein, and the protein samples were transferred to a PVDF membrane. After blocking, the membrane was incubated with Bax, Bcl-2 (1:800), Cyt C (1:1000) and β-actin (1:500) primary antibodies, respectively. The blots were treated with HRP-conjugated secondary antibodies, and then detected by Bio-Rad imaging system (Bio-Rad, USA), and then qualified with the Gel-Pro software.

2.5 Caspase-8 and Caspase-3 Activity Detection

Caspase-8 and Caspase-3 Colorimetric Assay Kit (Beyotime, China) was used to detect caspase activities of liver according to the manufacturer's manipulations. The liver lyses were incubated in ice-cold lysis buffer for 20 min, then centrifuged at 10,000 × g 2 min. The related results were showed as a ratio to control.

2.6 Statistical Analysis

Statistical analysis was performed with SPSS 11.0 statistical software. Data were analyzed using one-way ANOVA and expressed as means ± SD in triplicate.

3 Results

3.1 Protective Capacity of Tau on As-Induced Apoptosis in Mouse Hippocampus

Tunel staining was performed to detect the apoptotic cells in hippocampus. As shown in Fig. 1, an elevated quantity of TUNEL-positive cells were observed in As-intoxicated mouse hippocampus, whereas almost no Tunel-positive cells was observed in both saline and Tau alone-treated mouse hippocampus, indicating As intoxication induced apoptosis in hippocampus. Interestingly, compared with As

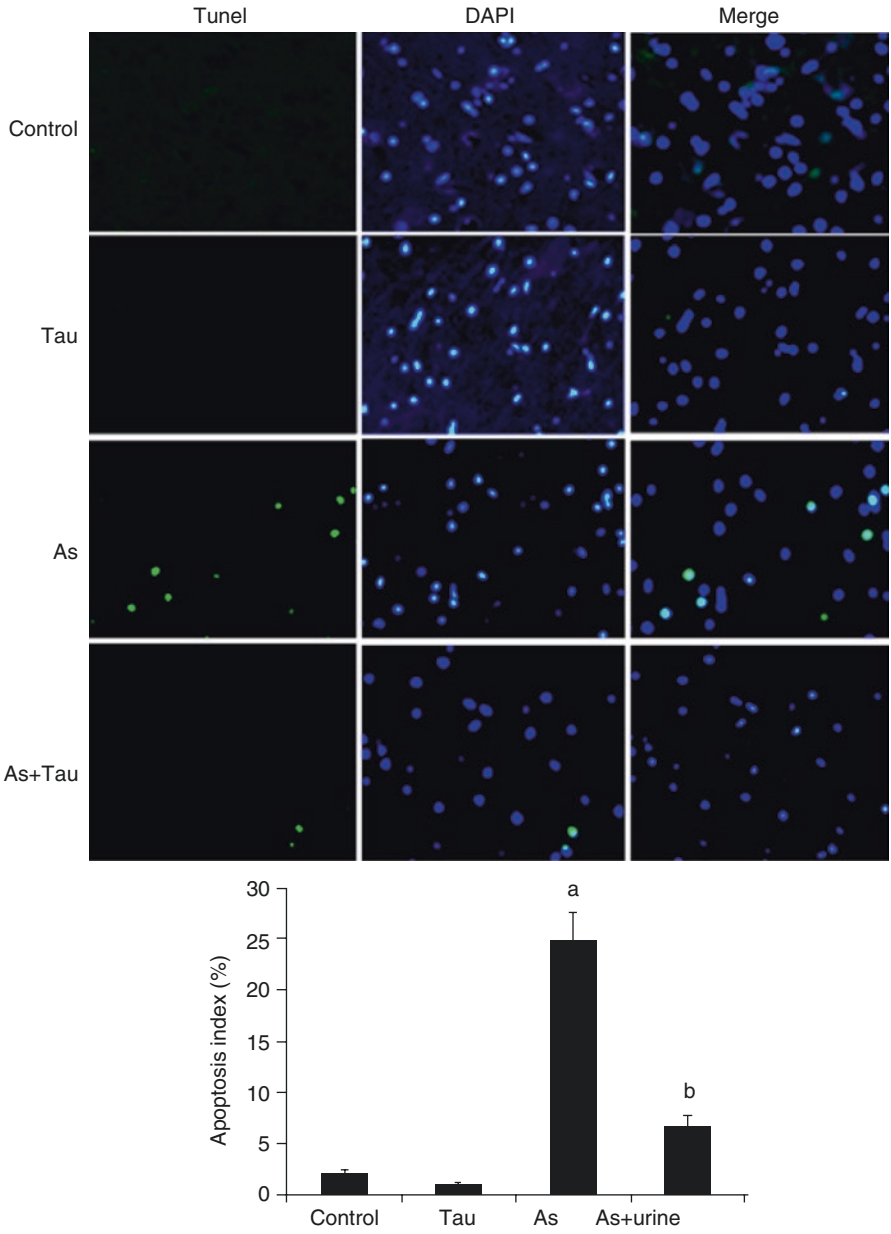

Fig. 1 Effect of Tau on apoptosis induced by As in mouse hippocampus. The apoptotic rates were determined by TUNEL staining. Green color indicates apoptosis. Counterstaining with DAPI, blue color, suggests cell nuclei. TUNEL-positive cell percent was quantified as apoptotic index. [a]$p < 0.05$, vs. control group; [b]$p < 0.05$, vs. As group

alone group, Tau treatment markedly reduced the quantity of Tunel-positive cells. The increased apoptosis induced by As was significantly reversed by Tau treatment, suggesting Tau attenuates As-induced apoptosis in mouse hippocampus.

3.2 *Protective Capacity of Tau on Bax and Bcl-2 Expression in As-Exposed Hippocampus*

Level of Bax and Bcl-2 were examined by both real time RT-PCR (Fig. 2a, b) and WB (Fig. 2c, d). As Fig. 2a showed that Bax gene expression was markedly increased in As group than that in control group. However, after treating with Tau, the gene expression of Bax was markedly decreased. On the other hand, compared with control group, As intoxication markedly decreased the gene expression of Bcl-2 in hippocampus, which was significantly reversed in protective groups (Fig. 2b). The similar effects also observed in protein level of Bax and Bcl-2 in Tau protect group (Fig. 2c, d).

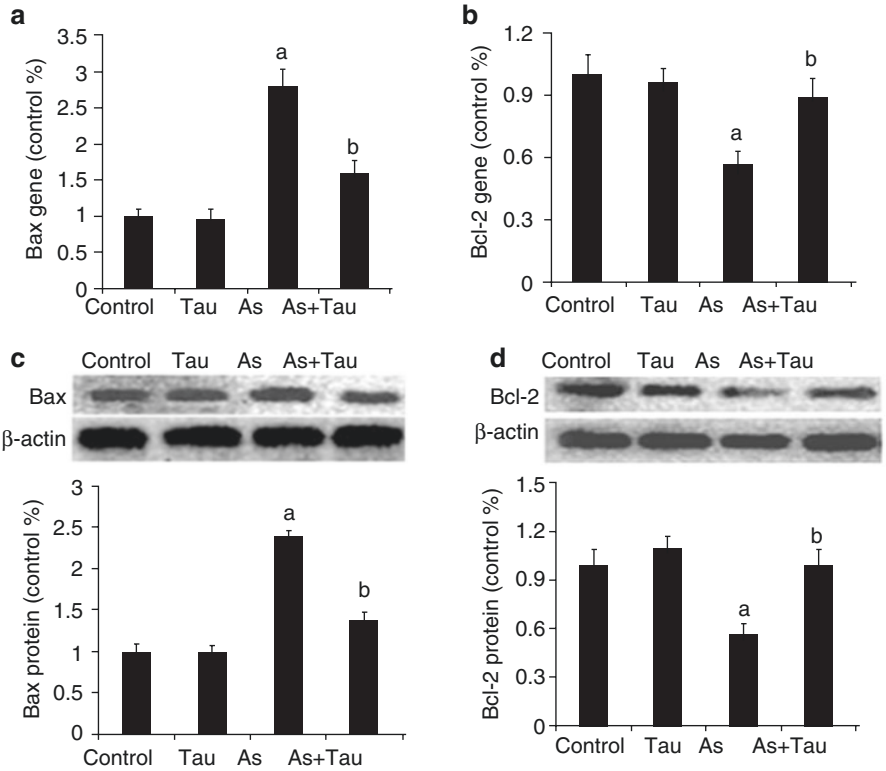

Fig. 2 Effect of Tau on Bax and Bcl-2 expression in As-exposed hippocampus. Real time RT-PCR (**a**, **b**) and WB (**c**, **d**) were used to examine Bax and Bcl-2 expression. $^{a}p < 0.05$, vs. control group; $^{b}p < 0.05$, vs. As group

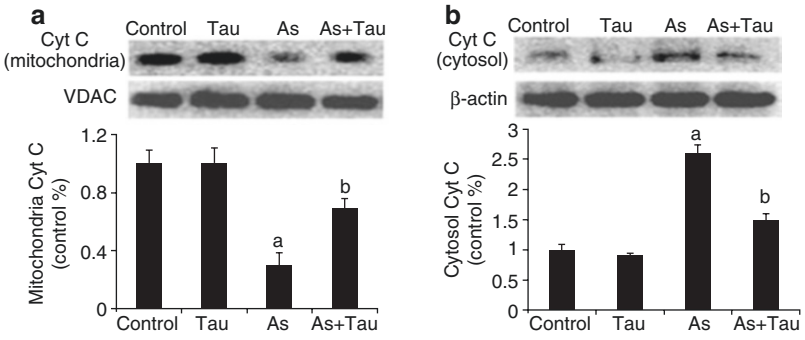

Fig. 3 Effect of Tau on cytochrome c level in mitochondria (**a**) and cytosol (**b**) in hippocampus of As-intoxicated mice. [a]$p < 0.05$, vs. control group; [b]$p < 0.05$, vs. As group

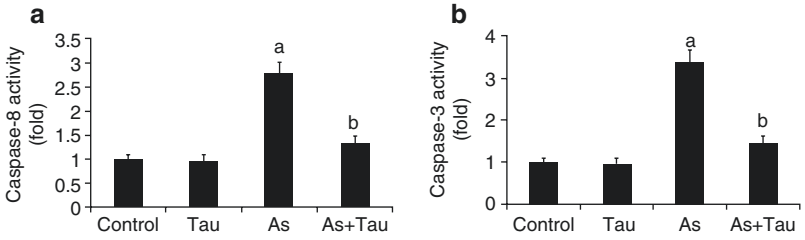

Fig. 4 Effect of Tau on caspase-8 and caspase-3 activity in As-exposed hippocampus. [a]$p < 0.05$, vs. control group; [b]$p < 0.05$, vs. As group

3.3 *Protective Capacity of Tau on Cyt C Expression in Hippocampus of As-Exposed Mice*

As Fig. 3a showed the protein level of Cyt C in mitochondria significantly decreased in As-exposed hippocampus compared to control group. On the other hand, the protein level of Cyt C in cytosol significantly increased in As-exposed hippocampus (Fig. 3b). These results indicate As exposure caused mitochondrial Cyt C release. Interestingly, the release was abolished when As-intoxicated mice were treated with Tau (Fig. 3a, b).

3.4 *Protective Capacity of Tau on Activities of Caspase-8 and Caspase-3 in As-Intoxicated Hippocampus*

Activities of caspase-8 and caspase-3 in As-intoxicated hippocampus were shown in Fig. 4. Compared with control group, caspase-8 activity in As-exposed hippocampus was markedly higher. Interestingly, Tau reversed the activation of caspase-8 in As-treated mice compared with As alone group. Consistent with that of caspase-8, As intoxication also induced the activation of caspase-3, which was blocked by Tau treatment (Fig. 4b).

4 Discussion

Increasing evidences showed that taking an apoptosis inhibitor may be a practical approach for the protection against nervous system disorders. Currently, the anti-apoptotic potential of Tau has been highlighted in many disorders (Hsu et al. 2008; Leon et al. 2009; Das et al. 2010; Taranukhin et al. 2010; Gao et al. 2011; Das et al. 2012a, b). Moreover, it was reported that Tau treatment could also prevent from apoptosis induced by various toxicants in vitro (Leon et al. 2009; Zhou et al. 2011; Chang et al. 2014). In the present study, the anti-apoptotic capacity of Tau was assayed in hippocampus of mice received As alone or with Tau by Tunel staining. As expected, As-induced elevation of Tunel-positivity cells in hippocampus was markedly blocked by Tau treatment, indicating that Tau has the efficacy to protect against As-induced apoptosis in hippocampus of mice.

Intrinsic pathway is the major intracellular signaling leading to apoptosis, which is accompanied by the dysregulation of Bcl-2 family protein and Cyt C release, where Cyt C eventually activates caspase-3 (Mikhailov et al. 2003; Wang et al. 2013). Bax and Bcl-2 are the representative regulators of Bcl-2 family, which play a vital role in mediating apoptosis process (Braun 2012). It was reported that As intoxication increased Bax level and decreased Bcl-2 level in cultured mesenchymal cells (Zhou et al. 2007). However, there are no reports that the MSCs or Tau treatment mediates the disturbed expression of Bax and Bcl-2 in As-intoxicated nerve cells. In the present study, the results suggested that Tau significantly inhibited the disrupted expression of Bax and Bcl-2 in hippocampus. Bax/Bcl-2 ratio was also assessed in this study, which is markedly decreased in hippocampus once As-intoxicated mice were treated with Tau. These results indicate a potential link between mediating the disturbed expression of Bax and Bcl-2 and the anti-apoptotic capacity of Tau in hippocampus of As-exposed mice.

Several evidences illustrated that the disturbance of Bax and Bcl-2 expression led to the efflux of mitochondrial Cyt C, which activates the downstream caspase cascade (Wang et al. 2013). Among the identified caspases, caspase-8 and caspase-3 are the important enzymes that induce the activation of apoptosis process (Braun 2012; Kadeyala et al. 2013). It was reported that Tau treatment significantly inhibited endosulfan-induced the activation of caspase-3 in rat testis (Aly and Khafagy 2014). In the present stud, we found that Tau significantly suppressed Cyt C release and the activation of caspases in As-intoxicated mouse hippocampus, indicating the trigger of apoptosis in As-intoxicated hippocampus was blocked by Tau. These studies and our results indicate that Tau represses intrinsic apoptosis pathway and the inhibited intrinsic pathway may take part in the prevention of Tau against As-induced apoptosis in mouse hippocampus.

5 Conclusion

In conclusion, the results indicated that taruine inhibits As-induced apoptosis in hippocampus of mice. Moreover, treatment of Tau significantly inhibited the disturbed expression of Bax and Bcl-2, the release of Cyt C and the activated caspases in

As-exposed hippocampus. These results indicate that Tau may protect against apoptosis in hippocampus via mediating As-disturbed intrinsic pathway. As for the more precise mechanism, further studies are required.

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Neuroprotective Functions Through Inhibition of ER Stress by Taurine or Taurine Combination Treatments in a Rat Stroke Model

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Abstract Taurine, as a free amino acid, is found at high levels in many tissues including brain, heart and skeletal muscle and is known to demonstrate neuroprotective effects in a range of disease conditions including stroke and neurodegenerative disease. Using in vitro culture systems we have demonstrated that taurine can elicit protection against endoplasmic reticulum stress (ER stress) from glutamate excitotoxicity or from excessive reactive oxygen species in PC12 cells or rat neuronal cultures. In our current investigation we hypothesized that taurine treatment after stroke in the rat middle cerebral artery occlusion (MCAO) model would render protection against ER stress processes as reflected in decreased levels of expression of ER stress pathway components. We demonstrated that taurine elicited high level protection and inhibited both ATF-6 and IRE-1 ER stress pathway components. As ischemic stroke has a complex pathology it is likely that certain combination treatment approaches targeting multiple disease mechanisms may have excellent potential for efficacy. We have previously employed the partial NMDA antagonist DETC-MeSO to render protection against in vivo ischemic stroke using a rat cerebral ischemia model. Here we tested administration of subcutaneous administration of 0.56 mg/kg DETC-MeSO or 40 mg/kg of taurine separately or as combined treatment after a 120 min cerebral ischemia in the rat MCAO model. Neither drug alone demonstrated protection at the low doses employed. Remarkably however the combination of low dose DETC-MeSO plus low dose taurine conferred a diminished infarct size and an enhanced Neuroscore (reflecting decreased neurological deficit). Analysis of ER stress markers pPERK, peIF-2-alpha and cleaved ATF-6 all showed decreased expression demonstrating that all 3 ER stress pathways were inhibited concurrent with a synergistic protective effect by the post-stroke administration of this DETC-MeSO-taurine combination treatment.

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Keywords Stroke • Taurine • Apoptosis • Endoplasmic reticulum stress • DETC-MeSO

Abbreviations

DETC-MeSO	S-methyl N,N-diethylthiocarbamate sulfoxide
ER stress	ER stress
MCAO	Middle cerebral artery occlusion

17.1 Introduction

Globally, stroke is reported to be one of the highest causes of death and severe disability. Ischemic stroke, the most common type of stroke, is responsible for 87% of the total stroke incidence (Go et al. 2014). To date, despite extensive research efforts, it has not been possible to develop effective treatments for this major disease (Go et al. 2014). Cerebral ischemia decreases the oxygen and glucose supply to neural tissues and elicits excessive release of glutamate pre-synaptically in conjunction with over activation of the post-synaptic glutamate receptors which in turn results in cell death through apoptosis and necrosis (Choi and Rothman 1990; Nicholls and Attwell 1990). The core of the ischemic infarct is subjected to the most major stresses through oxygen and nutrient deprivation and this region becomes rapidly necrotic (Kiewert et al. 2010). The ischemic penumbra which surrounds the core is a region with diminished perfusion but it remains metabolically active. Because apoptosis proceeds over an extensive period of time in the penumbra there is potential for preventing cell death in this region and rescuing this tissue through new therapeutic interventions (Broughton et al. 2009).

Hypoxia/ischemia results in accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) eliciting the unfolded protein response (UPR) (DeGracia and Montie 2004). The three major ER pathways that are triggered under conditions of UPR include the PKR-like endoplasmic reticulum kinase (PERK) signaling pathway, the transcription factor 6 (ATF6) signaling pathway and the inositol requiring enzyme 1 (IRE1) signaling pathway. When the UPR is overwhelmed these pathways activate specific signaling cascades which comprise the ER stress response (DeGracia and Montie 2004). The IRE-1 and ATF-6 pathways are known to increase expression of the ER-resident chaperone glucose regulated protein 78 (GRP78) and all three major ER stress pathways activate a pro-death transcription factor CHOP which decreases levels of anti-apoptotic Bcl-2 and increases levels of pro-apoptotic Bim (Gharibani et al. 2013). An additional mechanism underlying the ER stress response is activation by PERK of caspase-12 which in turn contributes to apoptosis (Nakagawa et al. 2000).

The amino acid taurine possesses multiple important cellular functions, as a neurotransmitter, a neuromodulator, in prevention of cellular calcium overload, in osmoregulation, in membrane stabilization and as a neuroprotector (Oja and Saransaari 1996; Okamoto et al. 1983; Kumari et al. 2013). Several investigations have shown that taurine administered post-stroke can elicit a reduction in neurological deficits, brain infarct size and expression of apoptotic components including caspase-3. We have previously reported that using the middle cerebral artery occlusion (MCAO) rat stroke model taurine was capable of preventing ER stress induced apoptosis through inhibiting the ER stress components ATF6, and IRE1 but not PERK (Gharibani et al. 2013).

DETC-MeSO is a metabolite of disulfiram (also known as Antabuse-TM) which has been in use as a treatment for alcoholism. We have recently demonstrated that DETC-MeSO is effective at preventing glutamate –induced toxicity using in vitro neuronal models. Importantly we have shown that DETC-MeSO acts as a partial antagonist of glutamate receptors and contributes to preventing excessive calcium influx and overload arising from over-activation of NMDA receptors. DETC-MeSO was shown to prevent seizures in mouse models induced by NMDA or by hyperbaric oxygen.

17.2 Materials and Methods

17.2.1 Animal Use

All animal procedures were carried out according to the NIH guide for care and use of animals using protocols and procedures that were reviewed and approved by the Institutional Animal care and Use Committee of Florida Atlantic University, Boca Raton, FL. Adult male Sprague Dawley rats (weighing 260–300 g, Harlan, Chicago, IL) were given free access to food and water. Before surgical procedures animals were anesthetized with ketamine hydrochloride and xylazine hydrochloride (administered IP) (McCollum et al. 2010). Animal temperature was maintained at 37 °C by a thermostatically regulated heating pad with a rectal temperature probe (CMA 450).

17.2.2 Middle Cerebral Artery Occlusion in Rats

Transient focal cerebral ischemia of the middle cerebral artery (MCA) for 2 h was created by the suture insertion technique (Longa et al. 1989; Sun et al. 2011; Modi et al. 2014; Gharibani et al. 2014). Briefly, the left common carotid artery and the left external carotid artery were accessed through a midline neck incision after which a 4.0 monofilament nylon suture was inserted in the external carotid

artery and carefully advanced to 17 mm from the carotid bifurcation into the internal carotid artery. Local cerebral blood flow (LCBF) was monitored in the cerebral cortex of the left hemisphere using a Laser Doppler flowmeter (LDF) (Perimed Inc., OH, USA). Reduction in LCBF could be demonstrated when the filament was inserted the appropriate distance. Animals that did not demonstrate a LCBF decrease of greater than 70% were excluded from the analysis (Mohammad-Gharibani et al. 2014). Reperfusion was carried out by removing the suture 2 h after MCAO.

17.2.3 Rat Treatment Protocols

For taurine studies animals (N = 15) were randomly assigned as controls (MCAO rats receiving only vehicle, 0.9% saline), experimental (MCAO rats receiving taurine 40 mg/kg) and sham-operated (receiving the same surgical procedure without insertion of the filament). Taurine was injected subcutaneously in the experimental group 24 h after the reperfusion for 4 days.

For the DETC-MeSO plus taurine investigations, groups received either DETC-MeSO (n = 9, 0.56 mg/kg) or taurine (n = 9, 40 mg/kg) either individually or in a combination (N = 9) 4 h after reperfusion (s.c.). Animals received treatment for 4 days before sacrifice. In the control group (n = 9) vehicle (1 mL saline 0.09%) was injected subcutaneously 4 h after reperfusion for 4 days before animals were sacrificed. The sham operated group received the same surgical procedure without insertion of the silicon filament. After surgery animals recovered from anesthesic and had free access to food and water. The animals were examined daily for body temperature and weight and those that had a body temperature of greater than 39 °C after 4 h were excluded from the experiment (Li et al. 1999; Gharibani et al. 2014).

17.2.4 Determination of Area of Infarction

Animals were sacrificed using isoflurane and brains were removed for 2,3,5-triplenyltetrazolium chloride (TTC) staining and for analysis in Western blot (Kramer et al. 2010). Using an adult brain slicer (Matrix, Zivic Instruments) brains were sectioned coronally into six 2 mm coronal slices located from 2 to 12 mm from the frontal pole and incubated for 5 min in TTC (J.T.Baker, Faridabad, India) under dark room conditions for staining (Kramer et al. 2010) followed by collecting samples for western blot (Kramer et al. 2010). TTC is reduced enzymatically by mitochondrial dehydrogenases to become formazan, which causes normal tissue to stain a deep red color (Bederson et al. 1986). Lesion volume was assessed as described previously (Gharibani et al. 2013). After the TTC analysis, while the sections were

on ice, the ischemic parts of the left hemisphere were quickly removed by dissection and snap frozen (Gharibani et al. 2013).

17.2.5 Measurement of Protein Expression by Western Blot Analysis

Rat brain samples were homogenized in RIPA buffer plus 1% mammalian protein inhibitor cocktail (SIGMA) and 1% (v/v) phosphatase inhibitor cocktail (Thermo Scientific). Western blot was carried out as described previously (Gharibani et al. 2013) with overnight incubation with the following primary antibodies: Abcam: GRP78, ATF4, p-IRE1, pXBP1, Cell Signaling Bax, Bcl-2, cleaved caspase 3, Akt, pAkt, peIF2–alpha, Santa Cruz: CHOP/GADD153, PERK, p-PERK. Membranes were then treated with ECL horseradish peroxidase conjugated anti-rabbit or anti mouse IgG (1:3000; GE Healthcare, UK) at room temperature for a further 90 min. Quantitative western blot results were obtained by densitometric analysis using image processing and analysis in Java (Image J). Sham operated core and penumbra samples served as control non-ischemic protein samples for comparison with the left (ischemic hemisphere samples).

17.2.6 Statistical Analysis with Post-hoc Tests

All data was expressed as the mean ± SEM and analysed with t test or one–way ANOVA. Dunnett post-hoc test or Tukey test was employed for comparison between groups.

17.3 Results

We had previously investigated the protective mechanisms of taurine administration acting on ER stress pathways in vitro on hypoxia/re- oxygenation exposure of primary neuronal cultures and in vivo using the MCAO rat stroke model (Gharibani et al. 2013). In cell cultures subjected to hypoxia/re-oxygenation we demonstrated that taurine could down-regulate the ATF6 pathway (as shown by decreased cleaved ATF6 to ATF6 ratio) as well as down-regulate the IRE-1 pathway as indicated by a decrease in phosphorylated IRE-1 (pIRE-1). In contrast to its effect on the ATF6 and IRE-1 pathways there was no effect of taurine on down-regulating the PERK pathway as determined by measurement of ATF4 or p-eIF-2-alpha (Gharibani et al. 2013; Pan et al. 2012). Using the MCAO rat stroke model we analyzed the effects of taurine administration 24 h after reperfusion on infarct volume on day 4 of reperfusion and

on expression levels of components of the PERK, ATF-6 and p-IRE-1 pathways. Our data indicated that in the MCAO model taurine administration resulted in a decrease in cleaved ATF-6 to ATF6 ratio and a decrease in p-IRE1 expression (Fig. 1a, b) but, by contrast, no down-regulation of the PERK pathway component ATF4 was observed (data not shown) using this in vivo model of stroke (Gharibani et al. 2013).

To analyze the effects of taurine administration on inhibition of apoptotic markers associated with the mitochondrial pathway of programmed cell death we employed Western Blot analysis to measure levels of the ratio of Bcl2 to Bax as well as expression of Caspase 3 in MCAO rats. Bcl2 family members comprise a set of homologous proteins that act as key regulators of the mitochondrial pathway of apoptosis. In cerebral ischemia Bax will translocate to the mitochondrion and elicit mitochondrial permeability transition pore opening together with release of cytochrome C and activation of downstream caspases including caspase 3. Our analysis in the MCAO rats indicated that with taurine administration there was a more than fourfold increase in Bcl2/Bax ratio which resulted in prevention of Caspase 3 activation (Fig. 1c, d). To test the effect of taurine on apoptosis caused by ER stress we measured levels of CHOP. Western blot analysis showed that taurine decreased levels of CHOP in vivo in the MCAO rat stroke model (Fig. 1e; Gharibani et al. 2013).

In our investigations on the N-Methyl-D-aspartate (NMDA) receptor partial antagonist S-M-ethyl-NN diethyl-thio-carbamate-sulfoxide (DETC-MeSO) we found that this agent was protective against tissue damage in the rat MCAO model of cerebral stroke. Specifically we found reduced infarct size as well as decreased ER stress and fewer behavioral deficits as seen as improved performance on the neuroscore test (Gharibani et al. 2014). In response to DETC-MeSO administration levels of the pro-apoptosis proteins Bak in the penumbra and Bad in both core and penumbra were decreased while expression of the anti-apoptotic component Bcl-2 was increased in the core and the penumbra. To assess whether ER stress pathways affected by ischemia and by treatment with DETC-MeSO we employed western blot to measure levels of components of the PERK, ATF6 and pIRE1 pathways after administration of DETC-MeSO in the MCAO model (Gharibani et al. 2014). Importantly expression of PERK pathway components increased after MCAO and dramatically declined in both core and penumbra after treatment with DETC-MeSO. To evaluate the effects on the IRE-1 pathway levels of p-IRE-1 were measured. Following MCAO levels of pIRE1 were increased in the core and the penumbra but treatment with DETC-MeSO did not modify expression levels of this ER stress component. Measurement of expression levels of cleaved ATF-6, a component of the ATF-6 pathway of ER stress revealed that DETC-MeSO administration had no effect on levels of cleaved ATF-6 in both the core and the penumbra (Gharibani et al. 2014).

To analyze the effects of post treatment with a combination of taurine plus DETC-MeSO on behavioral outcomes and expression of apoptotic and ER stress components in the rat MCAO model of cerebral stroke we injected taurine (40 mg/kg) or DETC-MeSO (0.56 mg/kg) or a combination of both drugs starting 24 h after reperfusion with injections continuing daily for 4 days.

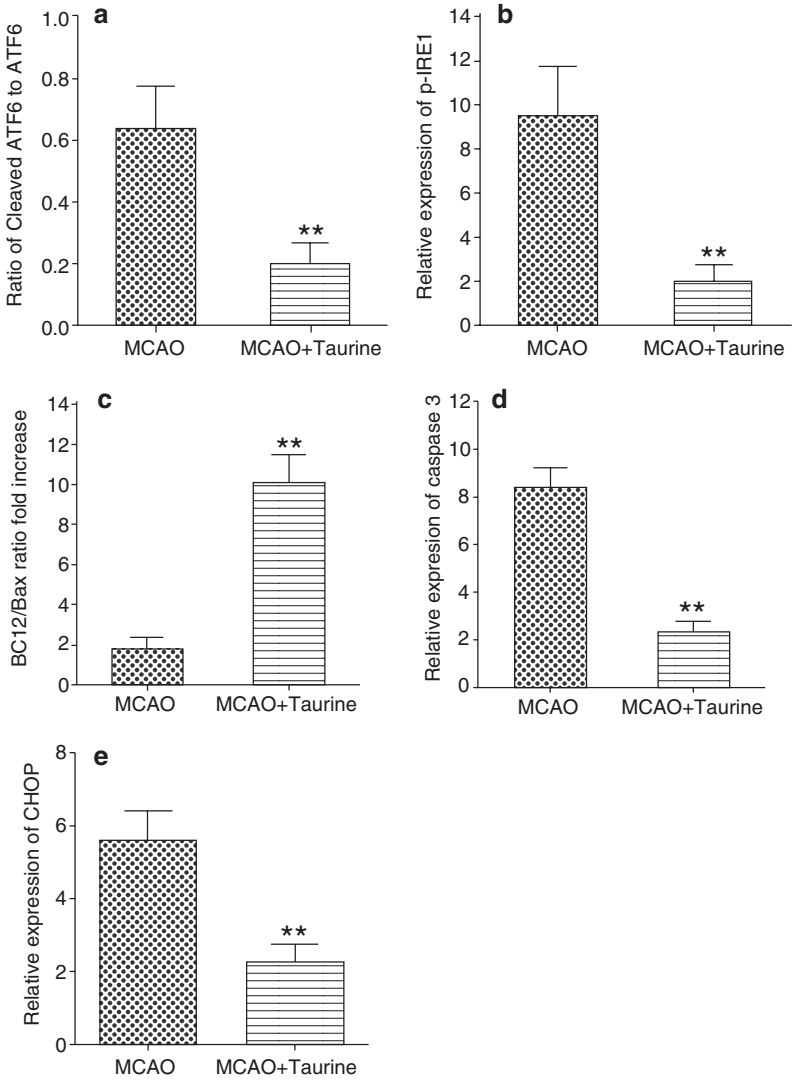

Fig. 1 Effects of taurine on ER stress signaling, apoptosis marker expression and CHOP levels in the rat MCAO stroke model. *MCAO*: middle cerebral artery occlusion for 2 h. followed by 4 days reperfusion. *MCAO + taurine*: middle cerebral artery occlusion for 2 h followed by 4 days reperfusion, with taurine injected 24 h after reperfusion subcutaneously and injections continued for 4 days. (**a**) ATF6 protein expression in the core of the ischemic infarct as determined by Western blot. The *bar graphs* present the ratio of cleaved ATF6 to ATF6 using densitometric data from the experiment using Western blot analysis. (**b**) P-IRE1 expression in the core of MCAO brain as determined by Western blot. The *bar graphs* present the densitometric analysis from the Western blot. Data is presented and mean ± SEM, n = 3, *p < 0.01 versus MCAO. (**c**) Bax and Bcl-2 levels in the core of MCAO brain as determined by Western blot in MCAO and MCAO + taurine. (**d**) Caspase 3 protein expression in the core of MCAO brain analyzed by Western blot in MCAO and MCAO + taurine. (**e**) CHOP protein levels on Western blot analysis in the core of the MCAO rat brain (After Gharibani et al. 2013)

To analyze infarct size 4 days after MCAO, brains were visualized after TTC staining. A clear infarct was seen in 2 mm sections from the left hemisphere of rats treated with vehicle whereas the infarct was significantly reduced in the drug treated groups. In taurine and taurine + DETC-MeSO treated groups a clear reduction in infarct size was seen compared to the vehicle treated group. Notably the maximum reduction was found in rats treated with the combination of taurine + DETC-MeSO with an approximate fivefold reduction by comparison to the vehicle treated group (data not shown; Gharibani et al. 2015).

For evaluation of behavioral deficits the neuroscore test was employed. There was no deficit observed in animals before surgery. Sham operated animals also showed no deficit. Animals that received taurine + DETC-MeSO showed a significant decrease in neurological deficits whereas those receiving taurine or DETC-MeSO showed no decrease in behavioral deficits compared to the vehicle treated group (Gharibani et al. 2015).

To determine whether our pharmacological treatments modulate the unfolded protein response in ER stress induced apoptosis we employed Western blot to measure UPR markers as well as the transcription factor CHOP in MCAO rats treated with taurine or DETC-MeSO or the combination of taurine + DETC-MeSO. Both treatment groups of taurine and taurine + DETC-MeSO showed decreased expression of GRP78 (Fig. 2b). The combination treatment group of taurine + DETC-MeSO showed a significant decrease in GRP78 expression compared to the other groups. As seen in Fig. 2c treatment with DETC-MeSO had no effect on levels of cleaved ATF6 in the penumbra of MCAO rats whereas taurine and taurine + DETC-MeSO both resulted in significant decreases in levels of cleaved ATF6 (Gharibani et al. 2015). We proceeded to analyze the PERK pathway in the brains of MCAO rats. Combined treatment with taurine + DETC-MeSO resulted in a major decrease in ATF4 in penumbra in comparison to the other treatment groups indicating that the combination effectively inhibited the PERK pathway (Fig. 2d). To analyze the contribution of the IRE-1 pathway we tested levels of expression of pIRE1 (the activated form of IRE1). The data indicated that phosphorylated IRE1 is expressed at high levels in the penumbra in the MCAO rat brain by comparison to the sham operated group. DETC-MeSO administration had no effect on expression of IRE1 (Fig. 2e). By contrast both treatment with taurine and treatment with the taurine + DETC-MeSO combination prevented IRE1 activation in the ischemic penumbra of the infarct area (Fig. 2e).

As PERK, IRE-1 and ATF6 pathways all converge on CHOP which can elicit an apoptotic response we wanted to test whether our treatments modulated ER stress induced apoptosis via CHOP activation (Kim et al. 2008). The data showed that levels of CHOP were up-regulated in the penumbra of MCAO rats by comparison to the sham operated group. Western blot analysis showed approximately a fourfold decrease in CHOP in penumbra with the taurine + DETC group compared to the other treatment groups, indicating that the combined therapy has a greater capacity for inhibiting apoptosis induced by ER stress in the MCAO stroke model (Fig. 2f).

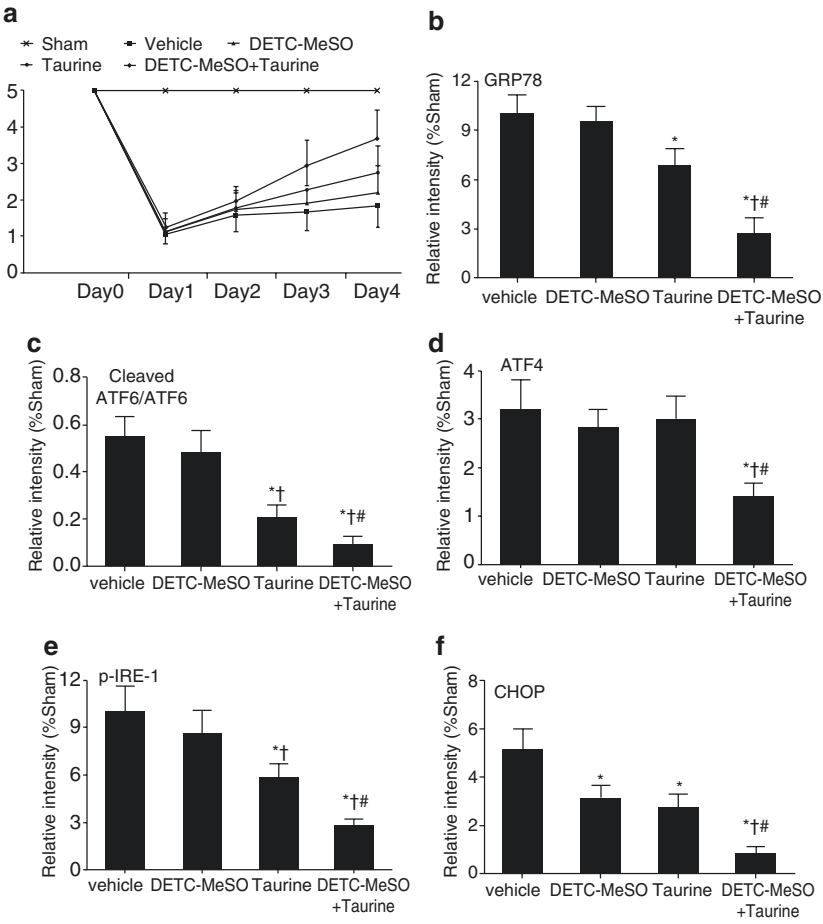

Fig. 2 Analysis of neuronal deficit and expression of UPR (GRP78) and ER stress proteins in the MCAO stroke model. (**a**) Effect of DETC-MeSO and taurine on neuro-score measurement in MCAO stroke model at 4 days. Recovery from neurological deficits is only observed in the group that received DETC + MeSO + taurine each day starting 24 h after reperfusion. All sham animals demonstrated no deficit scoring 5 on neuroscore. Values shown indicate mean ± SEM. * denotes statistical significance between DTEC-MeSO + taurine (0.56 mg/kg and 40 mg/kg, respectively) and vehicle treated group by Kruskal Wallis ANOVA, together with Dunn's test; N = 9, $p < 0.05$). (**b**) GRP78 levels in the penumbra of the MCAO brain were analyzed by Western blot. GRP78 levels were found to substantially decrease in the penumbra of combined DETC-MeSO + taurine treated group compared to the other treated groups. All three pathways of ER stress were analyzed in the penumbra of MCAO at 4 days of treatment. Combined treatment with DETC +taurine resulted in significant decreases in all 3 pathways of ER stress including ATF6 (**c**) ATF4 (**d**) and p-IRE1 (**e**) when compared to the other treatment groups. (**f**) Analysis by Western blot demonstrated that all treatment groups could significantly decrease expression of CHOP in the ischemic penumbra of rat brain. Notably combined therapy more effectively inhibited CHOP and apoptosis caused by ER stress compared to other treatment groups. Values in the graphs indicate mean ± SEM. *, † and # refer to statistical significance compared to vehicle control, DETC-MeSO and taurine treated groups respectively using ANOVA in addition to Tukey post hoc test (N = 5, $p < 0.05$). (After Gharibani et al. 2015)

17.4 Discussion

Numerous studies have demonstrated that taurine administration is protective against stroke, neurodegenerative disease and traumatic brain injury (TBI) Menzie et al. 2014). Furthermore we recently showed that DETC-MeSO, a partial NMDA antagonist is protective in the MCAO rat model of focal ischemia. Our recent experiments demonstrated that taurine or DETC-MeSO administration alone in the rat MCAO model ere both protective through down-regulating apoptotic pathways and that they differed in their activities by selectively regulating specific pathways from the three major ER stress pathways, PERK, ATF6 and IRE1. Our hypothesis was therefore that a combined drug treatment with taurine plus DETC-MeSO administered after the MCAO insult would elicit greater protection than administration of either drug alone.

The protective serine/threonine specific kinase Akt is known to be antiapoptotic and neuroprotective in both cell culture models and in vivo. Akt has been found in several investigations to promote neuroprotection in stroke models. In our previous studies we demonstrated that treatment with 5.6 mg/kg of DETC-MeSO elicited protection through inducing increased levels of p-Akt in the penumbra of the MCAO model at 4 days. However a low dose of DETC-MeSO (at 0.56 mg/kg) elicited no protection in this model and did not increase pAkt expression (data not shown). Administration of taurine or taurine plus DETC-MeSO elicited a significant induction of pAkt which occurred in parallel with elevated Bcl-2/Bax ratio reflecting enhanced neuroprotection and decreased apoptosis (Gharibani et al. 2015).

The endoplasmic reticulum plays important roles of maintaining intracellular calcium homeostasis as well as folding and processing of cellular proteins (Reddy et al. 2003). Cell stresses including hypoxia and glucose deprivation may lead to accumulation of unfolded or misfolded proteins in the ER which may subsequently elicit ER stress and downstream apoptotic cell death (Harding et al. 2000; Oyadomari and Mori 2004). In investigating the protective effects of taurine or DETC-MeSO or taurine plus DETC-MeSO on the activation of ER stress pathways we employed western blot analysis to measure levels of expression of components of the PERK, ATF6 and IRE-1 pathways. ER stress signaling through the PERK pathway elicits inhibition of cap dependent translation by phosphorylation of a subunit of eIF2-alpha which contributed to preventing a further increase in mis-folded proteins in the ER. PERK also signals to ATF-4 dependent genes which are in turn responsible for regulating amino acid biosynthesis and redox reactions as well as pro-apoptotic pathways through activation of the transcription factor CHOP. In the MCAO model of stroke our data indicated that there was no change in ATF4 following either DETC-MeSO or taurine treatment. However the combined treatment with DETC-MeSO plus taurine resulted in a significant decrease in ATF4 expression.

In our previous in vivo studies treatment with taurine decreased expression of components of two of the ER stress pathways, the ATF6 and IRE1 pathways but had no effect on the PERK pathway (Gharibani et al. 2013). Following activation of the ATF6 pathway ATF6 translocates to the nucleus to induce expression of chaperone

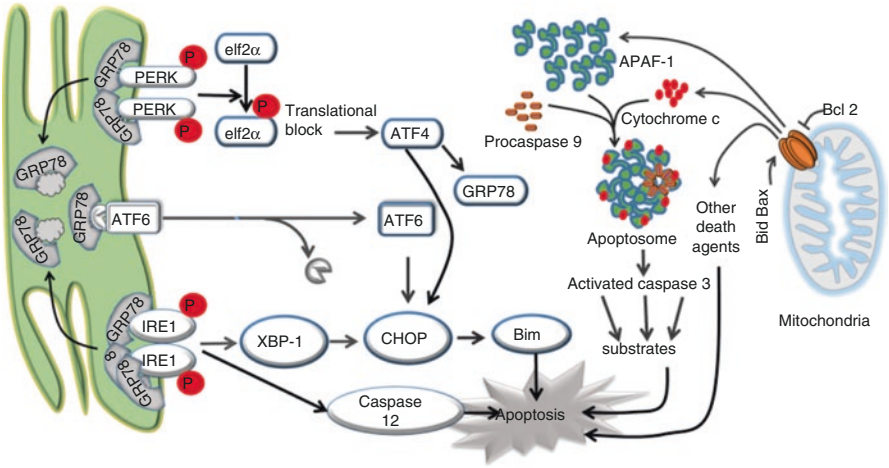

Fig. 3 Major Endoplasmic Reticulum Stress and Mitochondrial Stress pathways eliciting cell death in focal ischemia. ER stress initiated by misfolded or unfolded proteins in the ER is triggered by release of inhibition of PERK, ATF6 and IRE1 from GRP78 which dissociates from these partnering molecules in the ER. Subsequent steps involve dimerization and phosphorylation of PERK and of IRE-1 and cleavage of ATF6 (p90) to ATF6 (P50). Activated PERK phosphorylates eIF2-alpha leading to an increase in ATF-4. All three of these pathways upregulate the transcription factor CHOP resulting in cell death. Mitochondrial stress resulting from enhanced Bax to Bcl-2 ratio will elicit opening of the mitochondrial permeability transition pore and release from the mitochondrion of cytochrome C and the adaptor protein APAF1 which, in turn, comprise part of the apoptosome, an intermediate signaling complex. Following formation of the apoptosome activated caspase 9 then cleaves downstream caspase 3 culminating in apoptotic cell death

proteins as well as the pro-survival transcription factor XBP1. With post-ischemia administration, DETC-MeSO did not decrease levels of cleaved ATF6 in penumbra. By contrast treatment with taurine and with taurine plus DETC-MeSO resulted in a significant decrease in levels of ATF6.

The degree of inhibition of the IRE-1 pathway, the third ER stress pathway was evaluated by measurement of levels of pIRE-1 in the penumbra. Our data indicated that there was a significant decrease in pIRE-1 expression in the penumbra with post administration of DETC-MeSO. Notably our combined treatment strategy using both DETC-MeSO and taurine administration post-stroke resulted in a decrease in activity of all 3 ER stress pathways.

Neuronal cell death in stroke can occur through the mitochondrial pathway of apoptosis or through ER stress pathways that signal through CHOP and culminating in mitochondrial induced programmed cell death (Fig. 3) (Oyadomari and Mori 2004). Mitochondrial stress resulting in apoptosis is elicited through the action of Bcl-2 family members Bax or Bak as a trigger on the mitochondrial membrane, contributing to the release of cytochrome C from the inter-membrane space. Cytochrome C in conjunction with APAF1 and caspase 9 constitute part of the apoptosome, an intermediate complex that proceeds to signal to the downstream

caspase, caspase 3, culminating in programmed cell death. ER stress in cerebral ischemia will also result in cell death (Fig. 3) (Yoshida et al. 1998). Dissociation of GRP78 from the sensors of ER stress Perk, ATF6 and IRE-1 enable activation of the ER stress pathways culminating in signaling to CHOP, activation of Bim and subsequent ER stress induced apoptotic cell death. Notably, in the current studies our data demonstrates that both mitochondrial stress and ER stress are activated in the MCAO rat stroke model and furthermore, the key components of these two major types of neuronal stress are potently inhibited by taurine (Gharibani et al. 2013), or DETC-MeSO (Gharibani et al. 2014) individually as well as by synergistic effects of low dose administration of the combination therapy of taurine plus DETC-MeSO (Gharibani et al. 2015).

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Analysis of Neuroprotection by Taurine and Taurine Combinations in Primary Neuronal Cultures and in Neuronal Cell Lines Exposed to Glutamate Excitotoxicity and to Hypoxia/Re-oxygenation

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Abstract Ischemic stroke is one of the greatest contributors to death and long term disability in developed countries. Ischemia induced brain injury arises due to excessive release of glutamate and involves cell death due to apoptosis and endoplasmic reticulum (ER) stress responses. Despite major research efforts there are currently no effective treatments for stroke. Taurine, a free amino acid found in high concentrations in many invertebrate and vertebrate systems can provide protection against a range of neurological disorders. Here we demonstrate that taurine can combat ER stress responses induced by glutamate or by hypoxia/re-oxygenation in neuronal cell lines and primary neuronal cultures. Taurine decreased expression of ER stress markers GRP78, CHOP, Bim and caspase 12 in primary neuronal cultures exposed to hypoxia/re-oxygenation. In analyzing individual ER stress pathways we demonstrated that taurine treatment can result in reduced levels of cleaved ATF6 and decreased p-IRE1 levels. We hypothesized that because of the complex nature of stroke a combination therapy approach may be optimal. For this reason we proceeded to test combination therapies using taurine plus low dose administration of an additional drug: either granulocyte colony stimulating factor (G-CSF) or sulindac a non-steroidal anti-inflammatory drug with potent protective functions through signaling via ischemic preconditioning pathways. When primary neurons were pretreated with 25 mM taurine and 25 ng/mL G-CSF for I hour and then exposed to high levels of glutamate, the taurine/G-CSF combination increased the protective effect against glutamate toxicity to 88% cell survival compared to 75% cell survival from an individual treatment with taurine or G-CSF alone. Pre-exposure of PC12

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cells to 5 mM taurine or 25 μM sulindac did not protect the cells from hypoxia/re-oxygenation stress whereas at these concentrations the combination of taurine plus sulindac provided significant protection. In summary we have demonstrated the protective effect of taurine in primary neuronal cultures against hypoxia with re-oxygenation through inhibition of ATF6 or p-IRE-1 pathway but not the PERK pathway of ER stress. Furthermore the combinations of taurine plus an additional drug (either G-CSF or sulindac) can show enhanced potency for protecting PC 12 cells from glutamate toxicity or hypoxia/re-oxygenation through inhibition of ER stress responses.

Keywords Taurine • Neuroprotection • Hypoxia • Endoplasmic reticulum stress • Glutamate excitotoxicity

Abbreviations

ER stress	Endoplasmic reticulum stress
GCSF	Granulocyte colony stimulating factor

1 Introduction

Stroke is the third cause of mortality world-wide and is a leading cause of disability (Go et al. 2014). Despite intense research efforts there are to date no effective therapies for stroke. Cerebral hypoxia/ischemia results in depleted oxygen and glucose availability and induces excessive release of glutamate and other neurotransmitters. Subsequent activation of post-synaptic glutamate receptors acts as a trigger for activation of major downstream signaling cascades resulting in neuronal cell death (Nicholls and Attwell 1990).

Taurine (2-amino ethanesulfonic acid) is one of the most abundant amino acids found in mammalian brain, skeletal muscle and cardiac muscle (Sturman 1993; Huxtable 1992). Taurine has been employed in the treatment of a range of neurological diseases including Alzheimer's disease, Huntington's disease and ischemic stroke (Takatani et al. 2004; Paula-Lima et al. 2005; Takahashi et al. 2003). The physiological functions of taurine include neuro-modulation, prevention of cellular calcium overload, osmoregulation, neurotransmission and neuroprotection (Oja and Saransaari 1996; Okamoto et al. 1983; Kumari et al. 2013). Taurine has also been reported to contribute to membrane stabilization and detoxification and to counteract the effects of oxidative stress in the brain (Moran et al. 1987; Chen et al. 2001). We have previously demonstrated that taurine can protect primary cortical neurons from hypoxia and glutamate induced endoplasmic reticulum stress (ER stress) induced by oxidative stress (Pan et al. 2010b).

Granulocyte colony stimulating factor (GCSF) is a growth factor that is clinically in use for treatment of neutropenia (Metcalf 1990). GCSF can cross the blood brain barrier and GCSF demonstrates important actions in the CNS through binding to the GCSF receptor on Neuronal cells. Increasing evidence indicates that GCSF is neuroprotective as well as neuroregenerative and GCSF has been found to elicit protection in a number of neurological disease models including those for Parkinson's disease, Huntington's disease and ischemic stroke (Schäbitz et al. 2003). A further additional candidate of great potential value for use in neuro-protection in the CNS is sulindac, a well-known anti-inflammatory drug and inhibitor of COX1 and COX2. It has been demonstrated that sulindac acts as an anti-cancer agent while also possessing the property of protecting normal cells by pro-survival pathways that include ischemic preconditioning pathways (Tinsley et al. 2011; Moench et al. 2009).

In our previous investigations we demonstrated neuroprotection by taurine against ER stress induced by glutamate treatment of primary cortical neurons (Pan et al. 2010a, 2012). In our subsequent studies we have employed primary cortical neurons and PC12 cells to characterize the mechanisms of neuroprotection by taurine and taurine containing drug combinations (Pan et al. 2012). We have tested the hypotheses that other neuroprotective including sulindac and GCSF may demonstrate enhanced protection when combined with taurine using in vitro models of stroke (Pan et al. 2010b). To examine the potential of taurine plus sulindac to elicit protection of PC12 cells against hypoxia/re-oxygenation we have tested low doses of these drugs that show no protective effects individually to determine whether the combination of taurine and sulindac at these doses may elicit synergistic neuroprotective effects.

2 Materials and Methods

2.1 Primary Neuronal Cell Culture

Primary neuronal cultures were prepared by standard methods. Briefly pregnant rats were euthanized after isoflurane exposure and embryos at days E16–E18 were removed and brains were isolated from the fetuses. Brains were placed in Basal Medium Eagle supplemented with 2 mM glutamine, 6.8 mM glucose and 20% heat—inactivated fetal bovine serum. Cortices were dissociated by passing through a 14-G cannula. Cells were centrifuged at 300 g/min for 5 min at room temperature after which the pellet was re-suspended in GME for plating in tissue culture plates that had been pre-coated with 5 μg/mL of poly-D- lysine. Cells were then maintained for 1 h in a humidified incubator (37 °C, 99% humidity and 5% CO2) after which incubation medium was replaced with serum free neurobasal medium supplemented with 2% B27 and 500 μM glutamine. The cells were then maintained in an incubator for 12–18 days until they were ready for use in experimental analysis (Hartung 1998).

2.2 PC12 Cell Culture

PC12 cells were maintained at 37 °C/5% CO2 in F12-K medium supplemented with 2.5% (v/v) fetal bovine serum (FBS), 15% (v/v) penicillin—streptomycin solution. All experiments were performed on undifferentiated cells plated at a density of approximately 5 × 10e4 cells/ell for western blot for 4 h before starting the experiments. The 96 well plates or petri dishes were pre-coated with poly-d-lysine before plating.

2.3 Hypoxia and Re-oxygenation

To provide a hypoxic environment 14-day cultured neurons in 6 or 96 well plates were placed in a hypoxia chamber with oxygen levels maintained at 0.3–0.4%. The level of oxygen was continuously monitored using an oxygen electrode. Primary cortical neuronal cultures in the presence or absence of appropriate drug treatment conditions were subjected to 20 h of hypoxia. Re-oxygenation was carried out by removing the cultures from the hypoxia chamber and transferring them to a normal culture incubator for another 20 h. For taurine plus sulindac combination experiments, cells were pre-exposed to taurine or sulindac alone or both taurine and sulindac for 24 h prior to hypoxia/re-oxygenation exposure.

2.4 Glutamate Toxicity

To elicit glutamate induced toxicity neurons at 14 days in culture ere pre-incubated with different concentrations of drug treatment for 1 h. The neurons were then treated with 100 μM glutamate for another 1 h or 10 min.

2.5 ATP Assay for Measurement of Cell Viability

Primary cortical neuronal cells in 96 well dishes were subjected to drug treatment for 1 h and then cells were subjected to glutamate toxicity or to hypoxia/re-oxygenation to induce cell death. ATP solution (Promega) as added to each well and cells were incubated for 10 min after which the amount of ATP was quantified in a luciferase reaction. The luminescence intensity was measured using a luminometer with lysates in a standard opaque walled multi-well plate. The ATP content was determined by running an internal standard and expressed either in raw luminescence units or as a percentage of untreated cells (control).

2.6 Western Blot Analysis

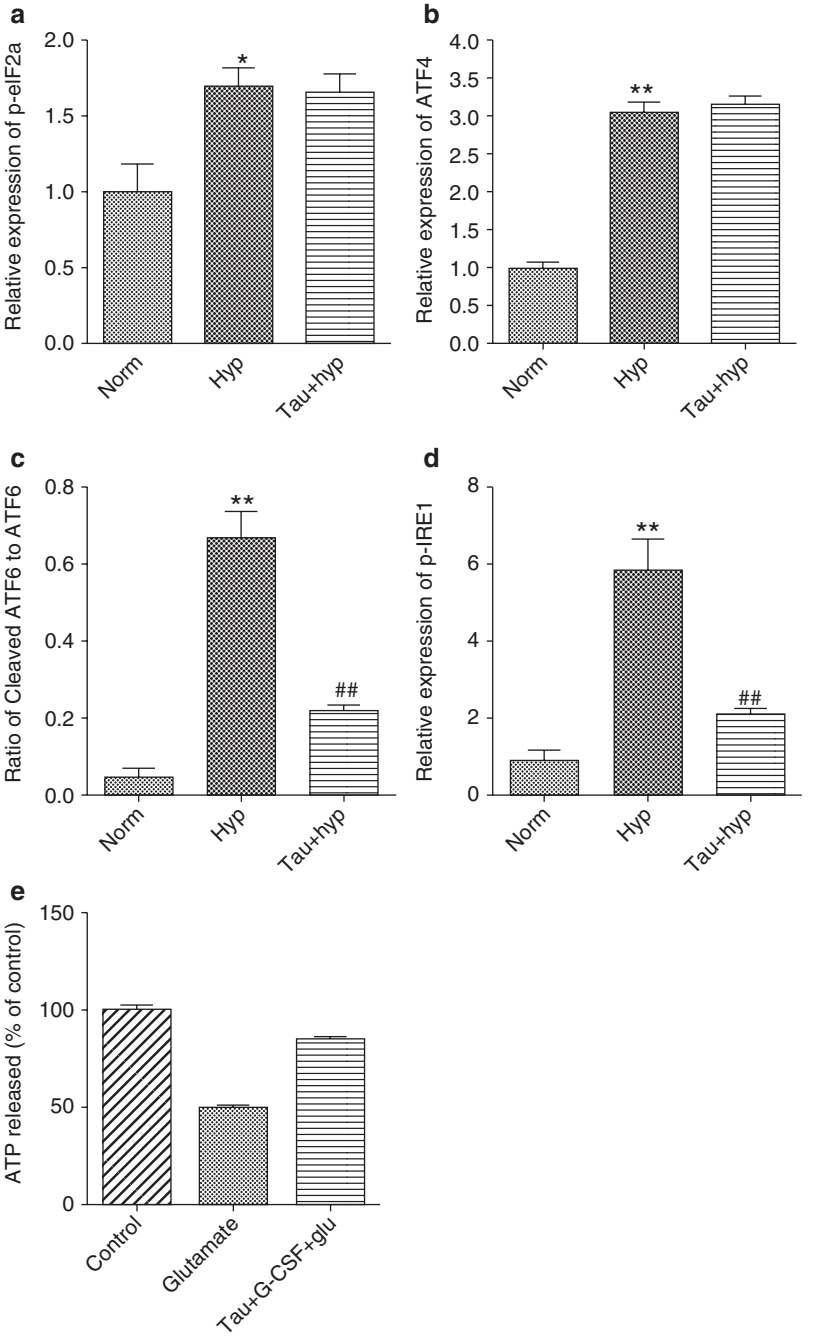
PC12 cultures were lysed in RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1% SDS) containing 1% mammalian protease inhibitor cocktail from Sigma and separated on SDS-PAGE followed by transfer to a nitrocellulose membrane. The membrane as then blocked in blocking buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, 5% milk) for 1.5 h at room temperature. After blocking, membranes were incubated with primary antibodies for 1 h followed by a 1 h incubation with the corresponding HRP-conjugated secondary antibody at room temperature. Extensive washes with blocking buffer were performed between each step. The protein immuno-complex was visualized using ECL detection reagent purchased from Thermo Scientific. Quantitative Western Blot results were obtained by densitometric analysis using image processing and analysis in Java (Image J).

2.7 Statistical Analysis

All data were expressed as mean ± SEM. The statistical significance of the data was determined with Student's t-test of by one- or two-way ANOVA combined with Dunnett post hoc or Tukey post hoc test to compare means between groups.

3 Results

In cortical neurons treated with hypoxia and re-oxygenation and tested with a range of doses of taurine we previously established that culturing in the presence of 10 mM taurine would increase cell survival as measured by ATP assay from 49% without taurine up to the level of 85% cell viability. On exposure of cultures to hypoxia/re-oxygenation, we showed that expression of CHOP, caspase 12 and cleaved caspase 12 were highly induced and that pretreatment with taurine resulted in a significant reduction of CHOP, caspase 12 and cleaved caspase 12 pointing to a decrease in apoptosis resulting from ER stress (Pan et al. 2012). The major ER stress induced signaling pathways PERK (as measured by quantification of eIF2-alpha and ATF4), ATF6 and IRE1 (shown as cleaved ATF6 to ATF6 ratio) and levels of IRE-1 as measured by levels of phosphorylated IRE1 (pIRE-1) were substantially increased by hypoxia/re-oxygenation (Fig. 1a, b, c, d). Taurine pre- treatment resulted in a large decrease in cleaved ATF6/ATF6 ratio. pIRE-1 levels fell to less than 40% of hypoxia/


a
Relative expression of p-eIF2a
2.0
1.5
1.0
0.5
0.0
*
Norm
Hyp
Tau+hyp
b
Relative expression of ATF4
4.0
3.5
3.0
2.5
2.0
1.5
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Norm
Hyp
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c
Ratio of Cleaved ATF6 to ATF6
0.8
0.6
0.4
0.2
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Norm
Hyp
Tau+hyp
d
Relative expression of p-IRE1
8
6
4
2
0
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Norm
Hyp
Tau+hyp
e
ATP released (% of control)
150
100
50
0
Control
Glutamate
Tau+G-CSF+glu

Fig. 1 Neuroprotective effect of taurine via hypoxia and glutamate induced ER stress pathways. Taurine has no effect on the PERK pathway (reflected in levels of P-eIF2-alpha and ATF4) after hypoxia/re-oxygenation and glutamate induced ER stress pathways. Taurine has no effect on the PERK pathway (reflected in levels of p-eIF2-alpha and ATF4) after hypoxia/re-oxygenation. Levels of expressed proteins were determined by Western blot and bar graphs reflect the densitometric data for the levels of the particular molecular target. (**a**) P-eIF-alpha Western blot results with arbitrary units. (**b**) ATF4 expression Western blot results with arbitrary units. (**c**) Ratio of cleaved ATF6 to ATF6 expression Western blot results with arbitrary units. (**d**) P-IRE-1 expression Western blot results with arbitrary units. (**e**) Neuroprotective effects of taurine plus GCSF against glutamate—induced excitotoxicity. Primary cortical neurons were pre-incubated with 25 mM taurine plus GCSF (25 ng/mL) for 1 h and then exposed to 100 μM glutamate for 4 h. Cell survival was measured by ATP assay. Values in bar graphs represent mean ± SEM, n = 3, *p < 0.05 and **p < 0.01 versus Normoxia, ##p < 0.01 versus Hypoxia (After Pan et al. 2010a and Pan et al. 2012)

re-oxygenation levels (Fig. 1c, d). In contrast to the evidence for inhibition of the ATF6 and IRE-1 pathways by taurine it was found that pre-treatment with taurine did not prevent the induction of the PERK pathway components p-eIF2-alpha or ATF4 (Fig. 1a, b).

To examine the potential of taurine or taurine in combination with GCSF to elicit protection of cultured neurons we preincubated cells for 1 h with taurine at a range of concentrations from 5 to 25 mM in combination with GCSF at 10 or 25 ng/mL and then subjected the cultures to excessive glutamate exposure to elicit excitotoxicity (data not shown). Exposure of cortical neurons to 100 μM glutamate for 4 h resulted in glutamate toxicity. Pre-exposure of cells to 25 mM taurine plus 25 ng/mL GSCF for 1 h resulted in protection against glutamate toxicity and increased cell survival to 88% compared to less than 75% cell survival with taurine or GCSF treatment alone (Fig. 1e).

To study the protection by taurine or taurine combination therapy in cultured PC 12 cells exposed to hypoxia/re-oxygenation we pre-incubated cells for 30 min with 5 mM taurine in combination with sulindac at 25 μM and then subjected the cultures to 24 h hypoxia followed by 24 h of re-oxygenation. Pre-exposure of cultures to 5 mM taurine plus 25 μM sulindac protected the PC12 cells against hypoxia/reoxygenation and increased cell survival significantly compared to levels obtained for 5 mM taurine or 25 μM sulindac alone (Fig. 2). Hence taurine and sulindac in combination demonstrated a synergistic effect of protection of PC12 cells subjected to hypoxia/re-oxygenation at the low doses employed in this study (Fig. 2).

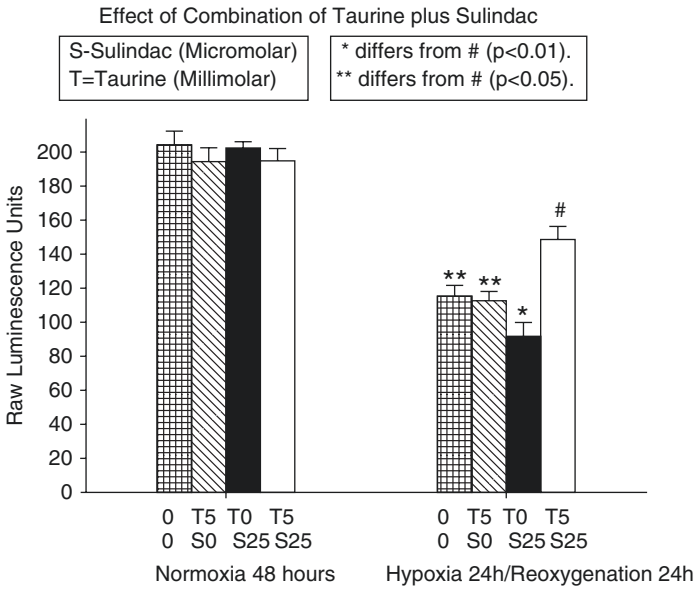

Fig. 2 Synergistic neuroprotective effect of taurine plus sulindac enhances cell survival of PC12 cells subjected to hypoxia and re-oxygenation. PC12 cells were preincubated for 30 min with 5 mM taurine or 25 μM sulindac or a combination of 5 mM taurine plus 25 μM sulindac or with growth medium without drugs and then maintained in normoxic conditions or subjected to 24 h hypoxia with 24 h re-oxygenation. Cell viability was measured by ATP assay and expressed in raw luminescence units. Significant differences determined by ANOVA with post-hoc Tukey test.: *differs from # ($p < 0.01$); **differs from # ($p < 0.05$)

4 Discussion

Taurine, the most abundant free amino acid in the CNS is known to elicit protection for stroke and neurodegenerative disease (Birdsall 1998; Sun and Xu 2008; Sun et al. 2011). Taurine protection by antioxidant mechanisms has been previously demonstrated in myocardial mitochondria subjected to hypoxia re-oxygenation or to Mn-superoxide dismutase inhibition (Chen et al. 2009). Through its action as a GABA agonist taurine has been shown to increase GABA levels as well as to activate GABA receptors (Paula-Lima et al. 2005; Tadros et al. 2005). Taurine can also protect through preventing the increase in intracellular free calcium resulting from glutamate excitotoxicity.

We previously demonstrated that ER stress inhibition may underlie the protection by taurine against glutamate excitotoxicity (Pan et al. 2010b). In subsequent studies we have employing primary cortical neuronal cultures we demonstrated clear protection by taurine administration against cell death caused by hypoxia and re-oxygenation (Pan et al. 2012). Here we present analyses of the signaling pathways underlying the protection of cortical neurons against hypoxia/re-oxygenation that leads to ER stress pathway activation. Furthermore we analyze the contribution

of individual ER stress pathways to hypoxia/re-oxygenation and determine the effect of taurine on inhibition of these pathways.

We have previously demonstrated that in primary cortical neurons treatment with excessive glutamate concentrations resulted in activation of intracellular components of an ER stress response including GRP78, CHOP, Caspase 12 and Bim (Pan et al. 2010a, 2012). The pro-apoptotic transcription factor CHOP is expressed at low levels in untreated cells and is known to be greatly induced by ER stress (Nemetski and Gardner 2007). We have shown that CHOP is increased by exposure to hypoxia/re-oxygenation and that taurine administration will decrease levels of CHOP to normoxic levels. Pro-caspase 12 resides on the ER membrane and activates caspase dependent apoptosis in response to ER stress. We demonstrated that caspase 12 or cleaved caspase 12 was induced by hypoxia/re-oxygenation and that taurine reduced levels of caspase 12 to levels found in normoxic conditions. Hence we have demonstrated the contribution of taurine to preventing cell death resulting from hypoxia/re-oxygenation through decreasing both caspase 12 and CHOP.

Treatment with combination therapies of drugs at low doses may show potential for achieving good efficacy while avoiding side effects that could result from high dose drug exposure. We have demonstrated that taurine in combination with GCSF is capable of eliciting protection of primary cortical neurons against glutamate excitotoxicity. We have extended our multi-drug studies to include pre-treatment of PC12 cells subjected to hypoxia/re-oxygenation with a combination of taurine plus sulindac. Individually 5 mM taurine and 25 μM sulindac did not show protection but the combination of taurine plus sulindac at these low doses showed significant protection. In conclusion taurine is effective in protecting neuronal cells against ER stress induced by glutamate toxicity or hypoxia/re-oxygenation and combination treatments using taurine plus GCSF and or taurine plus sulindac show very good potential for eliciting high level neuroprotection in cell culture models of glutamate excitotoxicity and hypoxia/re-oxygenation.

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Minor Adaptations of Ethanol-Induced Release of Taurine Following Chronic Ethanol Intake in the Rat

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Abstract Alcohol dependence is a puzzling brain disorder causing enormous suffering and financial costs world-wide. One of the few common denominators of all addictive drugs is activation of the mesolimbic dopamine system resulting in increased dopamine levels in the nucleus accumbens. In order to understand the development of addiction and find new efficient treatment strategies we need to understand how addictive drugs increase dopamine following acute and chronic administration of drugs. In the search for mechanisms underlying ethanol's ability to increase dopamine in the nucleus accumbens we have found taurine to be of major importance, although the complete picture remains to be disclosed. The aim of the present study was to explore whether chronic voluntary ethanol intake influences the ethanol-induced elevation of taurine. By means of in vivo micro-dialysis we found that voluntary intake of large amounts of ethanol for 12 weeks only had a modest influence on ethanol-induced elevations of taurine in the rat.

Keywords Alcohol • Dopamine • Self Administration • In vivo Microdialysis

Abbreviations

nAc Nucleus accumbens

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1 Introduction

Alcohol use and abuse is continuously causing disease, suffering as well as enormous costs for societies world-wide. If we are to prevent and treat alcohol use disorders, we need to understand the mechanisms underlying the development of addiction. One of the few common denominators among addictive substances is that they increase dopamine in the nucleus accumbens (nAc) (Di Chiara and Imperato 1988). This dopamine elevation has been linked to the rewarding and reinforcing properties (Koob 1992; Wise and Rompre 1989; Spanagel 2009), which is also why this neurotransmitter often is in focus in addiction research. Thus, understanding the mechanisms underlying ethanol's ability to increase dopamine is of high importance.

Apart from dopamine ethanol is also known to increase extracellular levels of the amino acid taurine (De Witte et al. 1994; Dahchour et al. 1996). In addition, local administration of taurine in the nAc increases dopamine via the same neuronal circuitry as ethanol (Ericson et al. 2006) involving glycine receptors in the nAc as well as nicotinic acetylcholine receptors in the ventral tegmental area (Söderpalm and Ericson 2013). Interestingly, we also found that in order for ethanol to produce an elevation of dopamine in the nAc an increase of extracellular taurine is required (Ericson et al. 2011). This would then implicate taurine as a major participant in the reinforcing properties of ethanol. Further studies are needed to validate or discard this hypothesis.

Several studies have linked chronic drug/alcohol intake to decreased endogenous levels of dopamine in the mesolimbic dopamine system (Diana et al. 1993; Volkow et al. 2007; Feltmann et al. 2016). This adaptation appears also to be a common denominator for addictive drugs but the underlying mechanism to this event is not known. Whether chronic intake of ethanol also produces altered extracellular levels of taurine is not well studied. Thus, in the present study we aimed to measure basal and ethanol-induced extracellular levels of taurine and dopamine in naïve rats and in rats with a history of high voluntary ethanol intake.

2 Methods

2.1 Voluntary Ethanol Intake

A total of 48 male Wistar rats (Taconic, Ejeby, Denmark) weighing 160–180 g at arrival were housed with regular 12 h light cycle and had ad lib access to tap water and standard rodent chow. Following 1 week of acclimatization to the animal facilities the rats were placed in single cages with continuous access to a bottle of water and a bottle of 6% (v/v) ethanol solution. After 3 weeks the rats with the highest ethanol consumption were selected (n = 8) and were administered 12% ethanol solution in the bottle in addition to the water bottle for another 8 weeks.

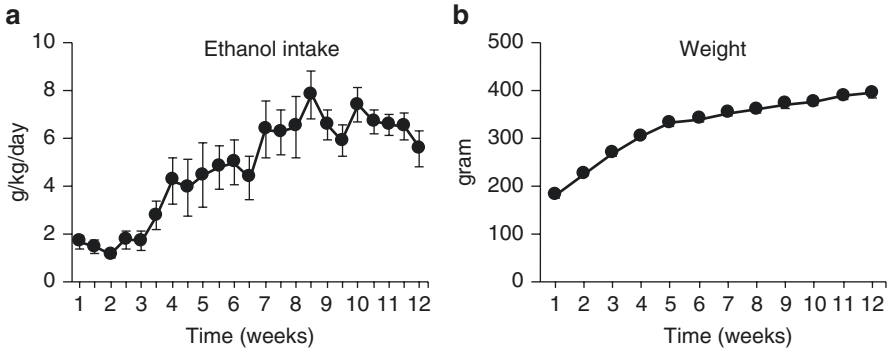

Fig. 1 Ethanol intake calculated as g/kg/day (**a**) and weight gain (**b**) for the eight selected ethanol high preferring rats placed in a voluntary ethanol consumption paradigm. The first 3 weeks the rats received 6% ethanol solution in addition to the regular water bottle and 12% ethanol solution for the remainder of the experiment

The bottles were weighted twice a week and body weight was measured once a week (Fig. 1). All experiments were approved by the Ethics Committee for Animal Experiments, Gothenburg, Sweden.

2.2 In Vivo Microdialysis

In vivo microdialysis was carried out in freely moving rats with or without a history of ethanol consumption (naïve). Dialysis probes were surgically implanted in the nAc as previously described (Clarke et al. 2014) using the coordinates (A/P: +1.85, M/L: −1.2 mm to bregma, V/D: −7.8 mm relative to dura; Paxinos and Watson 2007). Following 2 days of recovery the microdialysis experiment was initiated. The dialysis probe was connected to a microperfusion pump via a swivel and the probe was perfused with Ringer's solution at a rate of 2 μL/min. Dialysate samples were collected every 15 min and analyzed for dopamine and taurine using two separate high-performance liquid chromatography systems as previously described (Lido et al. 2009). After a stable baseline had been obtained the rats received ethanol (300 mM) in the perfusion medium for 45 min or were maintained on Ringer's solution (control animals). After the experiment the rats were sacrificed and the probe placement was verified.

2.3 Statistic Analysis

Statistical significance was determined by Student's t-test. Each value was expressed as the mean ± SEM. Differences were considered statistically significant when the calculated P value was less than 0.05.

3 Results

3.1 *Ethanol-Induced Elevation of nAc Taurine Is Modest in Ethanol-Experienced Rats*

In the present study we wanted to further explore the ethanol-induced elevation of extracellular taurine in the nAc. As we previously demonstrated an ethanol-induced elevation of dopamine needs to be preceded by an increase of extracellular taurine (Ericson et al. 2011) possible adaptations following chronic ethanol intake may be of importance. Here we found that voluntary consumption of rather high amounts of ethanol (6.22 ± 0.59 g/kg/day in average calculated from the last three recordings) did not have a major impact on ethanol's ability to increase extracellular levels of taurine (Fig. 2a).

Rats with a previous history of ethanol consumption responded with increased extracellular levels of taurine following acute ethanol administration (Ringer vs. ethanol exposed rats, $p < 0.001$). Only at time-point 30, i.e. 30 min following the

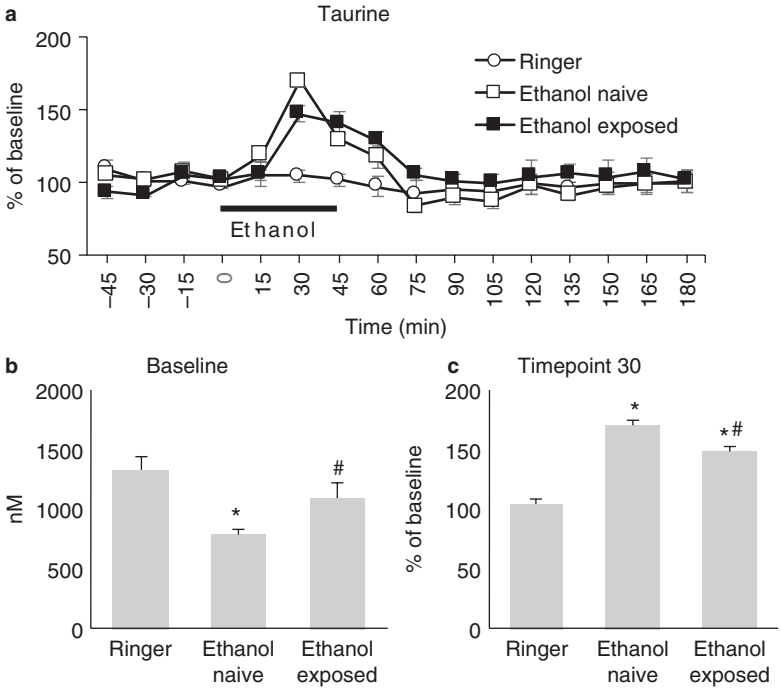

Fig. 2 Extracellular levels of taurine in the nAc as measured by in vivo microdialysis in naïve rats or rats with a high ethanol intake (n = 5–7). Ethanol (300 mM) was perfused via the dialysis probe as indicated by the *black line* in (**a**). *Baseline* levels of taurine is shown in (**b**), and the histogram in (**c**) highlights extracellular taurine levels at time-point 30. All data are expressed as mean ± SEM, * = $p < 0.05$ as calculated against the Ringer group; # = $p < 0.05$ as compared to the ethanol naïve group

initiation of ethanol perfusion, a blunted response was revealed (Fig. 2c; ethanol naïve vs. ethanol exposed, p = 0.006). When comparing baseline levels of extracellular taurine, we found that the two groups of naïve rats (Ringer and ethanol naïve groups) had significantly different extracellular levels of taurine (p = 0.004). However, when pooling the two groups with ethanol naïve rats their basal levels of taurine did not differ from rats with a previous history of voluntary ethanol consumption.

3.2 *Ethanol-Induced Elevation of nAc Dopamine Is Lower in Ethanol-Experienced Rats*

When analyzing dopamine, we found that the ethanol-induced elevation of dopamine was less prominent in rats chronically exposed to ethanol (Fig. 3a). Although the ethanol high preferring rats did respond with a significant elevation of dopamine following acute exposure (Ringer vs. ethanol exposed, p = 0.042) ethanol was able

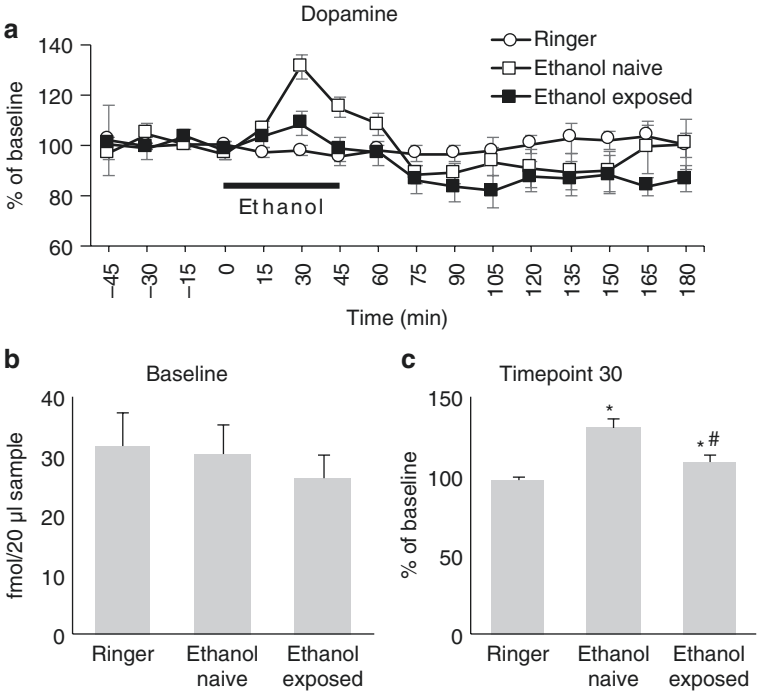

Fig. 3 Extracellular levels of dopamine in the nAc as measured by in vivo microdialysis in naïve rats or rats with a high ethanol intake (n = 6–7). Ethanol (300 mM) was perfused via the dialysis probe as indicated by the *black line* in (**a**). *Baseline* levels of dopamine is shown in (**b**), and the histogram in (**c**) highlights extracellular dopamine levels at time-point 30. All data are expressed as mean ± SEM, * = $p < 0.05$ as calculated against the Ringer group; # = $p < 0.05$ as compared to the ethanol naïve group

to produce a larger increase in the ethanol naïve rats (Fig. 3c; ethanol naïve vs. ethanol exposed, $p = 0.004$). When comparing basal levels of dopamine, no differences were found between the groups (Ringer vs. ethanol naive rats, $p = 0.427$, Ringer vs. ethanol exposed rats, $p = 0.210$).

4 Discussion

In the present study using rats with almost 3 months of high voluntary ethanol consumption, we found only very modest alterations of taurine in comparison to ethanol naïve rats. Ethanol has the ability to increase extracellular taurine both in naïve rats and rats with 3 months of high ethanol intake. This is in line with a study from Lallemand and co-workers (2011) where they only found a minor reduction in ethanol's ability to increase taurine in rats following 3 weeks of forced ethanol intake (gavage). This would then indicate that the ethanol-induced increase of extracellular taurine is a rather robust phenomenon or that a longer period of alcohol exposure is needed to induce adaptations. When dopamine was analyzed in the same samples we found a more pronounced difference in response to acute ethanol administration between naïve rats and rats that had consumed ethanol for 12 weeks. The ethanol-experienced rats displayed a significant but modest dopamine elevation. It is not possible to determine whether the diminished dopamine elevation is due to adaptations following ethanol consumption or if the animals voluntarily choose to consume large amounts of ethanol due to an unresponsive endogenous dopamine system. It has also been suggested that the time point for measuring dopamine following ethanol exposure is delicate. Immediately after withdrawal dopamine levels are low whereas after a few weeks of abstinence the dopamine levels are higher than normal (Hirth et al. 2016). Further studies addressing this question is needed.

Low levels of endogenous striatal dopamine were suggested to both be a cause for, as well as consequence of, excessive ethanol intake (Kashem et al. 2012). When monitoring the basal dopamine levels in the present study we found no differences between naïve animals and those that hade been drinking large amounts of ethanol. Since the naïve rats did not have the opportunity to demonstrate whether they were ethanol preferring rats or not, a spontaneously low dopaminergic tone could have masked any ethanol-induced adaptations in dopamine transmission. However, including more animals and utilizing another method (such as no-net-flux microdialysis) could in future studies shed light on these issues.

Analyzing basal levels of taurine and dopamine in the same samples produced an interesting result. The two groups with naïve rats differed substantially in basal levels of extracellular taurine. This would indicate that the basal extracellular levels of taurine can be very different between individuals, an interesting phenomenon that should be investigated more in depth. In the present study, although statistically significant findings, we are unable to make any conclusions regarding possible adaptations of a taurinergic tone following ethanol consumption. We will continue to investigate differences in extracellular taurine levels in relation to ethanol consumption and ethanol-induced dopamine elevation.

5 Conclusion

In the present study we found that ethanol high preferring rats, consuming ethanol for 12 weeks, displayed a modestly attenuated ethanol-induced elevation of taurine and a diminished increase of extracellular dopamine. Whether this is due to ethanol-induced adaptations or if this is an underlying factor as to why some rats choose to voluntarily consume large amounts of ethanol remains to be established.

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Taurine-Carbohydrate Derivative Stimulates Fibrillogenesis of Amyloid-β and Reduce Alzheimer-Like Behaviors

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Abstract Amyloid-β (Aβ) aggregates are a hallmark of Alzheimer's disease (AD). Through the misfolding process of Aβ in the brain, oligomeric forms of Aβ accumulate and significantly damage the brain cells inducing neuronal loss and cognitive dysfunctions that lead to AD. We hypothesized that decrease in Aβ oligomers during the aggregation process might be able to reduce Aβ-dependent brain damage. As taurine-like chemicals are often reported to have direct binding abilities to Aβ, we prepared a chemical library that consisted of taurine-carbohydrate derivatives to search for molecules that target Aβ and accelerate its fibrillogenesis. Here, we report that 1-deoxy-1-(2-sulfoethylamino)-D-fructose stimulates the formation of relatively less toxic Aβ fibrils leading to prevention of cognitive deficits in AD acute model mice.

Keywords Taurine-fructose derivative • Prevention of cognitive deficits

Abbreviations

AD Alzheimer's disease
Aβ Amyloid-β

1 Introduction

In the brains of Alzheimer's disease (AD) patients, amyloid-β (Aβ) peptides are generated through the sequential cleavage reactions on the amyloid precursor protein by β- and γ-secretases. The Aβ peptides misfold and gradually aggregate to form various toxic products in the brain. Thus, we hypothesized that regulation of Aβ misfolding process would be an attractive approach to prevent or treat AD (Wright 2006; Kim et al. 2015).

Since soluble oligomeric forms of Aβ are significantly more toxic to neurons and synapses than fibrillar forms of Aβ (Haass and Selkoe 2007; Verma et al. 2015), reducing toxic Aβ oligomers may prevent formation of Aβ-induced neurotoxic environments in AD brains. Considering that clinically diagnosed AD patients already have Aβ aggregates in their brains, reducing the exposure duration of brain tissues to toxic oligomers through stimulation of fibrillogenesis may help prevent further development of Aβ-dependent brain impairments. The stimulation of fibrillogenesis of Aβ will speed up Aβ fibril formation from toxic oligomers, leading to decreased concentration of toxic oligomers and decreased damage to the brain.

In our previous studies, we observed that taurine and taurine-like molecules directly bound to Aβ peptides and rescued Alzheimer-like behaviors in rodent models (Kim et al. 2010, 2014, 2015), suggesting a potential role of taurine in

regulating Aβ fibrillogenesis. In this study, we designed a library of taurine-carbohydrate derivatives to test how they modulate Aβ aggregation during the misfolding progression. We screened for potential molecules that accelerated Aβ fibrillization *in vitro* using thioflavin-T assay. We also confirmed that Aβ injection into the intracerebroventricular region of AD acute mouse model brain resulted in cognitive dysfunction (Kim et al. 2016). In the thioflavin-T assay, 1-deoxy-1-(2-sulfoethylamino)-D-fructose showed increased Aβ fibril formation. In addition, the cognitive function of the mice injected with an incubated sample of Aβ and 1-deoxy-1-(2-sulfoethylamino)-D-fructose showed a similar cognition level to that of the control mice, whereas mice injected only with Aβ aggregates showed AD-like abnormal behaviors. This can be explained by our hypothesis that plaques formation of Aβ in the brain might be a possible defense mechanism to minimize the fatal damages caused by toxic, soluble Aβ oligomers. Therefore, this study suggests that promotion of less-toxic species in the brains such as plaques by increasing the rate of aggregation of Aβ can be a powerful approach to reduce the Aβ-dependent impairments.

2 Methods

2.1 In Vitro *Test for Aβ Aggregation*

Aβ peptides (42 amino acids) were synthesized and purified in house using solid-phase peptide synthetic method (Choi et al. 2012). For thioflavin-T assay, Aβ was dissolved in DMSO at 5 mM. Each taurine derivative was dissolved in deionized water to make 10 mM. Aβ and taurine derivatives were mixed to make final concentration of Aβ (50 μM) and taurine derivatives (100, 50, 25, 12.5, 6.25 and 3.12 μM). The mixtures were seeded into a black 96-well plate and incubated at 37 °C. After 5 h of the incubation, thioflavin-T solution (5 μM) was added and incubated for 5 min then the fluorescent intensities were measured (excitation 450 nm/emission 485 nm) (Kim et al. 2010).

2.2 In Vivo *Test Using AD Acute Mouse Model*

All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals (8th edition) and the Animal Institutional Animal Care and Use Committee of KIST (Seoul, Korea).

To make a 100 μM of Aβ and 1-deoxy-1-(2-sulfoethylamino)-D-fructose mixture, 200 μM of Aβ (10% DMSO, 90% PBS) and 200 μM of 1-deoxy-1-(2-

sulfoethylamino)-D-fructose were prepared. Mixture was incubated for a week at 37 °C to obtain Aβ aggregates.

Imprinting Control Region (ICR) mice (6-week-old) were purchased from Orient Bio (Seoul, Korea) and habituated for 2 weeks (8-week-old). Prepared Aβ aggregates with or without 1-deoxy-1-(2-sulfoethylamino)-D-fructose were injected into the intracerebroventricular region of mice's brains (5 μL) to obtain Aβ-induced AD acute mice (Kim et al. 2016). Four days after the injections, Y-maze test was performed using a three identical arm maze with the symmetrical angles between the each arm. The number of total arm choices and the sequences of arm choices during 12-min exploration of each mouse was recorded, and then percent alternation was calculated, reflecting the level of cognitive dysfunction (Kim et al. 2013, 2015).

2.3 Statistic Analysis

Statistical Graphs were obtained with the excel program and statistical analyses were performed with Student's *t*-tests (*P < 0.05, **P < 0.01, ***P < 0.001; other comparisons were not significant). The error bars represent the sem.

3 Results

3.1 Taurine-Carbohydrate Derivative, 1-Deoxy-1-(2-Sulfoethylamino)-D-Fructose, Increases Aβ Aggregation

To screen for molecules regulating Aβ aggregation, we performed thioflavin-T assays and measured the level of mature β-sheet structure of Aβ aggregates. The taurine-carbohydrate derivatives (50 μM) were incubated with Aβ (50 μM), and we screened using ThT assay for a molecule that accelerated Aβ aggregation. We found that Aβ (50 μM) incubated with 50 μM of 1-deoxy-1-(2-sulfoethylamino)-D-fructose (Fig. 1a) showed increased fluorescent intensity compared to the control (Aβ without taurine-carbohydrate derivatives) (data not shown). We further confirmed the effect of 1-deoxy-1-(2-sulfoethylamino)-D-fructose on Aβ aggregation in various concentrations of 3.125, 6.25, 12.5, 25, 50, and 100 μM. The levels of thioflavin-T fluorescence were increased in a dose-dependent manner, suggesting that 1-deoxy-1-(2-sulfoethylamino)-D-fructose accelerates the Aβ aggregation (Fig. 1b).

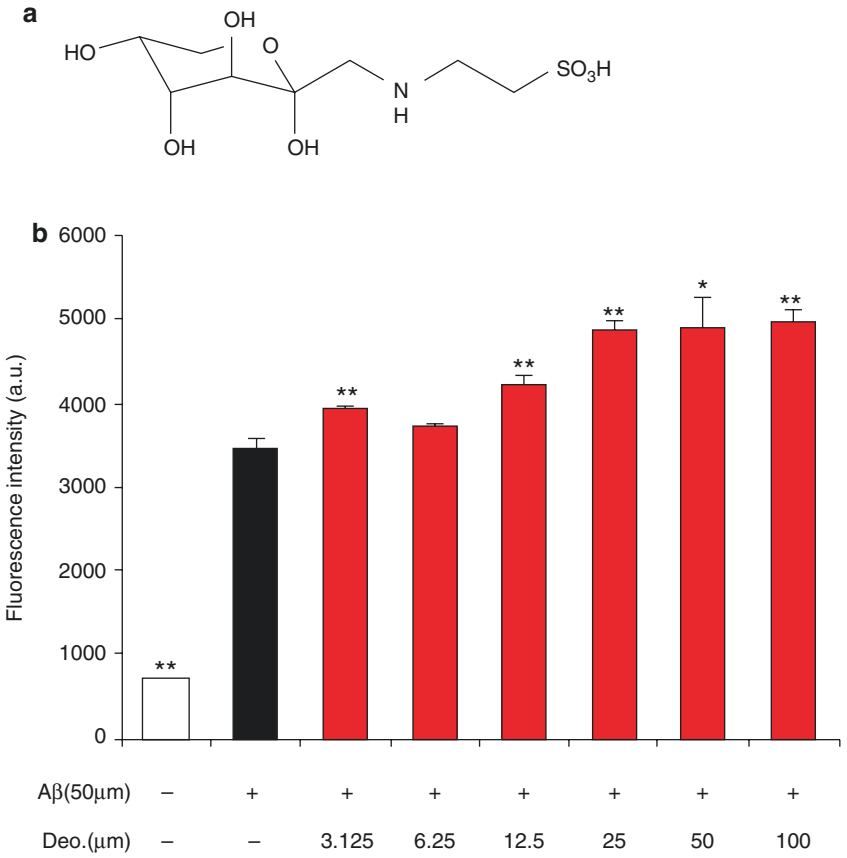

Fig. 1 1-Deoxy-1-(2-sulfoethylamino)-D-fructose increases the Aβ fibril formation. (**a**) Structure of 1-deoxy-1-(2-sulfoethylamino)-D-fructose. (**b**) Aβ peptides (50 μM) with 1-deoxy-1-(2-sulfoethylamino)-D-fructose (3.125, 6.25, 12.5, 25, 50, and 100 μM) were incubated for 5 h and their fluorescent intensities measured using thioflavin-T assay at 450/480 (ex/em) by EnSpire plate reader (Perkin-Elmer). The error bars represent the sem (*P < 0.05, **P < 0.01, ***P < 0.001; other comparisons were not significant). *Aβ* Amyloid-β, *Deo.* 1-deoxy-1-(2-sulfoethylamino)-D-fructose, *a.u.* arbitrary unit

3.2 Taurine-Carbohydrate Derivative, 1-Deoxy-1-(2-Sulfoethylamino)-D-Fructose, Blocks the Cognitive Impairment in Aβ-Infused AD Mice

Since Aβ plaques are less toxic than oligomeric forms (Haass and Selkoe 2007), acceleration of Aβ fibrilization into plaques by 1-deoxy-1-(2-sulfoethylamino)-D-fructose might lessen pathological symptoms in AD. To examine this hypothesis, we acutely induced AD-like symptoms in mice by injecting oligomeric forms of Aβ into the mouse brain. Aβ was incubated with or without

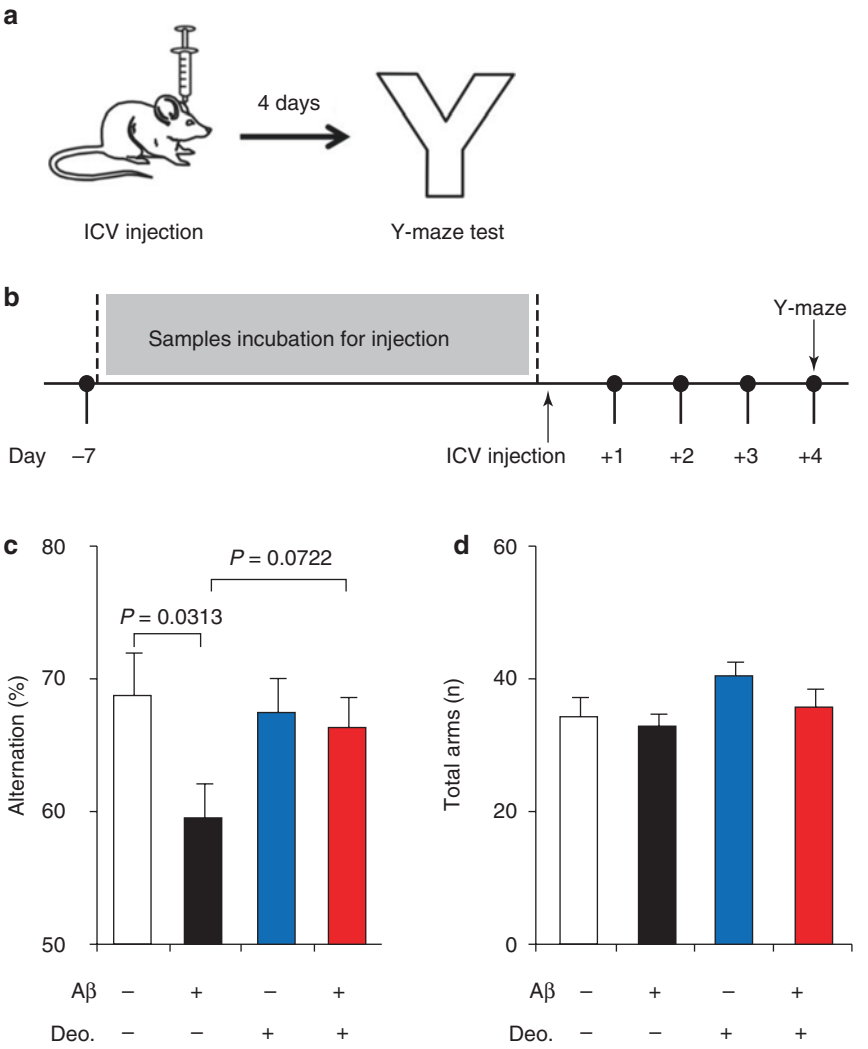

Fig. 2 Incubated Aβ with 1-deoxy-1-(2-sulfoethylamino)-D-fructose injected mice showed the normal cognitive function in Y-maze. (**a**) For AD acute mice preparation, Aβ was incubated for a week with or without 1-deoxy-1-(2-sulfoethylamino)-D-fructose to obtain the aggregated forms. Vehicle (10% DMSO, 90% PBS), Aβ aggregates, Aβ aggregates with 1-deoxy-1-(2-sulfoethylamino)-D-fructose, and 1-deoxy-1-(2-sulfoethylamino)-D-fructose were injected into the intracerebroventricular injection of ICR mice (8 weeks, male, n = 8 per group). (**b**) Y-maze tests were performed to obtain percent alternation reflecting cognitive functions and (**c**) total numbers of arm entries during the test. The error bars represent the sem (*P < 0.05, **P < 0.01, ***P < 0.001; other comparisons were not significant). *ICV* intracerebroventricle, *Aβ* Amyloid-β, *Deo.* 1-deoxy-1-(2-sulfoethylamino)-D-fructose

1-deoxy-1-(2-sulfoethylamino)-D-fructose for a week to obtain aggregated forms and injected into intracerebroventricular region of Imprinting Control Region (ICR) mice (8-week-old, male, n = 8) (Fig. 2a). We conducted Y-maze behavioral tests 4 days after the intracerebroventricular injection to assess spatial working memory (Fig. 2b). While mice injected only with Aβ showed significant cognitive impairment, mice injected with the mixture of Aβ and 1-deoxy-1-(2-sulfoethylamino)-D-fructose did not display the behavioral deficits (Fig. 2c) without alteration of total numbers of arm entries (Fig. 2d). These results imply that 1-deoxy-1-(2-sulfoethylamino)-D-fructose prevented the Aβ-induced cognitive impairments in the Aβ infusion model.

Considering both the thioflavin-T assay and the animal studies, we conclude that rapid aggregation of Aβ by 1-deoxy-1-(2-sulfoethylamino)-D-fructose decreased concentration of toxic species of Aβ and prevented cognitive deficits in acutely induced AD mice.

4 Discussion

In normal human brains, Aβ concentration is maintained through homeostasis for physiological functions. However, imbalance of the Aβ homeostasis leads to misfolding of the peptide—formation of oligomers, fibrils, and plaques—resulting in AD (Sadigh-Eteghad et al. 2015). Among the various misfolded Aβ species, oligomeric forms induce neuotoxicity which can disturb long-term potentiation and learning and memory (Shankar and Walsh 2009). Therefore, regulations of Aβ fibrillization, production, and clearance have been targeted as a treatment strategy of AD.

Here we report that a taurine-carbohydrate derivative, 1-deoxy-1-(2-sulfoethylamino)-D-fructose, accelerates the aggregation of Aβ oligomeric forms into mature fibrils. Faster aggregation of Aβ by the addition of our taurine derivative removes toxic oligomers that are highly correlated with cognitive deficits. This study supports our hypothesis that alternative method of clearing Aβ oligomers by stimulating fibrillogenesis in the brain will be able to block the Aβ-induced brain damages.

5 Conclusion

In summary, this study shows that 1-deoxy-1-(2-sulfoethylamino)-D-fructose stimulated rapid aggregation of Aβ into fibrils. Furthermore, AD mouse group intracerebroventricularly injected with a mixture of Aβ and 1-deoxy-1-(2-sulfoethylamino)-D-fructose resulted in normal behavior. The stimulatory effect of the taurine-carbohydrate, 1-deoxy-1-(2-sulfoethylamino)-D-fructose, on Aβ aggregation provides a novel strategy for approaching AD treatment.

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Taurine Directly Binds to Oligomeric Amyloid-β and Recovers Cognitive Deficits in Alzheimer Model Mice

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Abstract Alzheimer's disease (AD) is the most common cause of dementia leading to severe cognitive decline. During the progression of AD, amyloid-β (Aβ) monomers aggregate into neurotoxic soluble oligomeric Aβ that causes cognitive impairments. Our previous study indicates that oral supplementation of taurine at 1000 mg/kg/day significantly ameliorates hippocampal-dependent cognitive deficits in

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APP/PS1 transgenic AD mouse model. However, Aβ plaques and oligomeric Aβ levels are not affected after administration of taurine and the oral dosage of taurine was relatively high. Thus, in this study, we focused on direct correlation between taurine and oligomeric Aβ, causing memory deficits in a lower oral dosage of taurine, 250 mg/kg/day. We induced AD-like cognitive impairments to adult normal mice and orally administered taurine via drinking water for 10 days. We confirmed that taurine administration improved cognitive deficits in oligomeric Aβ-infusion mice in Y-maze and passive avoidance tests without activity alteration of mice. In addition, we found that taurine directly bound to oligomeric Aβ in surface plasmon resonance analyses. Our results propose that taurine can ameliorate cognitive impairment by directly binding to oligomeric Aβ in oral administration of 250 mg/kg/day for 10 days.

Keywords Alzheimer's disease • Amyloid-β • Taurine • Dementia

1 Introduction

Taurine, which is a natural endogenous small molecule, is involved in many physiological processes of human body, such as stabilization of protein, anti-inflammatory pathway and calcium homeostasis (Schaffer et al. 1995; Huxtable 1992; Miao et al. 2012). Due to its remedial properties, taurine has been used to treat various disorders, such as liver and heart failures (Matsuyama et al. 1983; Azuma et al. 1985). Our previous study reports that oral administration of taurine recovers cognitive deficits in the APPswe/PS1dE9 transgenic Alzheimer's disease (AD) mouse model (Kim et al. 2014). AD is a neurodegenerative disease characterized by aberrant deposition of amyloid-β (Aβ) in the brain. Aβ monomers aggregate into oligomeric Aβ, which plays critical role in disruption of cognitive function (Ferreira et al. 2015). This transgenic AD mouse model overexpresses mutant human amyloid precursor protein (APP) and presenilin-1 (PS1) protein (Jankowsky et al. 2001). These mutations, APPSwe and PS1dE9, are associated with abnormal production of Aβ. Although taurine administration improved AD-like cognitive decline, alteration of Aβ levels was barely found. Considering that APP/PS1 transgenic AD mice exhibit numbers of AD-like pathological phenotypes, such as gliosis as well as overproduction of various Aβ aggregates and truncates (Kamphuis et al. 2012), it is not suitable for investigating the clear correlation between taurine and oligomeric Aβ. Thus, in this study, we utilized an acute AD mouse model to tackle the issue. To induce acute AD-like cognitive deficits, we infused oligomeric Aβ into the lateral ventricle via intracerebroventricular injection in cognitively normal mouse brain. (Kim et al. 2016). Given that cognitive function of this acute AD mouse model is affected by direct injection oligomeric Aβ, it is possible to study the direct correlation between taurine and oligomeric Aβ. Taurine dissolved in drinking water was orally administered to oligomeric Aβ-infusion mice for 10 days. During administration of taurine, Y-maze and passive avoidance tests were performed to assess cognitive functions of oligomeric Aβ-infusion mice. In addition, we performed surface plasmon resonance analysis to investigate molecular interactions between taurine and oligomeric Aβ.

2 Methods

2.1 Materials

Dimethyl sulfoxide (DMSO) and taurine was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Phosphate buffered saline (PBS) was purchased from Gibco (Waltham, Massachusetts, USA). Deionized water was produced by Milli-Q plus ultrapure water system from Millipore (Darmstadt, Germany). Microsyringe was purchased from Hamilton company (Bonaduz, Switzerland).

2.2 Oligomeric Aβ-Infusion Mouse Model and Oral Administration of Taurine

All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals (8th edition) and the Animal Institutional Animal Care and Use Committee of KIST (Seoul, Korea).

C57Bl/6 mice (male, 6-week-old, n = 7) were purchased from Orient Bio Inc. (Seoul, Korea). To obtain oligomeric Aβ, Aβ42 (100 μM, 10% DMSO in PBS) was incubated at 37 °C for 1 week. Then, 5 μL of oligomeric Aβ or vehicle (10% DMSO in PBS) were injected into the lateral ventricle in the mouse brain via intracerebroventricular (ICV) injection. Aβ42 was synthesized with an automated peptide synthesizer (Choi et al. 2012). Oligomeric Aβ were injected on day 0 of the experiment, and taurine was orally administered from day −3 to day 7 at 250 mg/kg/day. For calculation of exact dosage of taurine, body weight and daily water consumption of mice were measured. In addition, health alterations, such as hair loss and body weight, were not observed.

2.3 Behavioral Test

Y-maze test. Y-maze test was used to evaluate spatial working memory of rodents. Y-maze apparatus was made of black plastic with 3-arms (40 L × 10 W × 12 H cm). The arms were arranged at 120° from each other. A mouse was placed at the end of one arm and allowed to explore y-shaped arms for 12 min. A successful entry was considered to occur when the whole tail of the mouse came within an arm. The sequences of arm entries were recorded.

Passive avoidance test. The Passive avoidance test was performed to assess fear-motivated hippocampal memory of rodents. The apparatus was composed of a dark chamber, equipped with a foot shock generator, and a light chamber. In the acquisition phase, a mouse was placed in the bright compartment. After 20 s, the door dividing the chambers was opened, and the mouse instinctually moved into the dark compartment. When all four limbs came within the dark chamber, the mouse

received an electric foot shock (0.2 mA, 1 sec, once). The next day, in the retention phase, each mouse was again placed in the bright area. The latency time was manually recorded (cutoff 500 s).

2.4 Surface Plasmon Resonance Analysis

The surface plasmon resonance analysis was conducted using Biacore T200 instrument and Series S carboxymethylated (CM5) dextran matrix sensor chips (Richter et al. 2010). HBS-EP$^+$ (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05% surfactant P20) was used as a running buffer at 25 °C. oligomeric Aβ (200 μg/mL) was diluted with 10 mM sodium acetate solution (pH 4.0 and pH 5.5, respectively) to make 40 μg/mL of oligomeric Aβ. Afterwards, oligomeric Aβ was covalently immobilized on the chip surface by amine coupling reaction. The remaining activated carboxymethyl groups on the surface were blocked by injection of 1 M ethanolamine (pH 8.0). Immobilization value of oligomeric Aβ was 9000 RU, and theoretical Rmax of oligomeric Aβ was 249.4 RU. Taurine was prepared in PBS-T running buffer (10 mM phosphate, 135 mM NaCl, 27 mM KCl, and 0.005% surfactant P20) as serial-diluted samples. For the binding and kinetics assay, Taurine was injected for 120 s at a flow rate of 30 μL/min.

2.5 Statistic Analysis

Statistical significance analysis between control and experiment groups was performed with Student's t-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, n.s.: no significance). The SEM values were indicated by error bars.

3 Results

3.1 Taurine Ameliorates Cognitive Deficits in Oligomeric Aβ-Infusion Mouse Model

In behavioral results from our previous study, we confirmed that taurine supplementation at 1000 mg/kg/day alleviates cognitive decline in APPswe/PSdE9 double transgenic mice. However, this concentration of taurine is relatively high to develop drug for AD patients. In order to verify therapeutic effect of taurine at a lower dasage, we adjusted administration dosage of taurine at 250 mg/kg/day. We orally administered taurine to oligomeric Aβ-infusion group (male, 7-week-old, n = 7) for

10 days. To investigate effects of taurine on cognitive decline caused by oligomeric Aβ, we induced acute AD-like cognitive impairments to wild-type C57Bl/6 mice (male, 7-week-old, n = 7) via intracerebroventricular injection.

To evaluate cognitive functions of vehicle and oligomeric Aβ-infusion mice, they were subjected to behavioral tests, including Y-maze and passive avoidance tests. Y-maze test was performed to assess the spatial working memory of rodent model. During this experiment, mice were allowed to explore y-shaped arms. The sequences of arm entries were recorded to calculate spontaneous alternation. We found that oral administration of taurine at 250 mg/kg/day significantly improved the spatial working memory of oligomeric Aβ-infusion mice (Fig. 1a) and that total arm entry number indicating behavioral activity of mice did not differ from other mice groups (Fig. 1b).

Following the completion of Y-maze test, we performed the passive avoidance test to evaluate fear-motivated hippocampal memory. The passive avoidance test was performed based on the instinctual preference of mice for dark conditions. Greater response latency in the retention phase indicated better hippocampal memory. In this study, we confirmed that taurine significantly enhances hippocampal memory in oligomeric Aβ-infusion mice (Fig. 1c)

3.2 Taurine Directly Binds to Oligomeric Aβ

Synaptic degeneration in the hippocampus and cortex is strongly correlated with cognitive impairments (Huang and Mucke 2012; Selkoe 2002; Sheng et al. 2012). Oligomeric Aβ induces memory loss by binding to mature synapses and inhibiting synaptic function (Lacor et al. 2004). Thus, we hypothesized that by directly binding to oligomeric Aβ, taurine may inhibit the synaptotoxicity of oligomeric Aβ. To examine molecular interactions between taurine and oligomeric Aβ, we carried out the surface plasmon resonance (SPR) analysis. Initially, oligomeric Aβ were immobilized on a CM5 sensor chip through the amine coupling reaction. Then, taurine was injected into the flow cell of Biacore T200 system at various concentrations (from 0.3 to 19.2 nM). In this analysis, we found that taurine directly binds to oligomeric Aβ with dose-dependent manner (Fig. 2a, b). This result suggests that taurine inhibits oligomeric Aβ-induced memory deficits by direct interacting with oligomeric Aβ.

Taken together, in behavioral studies, we confirmed that taurine supplementation at 250 mg/kg/day improves cognitive deficits in oligomeric Aβ-infusion mouse to a similar extent as the previously determined concentration of 1000 mg/kg/day. In addition, we found that taurine directly binds to oligomeric Aβ. Given that oligomeric Aβ induce cognitive impairment by binding to selective receptors in neuronal synapses, enhancement of learning and memory in oligomeric Aβ-infusion mouse model suggests that taurine may prevent memory deficits by direct binding to oligomeric Aβ.

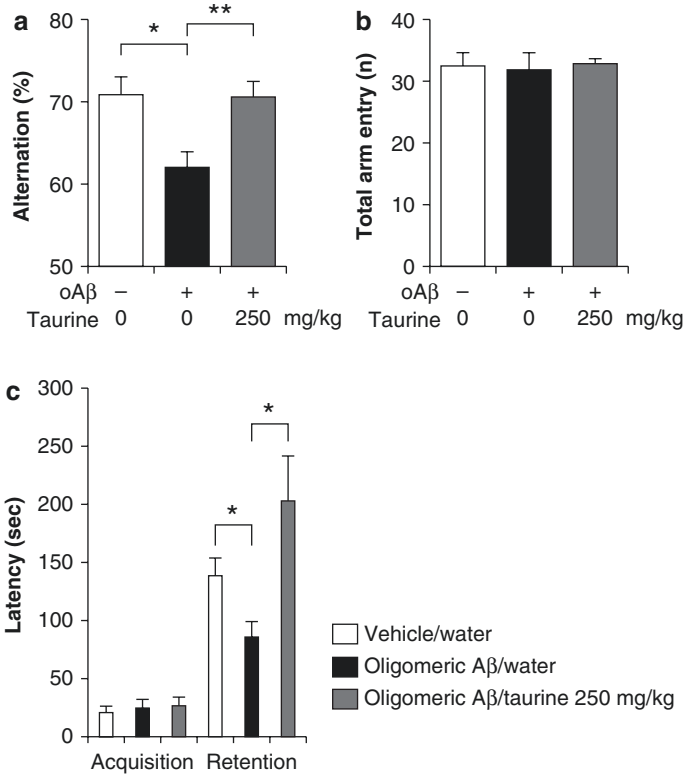

Fig. 1 Amelioration of cognitive impairments in oligomeric Aβ-infusion mouse model after taurine supplementation. Vehicle group and age-matched oligomeric Aβ-infusion group were orally administered water or taurine (250 mg/kg/day) for 10 days. (**a**, **b**) Y-maze test on vehicle and oligomeric Aβ-infusion mice. Average spontaneous alternation (%) of each group (**a**) and total arm entry number (**b**). (**c**) Passive avoidance test. Average response latency time for each group of mice. Error bars represent the SEM. (*P < 0.05, **P < 0.01, ***P < 0.001, other comparisons were not significant.) *oAβ,* oligomeric Aβ

4 Discussion

Here we report that (1) oral administration of taurine improves oligomeric Aβ-induced AD-like cognitive deficits in oligomeric Aβ-infusion mouse model, (2) taurine directly interacts with oligomeric Aβ, and (3) lowering the oral administration dosage, 250 mg/kg/day, and shortening the uptake duration, 10 days, of taurine is effective to prevent Alzheimer-like cognitive behaviors in mice.

In this study, we confirmed that taurine binds to oligomeric Aβ by using surface plasmon resonance analysis. These results follow our previous observation that taurine-like chemicals directly binds to Aβ and supports our hypothesis that taurine

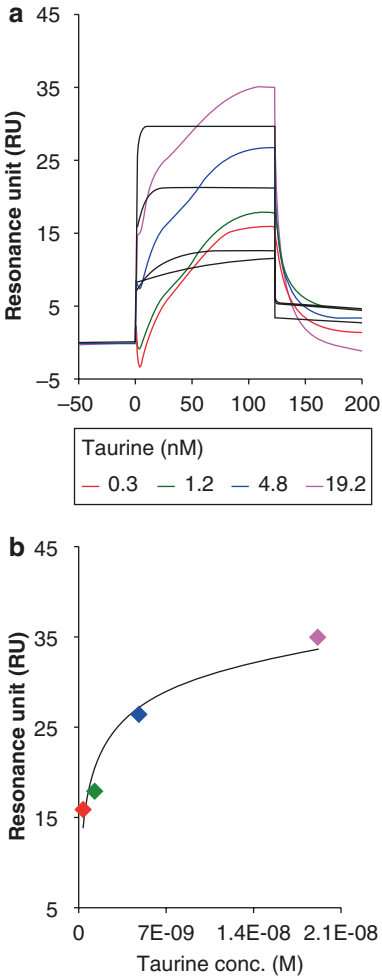

Fig. 2 Binding study on taurine and oligomeric Aβ. Surface plasmon resonance sensorgrams showing the binding of taurine toward immobilized oligomeric Aβ (**a**). The corresponding plot of steady stated response against various concentrations of taurine (**b**)

may interact with oligomeric Aβ. Thus, we propose that taurine can alleviate cognitive decline in oligomeric Aβ-infusion mouse model by direct binding to oligomeric Aβ. However, further studies are required to understand how taurine interacts with Aβ oligomers, as we only confirmed the ability of taurine to binds to oligomeric Aβ.

Our current study shows that taurine induces therapeutic effects at a lower dosage than previously studied. Therefore, these findings increase therapeutic potential of taurine as a drug to ameliorate cognitive deficits in AD patients. Furthermore, our findings about the molecular interactions between taurine and oligomeric Aβ may lead to further elucidation of the mechanisms underlying taurine mediated cognitive improvement.

5 Conclusion

Our current study suggests that oral administration of taurine at 250 mg/kg/day ameliorates cognitive impairment by directly binding to oligomeric Aβ.

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Neuroprotective Effect of Taurine-Rich Cuttlefish (*Sepia officinalis*) Extract Against Hydrogen Peroxide-Induced Oxidative Stress in SH-SY5Y Cells

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Abstract Oxidative stress mediates the cell damage in several neurodegenerative diseases, some of which are Alzheimer's disease (AD), multiple sclerosis and Parkinson's disease (PD). In this study, we investigated whether the taurine-rich cuttlefish extract could exert a protective effect on damaged human neuroblastoma SH-SY5Y cells induced by hydrogen peroxide (H_2O_2). Our results revealed that pre-treatment with cuttlefish extract effectively increased the cell viability by protecting the cells from intracellular reactive oxygen species (ROS) induced by H_2O_2 exposure. Furthermore, apoptosis related proteins Bcl-2 and Bax were investigated by western-blot analysis and results indicated that cuttlefish extract promoted the expression of anti-apoptotic Bcl-2 protein while inhibiting the expression of pro-apoptotic Bax protein. Therefore, cuttlefish extract containing the ability of scavenging excessive ROS, the capacity of anti-oxidative stress, could be employed in neurodegenerative disease prevention. In conclusion, the results suggest that cuttlefish extract could be used as a potential candidate for preventing several human neurodegenerative and other disorders caused by oxidative stress.

Keywords Neuroprotective effect • Cuttlefish • SH-SY5Y Cells

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1 Introduction

Oxidative stress is considered as one of the most significant candidates for the development of many kinds of diseases in humans (Kędziora-Kornatowska et al. 2010; Davies 1995; Matés et al. 2012). Since a long time, cell injury caused by oxidative stress through apoptosis or necrosis has been regarded as the main reason for neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Bhat et al. 2015: Yang et al. 2013). Therefore, antioxidants extracted from natural sources (fruits, vegetables or mushrooms) might be a possible strategy that could reduce the disease progression by proving neuroprotective effects through the scavenging of ROS and maintain a low cell oxidative stress (Haleagrahara et al. 2011; Hu et al. 2014).

Taurine is an organic compound, β-amino acid and it is distributed in high concentrations in mammalian tissues. A number of cytoprotective attributes are shown by this organic compound, taurine via its many roles such as a neurotransmitter, osmoregulator, neuromodulator, anti-oxidant, membrane stabilizer, anti-inflammation agent and neuroprotective agent (El Idrissi 2008; Schuller-Levis and Park 2004; Haas and Hosli 1973; Hussy et al. 1997). In recent years, many *in vitro* and *in vivo* brain injury model studies have been revealed that taurine has a neuroprotective activity (Zhu et al. 2016).

Common cuttlefish (*Sepia officinalis*) belongs to the class cephalopod and the genus *Sepia*. It is native to the Mediterranean Sea, the North Sea and Baltic Sea and distributed in some other parts of the world too. It is a rich source of taurine (Hmidet et al. 2011). However, many researchers have focused their studies on studying the functional activity of cuttlebone and muscle of *S. officinalis.*

In this study, we investigated the neuroprotective effect of taurine-rich cuttlefish extract against H_2O_2-induced oxidative stress in SH-SY5Y cells.

2 Materials and Methods

2.1 Reagent

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Industries Inc. (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), *N*-acetyl-L-cysteine (NAC), and dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (Missouri, USA). All other chemicals and reagents used in the experiments were of analytical grade.

2.2 Preparation of S. officinalis *Extract (SOE)*

In order to obtain the maximum yield of SOE, 100 g of *S. officinalis* was cut into small pieces and extracted with 1 L of distilled water at 95 °C for 1 h. Next, the water extract of *S. officinalis* was filtered through a Whatman No. 41 filter paper and

the resulted filtrate was evaporated on a rotary vacuum evaporator. Finally, the evaporated filtrate was lyophilized to obtain SOE in powder form.

2.3 Analysis of Chemical Composition Including Determination of Amino Acid Composition

Crude protein, crude fat (ether extract) and total ash content of SOE were determined according to the Association of Official Analytical Chemists (AOAC 1990) methods. Amino acid composition was analyzed using following steps. First, SOE was mixed with 10 mL of 6 N HCl. After that, the HCl acid mixed samples in the test tubes were purged with N_2 gas and then the samples were hydrolyzed in a dry oven at 110 °C for 24 h. The hydrolyzed samples were then evaporated and added with sodium-distilled buffer (pH 2.2). Next, samples were filtered through a syringe filter (0.45 μm) and analyzed for amino acid composition by measuring the absorbance at 440 and 570 nm.

2.4 Cell Culture

The human neuroblastoma SH-SY5Y cell line was obtained from the American Type Culture Collection (ATCC, CRL-2266™). It was cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin at 37 °C in a 5% CO_2 incubator.

2.5 Measurement of Cell Viability Using MTT Assay

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay was used to determine the cell viability. SH-SY5Y cells (1×10^4 cells/well) seeded in 96-well plates were treated with SOE and incubated for 24 h. After that, treated cells were again incubated with MTT solution at 37 °C for 3 h. For the demonstration of the potential protective effect of SOE, cells were pre-treated with SOE 1 h prior to H_2O_2 (150 μM) exposure for 24 h. After completion of H_2O_2 induction, MTT solution was added to each well and cells were further incubated at 37 °C for 3 h. The resulted formazan crystals were dissolved in DMSO and the absorbance at 540 nm was measured using a microplate reader (Thermo Scientific Multiskan GO, Thermo Fisher Scientific) to determine the cell viability. The cell viability of each sample was expressed as a percentage of untreated control cultures.

2.6 Determination of Morphological Changes

SH-SY5Y cells plated at a density of 2×10^5 cells per well in 6-well plates were pre-treated with SOE 1 h prior to H_2O_2 (150 μM) exposure for 24 h. Then, cells were observed under a phase-contrast microscope (Nikon, Japan) and 200× magnified images were recorded for close monitoring of morphological changes in the cells.

2.7 Measurement of Intracellular ROS Production

SH-SY5Y cells plated at a density of 2×10^5 cells per well in six well plates were incubated at 37 °C for 24 h. After the initial incubation period, cells were pretreated with SOE for 1 h before cells were exposed to H_2O_2 (200 μM) treatment. Intracellular ROS production was measured with the help of fluorescence probe DCFH-DA according to a previous demonstration (Hu et al. 2011). After 30 min of H_2O_2 exposure, cells were incubated with DCFH-DA (10 μM) fluorescence probe at 37 °C for 30 min under dark environment. After incubation, the cells were washed with PBS and the fluorescence was measured using a spectrofluorometer (SpectraMax M2/M2e; Molecular Devices).

2.8 Western Blot Analysis

After treatments, proteins were extracted and quantified using Bradford assay. Then the samples containing 25 mg of protein were subjected to SDS-polyacrylamide gel electrophoresis and separated proteins were transferred onto polyvinyldifluoride (PVDF) membranes. Then the membranes were blocked by non-fat dry milk (5% w/v) in TBST for 1 h at room temperature. Next, the membranes were incubated with specific primary antibodies at 4 °C overnight. After washing with TBST, the membranes were incubated again with the species appropriate HRP-conjugated secondary anti-bodies at room temperature for another 1 h. Finally, the blots were developed using ECL chemiluminescence detection reagent and they were visualised using a charge-coupled device system (LAS-3000; Tokyo, Japan).

2.9 Statistical Analysis

GraphPad Prism 5.0 was employed for statistical analysis of the data. Resulted values were analyzed statistically by using one way ANOVA, followed by Dunnett's tests. Each data was expressed as the mean ± SD. Significant level; $P < 0.05$ was set to consider statistically significances in values.

3 Results

3.1 Chemical Composition of SOE

The chemical composition of *S. officinalis* extract is given in Table 1. The percentages (w/w) of dry matter, crude protein, crude lipids, crude carbohydrates, and ash were 96.0%, 71.1%, 1.05%, 2.75% and 29.7%, respectively. Crude proteins were the most abundant compound available in the SOE.

3.2 Amino Acid Composition of SOE

The amino acid composition of *S. officinalis* extract is given in Table 2. According to the outcomes, the total amino acid concentration was 1352 μg/mL. Taurine, Arginine, and Glutamic amino acids were the major free amino acids available in

Table 1 Chemical composition of *S. officinalis* extract (%, dry matter basis)

Chemical composition	SOE
Dry matter	96.0 ± 0.16
Crude proteins	71.1 ± 0.48
Crude lipids	1.05 ± 0.23
Crude carbohydrates	2.75 ± 0.08
Ash	29.7 ± 0.52

Table 2 Amino acid composition of *S. officinalis* extract (μg/mL)

Amino acid composition	SOE
Taurine	460.56
Aspartic acid	37.46
Glutamic acid	98.93
Proline	41.51
Glycine	31.29
Alanine	69.55
Valine	41.60
Cysteine	13.36
Methionine	36.13
Isoleucine	34.50
Leucine	74.15
Tyrosine	49.20
Lysine	65.57
Histidine	13.69
Arginine	167.14
Serine	40.29
Phenylalanine	44.17
Threonine	32.90
Total	1352

S. officinalis extract. Most importantly, the taurine concentration was considerably high, even higher among other major free amino acids in *S. officinalis* extract, resulting 460.56 μg/mL.

3.3 Effect of SOE on Cell Viability

The effect of SOE on SH-SY5Y cell viability was assessed via MTT assay. SH-SY5Y cells were treated with SOE at different concentrations in between 0 and 0.2 mg/mL. After incubation for 24 h, the cell viability was determined and results were compared with control groups (0 μg/mL). SOE treated groups showed increased levels of cell viability in a dose-dependent manner (Fig. 1).

3.4 Protective Effect of SOE Against H_2O_2-Induced Oxidative Stress

To investigate the neuroprotective feature of SOE, cell viability was determined via MTT assay after secondary H_2O_2 treatment accompanied with SOE initial treatment on SH-SY5Y cells. As shown in Fig. 2, H_2O_2 (150 μM) treatment reduced the cell viability down to approximately 60%, but the SOE treatment could recover the damage and moreover it increased the cell viability in a dose-dependent manner. The significantly ($p < 0.01$) increased cell viability against H_2O_2-induced cell damage suggests that SOE possesses a protective effect for the oxidative stressed SH-SY5Y cells.

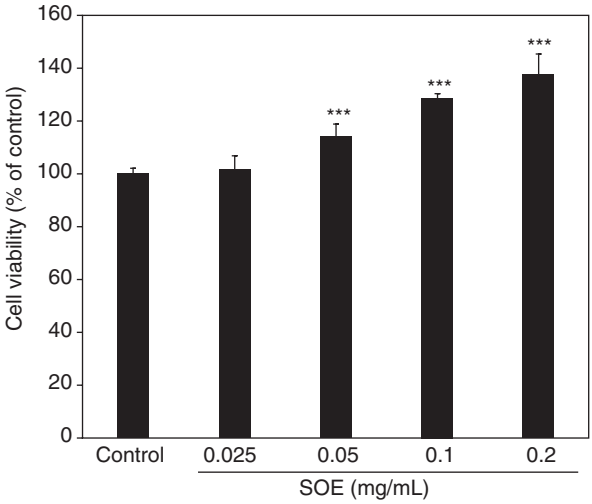

Fig. 1 The effect of *S. officinalis* extract on cell viability. SH-SY5Y cells were incubated with different concentrations of SOE for 24 h. Cell viability was then evaluated by the MTT assay. Data are presented as the mean ± SD ($n = 3$). $^{***}p < 0.001$ compared to control group

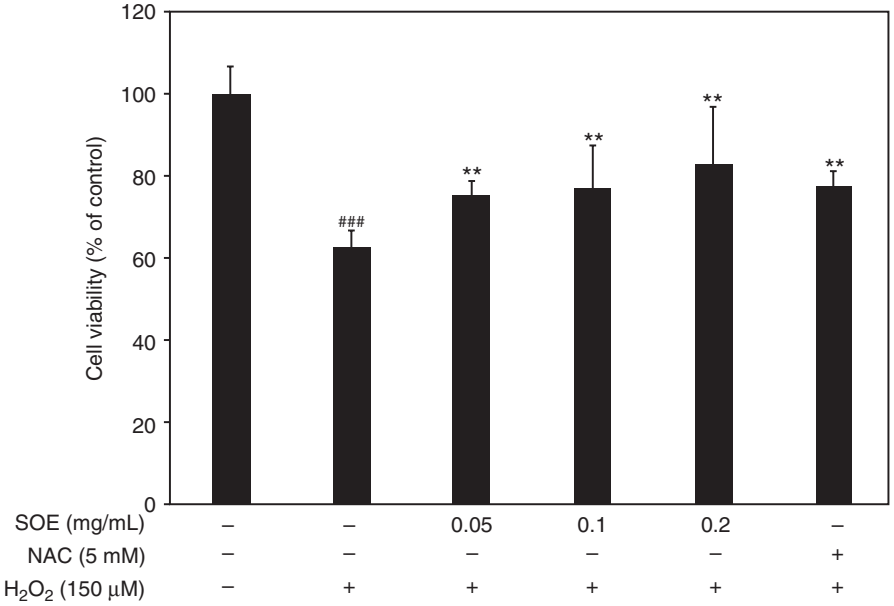

Fig. 2 Protective effect of *S. officinalis* extract on H_2O_2-induced oxidative stress cells. SH-SY5Y cells were pre-treated with different concentrations of SOE for 1 h before treatment of 150 μM H_2O_2 for 24 h. Cell viability was measured by the MTT assay. Data are presented as the mean ± SD ($n = 3$). $^{**}p < 0.01$ compared with H_2O_2 group, $^{###}p < 0.001$ compared with control group

3.5 *Effect of SOE on Inhibition of Intracellular ROS*

As shown in Fig. 3, when the cells were induced by H_2O_2 without SOE treatment, the fluorescence intensity increased significantly and when the cells were treated with SOE, the fluorescence intensity of cells decreased greatly. As the positive control, *N*-acetyl-L-cysteine (NAC) showed a strong inhibition of intracellular ROS production. The results suggest that SOE's protective effect on cells damage caused by H_2O_2 is associated with its inhibitory effect on ROS production.

3.6 *Effect of SOE on Cellular Morphology*

Cell morphology and growth were observed by using an inverted microscope. As shown in Fig. 4, adherent cells were grown well in dispersed manner and cell shapes were sharp and long. When cells were damaged by H_2O_2, it was found that adherent cells were deteriorated with cell aggregation and they became round in shape. However, administration of SOE exhibited significant improvement towards the healthy morphology.

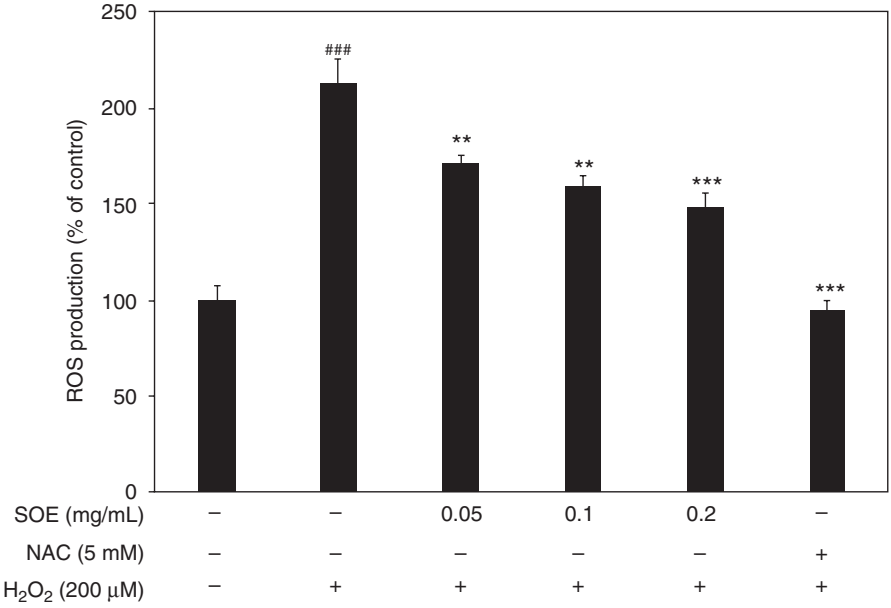

Fig. 3 Inhibitory effect of SOE on intracellular ROS production in H_2O_2-induced oxidative stress SH-SY5Y cells. SH-SY5Y cells were pre-treated with various concentrations of SOE for 1 h before treatment with 200 μM H_2O_2 for 30 min. ROS production was measured using a spectrofluometer. Data are given as the mean ± SD (n = 3). $^{**}p < 0.01$ compared with H_2O_2 group, $^{###}p < 0.001$ compared with control group

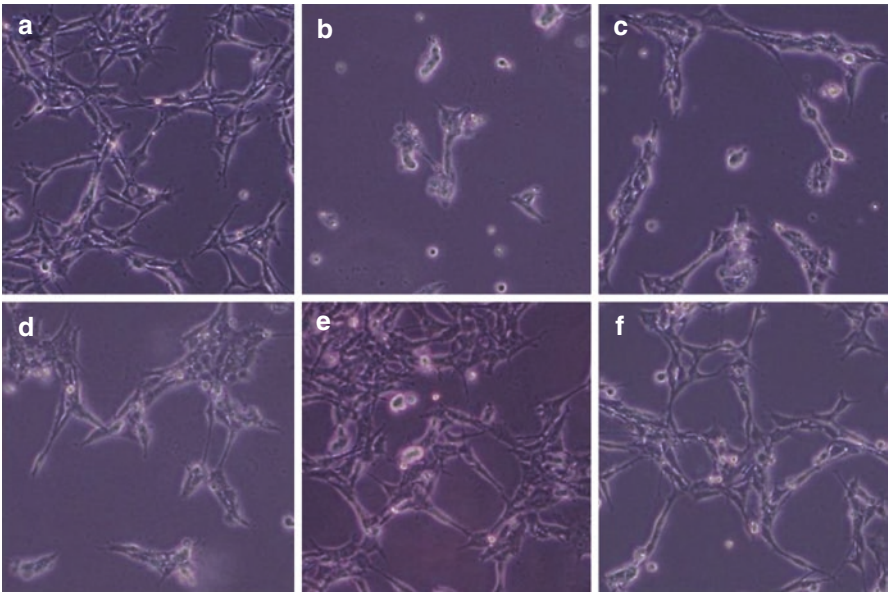

Fig. 4 Effect of SOE on cell morphology of H_2O_2-induced oxidative stress SH-SY5Y cells (**a**) Control, (**b**) H_2O_2 (150 μM), (**c**) 0.05 mg/mL of SOE + H_2O_2, (**d**) 0.1 mg/mL of SOE + H_2O_2, (**e**) 0.2 mg/mL of SOE + H_2O_2, (**f**) 5 mM of NAC + H_2O_2

3.7 *Effect of SOE on the Expression of Bcl-2 and Bax*

As shown in Fig. 5a, SOE significantly suppressed the expression level of pro-apoptotic Bax protein and it increased the expression level of the anti-apoptotic Bcl-2 protein. The present results reveal that SOE shows a significant protective effect against H_2O_2-induced cell damage in SH-SY5Y cells.

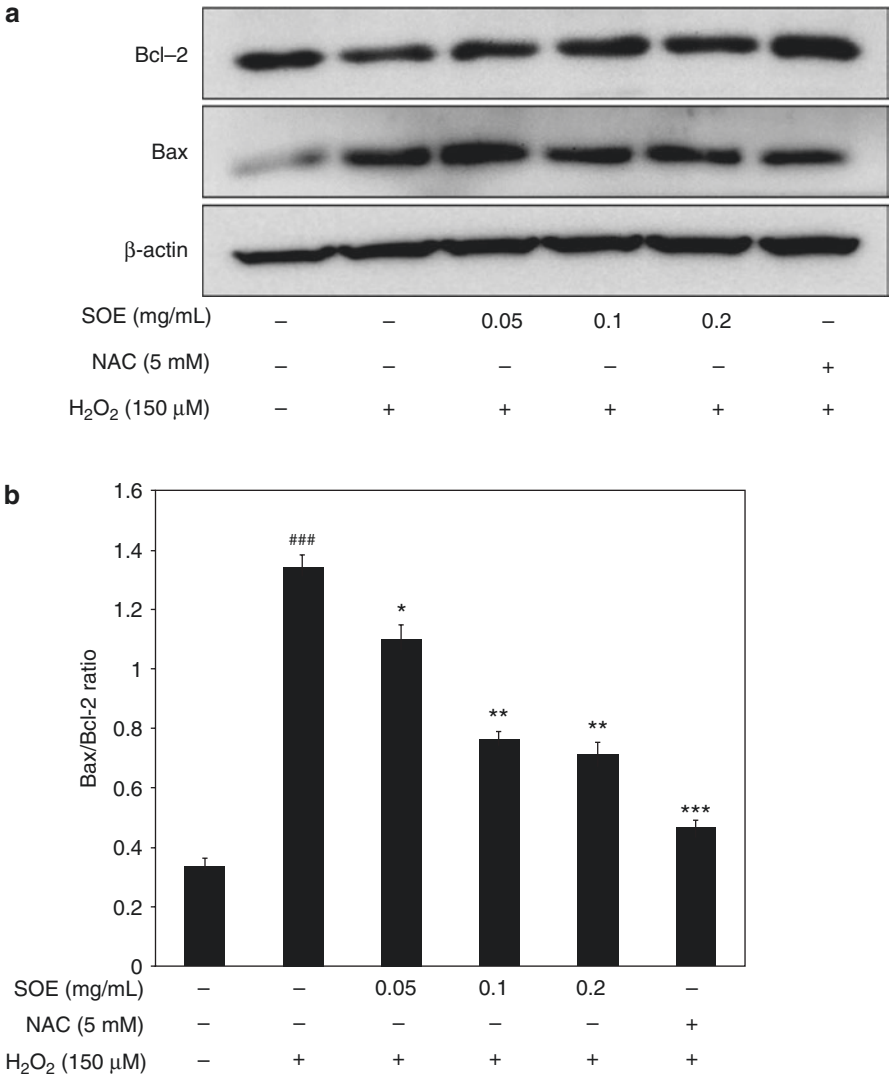

Fig. 5 Effect of SOE on the protein expression level of Bax/Bcl-2. Expression levels of Bax, Bcl-2 and β-actin were analyzed by using Western blot analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with H_2O_2 group, $^{\#\#\#}p < 0.001$ compared with control group

4 Discussion

Reactive oxygen species (ROS) which include hydrogen peroxide, hydroxyl radicals, superoxide anions and single oxygen possess a highly reactive chemical nature. Mostly, they are generated in mitochondrial electron pathway during normal cellular respiration. In addition, ROS are generated in metabolic processes and under various stress conditions (Finkel and Holbrook 2000; Vallet et al. 2005).

ROS are highly short-lived species and this nature of ROS can result in substantial damage to lipids, proteins, and DNA in cellular systems. Excessive ROS generation leads to break the balance between their production and elimination and this loss of homeostasis of ROS level is called as oxidative stress. Oxidative stress is an important condition which can damage the cell and promote the development of a variety of diseases such as cancer, hyperlipidemia, neurodegenerative diseases, etc. (Hopps et al. 2010; Li et al. 2016). Therefore, ROS scavenging ability is utmost important for protecting the cells against oxidative stress damage. Thus, antioxidative defence mechanisms are vital importance for fighting against ROS toxicity. The human body has antioxidative defence mechanisms through a family of antioxidant/detoxification enzymes which enhance cellular ROS-scavenging capacity and stimulation of this system is a key way to reduce oxidative damage (Malik and Storey 2009; Seifried et al. 2007).

The oxidative stress could result in many adverse cellular damages including oxidation of unsaturated fatty acid, proteins and damage to mtDNA which leads to morphological and functional abnormalities in mitochondria (Xu et al. 2015). Mitochondrial damage could worsen the cellular damage further by increasing ROS production and accelerating oxidative stress. Oxidative stress is found to play an important role in the pathogenesis of Parkinson's disease (PD) (Qin et al. 2011).

Taurine is considered as a semi-essential amino acid even though it is unable to make peptide bonds and it is involved in many physiological functions, including osmoregulation, membrane stabilization, cytoprotective effects, antioxidant activity, anti-inflammatory action, modulation of intracellular calcium concentration and ion channel function (De Luca et al. 2015; Oja and Saransaari 2007). Han et al. (2016) reported that taurine exhibited a protective effect against *N*-methyl-D-aspartate (NMDA)-induced neurone death.

Our present study suggested that taurine-rich SOE exerts neuroprotection against H_2O_2-induced oxidative stress in SH-SY5Y cells and it is accompanied with the reduction of Bax/Bcl-2 level and suppression of the production of intracellular reactive oxygen species (ROS).

5 Conclusion

This study reveals that the SOE is a rich source of taurine, and its neuroprotective effect on SH-SY5Y cells. Also, SOE markedly decreases the H_2O_2-induced neuronal apoptosis. This study outlines that the SOE significantly suppresses the

expression of pro-apoptotic Bax protein and increases the expression of the anti-apoptotic Bcl-2 protein leading to prevent apoptosis. Inhibition of ROS production possibly resulted in the neuroprotection of SOE against oxidative. Certainly, further investigations are required to investigate the more precise neuroprotective mechanisms of SOE.

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Protection of Taurine Against Impairment in Learning and Memory in Mice Exposed to Arsenic

Huai Guan, Zhewen Qiu, Xueying Zhou, Shuangyue Li, Xiaofeng Liu, Cong Zhang, and Fengyuan Piao

Abstract To evaluate protection of taurine against arsenic (As)-induced impairment of learning and memory as well as explore its protective mechanism, mice were divided into control, As and taurine protection groups. Mice of As exposure group exposed to drinking water containing 4 ppm As_2O_3. Mice of taurine protective group received both 4 ppm As_2O_3 and 150 mg taurine per kilogram. Mice of control group only drank double-distilled water. All animals were treated for 60 days. Morphology of brain was observed by HE staining. Morris water maze (MWM) tests and step-down passive avoidance task were performed to examine cognition function. Moreover, expressions of some genes and proteins related to regulation learning and memory in brain were tested by Real Time RT-PCR and Western Blot. As a result, abnormal morphologic changes in brain tissue and poor performance in cognition functions were observed in As-exposed mice. The expression of TRβ protein, a regulator of CaMK IV gene, significantly decreased in brains of As-exposed mice than in controls. By contrast, impairment in learning and memory, change in brain morphology and disturbance in protein expression were significantly mitigated in mice of taurine protective group. Our results suggest that taurine supplementation protects against neurotoxicity induced by As in mice.

[$]Huai Guan and Zhewen Qiu as co-first authors are contributed equally to this work.

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Keywords Arsenic trioxide • Neurotoxicity • Brain tissue • Taurine • Impairment in learning and memory • Arsenic • Learning • Memory • Protection

1 Introduction

Arsenic (As) is one of the most notorious toxicants known for thousands of years and now still affects more people than any other natural elements. Millions of people are living in areas polluted by high doses of As in more than 20 countries, the major affected areas including Bangladesh, India, China, Taiwan, Mexico, Argentina, Chile, and the USA (Bloom et al. 2014; Tyler and Allan 2014). It has been demonstrated that As is a neural toxicant. Epidemiological investigations in children suggested that chronic As exposure via drinking water was responsible for intellectual impairment (Wasserman et al. 2004). Several researches revealed that As exposure damaged learning and memory abilities as well as other neural behaviors at environmental relevant levels (Baldissarelli et al. 2012; Jing et al. 2012). We previously also found that subchronically exposed to As undermined learning and memory of mice (Wang et al. 2009). These studies indicated that brain is a vital target organ attacked by As exposure.

Long-term potentiation (LTP) and long-term depression (LTD) are the two key models for learning and memory (Malenka and Bear 2004; Mozzachiodi and Byrne 2010; Owen and Brenner 2012). Creb activation is essential to maintaining LTP and LTD which needs be phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase IV (CaMK IV) (Shaywitz and Greenberg 1999). We group had indicated that down-regulating CaMK IV gene and protein was a key link of As inducing learning and memory impairment (Wang et al. 2009; Guan et al. 2016). It has been illustrated that the binding of thyroid hormone receptor (TR) and thyroid hormone (TH) is essential to the activation of CaMK IV. Then this complex binds thyroid hormone-responsive element (TRE) (Li et al. 2004; Liu and Brent 2005; Morte et al. 2010). TH and TR are important regulators of CaMK IV gene.

Imbalance between free radical production and antioxidant leads to oxidative stress, which takes part in the pathogenesis of many disorders. As exposure has been reported to induce overproduction of reactive oxygen species (ROS) in tissues (Chattopadhyay et al. 2002). Increasing of ROS is supposed to be an important mechanism for As neurotoxicity. It was reported that taurine treatment antagonizes toxic effect of ROS caused by some toxic substances including As (Dogru-Abbasoglu et al. 2001, Tabassum et al. 2006). Our group previously found that taurine significantly alleviated As-lead to nucleic acid damage in mice brain (Piao et al. 2009). Therefore, we aim at exploring whether administration of taurine protects against As-induced impairment in learning and memory in mouse. We also deter-

mine antagonistic action of taurine on disturbance of As on TR expression in brain and TH level in serum.

In present study, learning and memory functions were examined. Expression of TR in brains was assessed the by qRT-PCR and Western blot. Serum levels of 3,5,3′-triiodothyronine (T3) and thyroxin (T4) were detected by radioimmunoassay (RIA). Moreover, HE staining was performed to observe the morphology of brain tissue.

2 Materials and Methods

2.1 Animals and Treatment

25.6–32.4 g male mice were selected as our experimental animals. They were purchased from Experimental Animal Center, Dalian Medical University. They were raised at condition of 22 ± 3 °C, 12-h light/dark cycle and relative humidity of 55 ± 15%. They were fed with common basal pellet diet (As concentration < 0.7 mg/kg). The animals were randomly divided into three groups. Each group has 12 animals. Group 1 (control group) only drank double-distilled water. Group 2 (As exposure group) exposed to drinking water containing 4 ppm As_2O_3. Group 3 (taurine protective group) received both 4 ppm As_2O_3 and 150 mg/kg taurine. Taurine was implemented by gavage twice a week, while As exposure continued for 60 days. After raising for 60 days, all animals underwent behavioral tests. After behavioral tests, all animals were killed. Through centrifugation of whole blood, serum samples were made for detecting THs concentrations. Brain tissues were collected for detection of biochemical indexes. For morphologic research, animals were perfused with saline followed with 4% paraformaldehyde. The animal experiments were carried out according to the guidelines of the committee of Dalian Medical University.

2.2 Behavioral Testing

2.2.1 Step-Down Passive Avoidance Task

We had previously reported the procedure in detail (Guan et al. 2016). In brief, when examining learning ability, error frequency to step down onto a foot-shocks floor within 180 s was the observation index. When examining memory ability, step-down latency was the observation index.

2.2.2 MWM Task

This test is used to examine spatial learning and memory. We had previously reported its methods and procedures in detail (Guan et al. 2016). In brief, our tank is black and round with 100 cm diameter. At the first stage, a hidden platform test was performed. It was used to examine spatial learning ability. Two parameters, escape latency and swimming distance to find hidden platform were the observation indexes. At the second stage, a probe test was performed. It was used to examine spatial memory ability. Two parameters, swimming time and distance in target quadrant were the observation indexes.

2.3 HE Staining

According to standard protocols, the fixed brain tissue was made into sections of 5 μm thickness. Then, they were stained with HE. Finally, they were observed under light microscope.

2.4 Real Time RT-PCR

Trizol® reagent (Takara, China) was used to extract gene sample according to the instructions. Transcriptor First Strand cDNA Synthesis Kit (Roche, \USA) was used to perform RT reactions. TP800 System and SYBR Green PCR kit (Takara, Japan) were used to carry out Real time RT-PCR. 95″C 5 min, followed by 95″C for 30 s, 40 cycles, then 55″C 30 s, 72″C 30 s were used as reaction conditions. The primers are as followed: TRα, GAC AAG GCC ACC GGT TAT CAC TAC, CAG CAG CTG TCA TAC TTG CAG; TRβ, AGC CAG AAC CCA CGG ATG AG, CGA TGG GTG CTT GTC CAAT G; β-actin, CAT CCG TAA AGA CCT CTA TGC CAA C, ATG GAG CCA CCG ATC CAC A.

2.5 Western Blot

Total proteins were extracted from cerebrum tissues with lysis buffer. BCA method was used to qualify protein concentration. SDS-polyacrylamide gel electrophoresis was carried out with same gram of loading sample protein, and the protein samples were transferred to a nitrocellulose membrane. After blocking with 10% milk for 30 min, the blots were incubated with (TRβ1, TRβ2 1:800; TRα1 1:1000; CaMK IV

1:1000; β-actin 1:350) primary antibodies, respectively. The blots were treated with horseradish peroxidase-conjugated secondary antibodies, and then detected by Bio-Rad ChemiDoc™ MP imaging system (Bio-Rad, CA, USA), and then qualified with the Gel-Pro software.

2.6 RIA

T3 and T4 concentrations in serum were detected with RIA kit (Santa, USA) according to the manufacturer's instructions.

2.7 Statistical Analysis

SPSS 17.0 for Windows was chose to analyze our data. Data were expressed as the mean ± SD. And analyzed by One-way ANOVA. p-value <0.05 was defined statistically significant.

3 Results

3.1 Brain Morphology

Abnormal morphological changes of mice brain of the three groups were examined by HE staining. Taking the frontal lobe as the example of cerebral tissue, in control mice, neuron cells were large and full. Their nuclei were large with karyolemma and nucleolus were clear (Fig. 1Aa). In the group exposed to 4 mg/L As_2O_3 alone, there were white and bright areas (white arrows) around nuclei in many neuron cells, suggesting cellular edema (Fig. 1Bb). In addition, there were metamorphotic nuclei (yellow arrows) in many neuron cells (Fig. 1Bb). These findings indicated that As exposure damaged cerebral morphology of mouse. In the group exposed to 4 mg/L As_2O_3 with taurine, there were also edema neurons and metamorphotic nuclei (Fig. 1Cc), but these changes were slighter than those in the group exposed to As alone. It indicate that treatment with taurine mitigated As-induced dam morphological changes in mouse cerebral cortex.

Taking the CA3 area as the example of cerebral hippocampal tissue, in control group, pyramidal cells were large and closed to each other in multilayer. Neuropil was dense and stained uniformly (Fig. 2Aa). In the mice of group exposed to 4 mg/L As_2O_3 alone, many pyramidal cells showed reduction in cell body and the number

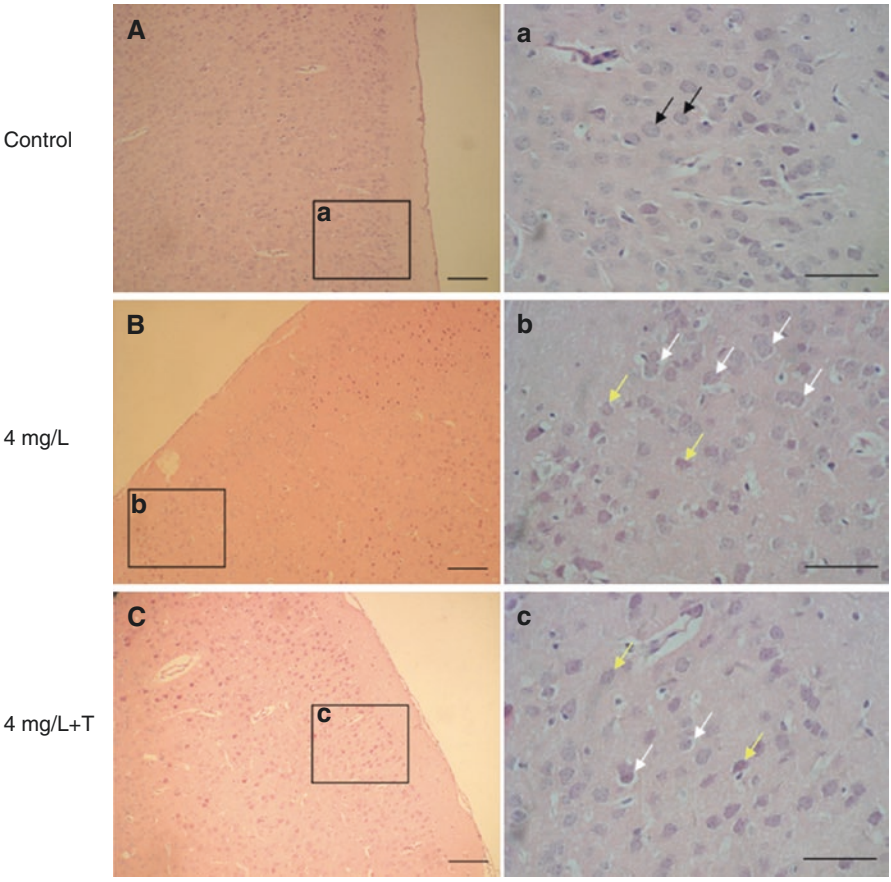

Fig. 1 Protective effects of taurine on As-induced morphological abnormity in mouse cerebrum. Group 1 (control group) orally received double distilled water. Group 2 (As exposure group) exposed to drinking water containing 4 ppm As_2O_3. Group 3 (protective group) received both As_2O_3 and taurine. All animals were treated for 60 days. Then, the brain was removed and made into sections. HE staining and light microscope were used to research brain morphology. Morphology of frontal lobe was taken as the example of cerebral cortex. (**A** and **a**) control group; (**B** and **b**) As exposure group; (**C** and **c**) protective group. *Black arrow*: normal neuron; *white arrow*: edematous neuron; *yellow arrow*: metamorphotic nucleus. Scale bars, 50 μm

of pyramidal cells was also reduced. In addition, there was some unoccupied space in the area of neuropil, even meshy neuropil (Fig. 2Bb). These findings indicate that As exposure induces abnormal morphological changes of mouse hippocampus. In the group exposed to 4 mg/L As_2O_3 with taurine, there were also the above changes (Fig. 2Cc), but they were slighter than those in the group exposed to As alone, indicating antagonistic action of taurine.

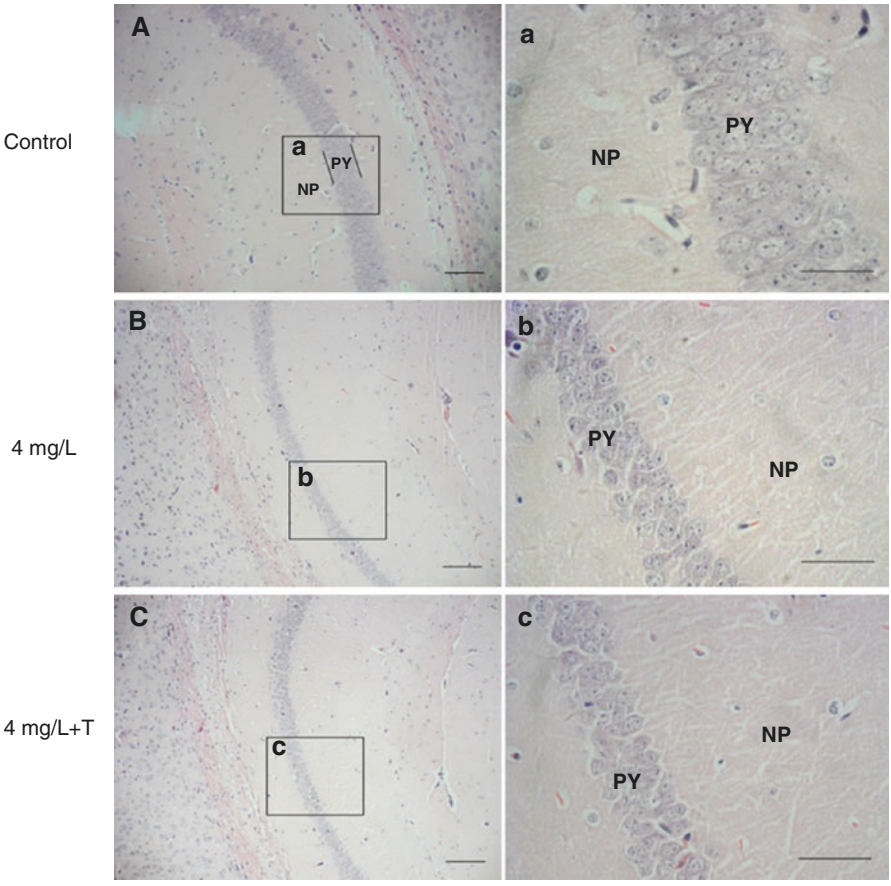

Fig. 2 Protective effects of taurine on As-induced morphological abnormity in mouse hippocampus. Group 1 (control group) orally received double distilled water. Group 2 (As exposure group) exposed to drinking water containing 4 ppm As_2O_3. Group 3 (protective group) received both As_2O_3 and taurine. All animals were treated for 60 days. Then, the brain was removed and made into sections. HE staining and light microscope were used to research brain morphology. Morphology of CA3 region was taken as the example of hippocampus. (**A** and **a**) control group; (**B** and **b**) As exposure group; (**C** and **c**) protective group. *PY* pyramidal cell layer, *NP* neuropil. Scale bars, 50 μm

3.2 Behavioral Tests

3.2.1 Step-Down Passive Avoidance test

In this test, average error frequency of group treated with 4 mg/L As_2O_3 alone was markedly higher than that of control group (Fig. 3a). Average latency of group treated with 4 mg/L As_2O_3 alone was markedly longer than that of control group (Fig. 3b).

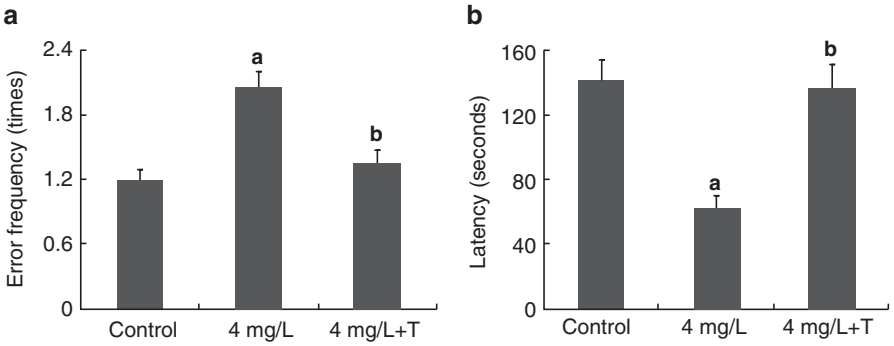

Fig. 3 Protective effects of taurine on As-induced impairment in state-dependent learning and memory in mice. Group 1 (control group) only received ddH_2O. Group 2 (As exposure group) exposed to drinking water containing 4 ppm As_2O_3. Group 3 (protective group) received both As_2O_3 and taurine. All animals were treated for 60 days. (**a**) Error frequency of step-down from a platform after foot-stock (times); (**b**) Step-down latency from the platform (seconds). $^{a}p < 0.05$, vs. control group; $^{b}p < 0.05$, vs. As exposure group

It suggested that As damaged state-dependent learning and memory. However, average error frequency of group treated with 4 mg/L As_2O_3 and taurine was similar to that of control group and significantly fewer than that of group exposed to 4 mg/L As_2O_3 alone (Fig. 3a). Average latency of group treated with 4 mg/L As_2O_3 and taurine was similar to that of control group and significantly longer than that of group exposed to 4 mg/L As_2O_3 alone (Fig. 3b). It suggested that taurine significantly alleviates As-induced impairment in state-dependent learning and memory.

3.2.2 MWM Tests

Hidden platform test was used to examine spatial learning ability of mouse. In this test, mice in each group showed a progressive reduction in swimming distance and escape latency during 5 training days, confirming that mice in each group learned to find the hidden platform across the training stage (Fig. 4a–c). Further analysis showed that on the first training day there was no significant difference in swimming distance or escape latency among mice of three groups ($p > 0.05$). On the 2nd, 3rd, 4th and 5th day of training, there was a significant difference in swimming distance or escape latency of mice between control and As exposure groups, the latter being longer than the former (all $p < 0.05$) (Fig. 4a–c). These results suggested that subchronic As intoxication damaged spatial learning ability of mouse. However, 2–5 days after training, the swimming distance and escape latency of mice in protective group were similar to those of control group ($p > 0.05$) and were markedly shorter than those in

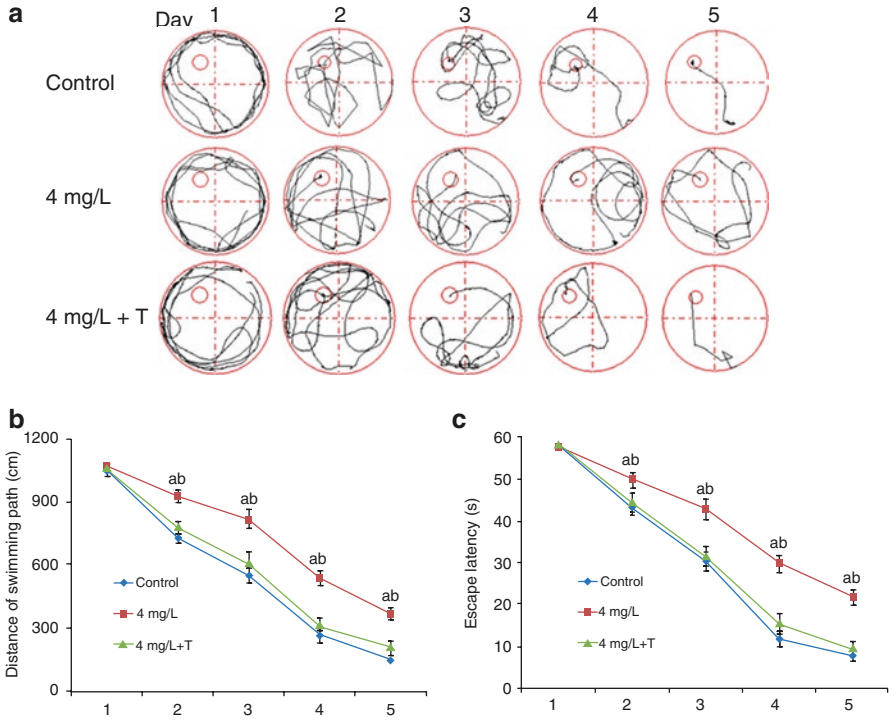

Fig. 4 Protective effects of taurine on As-induced impairment in spatial learning ability in mice. Group 1 (control group) orally received double distilled water. Group 2 (As exposure group) exposed to drinking water containing 4 ppm As_2O_3. Group 3 (protective group) received both As_2O_3 and taurine. All animals were treated for 60 days. Then, MWM tests were used to examine the spatial learning and memory functions. Data were expressed as mean ± SD (12 animals per group). (**a**) Swimming trace of mice in MWM device; (**b**) Swimming distance in hidden platform test (cm); (**c**) Escape latency in hidden platform test (s). $^{a}p < 0.05$, vs. control group; $^{b}p < 0.05$, vs. As exposure group

As exposure group ($p < 0.05$) (Fig. 4a–c). The above findings suggested that taurine reverses the impairment in spatial learning in As-intoxicate mice.

Probe test was used to examine spatial memory ability of mouse. In this test, similar trends were observed. Average time in target quadrant of As exposure group was significantly shorter than that of control group ($p < 0.05$) (Fig. 5a, b). Compared with control group, average swimming distance in target quadrant of As exposure group was markedly shorter t (Fig. 5a, c). It indicated that As induced impairment in memory retention in mouse. However, the two indexes of protective group were both similar to those of control group and both significantly longer than those of As exposure group (Fig. 5a–c). The above results suggested that taurine prevents As-induced impairment in memory retention in mouse.

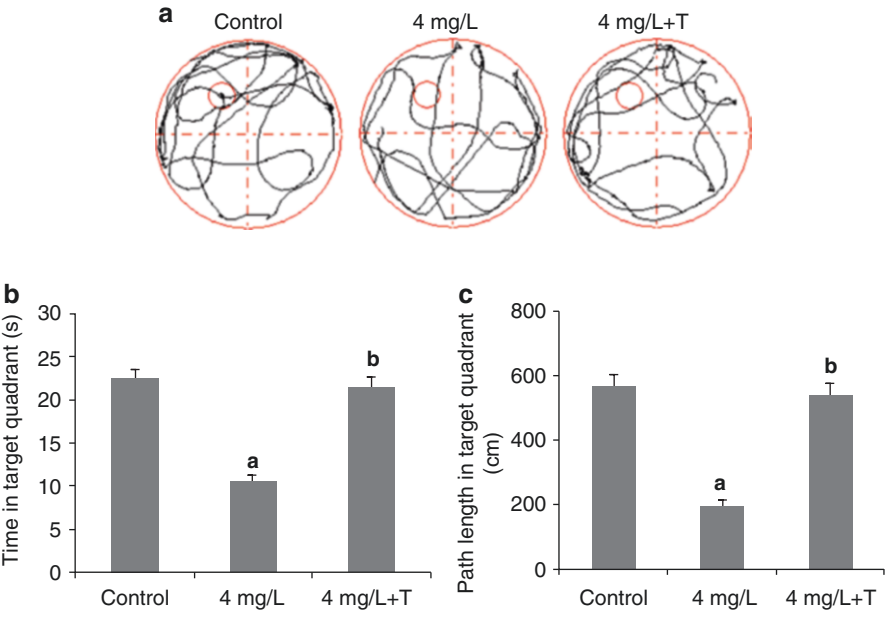

Fig. 5 Protective effects of taurine on As-induced impairment in spatial memory ability in mice. Group 1 (control group) orally received double distilled water. Group 2 (As exposure group) exposed to drinking water containing 4 ppm As_2O_3. Group 3 (protective group) received both As_2O_3 and taurine. All the animals were treated for 60 days. Then, MWM tests were used to examine the spatial learning and memory functions. Data were expressed as mean ± SD (12 animals for each group). (**a**) Swimming trace of mice in MWM device; (**b**) Time in target quadrant in probe test(s); (**c**) Distance in target quadrant in probe test (cm). $^{a}p < 0.05$, vs. control group; $^{b}p < 0.05$, vs. As exposure group

3.3 TR Expression in Mouse Brain

Figure 6a showed that mRNA expression of TRβ in brain of As-exposed mice was markedly decreased compared with that of control group. Moreover, expression of TRβ mRNA in brain of As exposure group was also markedly lower than that of protective group. Expression of TRβ mRNA in brain of protective group was similar to that of control group. It indicated that taurine alleviates down-regulation of TRβ mRNA induced by As in mouse brain. Among three groups, there was no significant difference in TRα mRNA expression in mouse brain.

As shown in Fig. 6b, protein expression of TRβ1 in brain of As-exposed mice was markedly lower than that of control mice. Moreover, expression of TRβ1 protein in brain of As exposure group was also significantly lower than that of protective group ($p < 0.05$). Expression of TRβ1 protein in brain of protective group was significantly lower than that of control group, but significantly higher than that of As

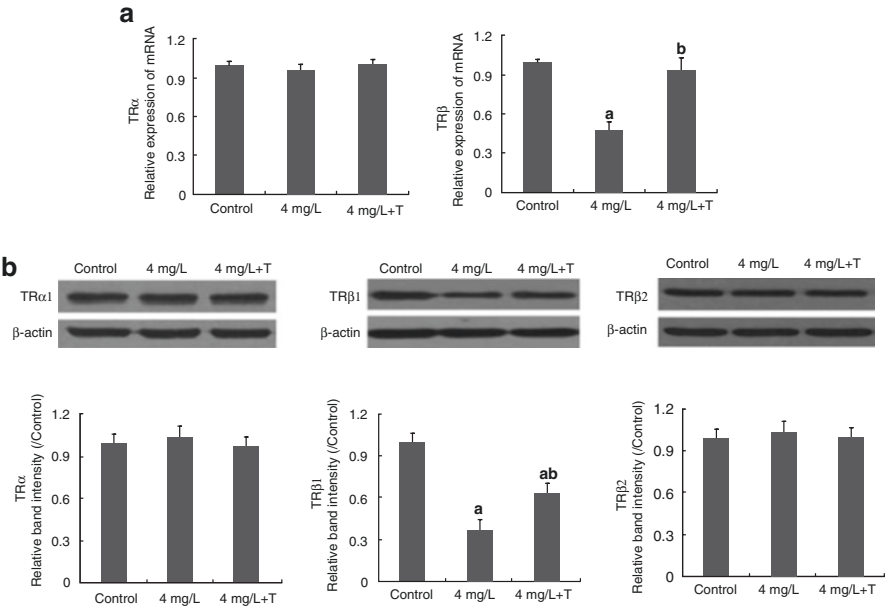

Fig. 6 Expression of TR gene (**a**) and protein (**b**) in mouse brain. Group 1 (control group) received ddH_2O. Group 2 (As exposure group) exposed to drinking water containing 4 ppm As_2O_3. Group 3 (protective group) received both As_2O_3 and taurine. All animals were treated for 60 days. Then, they were examined learning and memory capacities. Finally, they were examined some biochemical indexes. Real time RT-PCR and western blot were used to determine mRNA and protein, respectively. Data were expressed as mean ± SD (12 animals for each group). $^{a}p < 0.05$, vs. control group; $^{b}p < 0.05$, vs. As exposure group

exposure group. It indicated that taurine alleviates down-regulation of TRβ protein induced by As in mouse brain. There was no significant difference in TRα1 or TRβ2 protein level in mouse brain among three groups.

3.4 T3 and T4 Concentrations in Serum

RIA was used to determine T3 and T4 concentrations in mouse serum. As shown in Fig. 7, T3 levels in serum of control, As exposure and protective groups were (1.94 ± 0.06), (1.96 ± 0.07) and (1.97 ± 0.07) mmol/L, respectively. T4 levels in serum of control, As exposure and protective groups were (60.31 ± 7.12), (59.23 ± 6.41) and (60.54 ± 6.84) mmol/L, respectively. Among three groups, there was no significant difference in T3 or T4 level.

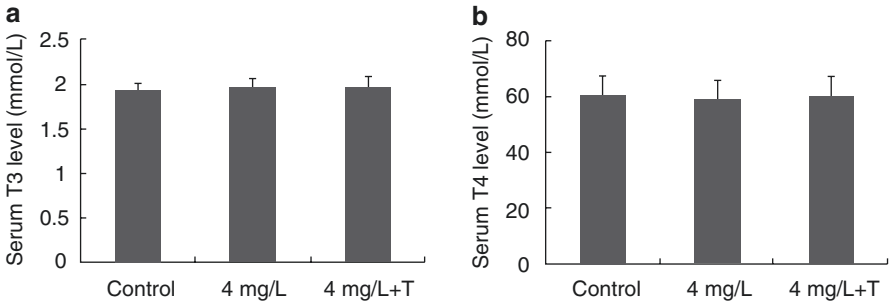

Fig. 7 T3 (**a**) and T4 (**b**) concentrations in mouse serum. Group 1 (control group) received ddH_2O. Group 2 (As exposure group) exposed to drinking water containing As_2O_3. Group 3 (protective group) received both As_2O_3 and taurine. All animals were treated for 60 days. Then, they were examined learning and memory capacities. Finally, they were examined some biochemical indexes. RIA was used to determine T3 and T4 concentrations in mouse serum. Data were expressed as mean ± SD (12 animals for each group)

4 Discussion

Epidemiological researches showed that As undermined learning (Rodríguez et al. 2003), memory and attention in humans (Tsai et al. 2003). Experimental studies proofed that As exposure impaired learning acquisition (Nagaraja and Desiraju 1994), locomotor behavior and spatial learning paradigm (Nagaraja and Desiraju 1994, Rodríguez et al. 2002). In the present study, we observed that, in step-down passive avoidance test, mouse of As exposure group showed higher error frequency and shorter latency than control. In MWM tests, mouse of As exposure group showed longer swimming distance and longer escape latency to find hidden platform. These findings indicated that As damaged state-dependent and spatial learning and memory functions in mice. Taurine intervention nearly reversed impairment in learning and memory in As exposure mice, which was reflected by the better performance of mice exposed to As with taurine than the mice exposed to As alone, as well as the similar performance of them to the controls. It suggests that taurine can antagonize neurotoxicity induced by As in some way, thereby protects neurological function of As-exposed mice.

Learning and memory are important cognitive functions. Hippocampus and cerebral cortex are proved main functional areas of them. In this study, we observed morphology of hippocampus and cerebral cortex to explore effects of As on mouse brain. Our results showed that, in cerebral cortex, As exposure induced neuron edema and nuclei abnormality. In hippocampal tissue, As resulted in shrinkage and reduction of pyramidal cells, as well as meshy neuropil. These findings suggested that As exposure induced abnormal morphology in mouse brain. However, taurine intervention significantly alleviated the above changes induced by As. It suggests that taurine protects against As-induced pathological damage in mouse brain.

It has been documented that LTP and LTD are two key models of learning and memory (Malenka and Bear 2004). The formation of them requires activation of CREB (Ahn et al. 1999; Casu et al. 2005; El Hajj et al. 2014). And signaling by CaMK IV cascade are involved in CREB activation (Lee et al. 2009). Our previous studies documented that subchronic As exposure depressed gene expressions of CaMK IV, CREB and other memory proteins in mouse brain (Wang et al. 2009). It hinted that down-regulated CaMK IV expression in brain induced by As might be associated with deficit of cognitive function.

It has been documented that binding with TH and TR are needed for transcriptional activation of CaMK IV (Li et al. 2004, Liu and Brent 2005, Morte et al. 2010). TR isoforms include TRα1, TRβ1 and TRβ2. They can bind to TH and target DNA sequence TREs in the CaMK IV gene 5′-flanking region (Liu and Brent 2005) and then activated CaMK IV gene. Therefore, we investigated effects of As on TR gene and protein expression in brain as well as TH level in serum. We also investigated protective effects of taurine as well as potential mechanism. Our study showed that As exposure markedly decreased TRβ mRNA expression in mouse brain. The result was further proved by Western blot examination. However, the decreased TRβ was significantly up-regulated in mouse of taurine protective group. It hinted that taurine supplementation significantly alleviated effect induced by As. On other hand, there was no significant difference in serum T3 or T4 level and TRα mRNA expression among three groups. These results hinted that As exposure disturbed expression of TRβ in brain tissue of mice. Moreover, it is also suggested that taurine protect brain against toxic effects induced by As. Taurine is a intracellular free b-amino acid. It exists in most mammalian tissues. It has many cytoprotective properties including anti-oxidation, osmoregulation, membrane stabilizatio. (Hansen 2001; Schaffer et al. 2003; Redmond et al. 1996). Taurine has been documented a protective agent which can antagonize oxidative stress-induced pathologies. It was reported that taurine might up-regulate anti-oxidant defences, form chloramines with HOCl, or bind free metal ions so as to scavenge oxygen free radicals. Thus, it play roles of protecting cells (Hansen 2001; Schaffer et al. 2003; Redmond et al. 1996). Therefore, protective effects of taurine against toxic effects of As may be attributed to its ability by its direct as well as indirect antioxidant activities.

5 Conclusion

In this study, As exposure induced an impairment in learning and memory, abnormal morphologic changes and down-regulated expression of TRβ in mouse brain. However, taurine supplementation significantly alleviated the toxic effects of As. Our results indicate that taurine protects against neurotoxicity induced by As. Meanwhile, it is also suggested that taurine may be potentially used as a therapeutic/protective agent against As-induced neurotoxicity. Further studies are needed to focus on determining exact molecular mechanisms of these protective effects.

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Conflicts of Interest The authors declare no conflict of interest.

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Effects of Taurine Supplementation on Neuronal Excitability and Glucose Homeostasis

Abdeslem El Idrissi, Fatiha El Hilali, Salvatore Rotondo, and Francoise Sidime

Abstract In this study we examined the role of chronic taurine supplementation on plasma glucose homeostasis and brain excitability through activation of the insulin receptor. FVB/NJ male mice were supplemented with taurine in drinking water (0.05% w/v) for 4 weeks and subjected to a glucose tolerance test (7.5 mg/kg BW) after 12 h fasting. We found that taurine-fed mice were slightly hypoglycemic prior to glucose injection and showed significantly reduced plasma glucose at 30 and 60 min post-glucose injection when compared to control mice. Previously, we reported that taurine supplementation induces biochemical changes that target the GABAergic system. Those studies show that taurine-fed mice are hyperexcitable, have reduced $GABA_A$ receptors expression and increased GAD and somatostatin expression in the brain. In this study, we found that taurine-fed mice had a significant increase in insulin receptor (IR) immuno-reactivity in the pancreas and all brain regions examined. At the mRNA level, we found that the IR showed differential regional expression. Surprisingly, we found that neurons express the gene for insulin and that taurine had a significant role in regulating insulin gene expression. We propose that increased insulin production and secretion in taurine-fed mice cause an increase activation of the central IR and may be partially responsible for the increased neuronal excitability observed in taurine supplemented mice. Furthermore, the high levels of neuronal insulin expression and its regulation by taurine implicates taurine in the regulation of metabolic homeostasis.

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Keywords Insulin receptor • Glucose • Excitability • Glucose tolerance • Insulin • Taurine • Hippocampus

Abbreviations

GAD	Glutamic acid decarboxylase
IGF	Insulin-like growth factor
IR	Insulin receptor
Tau	Taurine
WT	Wild type controls

1 Introduction

Taurine is a sulfur-containing semi-essential amino acid. Excitable tissues, including the brain, skeletal and cardiac muscles contain high levels of taurine. Taurine has been shown to play an important role in many physiological processes (Lambardini 1985; Solis et al. 1988; Saransaari and Oja 2000; Schaffer et al. 2000; Foos and Wu 2002). Additionally, taurine modulates both glutamate and GABA neurotransmission (Militante and Lombardini 1998; El Idrissi and Trenkner 1999, 2004). In the pancreas, taurine gestational taurine supplementation delays the onset of diabetes in non-obese diabetic mice (Arany et al. 2004). Furthermore, taurine has been shown to play a role in glucose homeostasis throughout life (Hansen 2001; Franconi et al. 2006).

Developing pancreas has been shown to undergo a significant level of remodeling, mediated by a balanced induction of cell proliferation and apoptotic cell death (Arany et al. 2004). Many factors have been shown to be implicated in this pancreatic remodeling, including IGF-II, inducible nitric oxide synthase (iNOS) and somatostatin (Scaglia et al. 1997; Liu et al. 1998; Petrik et al. 1998; El Idrissi et al. 2009). We have shown that taurine-fed mice have increased size and number of islets (El Idrissi et al. 2009). The role of taurine in pancreatic development has been postulated to be mediated by preventing or scavenging free radicals (Petrik et al. 1998), by inhibiting the expression of pro-inflammatory factors such as iNOS (Liu et al. 1998) and by promoting the expression of survival factors such as IGF-II and somatostatin (Scaglia et al. 1997; Petrik et al. 1998; El Idrissi et al. 2009).

We have shown that taurine supplementation increased islets size in the pancreas and insulin production by β cells (El Idrissi et al. 2009). These changes in pancreatic function are responsible for the increased resistance to glucose

challenges in taurine-fed mice. Furthermore, circulating insulin crosses the blood brain barrier and activates IR expressed on neurons. We suggest that this activation of IR receptors may be an additional mechanism for increased excitability in taurine-fed mice. This is consistent with the effects of taurine on GABAergic synapses. We have shown that chronic interaction of taurine with $GABA_A$ receptors induces a variety of alterations to the GABAergic system. These include increased GAD expression, decreased expression of $GABA_A$ receptors (El Idrissi and Trenkner 2004), and increased number of somatostatin-positive neurons (El Idrissi et al. 2009; Levinskaya et al. 2006). The changes induced by taurine supplementation to the GABAergic system are consistent with increased neuronal excitability. Coupled with these changes in the GABAergic system, here we report an increased expression and activation of the insulin receptors which will further enhance neuronal excitability.

2 Methods

2.1 Animals

All mice used in this study were 2-month-old FVB/NJ males. For taurine-fed mice, taurine was dissolved in water at 0.05%, and this solution was made available to the mice for 4 weeks beginning at 4 weeks of age. All mice were housed in groups of three in a pathogen-free room maintained on a 12 h light/dark cycle and given food and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee of the College of Staten Island/CUNY, and were in conformity with National Institutes of Health Guidelines. The number of mice used in these studies was sufficient to provide statistically reliable results.

2.2 Immunohistochemistry and Confocal Microscopy

Frozen sections were made as previously described (Levinskaya et al. 2006). Briefly, primary antibodies (Chemicon International) used were directed against insulin receptor (mouse host) and insulin (rabbit) diluted 1:500 and incubated overnight at 4 °C. Secondary antibodies were all raised in goat and conjugated to Alexa Fluor 488 or Cy5 (Invitrogen/Molecular probes). Images were obtained by confocal microscopy (Leica SP2 AOBS). Nuclei were counterstained with SlowFade with DAPI (Invitrogen). Immunoreactivity was quantified using Imaris ×64 (Bitplane).

2.3 Intraperitoneal Glucose Tolerance Test

Glucose test was performed as previously reported (El Idrissi et al. 2009). Briefly, mice were fasted overnight (12 h) and then injected intraperitoneally with 0.02 mL/g of body weight D-glucose (7.5% stock solution in saline). Blood samples were taken by tail venesection at the indicated times

2.4 RNA Preparation, cDNA Preparation and Real-Time PCR Analysis

RNA was prepared from tissue samples as described previously (Zhang et al. 2009). Equal amounts of RNA (0.5 μg) were used to prepare cDNA using the SYBR GreenER Two-Step qRT-PCR kit (Invitrogen 11,748–100) and analyzed by real-time PCR in a 7500-sequence detection system (Applied Biosystems). All experiments were repeated twice and, in each experiment, real-time PCR reactions were done in triplicate. Target DNA quantities were calculated as described previously (Zhang et al. 2009). Statistical significance was determined by Student's t-test. Each value was expressed as the mean ± SEM. Differences were considered statistically significant when the calculated P value was less than 0.05.

2.5 Statistical Analysis

Statistical significance was determined by Student's t-test. Each value was expressed as the mean ± SEM. Differences were considered statistically significant when the calculated P value was less than 0.05.

3 Results

3.1 Taurine-Fed Mice Exhibit Tolerance to Glucose Challenges

We have shown that taurine supplementation increased islets size in the pancreas and insulin production by β cells (El Idrissi et al. 2009). These changes in pancreatic function are responsible for the increased resistance to glucose challenges in taurine-fed mice. Control mice showed a significant increase in plasma glucose concentration 30 min after glucose injection with a gradual decrease thereafter. By 120 min, mice were slightly hypoglycemic relative to baseline (Fig. 1). In contrast, taurine-fed mice showed a drastically different response to glucose injection. There was a delayed peak of plasma glucose at 60 min post injection and the plasma glucose in these mice was significantly lower than controls at all times measured ($p < 0.001$).

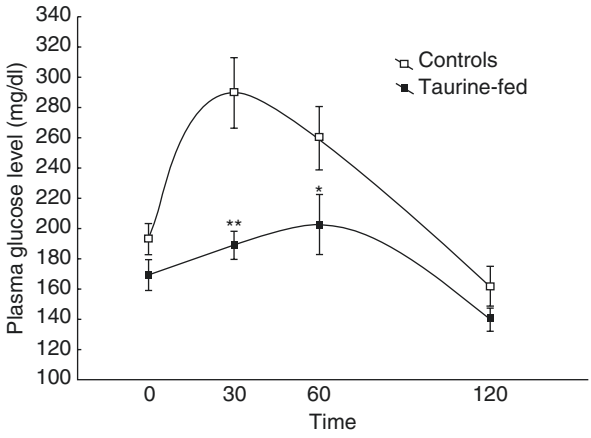

Fig. 1 Effect of taurine supplementation on glucose homeostasis. Taurine mice were significantly resistant to glucose injection (n = 12 in each group). Values are expressed as means ± S.E.M obtained from three experiments. $^{**}p < 0.01$, $^{*}p < 0.05$ when compared with taurine group

3.2 Taurine Supplementation Increases the Expression of Insulin Receptors in the Brain and Pancreas

Insulin is primarily a metabolic hormone functioning on muscle, fat and liver *via* activation of IR receptor. Insulin also function on other non-metabolic tissues such as the brain. Once insulin is secreted it crosses the blood-brain barrier by a transporter-mediated saturable mechanism. The IR is widely expressed in the brain at various levels (Unger et al. 1991). This regional specify implicates insulin, through activation of its receptor, in various brain function that are mediated by these brain structures. In this study, we examined the levels of IR expression in the pancreas and brain and found that taurine-fed mice have a significant increase in IR expression in all brain regions and pancreas compared to controls (Fig. 2).

3.3 Taurine Supplementation Alters Insulin and Insulin Receptor Gene Expression

To further investigate the functional significance increased insulin receptor expression in the brain, we examined mRNA levels of the IR and insulin. We found that the insulin receptor gene was differentially expressed in various brain regions and affected by taurine supplementation. In the cerebellum, taurine caused a significant decrease in IR gene expression, where as in the brain stem and hippocampus, there was a significant increase in IR expression. Interestingly, we found that taurine supplementation had a significant role in the regulation of insulin gene in the brain. Taurine caused a downregulation of insulin gene expression in all brain regions examined, except the diencephalon where taurine caused an increased in insulin gene expression.

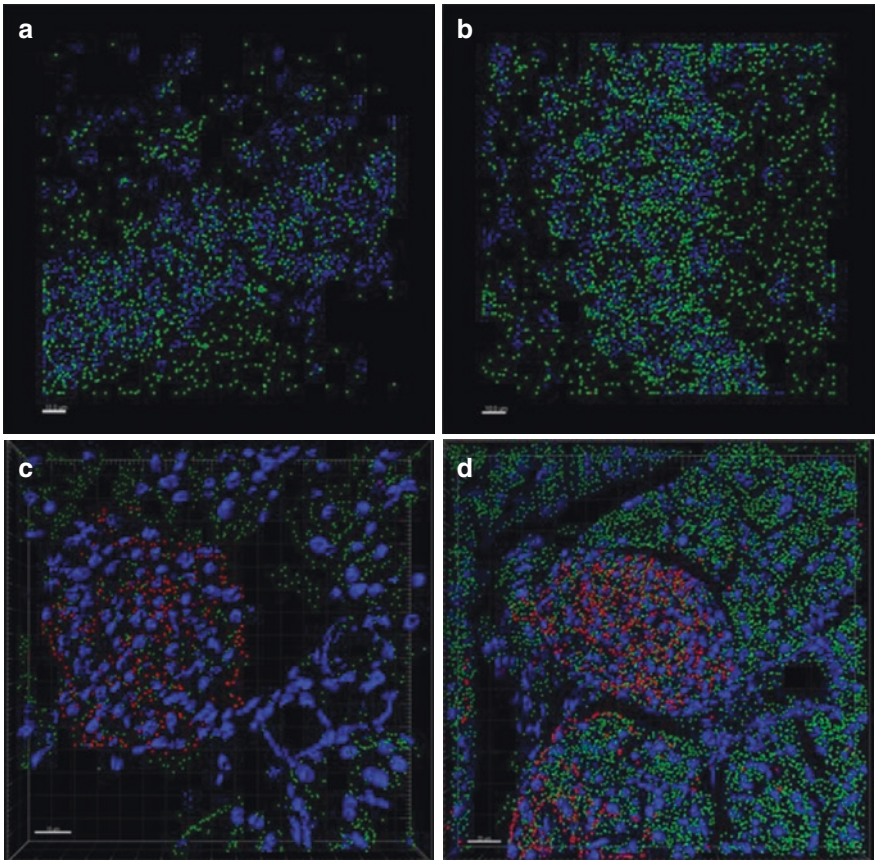

Fig. 2 Effect of taurine supplementation on IR expression in the brain and pancreas. Images depict Imaris reconstruction of z-stacks of confocal images obtained from 30 μm cryosections. *Upper panel* representative images showing insulin receptor (*green*) immunoreactivity in CA3 region of the hippocampus from control (**a**) and taurine-fed mouse (**b**). (**c, d**) are representative images obtained from the pancreas of controls and taurine-fed mice, respectively. *Red* is insulin, *green* is IR immunoreactivity. Hippocampi and pancreas from taurine-fed mice show a significant increase in immunoreactivity for insulin receptor. Scale bar 15 μm

4 Discussion

In this study we show that taurine, through supplementation drinking water, plays an important role in the function of the pancreas and neuronal excitability. Previously, we showed that taurine-fed mice have enlarged islets and increased insulin synthesis and secretion by β cells (El Idrissi et al. 2009). Additionally, here we show taurine regulates the expression of IR in both the brain and the pancreas (Figs. 2 and 3). We found that taurine supplementation caused an increase in IR expression notably in the brain stem and hippocampus (Fig. 3) with a decrease expression in the

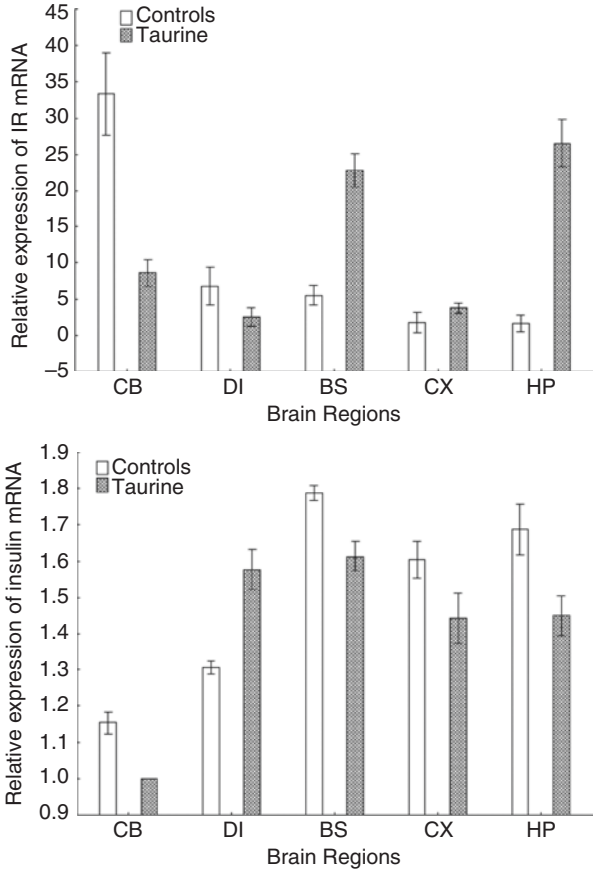

Fig. 3 Quantitative analysis of IR and Insulin mRNA in the brain of controls and taurine-fed mice. Summary of the real-time PCR analysis of IR and insulin mRNA relative to GAPDH mRNA expressed as a ratio to the control level. *CB* cerebellum, *DI* diencephalon, *BS* brain stem, *CX* cortex, *HP* hippocampus

cerebellum. IR is widely expressed in the brain and the expression pattern shows regional specificity (Havrankova et al. 1978; Unger et al. 1991). Interestingly, we found that the gene for insulin is also widely expressed in brain (Fig. 3). This is consistent with previous finding suggesting that insulin gene is expressed in certain regions of the brain (Devaskar et al. 1993). Here we show that insulin gene is expressed in neurons and its expression is regulated by taurine supplementation.

There are numerous studies demonstrating that IR signaling plays a role in both excitatory and inhibitory neurotransmission and that the expression of IR in the hippocampus is activity-dependent (Plum et al. 2005). The expression of potassium ion channel Kv1.3 in the olfactory bulb is increased in response to intranasal insulin delivery to mice (Marks et al. 2009). These changes led to increased cognitive function as measured by short- and long-term object recognition, suggesting the insulin

modulates neuronal activity and improves memory through changes in Kv1.3 expression levels. Furthermore, insulin has been shown to promote neuronal survival in the brain (Mielke et al. 2006) and prevent hippocampal cell death in response to glucose deprivation *in vitro* (Díaz et al. 1999).

Here, we showed that the expression of both insulin and its receptor are regulated by taurine supplementation. These effects are observed both in the brain and pancreas, suggesting a role for taurine in both regulation of glucose homeostasis and neuronal excitability.

5 Conclusion

In summary, taurine supplementation to mice in drinking water has a beneficial role on the function of the pancreas by increasing insulin production and secretion. Concomitant with this increased insulin secretion there is an increase in IR expression in both the brain and pancreas. Activation of IR on neurons would increase neuronal excitability. This is consistent with the increased excitability observed with taurine treatment. IRs are widely expressed in both the brain and the periphery. The regulation of IR expression by taurine may help explain the wide range of behavioral and physiological effects regulated by taurine.

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Putative Role of Taurine as Neurotransmitter During Perinatal Cortical Development

Werner Kilb

Abstract Neurotransmitters and neuronal activity affect neurodevelopmental events like neurogenesis, neuronal migration, apoptosis and differentiation. Beside glutamate and gamma-amino butyric acid, the aminosulfonic acid taurine has been considered as possible neurotransmitter that influences early neuronal development. In this article I review recent studies of our group which demonstrate that taurine can affect a variety of identified neuronal populations in the immature neocortex and directly modulates neuronal activity. These experiments revealed that taurine evoke dose-dependent membrane responses in a variety of neocortical neuron populations, including Cajal-Retzius cells, subplate neurons and GABAergic interneurons. Taurine responses persist in the presence of GABA(A) receptor antagonists and are reduced by the addition of strychnine, suggesting that glycine receptors are involved in taurine-mediated membrane responses. Gramicidin-perforated patch-clamp and cell-attached recordings demonstrated that taurine evokes depolarizing and mainly excitatory membrane responses, in accordance with the high intracellular Cl^- concentration in immature neurons. In addition, taurine increases the frequency of postsynaptic GABAergic currents (PSCs) in a considerable fraction of immature pyramidal neurons, indicating a specific activation of presynaptic GABAergic networks projecting toward and exciting pyramidal neurons. In summary, these results suggest that taurine may be critically involved in the regulation of network excitability in the immature neocortex and hippocampus via interactions with glycine receptors.

Keywords GABA(A) receptor • Glycine receptor • Cajal-Retzius cell • Subplate neuron • GABAergic interneuron

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Abbreviations

ACSF	Artificial cerebrospinal fluid
CNS	Central nervous system
CRc	Cajal-Retzius cells
GABA	γ Amino butyric acid
GES	Guanidinoethanesulfonic acid
GFP	Green fluorescent protein
PSC	Postsynaptic current
SPn	Subplate neurons

1 Introduction

The aminosulfonic acid taurine (2-aminoethanesulfonic acid) is among the most abundant organic molecules in the central nervous system (CNS) and has been attributed to a variety of physiological functions (for review Huxtable 1989; Oja and Saransaari 2015). For example, it has been demonstrated that taurine is involved in cell volume regulation (Lambert 2004), Ca^{2+} homeostasis (El Idrissi 2008b), mitochondrial translation (Suzuki et al. 2002), modulation of inflammation (Marcinkiewicz and Kontny 2014), and reduces apoptosis in the CNS (Taranukhin et al. 2008). In addition, taurine is an agonist of glycine and γ amino butyric acid (GABA) receptors, which can activate $GABA_A$ receptors with a very low affinity, glycine receptors with a low affinity, while it is a high-affinity ligand for $GABA_B$ receptors (Albrecht and Schousboe 2005). Thus, taurine is considered as endogenous neurotransmitter with inhibitory effects in the mature CNS, thus providing anticonvulsive actions (El Idrissi and L'Amoreaux 2008, but see Oja and Saransaari 2013) and antinociceptive effects (Pellicer et al. 2007). Taurine also augments learning and memory (El Idrissi 2008a). Taurine is rather a neuromodulator than as classical neurotransmitter, since no clear evidences for vesicular taurine release have been found (Kamisaki et al. 1996).

In the immature brain the total taurine concentration is considerably higher than in the adult nervous system, with a strong developmental downregulation after the first postnatal week in rodents (Huxtable 1989; Benitez-Diaz et al. 2003). In addition, the stimulated taurine release in immature brains is also significantly larger than in the mature CNS (Oja and Saransaari 1995). These observations suggest that taurine may play a particular important role during neuronal development. In agreement with this suggestion, it has already been demonstrated that the development of the visual cortex and the cerebellum is impaired in taurine deficient kitten (Palackal et al. 1986; Sturman et al. 1985). Since this seminal findings of John Sturman, a

number of experiments have been published that provide further evidence for the hypothesis that taurine is an important developmental neuromodulator.

The present article aims to summarize our published results that support the hypothesis that taurine is a relevant neuromodulator during neocortical development.

2 Methods

2.1 Slice Preparation

Tangential slices were prepared as described in detail elsewhere (Okabe et al. 2004). Animal handling was performed in accordance with national laws for the use of animals in research and approved by the local ethical committee. All efforts were made to reduce the number of animals and their suffering. Neonatal (within the first postnatal week) rats or mice were decapitated after deep anesthesia, their brain was quickly removed and stored for 1–2 min in ice cold artificial cerebrospinal fluid (ACSF), consisting of (in mM) 124 NaCl, 26 $NaHCO_3$, 1.25 NaH_2PO_5, 1.8 $MgCl_2$, 1.6 $CaCl_2$, 3 KCl 20 glucose (equilibrated with 95% O_2/5% CO_2; pH = 7.4, 336 mOsm). For the experiments either 400 μm coronal slices, prepared by means of a vibratom, or tangential slices, prepared manually from isolated and pia-striped hemispheres, were used. Slices were superfused with ACSF at a rate of 1–2 mL/min at 30–32 °C.

2.2 Electrophysiological Setup and Procedure

The electrophysiological setup consisted of an upright microscope with infrared Normarski interference contrast optics (Zeiss Axioskop or Olympus BX51WI) and a CCD camera (C5405, Hamamatsu, Japan). All potentials and currents were recorded by discontinuous voltage-clamp/current-clamp amplifiers (SEC05L, NPI, Tamm, Germany). Signals were amplified, low-pass filtered at 3 kHz, digitized online by an AD/DA-board (ITC-16, Heka, Lamprecht, Germany), recorded and processed with the software WINTIDA (Heka). Synaptic inputs were evoked by monopolar electrical impulses (1 mA, 100 μs) applied by bipolar tungsten electrodes (FHC, Bowdoinham, ME, Impedance 4–5 MOhm). Patch-pipettes were filled with a solution that mimics the high Cl^- concentration in immature neurons and contained (in mM) 86 K-gluconate, 44 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 EGTA, 10 HEPES, 2 Na_2-ATP, 0.5 Na-GTP, pH adjusted to 7.4 with KOH and osmolarity to 306 mOsm with sucrose. In addition, cell-attached and gramicidin-perforated patch-clamp experiments were performed according to published procedures.

2.3 Identification of Cells

Cells were identified according to their morphological appearance, electrophysiological properties and, in case of GABAergic interneurons, by molecular markers. Cajal-Retzius cells (CRc) were identified in tangential slices by their unique morphology and electrophysiological properties (Luhmann et al. 2000). Subplate neurons (SPn) were identified in coronal slices by their location in the subplate and their typical electrophysiological properties (Luhmann et al. 2000). Excitatory neurons in the cortical plate and immature neocortical layers were identified in coronal slices by their pyramidal-like appearance (Luhmann et al. 2000), while GABAergic interneurons in these layers were identified by the expression of green fluorescent protein (GFP) under control of the GABA synthesizing enzyme GAD67 (Tamamaki et al. 2003).

2.4 Statistics

All values are expressed as mean ± standard error of the mean. For statistical analysis student's t-test, Mann-Whitney U-test, Anova tests and Fisher exact test were used. Results were designated significant at a level of $p < 0.05$.

3 Results

Beginning with the seminal publication of Flint et al. (1998), several studies revealed that taurine influences neurons in the immature brain. In the following I will in detail review our studies investigating the action of taurine on distinct neuronal subpopulations and its impact on neuronal activity during early postnatal development.

3.1 Action of Taurine on Identified Neuronal Populations in the Developing Neocortex

Cajal-Retzius cells (CRc) are transient, early generated neurons which are located in the most superficial layer of the developing neocortex and play an essential role for cortical lamination (for review Kirischuk et al. 2014). In CRc taurine induces inward currents with a low amplitude of 66 ± 19 pA (n = 11, Fig. 1a$_1$), corresponding to about 20% of the maximal glycinergic amplitude (Kilb et al. 2002). The taurinergic inputs desensitize by only 9%, suggesting that taurine may be relevant for tonic currents. Dose-response relationship revealed that taurinergic responses are mediated by glycine receptors with an EC_{50} of 2.4 mM (Fig. 1a$_2$). However, the

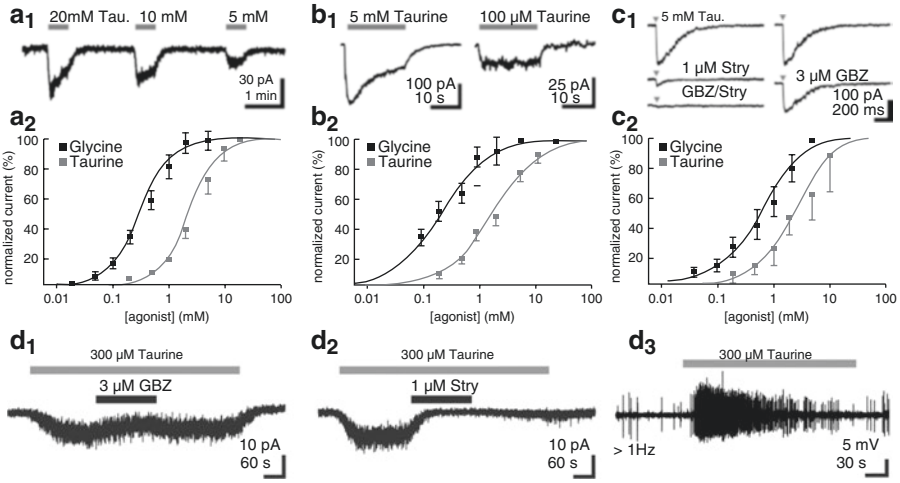

Fig. 1 Effect of taurine on identified neuronal populations in the immature neocortex. (**a**) Membrane currents (a_1) evoked by different taurine concentration in Cajal-Retzius cells and resulting dose response curve (a_2). (**b**) Membrane currents (b_1) evoked by 5 mM and 100 µM taurine in subplate neurons and resulting dose response curve (b_2). Note the stable tonic currents evoked by 100 µM taurine. (**c**) Pharmacological properties of inward currents evoked in immature pyramidal neurons by focal application of 5 mM taurine (c_1) and dose response curve (c_2) for taurinergic inward currents in these cells. (**d**) In GABAergic interneurons application of 300 µM taurine induces a tonic inward current that is partially attenuated by 3 µM gabazine (GBZ, d_1), inhibited to a larger extend by 1 µM strychnine (Stry, d_2) and enhanced action potential discharge frequency in cell-attached recordings (d_3). Figures modified with permission from Kilb et al. (2002), Okabe et al. (2004), Kilb et al. (2008), and Sava et al. (2014)

contribution of $GABA_A$ receptors to taurinergic responses was not evaluated in this study (Kilb et al. 2002). In line with the high intracellular Cl^- concentration in CRc (Achilles et al. 2007), gramicidin-perforated patch-clamp experiments revealed that activation of glycine receptors mediate a membrane depolarization in CRc (Kilb et al. 2002), that is sufficient to trigger action potentials and thus suggests an excitatory action of taurine.

Subplate neurons (SPn) populate the subplate, located between the white matter and the developing neuronal layers, and are critically involved in establishing correct brain circuits (for review Kanold and Luhmann 2010). In SPn focal taurine application evokes inward currents with a maximal amplitude of 264 ± 34 pA (n = 15; Fig. $1b_1$), which is ca. 50% of the maximal glycinergic current amplitude (Kilb et al. 2008). The taurinergic currents are mediated via glycine receptors with an affinity of 1.7 mM (Fig. $1b_2$), but for SPn the contribution of $GABA_A$ receptors to taurinergic membrane responses was also not tested (Kilb et al. 2008). While higher taurine concentrations evoke desensitizing responses, 100 µM taurine induces a tonic inward current in this cell type (Fig. $1b_1$). Gramicidin-perforated patch-clamp recordings revealed that also in SPn activation of glycine receptors mediate depolarizing responses. The tonic currents upon bath application of 100 µM

taurine evoke only small subthreshold membrane depolarizations of 4.5 ± 0.6 mV (n = 16) which, however, are sufficient to substantially lower the rheobase from 27.6 ± 3.3 pA to 19.5 ± 3 pA (n = 13), thus demonstrating an obvious excitatory effect (Kilb et al. 2008).

The first evidences that taurine can directly influence neurons in developing cortical plate came from Flint et al. (1998), who identified glycine receptors with a taurine affinity of ca. 1 mM in cortical plate neurons from late embryonal stages. Our experiments on visually identified neurons in the cortical plate of early postnatal rats revealed that taurine evokes inward currents of with a maximal amplitude of 27 ± 9 pA (n = 7), corresponding to ca. 20% of the maximal glycinergic current amplitude (Okabe et al. 2004). The inward currents evoked by focal application of 5 mM taurine are reduced by 80 ± 5.2% (n = 7) in the presence of the glycinergic antagonist strychnine (1 μM) and by 22 ± 2.5% (n = 8) in the presence of the GABAergic antagonist gabazine (3 μM; Fig. $1c_1$), indicating that taurinergic currents are mediated by glycine and to a lesser extent by $GABA_A$ receptors (Sava et al. 2014). Taurine activates glycine receptors with an affinity of 2.3 mM (Fig. $1c_2$; Okabe et al. 2004). Also in cortical plate neurons the activation of glycine receptors mediates an excitatory effect, as concluded from taurine-induced Ca^{2+} transients (Flint et al. 1998).

Usage of transgenic mice, in which GFP is ectopically inserted in one copy of the GAD67 allele (Tamamaki et al. 2003), allows the identification of GABAergic interneurons by GFP fluorescence. Our experiments revealed that in GABAergic interneurons focal application of 5 mM taurine induces an inward current of 159 ± 22 pA (n = 10), which is completely blocked by strychnine (n = 9), while the GABAergic antagonist gabazine (3 μM) has only a marginal effect (Sava et al. 2014). Long lasting application of 300 μM taurine induces a tonic inward current with an amplitude of 11 ± 2 pA (n = 14), which is attenuated by 49 ± 8% (n = 6) in the presence of 3 μM gabazine and by 70 ± 13% (n = 7) in the presence of the 1 μM strychnine (Fig. 1d). These pharmacological results suggest that taurinergic currents are largely mediated by glycine receptors and to a lesser extent by $GABA_A$ receptors. Cell-attached recordings, which enable the recording of action potential discharges under conditions of undisturbed intracellular Cl^- concentrations, revealed that 300 μM taurine massively increases the discharge frequency (Fig. $1d_3$), indicating an excitatory effect.

In summary, these results demonstrate that taurine can act as a putatively excitatory neuromodulator in a variety of identified neuronal populations in the developing neocortex, primarily via an interaction with glycine receptors.

3.2 Action of Taurine on Network Activity in the Developing Neocortex

To investigate systematically whether tonic glycinergic currents evoked by low taurine concentrations are capable to modulate neuronal activity levels, we performed whole-cell and cell-attached patch clamp recordings on glutamatergic pyramidal

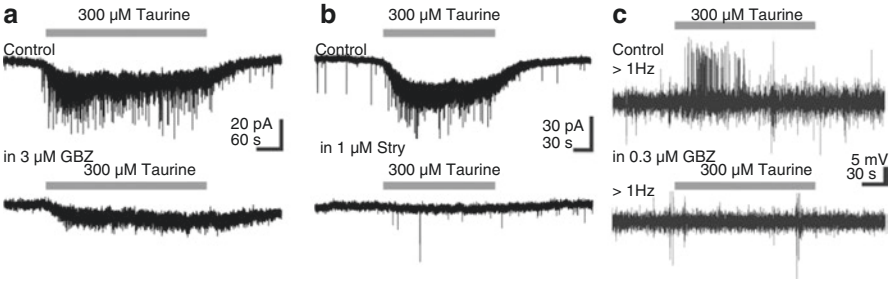

Fig. 2 Effect of taurine on postsynaptic currents in pyramidal neurons of the immature neocortex. (**a**) Bath-application of 300 µM taurine induces an inward current that is superimposed with a massive increase in postsynaptic current frequency. These currents are virtually blocked in the presence of 3 µM gabazine (GBZ). (**b**) The taurine-induced PSCs are abolished in the presence of 1 µM strychnine (Stry). Please note the persistence of spontaneous postsynaptic currents under this condition. (**c**) Cell-attached patch-clamp recordings revealed that taurine induces action potential discharges, which could be prevented in the presence of 0.3 µM gabazine. Figures modified from Sava et al. (2014) with permission

neurons and GABAergic interneurons in the cortical plate and developing layers using coronal neocortical slices of early postnatal mice (Sava et al. 2014). These experiments revealed, that a prolonged application of 300 µM taurine induces a tonic inward current in pyramidal neurons, which is associated with a significant increase in PSC frequency in 46.8% of 275 investigated cells. Interestingly, frequency and amplitude of the taurine-induced PSCs are unaffected by glutamatergic antagonists, indicating that most probably GABAergic synapses mediate this activity. And indeed, the taurine-evoked PSCs reversed around the expected reversal-potential for $GABA_A$ receptors and were completely abolished in the presence of 3 µM gabazine (n = 5; Fig. 2a). The glycinergic antagonist strychnine also abolishes taurine-induced PSCs (n = 12), but in contrast to gabazine does not affect spontaneous GABAergic PSCs (Fig. 2b). In combination, these results indicate that 300 µM taurine preferentially activates GABAergic interneurons, by its glycine receptor-dependent excitatory effect on these cells, and thereby massively increase the frequency of GABAergic PSCs. Since it has been shown that GABA mediate an excitatory effect in pyramidal neurons in the immature cortical plate (Rheims et al. 2008), we next performed cell-attached recordings, which revealed that application of 300 µM taurine significantly enhances the frequency of action potentials in 57% of all investigated pyramidal neurons (n = 35). The taurine-induced action potential activity is completely blocked in the presence of 0.3 µM gabazine (Fig. 2b), which at this low concentration selectively suppresses synaptic $GABA_A$ receptors, indicating that GABAergic postsynaptic events trigger these action potentials. In summary, the observations of our study indicate that the taurine-induced increase in GABAergic activity imposes an excitatory effect on immature neonatal circuits (Sava et al. 2014).

4 Discussion

The studies summarized in this article present experimental evidence that taurine can be considered as endogenous neuromodulator providing a putatively excitatory input on identified CRc, SPn, immature pyramidal neurons, and neocortical GABAergic interneurons. The taurinergic responses are mediated mainly via glycine and to a lesser extent via $GABA_A$ receptors, although not for all populations the exact contribution of $GABA_A$ receptors had been determined. In line with these observations, taurine induces GABAergic network activity in the cortical plate.

Although glycine receptors have been observed in virtually all investigated neuronal populations in the immature neocortex, no evidences for glycinergic synaptic currents (e.g. Flint et al. 1998; Okabe et al. 2004) or activity-dependent glycine release have been found (Qian et al. 2014). Thus it was suggested, that taurine might be the endogenous transmitter acting on this receptor. The results of the studies reviewed in this article, in combination with other studies (Flint et al. 1998), clearly demonstrate that taurine indeed mediate glycine receptor-dependent membrane responses in most cell populations of the immature neocortex. In addition, at least for SPn, pyramidal neurons and GABAergic interneurons already taurine concentration between 100 and 300 μM evoke physiologically relevant responses.

Taurine is among the most abundant neuroactive organic molecules (Huxtable 1989; Oja and Saransaari 2015), but it is necessary to consider that the majority of taurine is stored in the intracellular compartment, while only the interstitial taurine can mediate effects via membrane bound neurotransmitter receptors. Direct measurements of extracellular taurine concentrations in the immature brain in-vivo have not been published, but from the taurine concentration of 25 μM in the mature CNS, which was measured under zero-flow conditions by means of microdialysis probes (Molchanova et al. 2004), and the fact that the total taurine concentration is more than five times larger during the first postnatal week as compared to the adult CNS (Benitez-Diaz et al. 2003), it is reasonable to assumed that the interstitial taurine concentration in the immature CNS is in the range of 100 μM. Therefore the taurine concentrations between 100 and 300 μM used in our studies to emulate tonic taurinergic actions are probably within the physiological range.

Our results demonstrate that the effects of taurine on membrane responses and network activity are mainly mediated via glycine receptors. This observation is in line with the fact that taurine is a low affinity agonist of $GABA_A$ receptors, revealing affinities >10 mM (Kletke et al. 2013), while it acts with a considerably higher affinity in the range between 1 and 4 mM in glycine receptors (Schmieden et al. 1992; Flint et al. 1998; Okabe et al. 2004). However, recent studies demonstrated that the action of taurine depends on the subunit composition of $GABA_A$ receptors, with alpha4, alpha6 and delta subunits, which typically characterize extrasynaptic $GABA_A$ receptors, mediating higher taurine affinity and revealing already at μM taurine concentrations considerable currents (Hadley and Amin 2007). Although these observations indicate that specific subunit compositions of $GABA_A$ receptors can be sufficient to mediate membrane responses at physiologically relevant taurine

concentrations, their contribution to taurinergic membrane responses in the immature neocortex has not been systematically addressed yet. At least for pyramidal neurons, however, the fact that about 20% of the inward current induced by 300 μM taurine is blocked by GABAergic antagonists (Sava et al. 2014) argue for the functional expression of $GABA_A$ receptors with moderate taurine affinity.

In all cell types presented in this manuscript taurine causes a depolarizing action. This depolarizing responses mediated by glycine and $GABA_A$ receptors, both ligand-gated anion channels, reflect the elevated Cl^--concentration in immature neurons, which is due to the dominance of NKCC-1 mediated Cl^- accumulation (Yamada et al. 2004). Taurine either evoked action potentials or decreased excitation threshold, thus is must be assumed that taurine has a net excitatory effect in the immature CNS. In CRc subthreshold glycine-receptor dependent depolarizations suppress action potential generation (Kilb et al. 2002), which indicate that an activation of glycine-receptors via taurine can in principle also mediate depolarizing, but shunting inhibitory responses. However, recent experiments revealed that taurine enhances the propagation of depolarizing waves in the marginal zone (Qian et al. 2014), indicating an excitatory action in the marginal zone via its influence on CRc. In immature neocortical pyramidal neurons taurine evokes action potentials, clearly indicating an excitatory action. This effect is caused by activation of glycine receptors on GABAergic interneurons and the resulting increase in the frequency of excitatory GABAergic synaptic potentials in pyramidal neurons (Sava et al. 2014). To our knowledge this is the first direct evidence, that taurine is directly involved in the regulation of network activity in immature neocortical networks. In the immature hippocampus it has been shown that low concentrations of taurine also enhance network excitability, while higher taurine concentrations attenuate network excitability (Chen et al. 2014).

By its putative excitatory influence on different cells types and network activity taurine may directly influence neuronal development. It is well known that different neurotransmitter systems interfere with cortical development at different levels (see Owens and Kriegstein 2002 for review). Activation or inhibition of $GABA_A$ receptors influences neurogenesis (Haydar et al. 2000), controls neuronal migration (see Luhmann et al. 2015 for review) and modulates neuronal apoptosis, differentiation and synaptogenesis (Meier et al. 2003; Blanquie et al. 2016). Via interaction with $GABA_A$ and glycine receptors, taurine increases the proliferation of mice embryonic progenitor cells (Hernandez-Benitez et al. 2010) and interfere with radial migration in the neocortex (Behar et al. 2001; Furukawa et al. 2014). In addition, the taurine-induced enhancement of immature neuronal activity can also modulate the maturation of neuronal circuits (for reviews Hanganu-Opatz 2010, Luhmann et al. 2016).

5 Conclusion

In summary, the presented studies as well as studies by other groups provided experimental evidence that taurine can activate glycine receptors and to a lesser extent $GABA_A$ receptors in a variety of identified neuronal populations. Taurine has

an excitatory action on immature neocortical neurons and enhances intrinsic network activity of immature neocortical circuits. These observations can explain the essential role of taurine for neuronal development and indicate that any interference with the taurinergic system during pregnancy or early childhood bear the risk to provoke developmental disorders. In this respect, the effects of antiepileptic substances like vigabatrin on neuronal survival and taurine homeostasis already indicate adverse effects of such an interference (Jammoul et al. 2009).

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Part II
Effects of Taurine on Obesity and Diabetes

Perinatal Taurine Supplementation Prevents Metabolic and Cardiovascular Effects of Maternal Diabetes in Adult Rat Offspring

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Abstract This study tests the hypothesis that perinatal taurine supplementation prevents diabetes mellitus and hypertension in adult offspring of maternal diabetic rats. Female Wistar rats were fed normal rat chow and tap water with (Diabetes group) or without diabetic induction by intraperitoneal streptozotocin injection (Control group) before pregnancy. Then, they were supplemented with 3% taurine in water (Control+T and Diabetes+T groups) or water alone from conception to weaning. After weaning, both male and female offspring were fed normal rat chow and tap water throughout the study. Blood chemistry and cardiovascular parameters were studied in 16-week old rats. Body, heart, and kidney weights were not significantly different among the eight groups. Further, lipid profiles except triglyceride were not significantly different among male and female groups, while male Diabetes displayed increased fasting blood glucose, decreased plasma insulin, and increased plasma triglyceride compared to other groups. Compared to Control, mean arterial pressures significantly increased and baroreflex control of heart rate decreased in both male and female Diabetes, while heart rates significantly decreased in male but increased in female Diabetes group. Although perinatal taurine supplementation did not affect any measured parameters in Control groups, it abolished the adverse effects of maternal diabetes on fasting blood glucose, plasma insulin, lipid profiles, mean arterial pressure, heart rate, and baroreflex sensitivity in adult male and female

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offspring. The present study indicates that maternal diabetes mellitus induces metabolic and cardiovascular defects more in male than female adult offspring, and these adverse effects can be prevented by perinatal taurine supplementation.

Keywords Blood pressure • Diabetes mellitus • Hypertension • Perinatal life • Taurine • Rat

Abbreviations

BSHR	Baroreflex sensitivity control of heart rate
Control+T	Control plus taurine supplementation
Diabetes+T	Diabetes plus taurine supplementation
PE	Phenylephrine
SNP	Sodium nitroprusside

1 Introduction

The prevalence of metabolic syndrome among the offspring of maternal diabetes mellitus in all age groups is increasing (Pantham et al. 2015; Pereira et al. 2015; von Julia and von Versen-Hoynck 2016). Maternal diabetes increases the incidence of complications in both mothers and fetuses. Women with diabetes are at an approximately 75% increased risk for cardiovascular complications compared with non-diabetic women (Kanaya et al. 2005). Further, gestational hyperglycemia causes the adult offspring at high risk of dyslipidemia, diabetes mellitus, and hypertension. Lines of evidence indicate that exposure to a diabetic intrauterine environment during pregnancy induces dyslipidemia, subclinical vascular inflammation, and endothelial dysfunction in the young offspring, all of which may underlie cardiovascular disorders in adulthoods (Vrachnis et al. 2012). Thus, the prevention of diabetes mellitus during pregnancy and lactation is crucial to adult offspring function and disease.

The maternal effects in adult offspring seem to be sex specific. The incidence of hypertension is high in post-menopausal compared to pre-menopausal women and this can be prevented by estrogen replacement therapy (Hay et al. 2014; Kim et al. 2014; Yanes and Reckelhoff 2011). Further, men have a higher incidence of cardiovascular and metabolic disorders than age-matched pre-menopausal women, while the incident rate becomes reversed in menopausal women. Estrogen is reported to play a protective role in the women. Estrogen possesses many activities including inhibition of renin-angiotensin system and sympathetic nervous system, anti-oxidation, and anti-inflammation (Ashraf and Vongpatanasin 2006; Sandberg et al. 2016). However, the effects of estrogen on cardiovascular control are rather complicated. High estrogen levels during pregnancy increases water and salt

retention and arterial pressure by direct and indirect increases in angiotensin II and aldosterone (Oelkers 1996). In addition, oral contraceptives are reported to increase arterial pressure in susceptible women, and this can be abolished by inhibition of the renin-angiotensin system (Ashraf and Vongpatanasin 2006). Thus, it is hypothesized that the adverse effects of maternal diabetes in adult offspring is sex dependent, particularly the development of metabolic and cardiovascular disorders.

Taurine (2-aminoethanesulphonic acid) is a small sulfur containing amino acid that plays many physiological roles from prenatal to adult life (Huxtable 1992; Sturman 1993). During pregnancy and lactation, the maternal taurine synthesis is not sufficient to match the increased maternal requirement and fetal consumption. Thus, taurine supplementation from food is recommended during pregnancy and lactation. Taurine possesses many activities including anti-oxidation, growth promotion, anti-hypertension, anti-hyperglycemia, and anti-hyperlipidemia (Roysommuti and Wyss 2014). Thus, perinatal taurine supplementation seems to be more beneficial in maternal diabetes than other hypoglycemic agents. However, perinatal taurine excess and depletion are reported to affect adult function and disease. Prenatal taurine depletion induces low birth weight newborns that are prone to develop diabetes mellitus and hypertension in adults (Sturman 1993). Epidemiologic studies also report a high incidence of metabolic syndrome and cardiovascular disease in people consuming low taurine diets (Yamori et al. 2010). These adverse effects of perinatal taurine depletion are closely similar to those of maternal diabetes mellitus (Pereira et al. 2015; von Julia and von Versen-Hoynck 2016; Vrachnis et al. 2012). Although taurine supplementation may abolish these adverse effects of perinatal taurine deficiency, the taurine supplementation in late pregnancy induces insulin resistance and obesity in adult rat offspring (Hultman et al. 2007). The present study tests the hypothesis that perinatal taurine supplementation prevents diabetes mellitus and hypertension in adult offspring of maternal diabetic rats.

2 Methods

2.1 Animal Preparation

Male and female Wistar rats were bred at the animal unit of Suranaree University of Technology and maintained at constant humidity (60 ± 5%), temperature (24 ± 1 °C), and light cycle (06:00–18:00 h). All rats were fed normal rat chow and tap water *ad libitum*. Female rats were induced diabetes mellitus by a single intraperitoneal injection of freshly prepared streptozotocin (50 mg/kg of body weight) followed by 20% glucose in tap water for 24 h (to prevent initial drug-induced hypoglycemic mortality) (Diabetes group). Three days later, fasting blood glucose was confirmed to be 280–350 mg/dL. Then, these animals were subjected to a mating procedure. The control group was similarly treated without diabetic induction (Control group). After conception, the pregnant rats were caged individually and supplemented with

3% taurine in tap water (Control plus taurine supplementation, Control+T; Diabetes plus taurine supplementation, Diabetes+T) or water alone until weaning (Diabetes and Control groups). After weaning, both male and female offspring were fed the normal rat chow and tap water throughout the experiment (n = 8 each group).

All experimental procedures were approved by the Universities Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health guidelines.

2.2 Experimental Protocol

At 16 weeks of age, fasting blood glucose and plasma insulin levels were determined from blood samples drawn from rat tail vein. Two days later, all rats were anesthetized with thiopental (50 mg/kg of body weight, intraperitoneal), implanted with femoral arterial and venous catheters, and arterial pressure and heart rate were continuously recorded by the PowerLab (ADIntruments, Bella Vista, New South Wales, Australia), After baseline data recording, a baroreflex sensitivity control of heart rate was measured by an intravenous infusion of phenylephrine (to increase arterial pressure) and sodium nitroprusside (to decrease arterial pressure). Then, a blood sample was collected from the arterial catheter (about 2 mL) for measuring plasma lipid profiles. Finally, all rats were sacrificed by a high dose of anesthesia and heart and kidney weights were collected.

2.3 Data Analyses

Blood glucose was immediately measured by a glucometer (Accu-CHECK, Roche), plasma insulin level by Rat/Mouse Insulin ELISA kit #EZRMI-13K (Merck Millipore, Merck), and plasma lipid profiles by The Suranaree Hospital Chemical Analysis Unit (Suranaree University of Technology, Nakhon Ratchasima, Thailand). Mean arterial pressure and heart rate were analyzed by the PowerLap software, while the baroreflex sensitivity control of heart rate was calculated from the ratio of a change in heart rate to a change in mean arterial pressure following phenylephrine (BSHR-PE) or sodium nitroprusside infusion (BSHR-SNP).

2.4 Statistical Analysis

All data are expressed as mean ± SEM. Statistical comparisons among the eight groups were performed by using one-way ANOVA followed by the *post hoc* Duncan's Multiple Range test (StatMost32 version 3.6, Dataxiom, CA, USA). The significant criterion is $p < 0.05$.

3 Results

At 16 weeks of age, body, heart, and kidney weights were not significantly different among the eight groups, irrespective of sex differences (Table 1). Fasting blood glucose and triglyceride levels significantly increased and plasma insulin level decreased in male, but not in female, Diabetes compared to male and female Control and Control+T groups (Tables 2 and 3). Further, these effects of maternal diabetes were abolished by perinatal taurine supplementation (male Diabetes+T group). The perinatal taurine supplementation did not affect any blood chemistry parameters in female Diabetes and both sex Control groups.

Compared to Control, mean arterial pressures significantly increased in both male and female Diabetes groups (Fig. 1), while heart rates significantly decreased in male but increased in the female Diabetes group (Fig. 2).

In contrast to mean arterial pressures, the baroreflex sensitivity control of heart rate measured by either phenylephrine (Fig. 3) or sodium nitroprusside infusion (Fig. 4) significantly depressed in both male and female Diabetes compared to Control groups. Perinatal taurine supplementation abolished these adverse effects of maternal diabetes mellitus without any effect on hemodynamic parameters in the Control groups.

Table 1 Body, kidney, and heart weights in male and female rats

	Body weight (g)		Heart weight (g)		Kidney weight (g)	
Treatment	Male	Female	Male	Female	Male	Female
Control	383 ± 5	259 ± 3	0.34 ± 0.02	0.90 ± 0.07	1.38 ± 0.04	0.90 ± 0.03
Control+T	386 ± 7	250 ± 3	0.33 ± 0.01	0.80 ± 0.05	1.31 ± 0.05	1.01 ± 0.03
Diabetes	368 ± 5	243 ± 4	0.36 ± 0.02	0.89 ± 0.11	1.29 ± 0.03	0.93 ± 0.02
Diabetes+T	370 ± 7	241 ± 5	0.35 ± 0.03	0.88 ± 0.07	1.34 ± 0.02	0.94 ± 0.03

Data are means ± SEM. There were no significant differences among groups (*Control+T* control plus taurine supplementation, *Diabetes+T* diabetes plus taurine supplementation; n = 8 each group)

Table 2 Fasting blood glucose and plasma insulin levels in male and female rats

	Fasting blood glucose (mg/dL)		Fasting plasma insulin (ng/dL)	
Treatment	Male	Female	Male	Female
Control	82.5 ± 2.1	82.6 ± 3.4	6.9 ± 0.7	7.0 ± 0.6
Control+T	82.4 ± 2.2	86.9 ± 2.7	6.0 ± 0.6	6.2 ± 0.7
Diabetes	116.1 ± 4.4*	90.1 ± 3.1	4.2 ± 0.6*	6.2 ± 0.4
Diabetes+T	85.1 ± 1.6	82.6 ± 1.4	6.4 ± 0.7	6.1 ± 0.5

Data are means ± SEM (*P < 0.05 compared to all other groups; *Control+T* control plus taurine supplementation, *Diabetes+T* diabetes plus taurine supplementation; n = 8 each group)

Table 3 Plasma lipid profiles in male and female rats

	Total cholesterol (mg/dL)		Triglyceride (mg/dL)		HDL (mg/dL)		LDL (mg/dL)	
Treatment	Male	Female	Male	Female	Male	Female	Male	Female
Control	80.2 ± 4.9	80.8 ± 3.9	130.0 ± 5.1	123.0 ± 4.3	62.0 ± 1.4	68.0 ± 3.1	103.0 ± 3.1	102.6 ± 4.3
Control+T	77.9 ± 0.4	75.4 ± 4.1	124.0 ± 4.1	117.6 ± 3.9	60.6 ± 1.7	66.5 ± 3.1	105.0 ± 3.1	97.1 ± 3.5
Diabetes	85.3 ± 6.6	78.4 ± 3.9	166.0 ± 7.0*	129.5 ± 5.0	56.8 ± 2.2	61.8 ± 2.1	108.0 ± 3.1	106.8 ± 4.1
Diabetes+T	80.2 ± 4.4	76.8 ± 4.2	132.0 ± 6.8	125.4 ± 6.0	61.5 ± 1.5	61.8 ± 3.2	102.0 ± 3.4	105.7 ± 2.0

Data are means ± SEM (*P < 0.05 compared to all other groups; *Control+T* control plus taurine supplementation, *Diabetes+T* diabetes plus taurine supplementation, *HDL* high density lipoprotein, *LDL* low density lipoprotein; n = 8 each group)

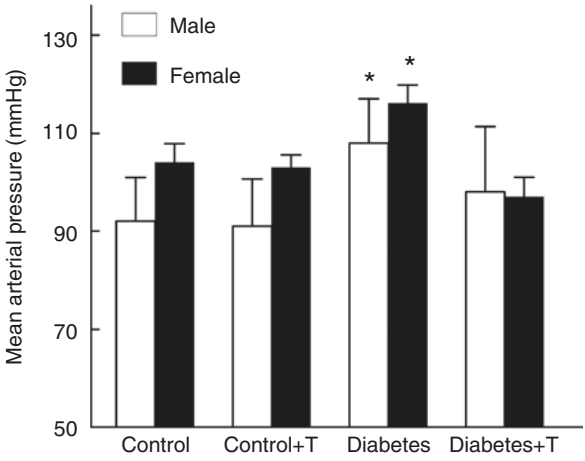

Fig. 1 Mean arterial pressures in male (*white bars*) and female rats (*black bars*) (*P < 0.05 compared to male or female Control; *Control+T* Control plus taurine supplementation, *Diabetes+T* diabetes plus taurine supplementation; n = 8 each group)

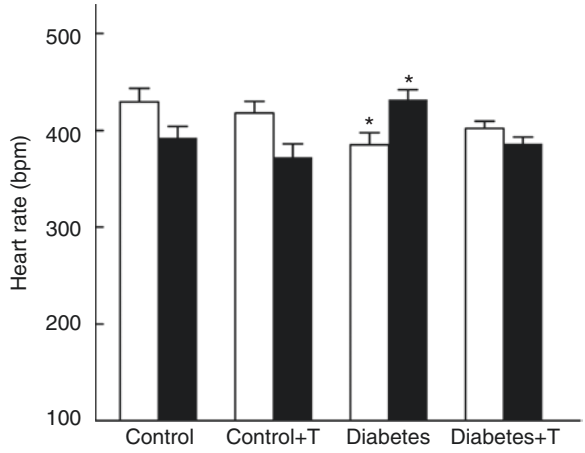

Fig. 2 Heart rates in male (*white bars*) and female rats (*black bars*) (*P < 0.05 compared to male or female Control; *Control+T* control plus taurine supplementation, *Diabetes+T* diabetes plus taurine supplementation; n = 8 each group)

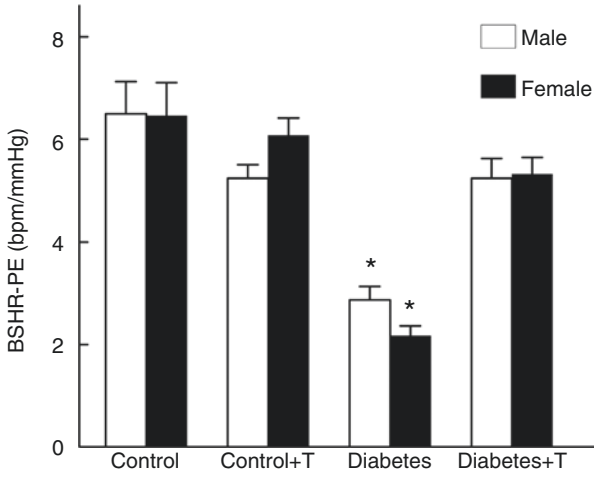

Fig. 3 Baroreflex sensitivity control of heart rate measured by phenylephrine infusion (BSHR-PE) in male (*white bars*) and female rats (*black bars*) (*P < 0.05 compared to male or female Control; *Control+T* control plus taurine supplementation, *Diabetes+T* diabetes plus taurine supplementation; n = 8 each group)

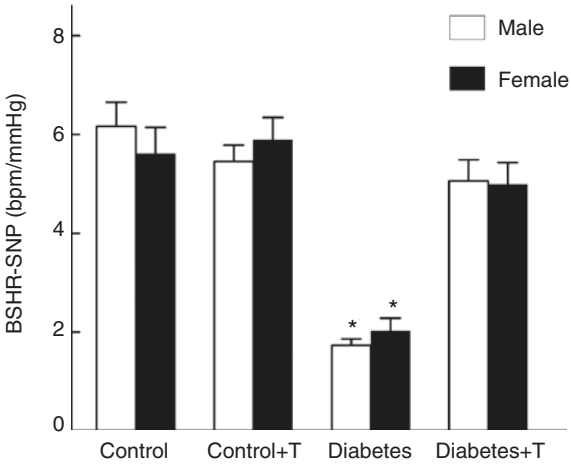

Fig. 4 Baroreflex sensitivity control of heart rate measured by sodium nitroprusside infusion (BSHR-SNP) in male (*white bars*) and female rats (*black bars*) (*P < 0.05 compared to male or female Control; *Control+T* control plus taurine supplementation, *Diabetes+T* diabetes plus taurine supplementation; n = 8 each group)

4 Discussion

Several lines of evidence report that maternal diabetes mellitus predisposes the offspring at high risk of dyslipidemia, diabetes mellitus, and hypertension in adult life (Pereira et al. 2015; von Julia and von Versen-Hoynck 2016; Vrachnis et al. 2012). The present study indicates that these adverse effects of maternal diabetes mellitus in adult offspring are sex dependent. Diabetic mothers' male offspring displayed dyslipidemia, hyperglycemia, and hypertension (three of them indicate the development of metabolic syndrome), while their female offspring developed hypertension without hyperglycemia and dyslipidemia. In addition, the present data demonstrate for the first time that perinatal taurine supplementation prevents all of these adverse effects of maternal diabetes in adult offspring independent of sex differences.

Taurine possesses glucose lowering activity in diabetes mellitus but not in normal subjects (Imae et al. 2014; Murakami 2015). This taurine's beneficial effect is mainly supported by animal experiments. In agreement with previous reports (Roysommuti et al. 2009; Roysommuti et al. 2013), the present study indicates that perinatal taurine supplementation alone does not affect fasting blood glucose, plasma insulin, lipid profiles, and hemodynamic parameters in male and female Control rats, while it normalized all adverse effects of maternal diabetes in adult male and female offspring. It is possible that the perinatal taurine supplementation may normalize or reduce hyperglycemia of the diabetic mother from conception to weaning; thus, the in utero effect of hyperglycemia on fetal and early postnatal growth and development may be abolished or compromised. Unfortunately, we could not measure plasma glucose and/or insulin levels in the rat mothers during pregnancy and lactation. Further experiments need to explore this hypothesis.

Perinatal environment has long-term effect on adult function and disease. The lesser adverse effects of female than male offspring in adult life may be due to a

difference in sex hormones. Hypertension is more common in men than age-match pre-menopausal women, but this begins to reverse after women reach menopause (Ashraf and Vongpatanasin 2006; Oelkers 1996; Yanes and Reckelhoff 2011). In young adult spontaneously hypertensive rats, mean arterial pressure is higher in males than in females, and inhibition of the renin-angiotensin system eliminates this sex difference (Yanes et al. 2006). Hypertension in ovariectomized animals also can be prevented by diets with phytoestrogens or estrogen replacement (Carlson et al. 2008). Although estrogen may play a protective role on glucose-insulin regulation and lipid metabolism in the female offspring from diabetic mothers, its action cannot explain the similar hypertension and blunted baroreflex function in both male and female offspring. Together with the data that these male offspring developed bradycardia and the female ones developed tachycardia, this study suggests that the hypertension in males and females may result from different mechanisms. This notation is also supported by the data that hyperglycemia and dyslipidemia were observed in the male but not the female offspring. Both hyperglycemia and dyslipidemia are common risk factors of cardiovascular disease particularly hypertension (Chen et al. 2016; Imae et al. 2014).

In the present study, maternal diabetes mellitus blunted baroreflex control of heart rate in both male and female offspring and these effects were completely prevented by perinatal taurine supplementation. It is possible that taurine may preserve the normal growth and development of neural pathways for baroreceptor reflex function in these adult offspring. Intrauterine growth restriction is reported to decrease neurogenesis in many brain areas of animals (Liu et al. 2011; Maliszewski-Hall et al. 2015) including tractus solitary nucleus (the main nucleus receiving peripheral sensory signals from baroreceptors) (Scabora et al. 2015). These changes are suggested to underlie the blunted baroreflex function and increased arterial pressure in adults that are intrauterine growth restricted. These adverse effects of intrauterine growth restriction are, at least in part, prevented by perinatal taurine supplementation. Maternal diabetes is also reported to suppress neurogenesis in adult offspring (Xu et al. 2013; Yang et al. 2016). Whether perinatal taurine supplementation can prevent the maternal diabetes-suppressed neurogenesis, particularly at the brain areas related to arterial pressure control, has to be further studied.

5 Conclusion

Maternal diabetes mellitus has long-term effects on metabolic and cardiovascular diseases in adult offspring. The present study demonstrates that these adverse effects are sex dependent, such that male offspring are more susceptible to maternal hyperglycemia than females do. However, perinatal taurine supplementation is very powerful to prevent maternal diabetes-induced metabolic and arterial pressure dysregulation. Therefore, the present data suggest that taurine supplementation or diets high in taurine could be an alternative treatment of maternal diabetes mellitus in humans.

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Taurine Improves Sexual Function in Streptozotocin-Induced Diabetic Rats

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Abstract Previous studies have identified that diabetic erectile dysfunction is associated with androgen and nitric oxide deficiency resulting from hyperglycemia. It has been demonstrated that taurine can stimulate testosterone secretion, increase nitric oxide synthase (NOS) activity and nitric oxide (NO) production, and reduce blood glucose levels in the diabetic animals. Furthermore, recent studies have found that taurine relaxes both the corpus cavernosum and the vasculature. Accordingly, we hypothesized that taurine might exert beneficial effects on erectile function of the diabetic rats. Here, we assessed the effects of taurine on sexual function in streptozotocin (STZ) -induced diabetic male rats. We observed that taurine treatment could markedly increase sexual response and mating ability of STZ-diabetic rats. The serum concentration of gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone (T) were also significantly increased by taurine administration. Importantly, taurine supplementation notably increased mRNA levels and activity of endothelial NOS (eNOS) and neuronal NOS (nNOS), as well as NO and cGMP content, in the corpus cavernosum of the diabetic rats. In conclusion, the present data indicate that taurine can increase sexual function of STZ-induced diabetic male rats mainly by correcting the diabetes, increasing sexual desire, which is implicated in ameliorating the hypothalamic-pituitary-testicular axis function, and by improving penile erection, which requires increased signaling from the penile endothelial- and neuronal-dependent NO-cGMP pathway.

Keywords Taurine • Male sexual function • Hypothalamic-pituitary-testicular axis • NO-cGMP signaling • Diabetic rats

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Abbreviations

cGMP	Cyclic guanosine monophosphate
EJF	Ejaculation frequency
EL	Erection latency
eNOS	Endothelial NOS
ERF	Erection frequency
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
HbA1c	Glycosylated hemoglobin
IF	Intromission frequency
iNOS	Inducible NOS
LH	Luteinizing hormone
MF	Mount frequency
ML	Mount latency
NANC	Non-adrenergic and non-cholinergic
nNOS	Neuronal NOS
NO	Nitric oxide
NOS	Nitric oxide synthase
STZ	Streptozotocin
T	Testosterone

1 Introduction

Reproductive impairment is associated with diabetes in human and experimental rodents. It has been reported that approximately 90% of people with diabetes exhibit sexual dysfunction that is characterized by disturbances in libido and psychophysiological changes that are related to the sexual response cycle, including decreased erection, ejaculation, fertility and sexual satisfaction (Jiang 1996). In males, diabetes has long been identified as a major risk factor for erectile dysfunction. The prevalence of penile dysfunction in diabetic men was threefold higher than those without diabetes, with the effect being age-dependent. Although the mechanism of diabetes-induced male sexual dysfunction remains under investigation, increasing evidence suggests that the neurological, vascular, hormonal, muscular and psychogenic changes resulting from hyperglycemia may contribute to the problem (Sexton and Jarow 1997).

Recent studies have demonstrated that diabetes-induced hypogonadism is also associated with impaired erectile function (Costabile 2003). A cross-sectional survey has found that the prevalence of hypogonadism in diabetic men was 20–60%, which increased in the elderly (Barrett-Connor et al. 1990). In a survey of more than 1200 Italian men with erectile dysfunction, Corona et al. (2006)

have found that the incidence of hypogonadism in men under 60 years of age was significantly increased compared with men who did not have diabetes. A large body of evidence has demonstrated that abnormalities in the hypothalamic-pituitary-gonad axis may be responsible for reproductive dysfunction (Betancourt-Albrecht and Cunningham 2003). Androgen deficiency is common in men with diabetes, as androgen function plays a regulatory role in penile erection at both the central and peripheral level, it may have contributed to diabetic erectile dysfunction (Mills et al. 1996). In the central nervous system, androgens are aromatized to estradiol, which then regulates sexual behavior. In the penis, androgens primarily stimulate or maintain the activity of nitric oxide synthase (NOS), which catalyzes the conversion of arginine to nitric oxide (NO), an important neurotransmitter and mediator of sexual behavior by relaxing the nerve fibers that innervate the blood vessels and corpus cavernosum of the penis, resulting in penile erection (Burnett et al. 1992).

Taurine, also known as 2-aminoethanesulfonic acid, was first isolated from ox bile in 1827 and has been shown to be an important free amino acid that participates in several physiological functions in human and animals, such as bile formation, neurotransmission or neuromodulation, osmotic adjustment, calcium homeostasis, membrane stabilization, antioxidant activity, and nutritional functions in central nervous development (Huxtable 1992). Although it is believed that the liver is the main source of taurine biosynthesis, previous studies have found that the male reproductive organs can also synthesize taurine, suggesting that taurine might be involved in male reproduction. In the testes, taurine is one of the most abundant free amino acids, and it is mainly localized in the Leydig cells, vascular endothelial cells and interstitial cells (Lobo et al. 2000). Our previous studies found that taurine can stimulate testosterone secretion by acting on the hypothalamic-pituitary-testicular axis, thus enhancing sexual function in aged rats (Yang et al. 2013). Moreover, we also found that taurine can decrease glucose levels, improve lipid metabolism and inhibit pancreatic islet cell apoptosis in STZ-treated rats (Lin et al. 2013). In addition, recent studies have demonstrated that taurine can induce rat corpus cavernosum relaxation through the NO pathway, which indicates that taurine may have a positive effect on erection (Dalaklioglu-Tasatargil 2012). Accordingly, we hypothesized that taurine may be beneficial for treating male diabetics with sexual dysfunction.

The aim of this study was to evaluate the effects of taurine on sexual response and mating ability of streptozotocin (STZ)-induced diabetic male rats and decipher its mechanism. We found that taurine enhances erectile function and mating ability; increases the levels of gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone (T) while elevating the activity/concentration of penile endothelial NOS (eNOS), neuronal NOS (nNOS), NO and cyclic guanosine monophosphate (cGMP) in diabetic rats. The present data suggest that taurine primarily increases male sexual function in STZ-induced diabetic rats by improving the diabetes, stimulating testosterone secretion and increasing penile NO-cGMP signaling.

2 Materials and Methods

2.1 Animals and Treatments

After being acclimatized to the laboratory environment for 1 week, adult (8 weeks old) male Sprague-Dawley rats weighing approximately 180–220 g were injected i.p. with streptozotocin (STZ, Sigma, USA, 50 mg/kg body weight, i.p.) or vehicle (0.1 M citrate buffer, pH 4.6). Fasting blood glucose levels were analyzed 72 h after the injection using blood glucose monitoring test strips; only rats with glucose concentrations higher than 16.7 mmol/L were considered diabetic and were used in the experiment. Diabetic rats were randomly divided into two groups of 15 animals each: diabetic (DM) and diabetic + taurine (DM + Tau). Control rats (injected with vehicle) and DM rats were provided tap water for 12 weeks, whereas the DM + Tau rats were provided water containing 2% taurine (Capital Commercial Source Co., Ltd., Beijing, China). All rats were maintained under controlled light (14-h light, 10-h dark) and temperature (22 ± 2 °C) conditions, and were allowed free access to a rat diet and water. All experimental protocols were approved by the Shenyang Agricultural University Ethical Committee and were in compliance with the Declaration of Helsinki.

2.2 Sexual Response and Mating Ability Tests

The animals' sexual response was tested 8 weeks after taurine treatment using apomorphine (Sachs et al. 1994). The erection latency (EL) and erection frequency (ERF) were recorded. Two weeks after sexual response testing, the animals' mating ability was determined as previously described (Clark et al. 1987). The mount latency (ML), mount frequency (MF), intromission frequency (IF), and ejaculation frequency (EJF) were recorded.

2.3 Biochemical Assay

After 12 weeks of treatment, all animals were euthanized under anesthesia, and blood and corpora cavernosa were collected. The blood samples were used to measure glycosylated hemoglobin (HbA1c), fasting serum glucose and serum GnRH, FSH, LH and T levels. The corpora cavernosa were homogenized in cold PBS with a homogenizer and used to analyze the levels of inducible NOS (iNOS), eNOS, nNOS, NO, cGMP and total protein. All of the parameters were analyzed by ELISA according to the instructions of the respective kit (Nanjing Jiancheng Bioengineering Institute, China).

2.4 Real-Time PCR

Total RNA was extracted from the corpora cavernosa using RNAiso Plus according to the manufacturer's procedure (TaKaRa, China). RNA purity and quality were determined spectrophotometrically. After first-strand cDNA synthesis (AMV First Strand cDNA Synthesis Kit, Sangon, China), Real-time PCR was performed on a Bio-Rad iQTM5 system using the SYBR Green PCR Master Mix (ABI). The primers were: iNOS forward primer: 5′-TCACAAGCATCAAAATGGTTF-3′, reverse primer: 5′-GAAGGGTGTCGTGAAAAATCT-3′; eNOS forward primer: 5′-GCGCCAGGCTCTCACTTACTT-3′, reverse primer: 5′-TGCCACGGATGGAAA TTGTT-3′; nNOS forward primer: 5′-AGCCAAAGCAGAGATGAAAGAC-3′, reverse primer: 5′-TCCTTCTCTGAATACGGGTTGT-3′; β-actin forward primer: 5′-TCGTGCG TGACATTAAAGAG-3′, reverse primer: 5′- ATTGCCGATAGTGATGACCT-3′. The relative gene expression levels were calculated using the $2^{-\triangle\triangle Ct}$ method and normalized to the expression of β-actin in the same sample. The data were presented as the relative fold-change compared with rats in the control group.

2.5 Statistical Analysis

SPSS 16.0 software was used for the statistical analysis. The data were analyzed by one-way ANOVA followed by the Student-Newman-Keuls' (SNK) multiple comparison test. All values are expressed as means ± SE. P values less than 0.05 were considered statistically significant.

3 Results

As shown in Fig. 1, final body weights of the STZ-induced diabetic rats were significantly decreased compared with those of the control groups ($P < 0.01$) but could be remarkably increased by taurine administration ($P < 0.05$). Fasting serum glucose and blood HbA1c levels in the diabetic animals were significantly increased ($P < 0.01$) but were reduced by taurine treatment ($P < 0.05$). These data suggest that taurine has a beneficial effect on STZ-induced diabetic rats.

The effect of taurine on sexual behavior of the diabetic animals is presented in Fig. 2. The diabetic rats displayed reduced erection frequency (1.24 ± 0.23 events, $P < 0.05$) and a longer erection latency (479.72 ± 29.53 min, $P < 0.01$) than the control rats (4.75 ± 1.23 events and 303.5 ± 48.31 min, respectively. Fig. 2a). However, taurine administration significantly increased the number of erection events and reduced the latency to the first erection compared with those of the diabetic animals ($P < 0.05$, Fig. 2b). According to the mating ability test, the diabetic

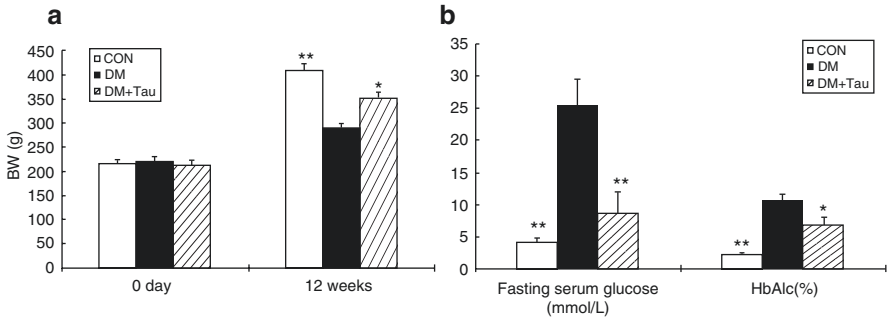

Fig. 1 Effects of taurine on body weight, the fasting serum glucose and blood HbAlc levels. The values are expressed as means ± SE (n = 10). $^{*}P < 0.05$ vs. DM, $^{**}P < 0.01$ vs. DM. Zero day is the first day of the experiment

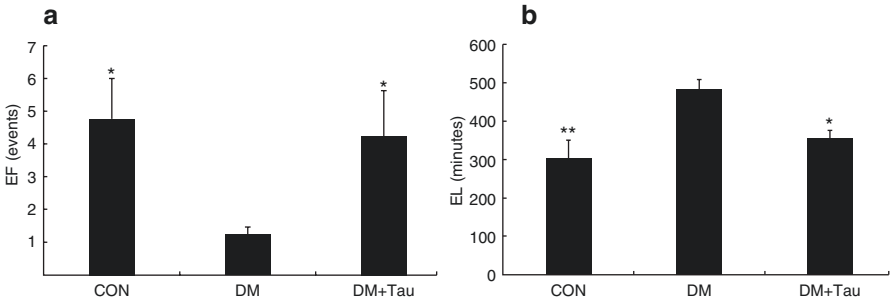

Fig. 2 Effects of taurine on the sexual response in STZ-induced diabetic rats. *EF* erection frequency, *EL* erection latency. Values were expressed as means ± SE (n = 10). $^{*}P < 0.05$ vs. DM, $^{**}P < 0.01$ vs. DM

rats exhibited a ten and fourfold increase in mount latency and intromission latency relative to those of the control animals, respectively ($P < 0.01$), while the mount and intromission frequency were decreased approximately threefold ($P < 0.01$, Fig. 3a). Maurine treatment, however, notably decreased the mount and intromission latency ($P < 0.01$), whereas it increased mount and intromission frequency twofold compared with the DM groups ($P < 0.05$, Fig. 3b). The present results suggest that taurine restores diabetes-induced erectile and mating dysfunction.

Although the mechanism of sexual dysfunction in diabetic males is still unclear, it has been demonstrated that androgen deficiency resulting from hypogonadism is clearly associated with diabetic mellitus. Therefore, we analyzed the effects of taurine on the hypothalamic-pituitary-testicular axis in diabetic animals. As shown in Fig. 4, serum GnRH, LH, FSH and T levels of diabetic rats were significantly reduced compared with those of the control rats ($P < 0.05$), whereas taurine treatment prevented the diabetes-induced decrease in the levels of the reproductive hormones ($P < 0.05$). The experimental results indicated that taurine increases the

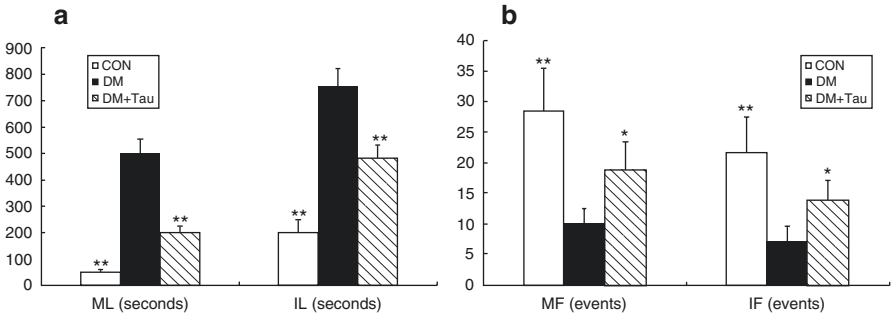

Fig. 3 Effects of taurine on the mating ability of STZ-induced diabetic rats. *ML* mount latency, *IL* intromission latency, *MF* mount frequency, *IF* intromission frequency. The values are expressed as means ± SE (n = 10). $^{*}P < 0.05$ vs. DM, $^{**}P < 0.01$ vs. DM

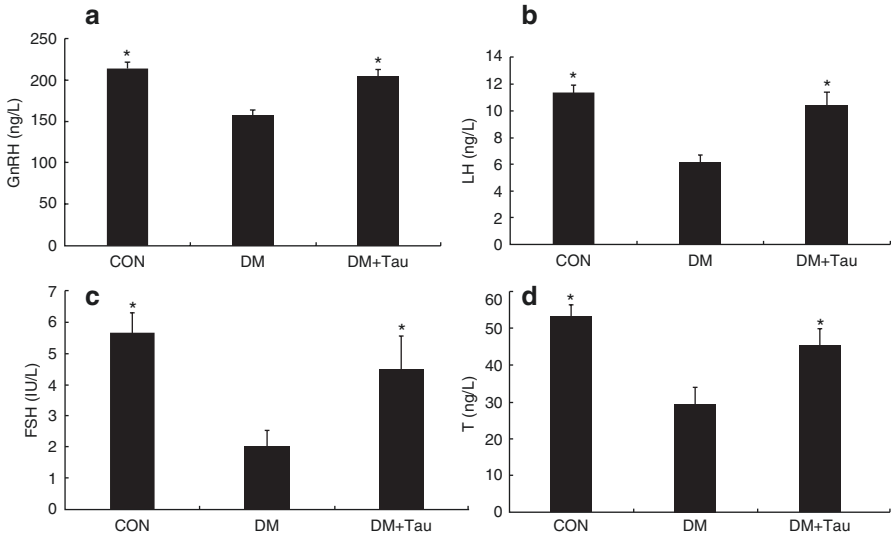

Fig. 4 Effects of taurine on the serum levels of reproductive hormones in STZ-induced diabetic rats. The values are expressed as means ± SE (n = 10). $^{*}P < 0.05$ vs. DM, $^{**}P < 0.01$ vs. DM

androgen concentration by enhancing the function of the hypothalamic-pituitary-testicular axis in diabetic rats.

Sexual behavior is a complex physiological event that is regulated by the central nervous system and local factors, with NO playing a crucial mediator role in penile erection, primarily through the cGMP/PKG signaling pathway. Fig. 5 shows the changes in NOS activity and NO levels in the corpora cavernosa of the experimental animals. The levels of eNOS, nNOS and NO were significantly decreased in the diabetic animals compared with those of the control rats, while taurine treatment restored the three parameters to the baseline values (Fig. 5a).

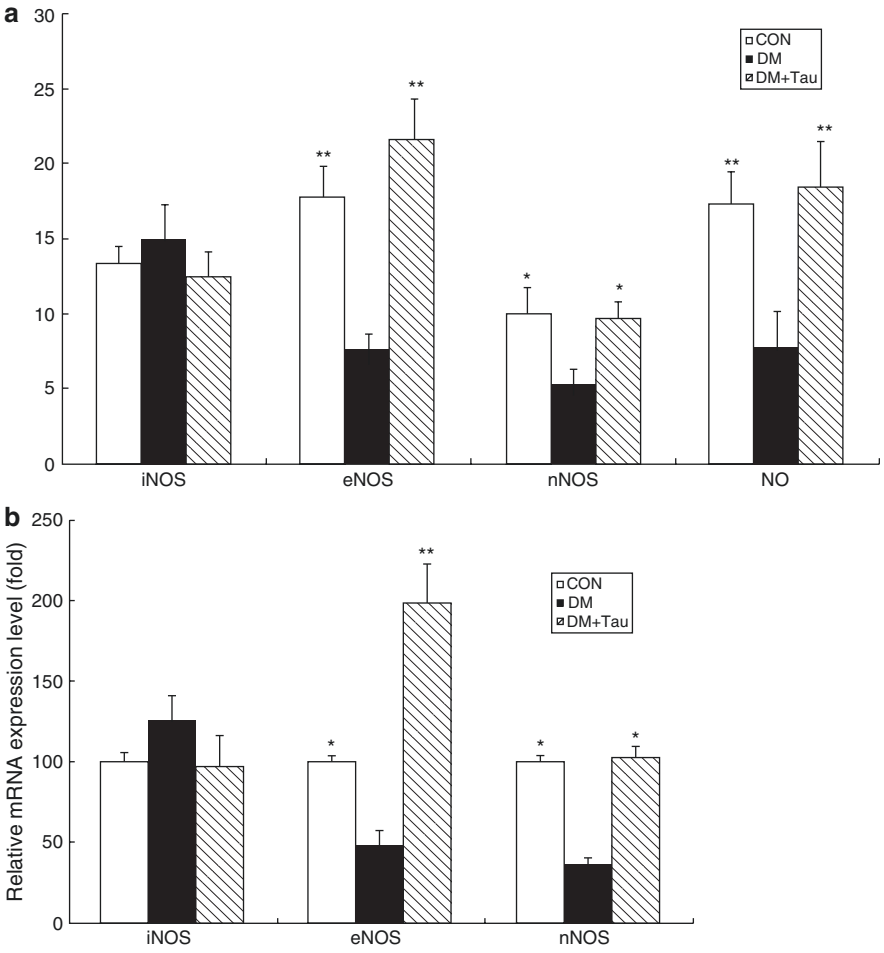

Fig. 5 Effects of taurine on NOS and NO levels in the corpus cavernosum of STZ-induced diabetic rats. (**a**) the activity/concentration of iNOS, eNOS, nNOS and NO; NOS (U/mg protein), NO (mol/g protein); (**b**) the expression levels of iNOS, eNOS and nNOS mRNAs. The values are expressed as means ± SE (n = 10). $^{*}P < 0.05$ vs. DM, $^{**}P < 0.01$ vs. DM

Moreover, we demonstrated that the expression of penile eNOS and nNOS mRNAs were remarkably decreased in diabetic rats, but taurine administration significantly elevated eNOS and nNOS expression of the diabetic rats ($P < 0.05$, Fig. 5b). Notably, cavernosal cGMP content of the diabetic rats was significantly increased in response to taurine treatment ($P < 0.05$, Fig. 6). These findings suggest that taurine may elevate the levels of NO and activate the NO-cGMP signaling pathway in the corpora cavernosa by increasing local eNOS and nNOS expression in diabetic rats.

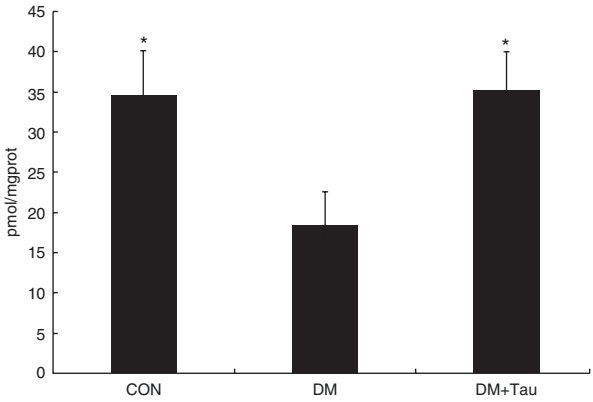

Fig. 6 Effect of taurine on the cGMP concentration in the corpus cavernosum of STZ-induced diabetic rats. The values are expressed as means ± SE (n = 10). *P < 0.05 vs. DM

4 Discussion

Several studies have demonstrated that taurine can decrease blood glucose levels of diabetics, mainly through its insulin-like activity and protection/stimulation of islet β-cell function (Nakaya et al. 2000). To investigate the effects of taurine on STZ-induced diabetic rats, we first analyzed its effects on body weight and fasting serum glucose and blood HbA1c levels, which are accepted as specific parameters of diabetes. Similar to previous studies (Askwith et al. 2012), our data showed that taurine supplementation effectively increases final body weight and decreases fasting serum glucose and blood HbA1c levels of the STZ-induced diabetic experimental animals, which indicate that taurine is an effective hypoglycemic agent.

A large number of investigations have demonstrated that either human diabetics or spontaneous/experimental diabetic animals show penile erectile impotence and a decrease in copulatory behavior (Escrig et al. 2002). It has also been determined that taurine can ameliorate the effects of diabetes mellitus, including retinopathy, nephropathy, neuropathy, atherosclerosis and cardiomyopathy (Franconi et al. 2006; Ito et al. 2012). However, until now, no studies have assessed the effects of taurine on male sexual function in diabetes. To determine whether taurine can ameliorate the sexual behavior of diabetic rats, the effects of taurine on the sexual response and mating ability of STZ-induced diabetic rats were analyzed. The results showed that taurine treatment significantly increases the number of erections, mount and intromission events, while it decreases the erection, mount and intromission latency of the experimental diabetic animals. The results suggested that taurine can increase libido and penile erection in STZ-induced diabetic rats. Our previous study also showed that taurine can enhance sexual behavior in aged male rats, although the experimental animals are different (Yang et al. 2013).

The mechanism of erectile dysfunction in diabetes is usually multifactorial and may be associated with neurological, vascular and hormonal disorders. The level

of testosterone in diabetic rats has been shown to be abnormally low (Howland and Zebrowski 1976), which may be due to inadequate, hyperglycemia-induced GnRH release, reduced pituitary response to GnRH, and abnormal reproductive hormone feedback on the hypothalamic-pituitary axis, which results from a decrease in pituitary sensitivity and disrupted steroid transport into target cells (La Marca et al. 1999). It is thought that androgen deficiency is the main risk factor for male sexual dysfunction in diabetes (Kalyani and Dobs 2007), as androgen not only regulates sexual desire but also plays an important role in maintaining the normal structure of the erectile tissue (Pan et al. 2006). The present study demonstrated that taurine treatment increases serum testosterone levels by stimulating GnRH, LH and FSH secretion in STZ-diabetic rats. Previous studies have demonstrated that taurine can stimulate testosterone secretion in male rats by acting on the hypothalamic-pituitary axis or relieving diabetes-induced testicular damage (Yang et al. 2013; Tsounapi et al. 2012). The taurine-induced increase in testosterone in diabetic rats may be due to its ability to protect gonadal structure and biological function through its anti-oxidative and hypoglycemic activities (Schaffer et al. 2009).

Several studies have demonstrated that male sexual function is androgen-dependent and mediated by NO and that androgen regulates erection by stimulating libido at the central level and increasing NO content at the local level. In the penis, NO is produced in the endothelium and non-adrenergic and non-cholinergic (NANC) nerves by eNOS- and nNOS-mediated catalysis, respectively. The function of NO is to relax the vascular smooth muscle and corpus cavernosum, which increases arterial inflow and results in penile erection. It has been demonstrated that the levels of eNO and nNOS activity were reduced in the corpus cavernosum of the diabetic rats (Bivalacqua et al. 2004; Vernet et al. 1995). The present study showed that taurine treatment effectively increased mRNA levels and activities of eNOS and nNOS, thereby elevating NO concentration in the corpus cavernosum of the STZ-induced diabetic rats. The results are consistent with the studies of Dalaklioglu and colleagues who reported that chronic supplementation with taurine improved eNOS and nNOS expression in the corpus cavernosum of STZ-induced diabetic rats, as demonstrated by immunohistochemistry (Dalaklioglu et al. 2014). It is widely accepted that the NO-cGMP signaling pathway is the most important mechanism that regulates the relaxation of the corpus cavernosum smooth muscle and penile erection (Burnett 1995). In addition, it has been identified that both the formation and degradation of cGMP were affected by testosterone. The present study demonstrated that taurine supplementation increased serum testosterone and corpora cavernosa cGMP levels in STZ-induced diabetic animals. These data indicated that taurine may improve penile erection of the diabetic rat by elevating sexual desire and enhancing the endothelial- and nonadrenergic, noncholinergic-dependent NO-cGMP signal transduction pathway. In addition, it is interesting to note that taurine itself exerts relaxant activity on the corpus cavernosum and vasculature, according to previous studies (Dalaklioglu-Tasatargil 2012; Abebe and Mozaffari 2011). All of these results suggested that taurine may increase sexual function through multiple mechanisms.

5 Conclusion

In summary, our results, combined with previous reports, demonstrated for the first time, that taurine improves male sexual function in STZ-induced diabetic rats, and the mechanism may be associated with its hypoglycemic and relaxant activities, increased sexual desire regulated by the hypothalamic-pituitary-testicular axis and increased testosterone secretion, and improved penile erection regulated by increasing the activity of the penile endothelial- and neuronal-dependent NO-cGMP signaling pathway. The present results provided important insights into the application of taurine for treating erectile dysfunction in diabetes.

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Taurine Increases Insulin Expression in STZ-Treated Rat Islet Cells In Vitro

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Abstract This research aims at figure out the effects and the pathway of taurine on insulin in islet cells cultured in vitro treated by STZ. In the experiment, islet cells were isolated from pancreatic tissue by in situ perfusion with collagenase V. The pancreatic islet cells, maintained in RPMI 1640 culture medium were divided into six groups: C: control, E: supplemented with 10 mmol/L of taurine, group M, T1, T2 and T3 was treated with STZ (0.5 mmol/L), at the same time, taurine were added in group T1,T2 and T3 for 30 min, and then culture medium were collected by centrifugation and then insulin levels were detected by radioimmunoassay, the cells were then rinsed with Hanks, and 0,10, 0, 5, 10, 20 mmol/L of taurine in group C, E, M, T1, T2 and T3 were added for 24 h respectively. Total RNA was extracted, then insulin gene and its transcription regulator such as PDX-1, NeuroD1 were amplified by semi-quantitative RT-PCR. The results showed that, the release of insulin from islet cells treated by STZ could be inhibited by taurine, gene expression of insulin, PDX-1 and NeuroD1 in STZ group decreased significantly, which were up-regulated by taurine administration. In conclusion, taurine exerts a certain degree of protective and reconstructive effects on islet cells treated by STZ.

Keywords Taurine • Insulin expression • STZ • Islet cells • Rat

Abbreviations

NeuroD1	Norvegicus neurogenic differentiation 1
PDX-1	Pancreatic and duodenal homeobox 1
STZ	Streptozotocin

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1 Introduction

Taurine (2-aminoethylsulphonic acid) is a non-protein amino acid which presents the most abundant free intracellular in all mammalian tissues. Taurine is involved in numerous physiological functions, including antioxidation, osmoregulation, modulation of ion movement, anti-inflammatory, etc. (Schaffer et al. 2000, 2009; Lin et al. 2013; Haojun et al. 2012). More and more evidence indicated that taurine supplementation could improve insulin dependent diabetes mellitus, non insulin dependent diabetes mellitus and insulin resistance (Nakaya et al. 2000; Anuradha and Balakrishnan 1999; Di Leo et al. 2004; Ito et al. 2011; Xiao et al. 2008). Diabetes mellitus is a chronic disease that is characterized by a relative or absolute lack of insulin, which could result in hyperglycaemia. Insulin, the only hypoglycemic hormone in the body, reduces blood glucose through promoting the transportation of glucose from blood into the liver and skeletal muscle to synthesis glycogen, inhibit glycogenolysis and gluconeogenesis.

STZ is one of the main compounds used to induce diabetes. Due to their similarity in structure to glucose, glucose can compete with STZ, and thus, fasting animals tend to be more susceptible to them. STZ, synthesized by streptomycetes achromogenes could enter the pancreatic β-cells through the Glut-2 transporter and causes alkylation of the DNA (Szkudelski 2001). Subsequent activation of PARP leads to NAD+ depletion, a reduction in cellular ATP and the inhibition of insulin production (Sandler and Swenne 1983). In addition, STZ is a source of free radicals that can also contribute to DNA damage and cell death. STZ were used in the establishment of diabetes models on different animals to destroy islet β cells (Rood et al. 2006; Jin et al. 2010; Hara et al. 2008; Ma and Long 2016), which consequently increase blood glucose. There are reports that taurine could decrease blood glucose of diabetic animal models induced by STZ and protect islet β cells (Alvarado-Vásquez et al. 2003; Tokunaga et al. 1983; Chang 2000; Colivicchi et al. 2004), but at present, there is lacking of the research on the effect of taurine on STZ treated islet β-cells cultured in vitro.

Radioimmunoassy were applied to assay extracellular levels of insulin in order to detect the protection effect of taurine on STZ destroyed islet β cells acutely. Semi-quantitive RT-PCR were applied to detect the gene expressions of insulin and insulin transcription regulators PDX-1 and NeuroD1 and discuss the recovery effect of taurine on STZ treated islet cells.

2 Materials and Methods

2.1 Cell Culture

Male Wistar rats (230–250 g) were anaesthetized with ethylether, and pancreatic islets were isolated using collagenase V (in Hank's solution), followed by

hand-picking under a stereomicroscope. Average diameter of isolated islets used throughout the study was 125 μm. The pancreatic islet cells, maintained in RPMI 1640 medium containing 10% FBS and 5.6 M glucose, were divided into six groups in a 24-well plate with 50 cells/well in triplicates as follows: group C was used as control, group E was taurine control group which was supplemented with 10 mmol/L taurine, group M was model group which was treated with STZ (0.5 mmol/L) for 30 min, groups T1, T2 and T3 were treated with 5, 10, 20 mmol/L of taurine and STZ (0.5 mmol/L) for 30 min. After centrifugation, the supernatants were collected to detect the level of insulin by radioimmunoassay, the cells of group C, E, M, T1, T2 and T3 in the subsidence were rinsed with Hanks, and cultured with taurine (0, 10, 0, 5, 10, 20 mmol/L) for 24 h. Islet cells were collected by centrifugation, total RNA was extracted, gene expressions of insulin and its transcription factors including PDX-1 and NeuroD1 were detected by semi-quantitative RT-PCR.

2.2 Radioimmunoassay

The culture medium was collected by centrifugation at 1000 rpm for 10 min at 4 °C. The supernatant was collected to measure the levels of insulin using the insulin RIA kit (Institute of Atomic Energy, China National Research Academy).

2.3 Semi-Quantitative RT-PCR

Total RNA were extracted by RNAiso Reagent (Takara) and quantified by spectrophotometry at 260 and 280 nm. RNA (1 μg) was subjected to the reverse transcription with RNA PCR Kit (AMV) Ver3 (Takara). PCR amplifications were carried out using primers specific for insulin (forward, 5′-CCTGCCCAGGCTTTTGTC-3′, reverse, 5′-TTGCGGGTCCTCCACTTC-3′), PDX-1 (forward: 5′-AACGCTGGAACAGGGAAG-3′,reverse:5′-CACGGGAAAGGGAGATGA-3′), NeuroD1 (forward: 5′-TTCACGATTAGAGGCACG-3′, reverse: 5′-TCCAAAGGCAGTAACGAC-3′), β-actin (forward: 5′-TTGTAACCAACTGGGACG-3′, reverse: 5′- GATATTGATCTTCATGGTG-3′). After PCR reaction, the PCR products were gel purified, cloned into PMD18-T vector and sequenced. The sequencing results were analyzed using NCBI blast software. Each PCR product was quantified and presented as the ratio of the target gene to β-actin. Semi-quantitive PCR reaction conditions: insulin (94 °C, 3 min; 94 °C, 30 s; 56.5 °C, 30 s; 72 °C, 30 s; 72 °C, 5 min, for 35 cycles), PDX-1 (94 °C, 3 min; 94 °C, 30 s; 49 °C, 30 s; 72 °C, 30 s; 72 °C, 5 min, for 36 cycles), NeuroD1 (94 °C, 3 min; 94 °C, 30 s; 56 °C, 30 s; 72 °C, 30 s; 72 °C, 75 min, for 38 cycles) (Table 1).

Table 1 Reverse transcription reaction system

Composition	Quantity (μL)
10 × PCR Buffer	1
dNTP Mixture	1
$MgCl_2$	2
RNase Inhibitor	0.25
Oliga dT	0.5
Template RNA (1 μg/μL)	1
AMV Reverse Transcriptase	0.5
ddH_2O	3.75
Total	10

2.4 Statistic Analysis

Data were presented as the mean ± SE and significant differences were determined by Duncan's multiple range test using SPSS 16.0 statistical analysis software. P values less than 0.05 were considered as significant.

3 Results

3.1 Effect of Taurine on Insulin Levels in Culture Medium in STZ-Treated Islet Cells

The results showed that insulin level in islet extracellular fluid in group M was significantly increased than group C ($P < 0.05$), but no significant differences were found between group C and the control group E ($P > 0.05$). In taurine groups, insulin level was obviously decreased than group M ($P < 0.05$), and there were no obvious differences among taurine groups ($P > 0.05$) (Fig. 1).

3.2 Effect of Taurine on Gene Expressions in Islet Cells

As was shown in Figs. 2, 3 and 4, mRNA expressions of insulin, PDX-1 and NeuroD1 in group M were significantly decreased compared with groups C and E. After taurine administration for 24 h, the expression levels of these genes in group T3, T2 and T1 increased compared with group M, among which, group T3 showed significant changes ($P < 0.05$) but there were no obvious differences among taurine groups ($P > 0.05$).

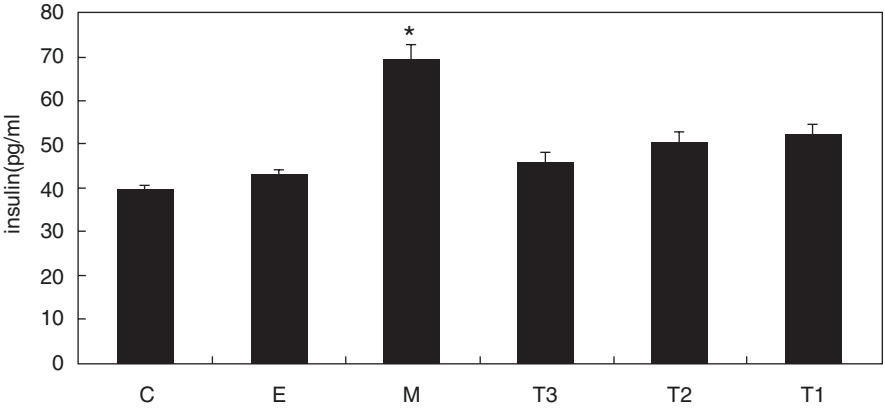

Fig. 1 Effect of taurine on insulin level of in STZ- treated rats islet cells in culture medium. Results are presented as mean ± SE (n = 6). *: significantly different compared with the control group ($p < 0.05$)

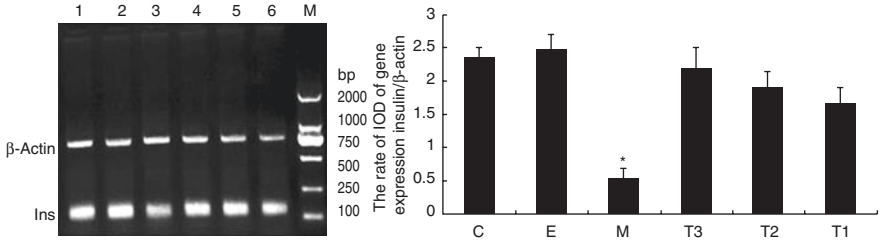

Fig. 2 Gene expression of insulin in STZ-treated pancreatic islet cells. 1 (C): control group, 2 (E): 10 mmol/L of taurine group, 3 (M): STZ group (0 mmol/L of taurine group), 4 (T3): 20 mmol/L of taurine group, 5 (T2): 10 mmol/L of taurine group, 6 (T1): 5 mmol/L of taurine group. Results are presented as mean ± SE (n = 6). Results are presented as mean ± SE (n = 3). *: significantly different compared with the control group ($p < 0.05$)

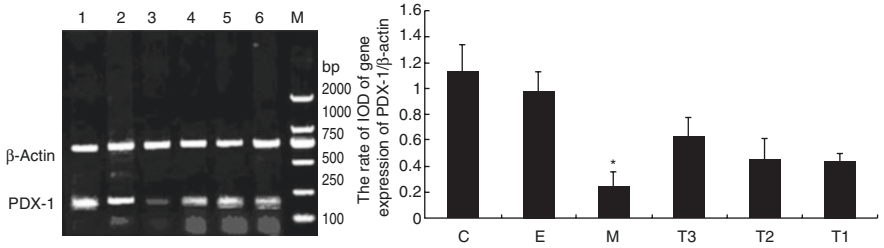

Fig. 3 Gene expression of PDX-1 in STZ-treated pancreatic islet cells. 1 (C): control group, 2 (E): 10 mmol/L of taurine group, 3 (M): STZ group (0 mmol/L of taurine group), 4 (T3): 20 mmol/L of taurine group, 5 (T2): 10 mmol/L of taurine group, 6 (T1): 5 mmol/L of taurine group. Results are presented as mean ± SE (n = 3). *: significantly different from control group ($p < 0.05$)

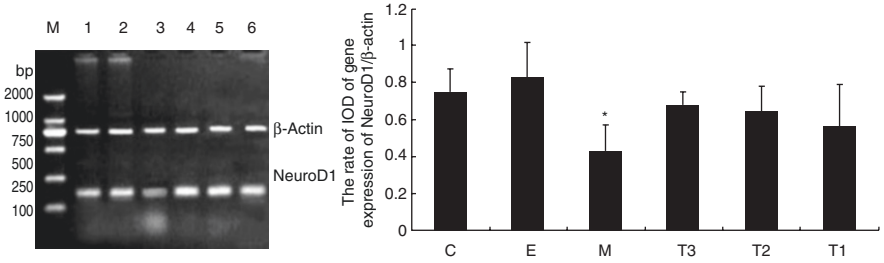

Fig. 4 Gene expression of NeuroD1 in STZ-treated pancreatic islet cells. 1 (C): control group, 2 (E): 10 mmol/L of taurine group, 3 (M): STZ group (0 mmol/L of taurine group), 4 (T3): 20 mmol/L of taurine group, 5 (T2): 10 mmol/L of taurine group, 6 (T1): 5 mmol/L of taurine group. Results are presented as mean ± SE (n = 6). *: significantly different from control group ($p < 0.05$)

4 Discussion

Taurine is the most abundant free amino acid presenting in the plasma and tissues of mammals, particularly in the pancreas, which accounts for approximately 0.1% of total human body weight (Bustamante et al. 2001; Ribeiro et al. 2010). taurine in human is derived from biosynthesis and dietary intake of meat and seafood (Pasantes-Morales et al. 1980; Rana and Sanders 1986). Taurine content is influenced by diet, as well as disease and aging (Wallace and Dawson 1990). The previous results showed that taurine level in plasma declined in patients and animals with diabetes (Franconi et al. 1995; De Luca et al. 2001; Franconi et al. 1996; Trachtman et al. 1995). Moreover, taurine levels were lower in the liver in high-fructose-fed rats (Nandhini et al. 2005), suggesting that diabetes also can be considered as a taurine-deficient disease. Therefore, taurine deficiency may be one reason for the occurrence and development of diabetes (Imae et al. 2014). Meanwhile, It could be hypothesized that exogenous taurine supplement is beneficial to the prevention and treatment of diabetes. Researches have also proved that taurine administration could prevent and cure the development of diabetes and its complications (Chauncey et al. 2003; Chang 2000; Elizarova and Nedosugova 1996; El Idrissi et al. 2009, 2015; Gavrovskaya et al. 2008; El Mesallamy et al. 2010; Ito et al. 2012; Ripps and Shen 2012).

Insulin produced by islet β-cells is important to control blood glucose. The present results showed that STZ destroyed cell wall of islet cells and consequently increase the insulin level in the cell culture medium, which is in accordance with the previous studies in vivo (West et al. 1996; Manell et al. 2014). Taurine supplement at the same time of STZ treatment could protect islet cells, decrease insulin level in the cell culture medium, indicating that taurine could protect islet cells from acute injury caused by STZ by protecting the intact structure of islet cells.

It has been reported that taurine supplemented at started from the time-point with streptozotocin (60 mg/kg i.p.) and lasted for 14 days decreased the blood glucose and plasma glucose of diabetic rats and promoted the insulin level to 50% above the diabetes value at the end of 6 weeks of treatment (Pandya et al. 2013). Inadequate intake of protein is also one reason for the dysfunction of islet cells. In protein-malnourished rodents, taurine restored the secretion capacity of insulin, almost at

the same time, similar results were obtained by Acharya M and Lau-Cam CA (Batista et al. 2012, 2013a; Acharya and Lau-Cam 2013). In addition, islets isolated from low-protein diet rats induced by high glucose concentrations showed decreased insulin secretion which was increased by taurine supplementation (Batista et al. 2012). Similarly, taurine supplementation increases insulin secretion in islets from malnourished mice fed on a high-fat diet (Vettorazzi et al. 2014). The above results illustrated that long term supplement of taurine can repair dysfunction of islet cells induced by different reasons, promote insulin secretion, and partially recover islet function. The transcription level of insulin were detected in the present study, the results showed that taurine administered for a long time could promote the repair of tissue acutely damaged by STZ, which was essentially in accordance with the above studies.

Many transcriptions could strengthen or weaken insulin gene transcription activity independently or combined with each other. These transcription factors include homeodomain (HDs) family, consisting of pancreatic duodenal homeobox-1 (PDX-1), HNFs, Hb9, Nkx, MafA and bHLH (basic helix-loop-helix) family consisting of Ngn3, neural differentiation factor 1 (NeuroD1). PDX-1, NeuroD1 and MafA are considered to regulate the transcription of insulin through combination with upstream region of insulin gene promoter (Qiu et al. 2002; Kataoka 2007; Aramata et al. 2007). PDX-1 which is the switch of pancreatic development mainly regulates the differentiation of embryonic pancreas and maintains the physiological function of islet cell (Kaneto et al. 2008; Melloul 2004). PDX-1 gene also has anti-apoptosis and proliferation promoting effect on insulin secreting cells (Hernandez-Sanchez et al. 2006). NeuroD1 also plays a key role for the development and physiological function on pancreatic β-cells (Kim et al. 2004). NeuroD1 gene knockout fetal mice died or showed severe hyperglycemia after birth because of the death of most pancreatic β-cells and the incomplete structure of islet. The insulin level also declined to about 5% of the normal mice (Huang et al. 2002). It was reported that islets from mice supplemented with taurine for 30 days showed increased insulin secretion stimulated by glucose and exhibited significant increased PDX-1 expression (Carneiro et al. 2009). Moreover, islet area, β-cell area and number of islets per section increased in mice administered with 5% taurine in drinking water. Likewise, mRNA levels of MafA, Ngn3 and NeuroD1 were also enhanced in mice treated with taurine (Santos-Silva et al. 2015). Similar results were found in the present study that taurine can increase the gene expressions of insulin transcription factors PDX-1 and NeuroD1 in rats islet cells cultured in vitro destroyed by STZ, indicating that taurine could repair islet cells and promote the synthesis of insulin.

5 Conclusion

Taurine can alleviate the acute injury of islet cells caused by STZ and partially promote the recovery of islet cells to synthesize insulin by up-regulating gene expressions of insulin and its transcriptional regulators to protect and repair STZ treated islet cells, the deeper mechanism of which needs to be further studied.

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Metabolomics Profiling of the Effects of Taurine Supplementation on Dyslipidemia in a High-Fat-Diet-Induced Rat Model by ^{1}H NMR Spectroscopy

Kyoung Soo Kim and Eunjung Bang

Abstract Metabolomics, the comprehensive study of metabolites, has merged as a potent tool for analyzing complex phenotypes and identifying biomarkers of specific physiological responses and has the potential to lead to innovative therapeutic and diagnostic schemes for many diseases. In a former report, we showed that taurine supplementation considerably ameliorated dyslipidemia in rats fed a high-caloric diet. In this work, we examined the metabolic changes that occur in rat serum after they were fed a normal diet, a high-fat diet, and a high-fat diet containing 2% taurine (tau) by NMR spectroscopy combined with a multivariate statistical analysis containing PCA, PLS-DA, and OPLS-DA. We obtained ^{1}H–NMR spectra of rat serum and used pattern recognition to identify key metabolites related to taurine supplementation. We found significant changes in creatine, methionine, glutamine, and threonine as well as in lipids, all of which decreased in the Tau group. To use these changes in metabolites as novel therapeutic and diagnostic markers, it should first be investigated whether these results are reproducible in future experiments. Next, researchers should determine how these changes affect serum lipid changes. This study identified some changes in serum metabolites and demonstrated the possibility of using an NMR-based metabolomics method to explore the effects of a taurine supplement on dyslipidemia in a high-fat-diet-induced rat model.

Keywords Metabolomics • Dyslipidemia • NMR spectroscopy • High-fat diet-induced rat model

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Abbreviations

HDL-C	High-density lipoprotein cholesterol
HF	High fat
LDL-C	Low-density lipoprotein cholesterol
NMR	Nuclear magnetic resonance
PLS-DA	Projection to latent structures discriminant analysis

1 Introduction

Excessive food intake and desk-based lifestyles have quickly increased the incidence of obesity and associated diseases, such as dyslipidemia, metabolic syndrome, and type 2 diabetes (Angulo 2002). More specifically, dyslipidemia refers to high levels of low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), and triglycerides (TG) and a low level of high-density lipoprotein cholesterol (HDL-C) in the blood. Cardiovascular disease (CVD) is a primary cause of disease and death worldwide and is thought to be induced by dyslipidemia (Kostis 2007). By 2020, it is expected that CVD will be the leading cause of death worldwide (Levenson et al. 2002). In contrast, CVD occurrence is considerably lower in Asian populations than in Western nations (Adlercreutz 1990). One reason for the reduced occurrence of CVD in Asian countries is thought to be due to the consumption of more fish and less meat compared to that in Western populations.

Taurine, which is plentiful in fish and shellfishes, was reported to decrease serum cholesterol levels and inhibit atherosclerosis progression in animal models (Matsushima et al. 2003; Murakami et al. 1999; Petty et al. 1990). We also previously reported that taurine ameliorated dyslipidemia and hyperglycemia by decreasing leptin levels and insulin resistance in type 2 diabetic rats (Kim et al. 2012). Taurine's effect on cholesterol level involves the modulation of bile acid and cholesterol homeostasis. The probable molecular and metabolic mechanisms of taurine's cholesterol-decreasing effect of taurine involves at three aspects; blood cholesterol clearance, cholesterol conversion to bile acid, and excretion of bile acid and cholesterol from the guts (Chen et al. 2012). However, no studies have yet considered specific metabolite changes associated with cholesterol reduction in an animal model.

Metabolomics, the comprehensive study of metabolites, has arisen as a powerful approach for embodying complex phenotypes and recognizing biomarkers of specific physiological responses. To identify biomarkers for dyslipidemia, we show that taurine supplementation ameliorates dyslipidemia and indicate how taurine supplementation changes the metabolite profile in the serum of rats fed a high-fat diet.

2 Methods

2.1 Animals

Four-week-old male SD rats were purchased and kept in animal housing at Kyung Hee University. Animal care followed recommendation guidelines. After 7 days of adaptation being fed a pellet diet, rats were fed one of the following diets for the next 12 weeks; N group, normal diet; HF group, high- fat diet,; Tau group, and high-fat diet +2% taurine. Taurine (2%) was added by mixing it into the food. The diet preparation was founded on AIN-93, as previously described (Reeves 1997).

2.2 Chemical Analysis of Samples

After a 12 h starvation period, whole blood was obtained from a heart puncture. Serum was acquired and kept at −70 °C. TG and TC levels from the serum were examined with an automatic analyzer from BPC BioSed srl. HDL-C was obtained from serum with a HDL precipitating reagent (Asan Pharm CO., Gyeonggi, Korea) after precipitation of LDL and VLDL for 10 min at 3000 rpm. The HDL-C levels were examined by the same method that was used for TC. The LDL-C value was calculated by using the Friedewald formula (Friedewald et al. 1972), as follows: LDL-C = TC − (HDL − C + TG/5).

We used an assayed chemistry control (Bio-Rad Laboratories, Irvine, CA, USA) for calibration and expressed all results as mg/dL serum.

2.3 NMR Spectroscopy and Data Analysis

We thawed 28 serum samples at room temperature and then mixed 25 μL of serum with 0.9% NaCl, adding 20% D_2O to a final volume of 50 μL and transferring each resulting sample to a NMR tube with a 1.7-mm diameter. ^{1}H NMR spectra were obtained on a Bruker Avance III furnished with a 1.7-mm probe working at 700.19 MHz at 298 K. The ^{1}H NMR spectra were acquired using a T_2-weighted (Carr-Purcell-Meiboom-Gill, CPMG) pulse sequence. Macromolecules, such as proteins and lipids, give broad signals that have a short delay, (T_2); these signals can be suppressed by a spin-echo sequence that has a long echo delay before data acquisition, which improves observations of low-molecular-weight metabolites. A total of 128 transients over a spectral area of 14,000 Hz were obtained, and the total number of data points was 128 k. The T_2 signal was filtered using a spin echo delay (τ) of 300 μs that was repeated 126 times (n), resulting in a total echo time of 76 ms for the CPMG pulse sequence $[90 - (\tau - 180 - \tau)_n]$. The ^{1}H NMR spectra were allocated by matching them with the Chenomx NMR Suite 6.0 library and with

previously published data (Chao et al. 2014; Merrifield et al. 2011). In addition, we used two-dimensional ^{1}H-^{1}H correlation spectroscopy and total correlation spectroscopy to confirm some of the assignments. We analyzed the data according to previously described methods (Nam et al. 2015).

2.4 Statistical Analysis

We expressed the data as the mean ± S.E.M and statistically analyzed them by one-way analysis of variance and Duncan's multiple range tests at $p < 0.05$. SPSS 17.0 was used for all analyses.

3 Results and Discussion

3.1 Effect of Taurine on the Level of Serum Lipids in Rats Fed a High-Fat Diet

TG, TC, and LDL-C serum levels in the high-fat (HF) group were significantly increased compared to those of the normal (N) group after 12 weeks of feeding. However, the lipid levels in the serum of the HF group were considerably lower in the Tau group (Kim et al. 2015). The TG and TC levels in the Tau group were even lower than those of the N group. The serum level of HDL-C in the HF group was not increased, but the level in Tau group was significantly decreased compared to that of the N group.

3.2 Effect of Taurine on Metabolite Changes in Rat Serum

To provide insight into the molecular mechanisms by which taurine decreases the lipid level in the serum of rats fed a high-fat diet, the serum metabolite profiles were compared in the three groups by ^{1}H NMR spectroscopy (Fig. 1). We used those spectra to detect numerous endogenous metabolites, such as lipids, glucose, amino acids, acetoacetate, and acetone. We used PLS-DA to explore the extent of metabolite differences of rats in the N, HF, and Tau groups (Fig. 2). PLS-DA plots from the NMR data show clear separation among the groups. We identified and then quantified endogenous metabolites from the ^{1}H NMR spectra. The 20 metabolites that varied among the groups (N, HF, and Tau) are listed in Table 1. The creatine level of the HF group was increased about twofold compared to that of the N group. The extent of this increase was considerably less in the Tau group. The change in creatine level was the most conspicuous difference among the

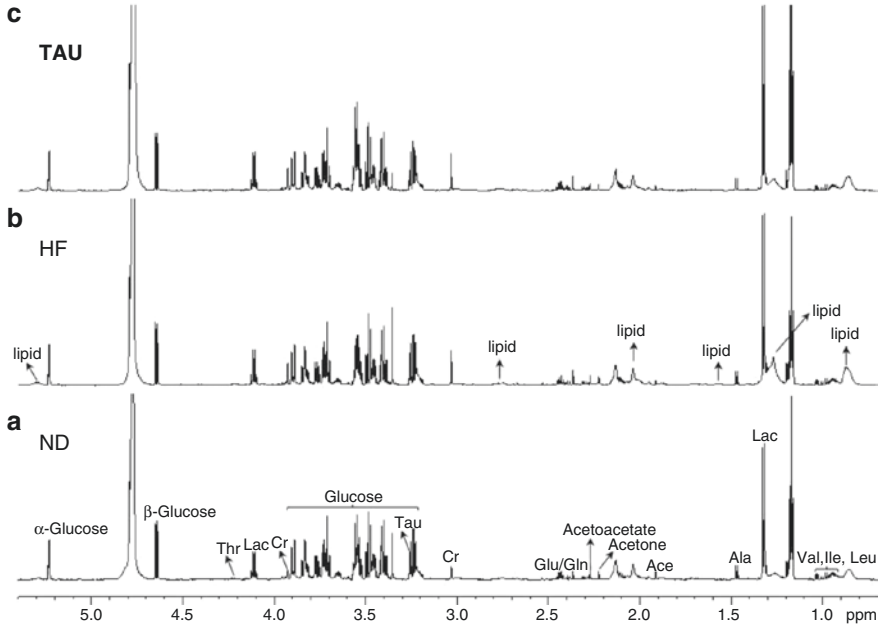

Fig. 1 Representative ^{1}H nuclear magnetic resonance (NMR) spectra obtained from serum from SD rats fed (**a**) a normal diet (ND), (**b**) a high-fat diet (HF) or (**c**) a high- fat diet +2% taurine (Tau)

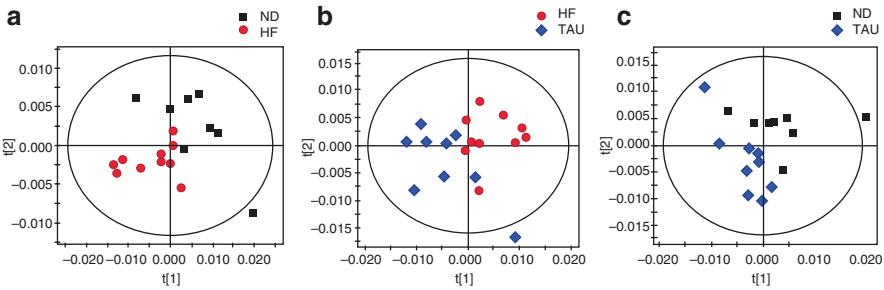

Fig. 2 Projection to latent structures discriminant analysis plots derived from the ^{1}H NMR spectra of serum from (**a**) ND (*squares*) and HF (*circles*) groups, (**b**) HF (*circles*) and Tau (*diamonds*) groups, and (**c**) ND (*squares*) and Tau (*diamonds*) groups

metabolites and could indicate renal dysfunction induced by the HF diet. Nonetheless, the HF group showed no kidney damage because albumin leakage was not detected in the urine. The increase in serum creatine level could be caused by a change in metabolism.

As with the creatine levels, the levels of lipids and methionine in the HF group were significantly higher than in the N group. The extent of this increase was considerably less in the Tau group. In contrast, the valine level was considerably lower in the HF group than in the N group, and the lower level was not recovered in the

Table 1 Metabolites that varied among rats fed a normal diet (N), a high-fat diet (HF), or (C) a high-fat diet +2% taurine (Tau)

	N vs. HF vs. TAU									
	Normalized peak intensity × 1000									
		Mean			SD			Fold change		
	Chemical shift (ppm)	N	HF	TAU	N	HF	TAU	HF/N	TAU/HF	TAU/N
3HB	2.30	3.44	3.52	3.03	1.24	0.67	0.92	1.02	0.86	0.88
	2.39	4.03	4.39	3.77	1.23	0.93	1.42	1.09	0.86	0.94
	4.15	1.83	1.78	1.40	0.80	0.53	0.61	0.97	0.79	0.77
Acetate	1.91	2.67	2.13	1.85	1.03	0.48	0.53	0.80	0.87	0.69
Acetoacetate	2.27	4.49	3.83	2.95	2.20	1.47	1.38	0.85	0.77	0.66
Acetone	2.22	2.69	2.62	1.88	0.89	0.46	0.38	0.97	**0.72**	**0.70**
Alanine	1.47	6.37	5.71	6.63	1.13	1.05	1.05	0.90	1.16	1.04
Citrate	2.53	1.38	1.41	1.54	0.23	0.20	0.37	1.02	1.09	1.12
	2.68	2.18	2.32	2.45	0.21	0.17	0.34	1.06	1.06	1.12
Creatine	3.03	4.58	10.62	6.71	1.38	5.44	2.97	**2.32**	0.63	1.47
	3.93	3.66	7.53	5.23	0.72	3.54	1.85	**2.06**	0.69	**1.43**
Glucose	3.89	30.04	29.73	31.38	4.31	2.29	2.66	0.99	1.06	1.04
	4.64	23.00	22.63	23.91	3.65	1.72	2.34	0.98	1.06	1.04
	5.23	16.89	16.61	18.08	2.62	1.52	1.98	0.98	1.09	1.07
Glutamate	2.34	2.10	2.21	2.02	0.30	0.48	0.40	1.05	0.91	0.96
Glutamine	2.43	7.92	9.13	8.22	0.69	1.22	1.14	**1.15**	0.90	1.04
Glycogen	5.38	0.26	0.29	0.26	0.08	0.08	0.09	1.12	0.90	1.00
Isoleucine	1.00	1.97	1.79	1.65	0.30	0.17	0.18	0.91	0.92	**0.84**
Lactate	1.33	63.24	65.36	66.18	18.79	15.82	30.56	1.03	1.01	1.05
	4.11	17.87	18.40	18.82	5.01	4.46	8.27	1.03	1.02	1.05
Lipid	0.84	41.51	50.25	46.48	6.22	8.04	7.89	**1.21**	0.92	1.12
	1.26	49.24	71.93	58.79	12.55	15.39	12.25	**1.46**	0.82	1.19
	1.56	2.72	3.83	3.02	1.00	1.12	0.82	**1.41**	0.79	1.11
	2.77	7.44	8.70	8.16	1.31	1.09	1.10	**1.17**	0.94	1.10
	5.30	6.40	8.09	7.53	1.37	1.41	1.76	**1.26**	0.93	1.18
Methionine	2.63	0.93	1.27	0.99	0.18	0.31	0.24	**1.37**	**0.78**	1.06
NAG	2.04	18.52	18.86	18.37	1.73	1.03	1.21	1.02	0.97	0.99
	2.15	23.33	23.80	23.00	2.43	1.49	1.21	1.02	0.97	0.99
Pyruvate	2.37	2.35	2.42	2.85	0.80	0.40	0.76	1.03	1.18	1.21
Taurine	3.26	6.87	6.72	4.87	1.47	0.76	0.75	0.98	**0.72**	**0.71**
	3.42	5.06	4.91	5.27	0.71	0.40	0.49	0.97	1.07	1.04
Threonine	4.21	3.69	3.88	3.32	0.22	0.43	0.33	1.05	**0.86**	**0.90**
Valine	0.98	4.41	3.55	3.51	0.41	0.27	0.22	**0.80**	0.99	**0.80**
	1.03	3.26	2.76	2.68	0.38	0.27	0.15	**0.85**	0.97	**0.82**

Bold P-value <0.05

Tau group. In addition, the levels of acetone, taurine, and threonine were higher in the HF group and considerably lower in the Tau group.

4 Conclusion

Taurine supplementation changed the serum metabolite levels of rats. In particular, metabolites that increased with a high-fat diet decreased with taurine supplementation. However, the significance of these changes remains to be discovered, along with the molecular mechanism underlying the taurine-induced decrease in the levels of serum lipids.

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The Effect of Taurine and Its Immediate Homologs on Diabetes-Induced Oxidative Stress in the Brain and Spinal Cord of Rats

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Abstract This study has examined the acute effects of taurine (TAU) and of its two immediate homologs aminomethanesulfonic acid (AMSA) and homotaurine (HTAU) on the oxidative stress that develops in the brain of rats as a result of type 2 diabetes mellitus. Male Sprague-Dawley rats, 220–225 g in weight, were divided into groups of 6 each, and treated with a single intraperitoneal (i.p.) dose of streptozotocin (STZ) in 10 mM citrate buffer pH 4.5 (60 mg/kg). The treatment compound (AMSA, HTAU or TAU) was administered by the i.p. route in two equal doses (1.2 mM/kg each) at 75 and 45 min before STZ. Control rats received only 10 mM citrate buffer pH 4.5 or only STZ by the i.p. route. The rats were sacrificed at 24 h after a dosing with STZ under general anesthesia, and their brains and spinal cords collected by the freeze clamp technique. A portion of brain, of a brain area (cerebellum, cortex, brain stem) or of spinal cord from each animal was extracted into 0.1 M PBS pH 7.4, and the extract was used for the assay of malondialdehyde (MDA), nitric oxide (NO), catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD). An extract for the assay of the reduced (GSH) and disulfide (GGSG) forms of glutathione was prepared in similar manner but using 2% metaphosphoric acid plus 0.1 M PBS pH 8.0 as the extracting medium. Diabetes was found to markedly increase the formation of MDA (by 160–202%), NO (by 138–313%) and GSSG (by 103–241%), and to lower the values of GSH (by 57–65%), GSH/GSSG ratio (79–89%) and activities of CAT (by 61–69%), GPx (by 52–66%) and SOD (by 55–68%) in the brain, brain areas and spinal cord relative to corresponding control values (all at $p < 0.001$). These effects were reduced to values that were generally at least one-half of those seen in untreated diabetic rats, with TAU providing a greater attenuation of the formation of MDA and NO, an about similar action on the depletion of GSH, and a lower action on the decrease in the GSH/GSSG ratio caused by diabetes than either AMSA or HMTAU. In contrast AMSA and HMTAU were about equipotent with each other and more potent than TAU in preventing the loss of antioxidant enzyme activities associated with diabetes.

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In short, pretreating diabetic rats with AMSA, HMTAU or TAU is found to protect the brain against changes in biochemical parameters indicative of oxidative stress, with potency differences among the test compounds varying within a narrow range.

Keywords Diabetes • Rats • Brain • Spinal cord • Oxidative stress • Aminomethanesulfonic acid • Homotaurine • Taurine

Abbreviations

AMSA	Aminomethanesulfonic acid
CAT	Catalase
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Glutathione disulfide
HTAU	Homotaurine
MDA	Malondialdehyde
NO	Nitric oxide
SOD	Superoxide dismutase
STZ	Streptozotocin
TAU	Taurine

1 Introduction

Two of the long term effects of diabetes on the brain are dysfunction and cell death, events that have been related to a reduction of antioxidant defenses and to a concomitant increase in free radicals, and which, in the long run, will lead to altered redox potential in brain cells, to tissue damage and to accelerated cognitive impairment, brain atrophy (Manschot et al. 2006) and brain ageing (Mastrocola et al. 2005).

The hyperglycemia of diabetes overloads metabolic pathways and, in this way, can lead to a reduction of antioxidant defenses and the overproduction of free radicals, events which are known to mediate oxidative stress (Vincent et al. 2004). In this connection, the brain is recognized to be especially vulnerable to oxidative damage because of a high oxygen consumption rate, a high lipid content, and a relative paucity of antioxidant enzymes when compared to other tissues (Muriach et al. 2014). As a result, an imbalance develops between the levels of reactive oxygen and nitrogen species and cellular antioxidant mechanisms, including a decreased activity of antioxidant enzymes, peroxidative damage to lipids and proteins (Muriach et al. 2014), altered redox metabolism, mitochondrial dysfunction, altered cell signaling and compromised energy metabolism (Raza et al. 2015).

Additional mechanisms that may contribute to central oxidative stress by diabetes are glucose autoxidation, lipid peroxidation (LPO), decreased tissue levels of low molecular weight antioxidants such as vitamin C, and reduced glutathione levels (Asmat et al. 2016). Indeed, in the brain of rats made diabetic with streptozotocin increases in the levels of malondialdehyde, a product of lipid peroxidation, are accompanied by a decreased activity of the antioxidant enzyme superoxide dismutase and of the ratio of reduced glutathione-to-glutathione disulfide (Mastrocola et al. 2005).

Previous work in this laboratory has shown that taurine can effectively attenuate the state of oxidative stress that develops in the heart (Mathew et al. 2013) and kidney (Pandya et al. 2013, 2015) of diabetic rats. Based on this evidence, the present study was designed to investigate in diabetic rats: whether (a) the antioxidant actions of taurine observed in the periphery are also extended to the brain; and (b) increasing or decreasing the carbon-chain length of taurine by one carbon will qualitatively and/or quantitatively influence such actions.

2 Materials and Methods

2.1 Treatment Compounds

Aminomethanesulfonic acid (AMSA), homotaurine (HTAU), taurine (TAU) and streptozotocin (STZ) were obtained from Sigma-Aldrich, St. Louis, MO, USA.

2.2 Animals

Male Sprague-Dawley rats, 200–225 g in weight, obtained from Taconic Farms Inc., Germantown, NY, USA, housed in a temperature- (23 ± 1 ℃) and humidity controlled room on a 12 h light-12 h dark cycle, and used after a 5 day acclimation period, during which they had free access to a commercial rodent diet (LabDiet® 5001, PMI Nutrition International, Brentwood, MO, USA) and filtered tap water. The rats were randomly assigned to groups of 6 each.

2.3 Treatments

Rats were pretreated with either AMSA, HTAU or TAU (1.2 mM/kg/2 mL), as an aqueous solution, at 75 and 45 min before receiving a dose of the diabetogen STZ in 10 mM citrate buffer pH 4.5. Control rats received only 10 mM citrate buffer pH 4.5 or only STZ. All the treatments were conducted by the intraperitoneal route.

2.4 Sample Collection and Preparation

The rats were sacrificed by decapitation at 24 h after the administration of STZ, The skulls were cut open with a Friedman rongeur to expose the brains and spinal cords, which were removed without delay by the freeze clamp technique of Wollenberger et al. (1960). A portion of each brain, brain area (cerebellum, cortex, stem) or spinal cord was made into a 1:30 (w/v) homogenate in ice-cold 0.01 M phosphate buffered saline pH 7.4 containing 0.05 M EDTA disodium using a hand held electric tissue homogenizer (Tissue-Tearor™, Bio Spec Products Inc., Bartlesville, OK), which was subsequently centrifuged at 3000 × *g* and 4 °C for 30 min to obtain a clear supernatant suitable for biochemical assays.

2.5 Assays

2.5.1 MDA Levels

The concentration of malondialdehyde (MDA) was measured as thiobarbituric acid reactive substances (TBARS) by the end point assay method of Buege and Aust (1978). The amount of TBARS was calculated by reference to a calibration curve of MDA prepared from serial dilutions of a 1,1,3,3-tetramethoxypropane stock solution, and was reported as nM/mg tissue.

2.5.2 NO Levels

The NO content was measured spectrophotometrically as the nonvolatile breakdown product nitrite using the experimental conditions of Fox et al. (1981), which is based on a diazotization reaction with the Griess reagent. The results were reported as nM/g of tissue.

2.5.3 GSH and GSSG Levels

The brain levels of GSH and GSSG were measured fluorometrically by the method of Hissin and Hilf (1976), which is based on the reaction of GSH with *ortho*-phthalaldehyde (OPT) at pH 8.0 and of GSSG with OPT at pH 12.0. Prior to the measurement of GSSG, any interfering GSH was complexed with N-ethylmaleimide according to the method of Güntherberg and Rost (1966). The concentrations of GSH and GSSG were expressed as mM/g of tissue.

2.5.4 CAT Activity

The CAT activity was measured using the spectrophotometric method of Aebi (1984), which is based on the rate of conversion of exogenously added hydrogen peroxide to water and oxygen. The results were expressed in U/mg tissue/min.

2.5.5 GPx Activity

GPx catalyzes the conversion of GSH to GSSG. In the presence of an excess of β-NADPH and glutathione reductase, GSSG is reduced to GSH and NADPH is reoxidized to $NADP^+$. The measurement of the GPx activity, based on the conditions described by Günzler and Flöhe (1985), is dependent on the rate of decrease in absorbance at 340 nm as a result of the conversion of NADPH to $NADP^+$. The results were expressed as U/mg tissue/min.

2.5.6 SOD Activity

The activity of copper-zinc SOD was measured using the spectrophotometric method of Misra (1985), which is based on the ability of the enzyme to inhibit the autoxidation of colorless epinephrine to colored adrenochrome in an alkaline medium. The results were expressed as U/mg tissue/min.

2.5.7 Statistical Analyses

The experimental results are reported as the mean ± SEM for n = 6. They were analyzed for statistical significance using unpaired Student's t-test followed by Dunnett's post-hoc test and a commercial computer software (InStat, GradPad Software, Inc., La Jolla, San Jose, CA). Intergroup differences were considered to be statistically significant at $p \leq 0.05$.

3 Results

3.1 Brain and Spinal Cord

Impairment of β-pancreatic cells by STZ caused a state of oxidative stress in the brain (Figs. 1, 2 and 3) manifested by a marked increase of the MDA (+166%), NO (+174%) and GSSG (+160%) levels, by drastic decreases of the GSH (−60%) and GSH/GSSG ratio (−85%) values, and by significant losses of CAT (−64%), GPx

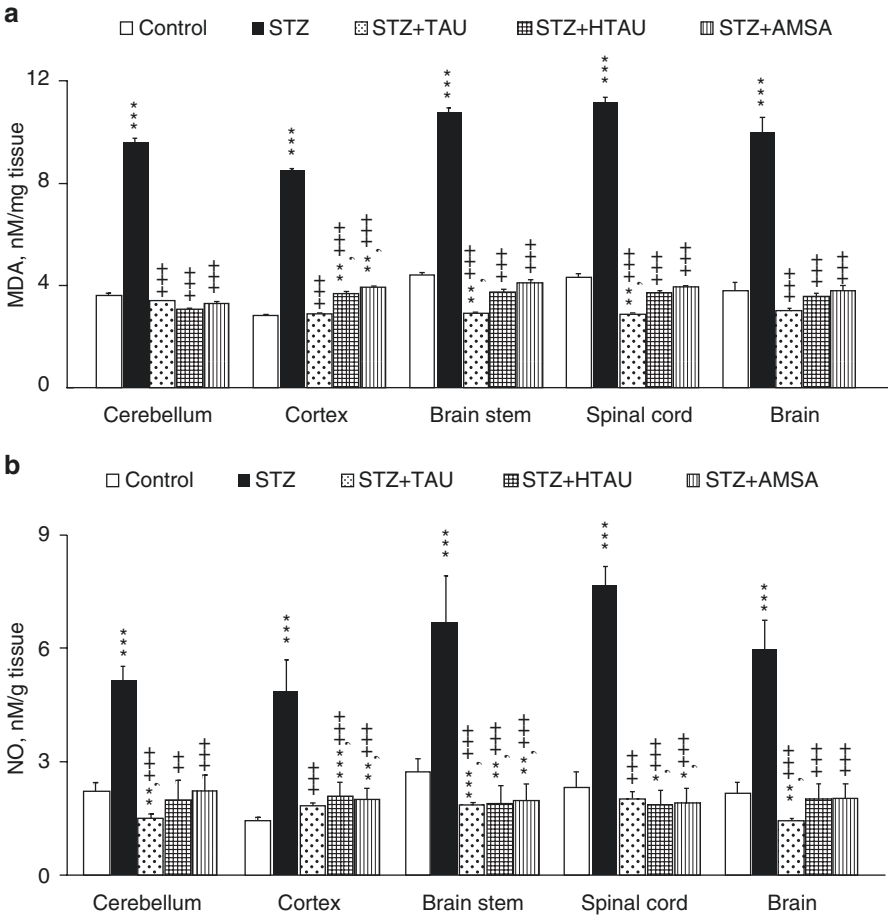

Fig. 1 The effects of AMSA, HTAU and TAU on the (**a**) MDA and (**b**) NO levels in brain areas, spinal cords and brains of rats made diabetic with STZ. Results are shown as the mean ± SEM for n = 6. Differences were significantly different from Control at $^{**}p < 0.01$ and $^{***}p < 0.001$; and from STZ at $^{++}p < 0.01$ and $^{+++}p < 0.001$

(−61%) and SOD (−61%) activities ($p < 0.001$ in all instances vs. corresponding control values).

Treating the rats with a sulfur-containing compound prior to a dose of STZ led to a clear protection in the brain against the changes of markers of oxidative stress induced by STZ, with HTAU usually appearing as the most potent and TAU as the weakest (Figs. 1, 2 and 3). Thus, AMSA (no change) and HTAU (−7%) were more protective than TAU (−21%, $p < 0.05$) against the formation of MDA, although the difference was not statistically significant (Fig. 1). Similarly, AMSA and HYTAU were usually more protective than TAU in preventing the increases of NO (−7% with AMSA, −9% with HTAU, −10% with TAU) and GSSG (−15% with HTAU, +24% with AMSA, +75% with TAU) levels; and the decreases of GSH (−21% with

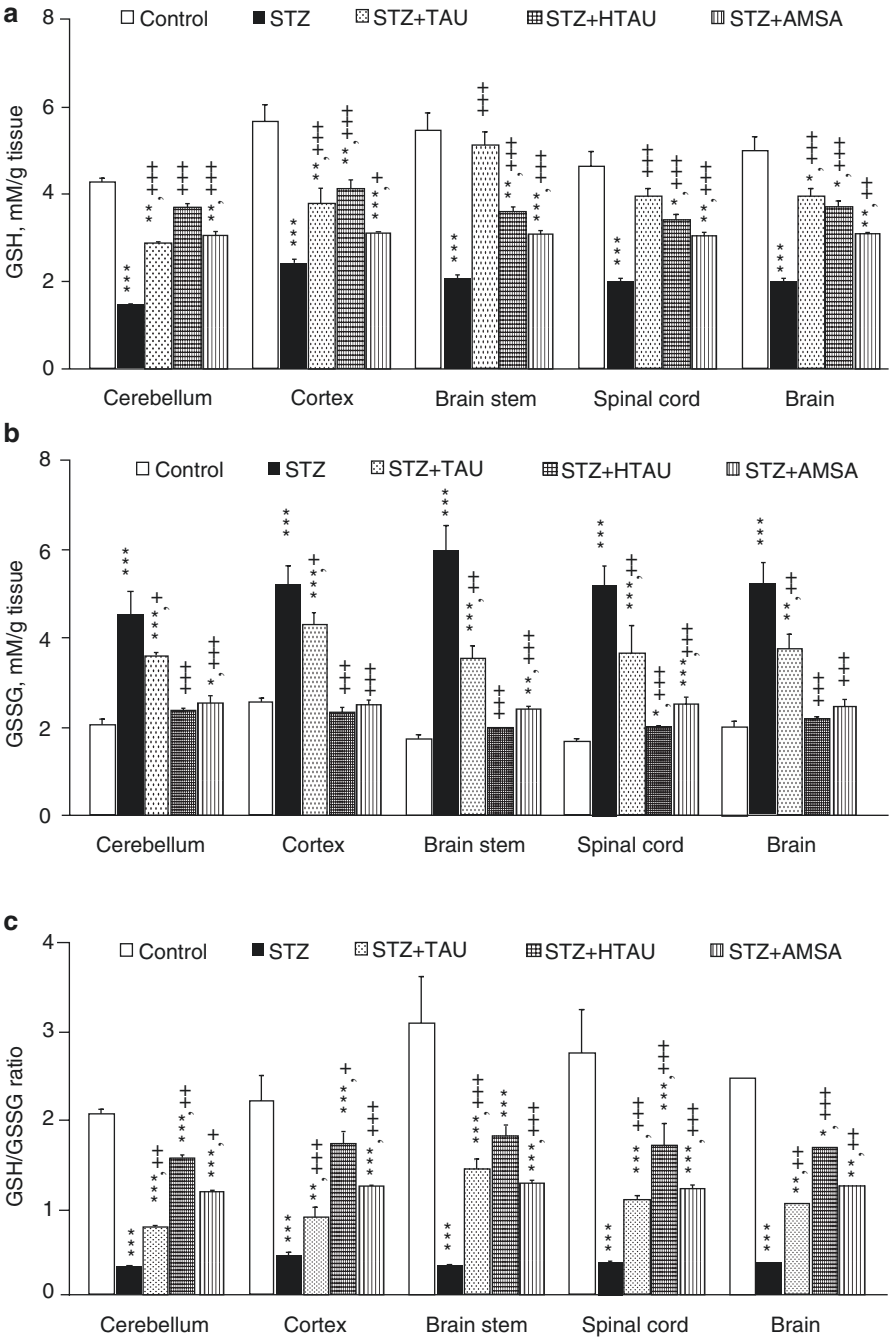

Fig. 2 The effects of AMSA, HTAU and TAU on (**a**) GSH and (**b**) GSSG levels and on (**c**) the GSH/GSSG ratio in brain areas, spinal cords and brains of rats made diabetic with STZ. Results are shown as the mean ± SEM for n = 6. Differences were significantly different from Control at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; and from STZ at $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$

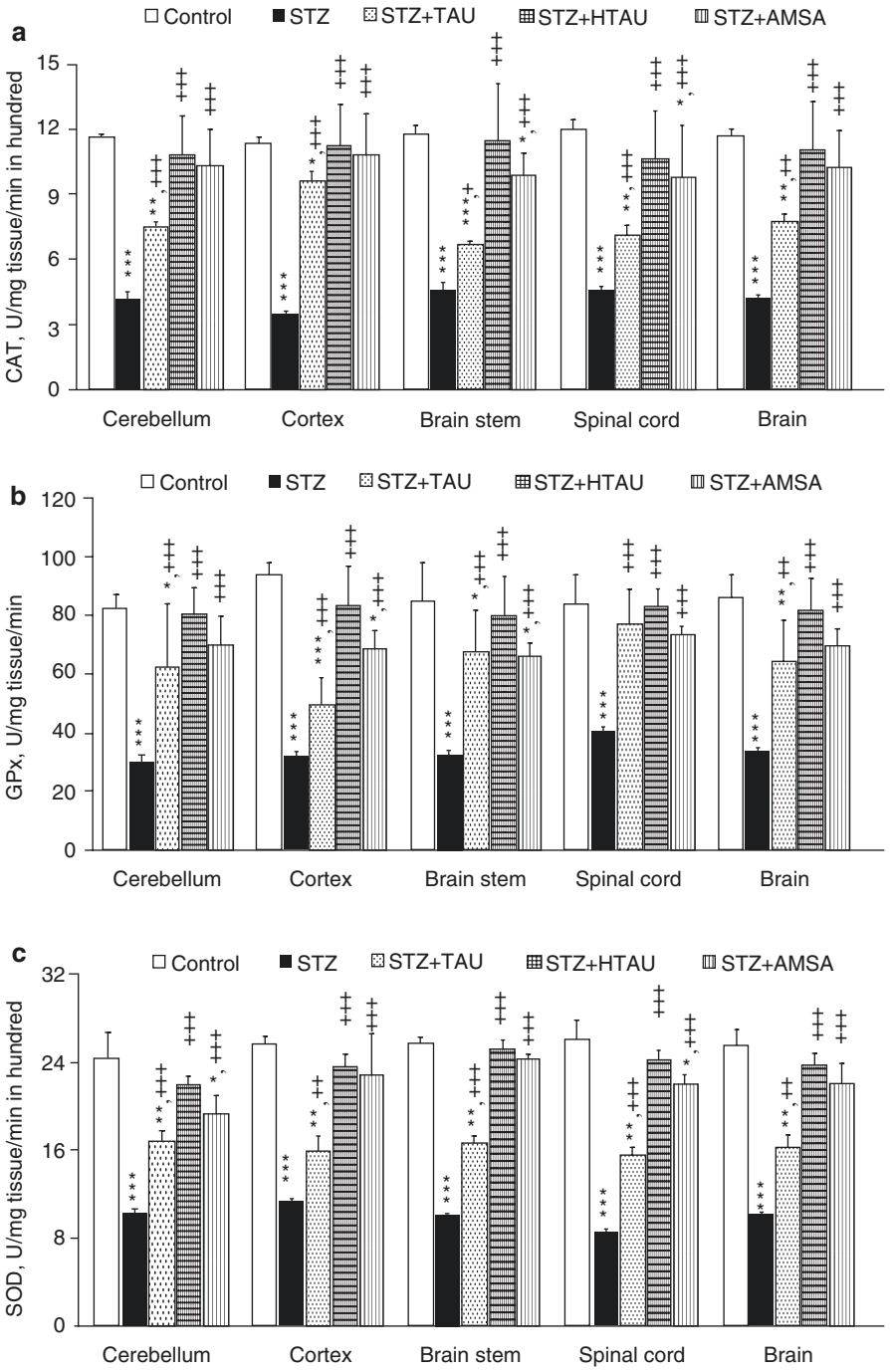

Fig. 3 The effects of AMSA, HTAU and TAU on the activities of (**a**) CAT, (**b**) GPx and (**c**) SOD in brain areas, spinal cord and brains of rats made diabetic with STZ. Results are shown as the mean ± SEM for n = 6. Differences were significantly different from Control at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; and from STZ at $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$

TAU, −26% with HTAU, −39% with AMSA) and GSH/GSSG ratio (−33% with HTAU, −51% with AMSA, −59% with TAU) and activities of CAT (−5% with HTAU, −13% with AMSA, −36% with TAU), GPx (−6% with HTAU, −19% with AMSA, −36% with TAU), and SOD (−7% with HTAU, −9% with AMSA. −37% with TAU).

By analogy to changes in the brain, STZ also drastically altered the indices of oxidative stress in the spinal cord (Figs. 1, 2 and 3). In addition to massive rises in the MDA (+160%), NO (+229%) and GSSG (+210%) values (Figs. 1a, b and 2b, respectively), there were also large decreases of GSH (−57%), the GSH/GSSG ratio (−85%) (Fig. 2a, c, respectively), and the activities of CAT (−62%), GPx (−52%) and SOD (−68%) (Fig. 3a–c, respectively), with the differences from corresponding control values being, in all instances, significant ($p < 0.001$).

In common with the results seen in the brain, all the treatment agents were also found to protect the spinal cord against STZ-associated oxidative stress, with the pattern of protection generally paralleling that seen in the brain (Figs. 1, 2 and 3). In this case, AMSA (−9%) and HTAU (−14%) were more protective than TAU (−34%, $p < 0.01$ vs. control) in preventing the formation of MDA (−9%, −14%, −34%, $p < 0.01$, respectively, Fig. 1a) and of NO (−7%, −9%, −36% $p < 0.01$, respectively, Fig. 1b) induced by STZ; but TAU was more protective than their analogs in preventing the accompanying decrease of GSH (−14% with TAU, −26% with HTAU, −34% with AMSA, Fig. 2a) and GSH/GSSG ratio (−27% with TAU, −33% with HTAU, −52% with AMSA) (Fig. 2c). While all three compounds provided an equivalent protection against the loss of GPx activity caused by STZ (−1 to −12%), HTAU and AMSA, in that order, were more protective against the loss of CAT (−12% and −18%, respectively) and SOD (−7 and −16%) than TAU (−41% in both instances, $p < 0.01$ vs. control values) (Fig. 3).

3.2 Brain Areas

To determine the extent of oxidative stress caused by STZ in different areas of the brain, the cerebellum, cerebral cortex and brainstem were isolated and analyzed for indices of oxidative stress.

In general. STZ caused an increase in MDA in all brain areas examined, being greatest in the cortex (+202%) than in the cerebellum (+167%) and brain stem (+147%, with the levels being significantly greater than corresponding control values (all at $p < 0.001$) (Fig. 1a). While generally all three treatment agents reduced MDA formation to insignificant concentrations in the cerebellum and brain stem (+6 to +15%), TAU (+2%) appeared more potent than either HTAU (+30, $p < 0.05$) or AMSA (+40%, $p < 0.01$) in the cortex (Fig. 1a).

STZ increased the NO levels in all brain areas examined ($p < 0.001$ vs. corresponding control values), being greater in the cortex (+313%) than in the cortex (+68%) and brain stem (+143%) (Fig. 1b). TAU was also more potent than either HTAU or AMSA in preventing the increase in NO caused by STZ in the cerebellum (−32%, $p < 0.01$, −10% and −2%, respectively), cortex (−14%, +42%, $p < 0.01$, and +55%, $p < 0.001$, respectively) and brain stem (−70%, $p < 0.001$, −28%,

$p < 0.05$, and −31%, $p < 0.01$, respectively) when compared to corresponding control values (Fig. 1b).

A treatment with the diabetogen STZ lowered the levels of GSH in the three brain areas examined, being slightly greater in the cerebellum (−66%) and brain stem (−62%) than in the cortex (−57%). At the same time, the GSSG levels increased to a significant extent ($p < 0.001$ vs. controls) in all brain areas examined, being greater in the brain stem (+241%) than in the cerebellum (+120%) or cortex (+103%). A pretreatment with a sulfur-containing compound led to a significant protection against these changes, with the potency varying according to the brain area examined. For example, while TAU attenuated the decrease in GSH in the brain stem (only −6%) to a greater extent than either AMSA (−23%, $p < 0.05$) or HTAU (−34%, $p < 0.01$) compared to corresponding control values, HTAU appeared more protective in the cerebellum (−14%) and cortex (−27%) than TAU (−33% in both areas, $p < 0.01$) or AMSA (−29%, $p < 0.05$ and −46%, $p < 0.001$, respectively) (Fig. 2a). On the other hand, HTAU was found insignificantly more potent than AMSA in preventing the accumulation of GSSG in the cerebellum (−15% vs. −24%, $p < 0.05$), cortex (−7% vs. 13%) and brain stem (−13% vs. +37%, $p < 0.01$) as compared to control values. TAU was also highly protective but not to the same extent as HTAU or AMSA (+75%, +69% and +102%, respectively, all at $p < 0.001$ vs. control values) (Fig. 2b). Based on these results, it was determined that HTAU was more potent than AMSA and TAU, in that order, in preventing the decrease of the corresponding GSH/GSSG ratios in the various brain areas examined (Fig. 2c).

In general, STZ lowered the activities of the antioxidant enzymes CAT, GPx and SOD to a significant ($p < 0.001$) extent in the three brain areas studied. In the case of CAT the activity was lower in the cortex (−69%) than in either the cerebellum (−64%) or brain stem −61%). Similarly, the GPx activity was narrowly lower in the cortex (−66%) than in the cerebellum (−64%) and brain stem −62%); and the SOD activity was lower in the brain stem (−61%) than in the cerebellum (−59%) and cortex (−54%) (Fig. 3). In the case of the CAT activity, a pretreatment with HTAU $\leq -4\%$) usually resulted in a greater attenuation of the lowering effect of STZ than with AMSA (−4 to −15%), which in turn was more protective than TAU (−15 to −52%) in all brain areas examined (Fig. 3a). A similar trend of results was observed for both the GPx activity, which was preserved to a greatest extent by HTAU ($\leq$ −12%) than by either AMSA (−15 to −29%) or TAU (−20 to −48%) (Fig. 3b), and for the SOD activity, which was higher with HTAU (−2 to −10%) than with either AMSA (−6 to −19%) or TAU (−31 to −38%) (Fig. 3c).

4 Discussion

In diabetes, the brain is considered to be vulnerable to oxidative damage due to a high total oxygen consumption, a high content of peroxidizable membrane polyunsaturated fatty acids, an increased glycoxidation and lipoxidation of proteins, and a

reduction of enzymatic and nonenzymatic antioxidant defenses (Baynes and Thorpe 1999; Low et al. 1997; Yildrim and Kilic 2011).

In view of the damaging role of oxidative stress in the brain of diabetics, several compounds possessing antioxidant properties have been investigated as brain protectants (Al-Khadem et al. 2015; Kahya et al. 2015; Peker et al. 2010; Ulusu et al. 2003). In the present study, the sulfur-containing amino acid TAU was investigated for the ability to protect the brain and spinal cord against diabetes-induced central oxidative stress since it was previously found to reduce LPO and to enhance GSH levels in the brain of rats subjected to D-galactose-related stress (Aydın et al. 2016). Also, since previous work from this laboratory has shown that the antioxidant activity of TAU-like compounds is dependent on the chain length (Gossai and Lau-Cam 2009; Pokhrel and Lau-Cam 2000), the TAU homologs AMSA and HTAU were included in the present study. Furthermore, the effects of these compounds in at least three regions of the brain was considered of interest since there is at least one report indicating that variations in the extent of oxidative damage exist within the central nervous system (Yildrim and Kilic 2011).

The concentration of MDA, a product of free radical-mediated LPO, was found to be markedly increased in both the brain and spinal cord of diabetic rats, with the elevations being rather comparable. In contrast, within the brain higher levels of MDA were detected in the cortex than in the cerebellum and stem in that order. Without exceptions, a pretreatment with a sulfur-containing compound reduced the formation of MDA induced by diabetes to levels that were within or below the control values and with TAU providing an insignificantly higher effect than AMSA or TAU. An assessment of the individual effects of these compounds on MDA generated in different brain areas resulted in a potency patterns that generally agreed with those seen in the whole brain, namely that TAU was either more potent than (cortex, brain stem) or about equipotent with (cortex) HTAU and AMSA. In contrast to the diabetic MDA content, that of NO was clearly higher in the spinal cord than in the brain, an effect that was effectively attenuated and to similar extents by TAU, HTAU and AMSA. Within the brain, the cortex showed the greatest increase, followed by the brain stem and cerebellum. These results lend credence to the concept that TAU and structurally-related compounds can protect against MDA formation by preventing LPO of membrane phospholipids (Pasantes-Morales and Cruz 1985; Pasantes-Morales et al. 1985) although alternative explanations unrelated to a direct antiperoxidative action have also been suggested (Nakamura et al. 1993).

As demonstrated for the MDA values, those of NO were also drastically increased by diabetes, more in the spinal cord than in the brain. Furthermore, within the brain the increase in NO was found highest in the cortex, followed by the stem and cerebellum in that order. Although all the treatment compounds were able to inhibit NO formation to a significant extent, TAU appeared to be more potent than AMSA which, in turn, was marginally more potent than HTAU. In general, all three compounds were able to reduce the diabetic tissue NO levels to below control values in the cerebellum and brain stem. The increase in brain NO may have physiological implications in the development of cognitive deficit in diabetes, a situation that may be the result of an increased expression of inducible NO synthase triggered by

cytokines and which, in turn, leads to an excessive synthesis of RNS in general and of NO in particular (Küçükatay et al. 2009). Furthermore, an increase of NO may lead to LPO upon its interaction with the superoxide anion radical to yield the more potent oxidant peroxynitrite (Pacher and Szabo 2008), which can not only lower the stores of GSH but also disrupt membranes through the production of phospholipid hydroperoxides (Küçükatay et al. 2009).

The contents of GSH and GSSG were assayed in the brain and spinal cord of STZ-treated rat to serve as a measure of antioxidant status and were compared with corresponding values for nondiabetic rats. In the present study, the intracellular levels of GSH were decreased by more than 55% of the normal values both in the brain and spinal cord of STZ-treated rats. In diabetes this decrease has been related to a limited availability of the GSH precursors glycine and cysteine (Sekhar et al. 2011), but it may also be the result of a low activity of the enzyme GR, which converts GSSG back to GSH (Mukherjee et al. 1994). While HTAU and TAU were able to reduce the losses of brain GSH to a significant extent in all brain areas examined, AMSA was only significantly effective in the cerebellum and brain stem. In addition, although HTAU was the most potent and AMSA the least among the three test compounds, TAU was found to virtually normalize the GSH in the brain stem. In contrast to the decreased levels of GSH in the brain and spinal cord of STZ-treated rats, those of GSSG were greatly elevated, more in the spinal cord than in the brain, with the trend of the effects paralleling those found in the plasma of humans with type 2 DM (Calabrese et al. 2012). A pretreatment of STZ-treated rats with a sulfonate compound led to a clear reduction in GSSG formation in both parts of the central nervous system, with HMTAU providing a greater reducing effect than AMSA, and TAU providing the weakest effects. A similar trend of potencies was observed in each of the brain areas examined.

GSH is regarded as one of the most important scavengers of ROS and its ratio with GSSG has been used as a marker of oxidative stress (Zitka et al. 2012). Treating rats with STZ led in a profound decrease (~85%) of the GSH/GSSG ratio in the brain and spinal cord at 24 h post treatment. Administering TAU or a TAU analog prior to STZ led to a significant increase of the GSH/GSSG ratio in both the brain and spinal cord, with the effect being greater in rats receiving HTAU rather than AMSA or TAU. When comparing the GSH/GSSG ratios of the three brain areas of interest, the highest decrease occurred in the brain stem, followed by the cerebellum and cortex. Again, a pretreatment with HTAU was found to increase the redox ratio to the greatest extent, followed in potency by AMSA, and TAU showing the least effect.

CAT, GPx and SOD are three antioxidant enzymes that are capable of protecting cells against hydrogen peroxide, hydroperoxides and superoxide anion, respectively. In spite of the widespread variability of activities reported for these enzymes in the brain and other organs of diabetic humans and laboratory animals, the present findings are in agreement with those previously found by this laboratory in erythrocytes and plasma (Budhram et al. 2013) and by Pari and Latha (2004) in the brain of rats made diabetic with STZ, namely a marked (>50%) decrease of the three activities in both the brain and spinal cord. Furthermore, whereas in the brain

diabetes reduced the activities of these enzymes to about the same extent (61–64%), in the spinal cord they vary according to the particular enzyme, being greatest for SOD, intermediate for CAT, and least for GPx. Without exceptions, these losses were attenuated to a significant extent by all the sulfur-containing compounds, with HTAU being the most protective and TAU the least. Except for the GPx activity, which was raised to almost normal values by all three pretreatments, in the spinal cord HTAU again provided the greatest protection followed closely by AMSA, with TAU being much weaker. In general, the extent of the protection offered by each compound in each brain area paralleled that seen for the whole brain, with HTAU appearing somewhat more protective than AMSA, and TAU providing a significant although weaker protection.

5 Conclusions

The present study finds that TAU and its immediate homologs AMSA and HTAU can all protect the brain and the spinal cord against the oxidative stress induced in the brain by the diabetogenic agent STZ. Also, this study finds that, with a few exceptions, a correlation exists between the antioxidant potency and the carbon chain length, with the higher homolog HTAU generally providing a greater protection than the lower homolog AMSA, and with both compounds usually appearing more potent than the parent compound TAU.

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The Effect of Metformin and Taurine, Alone and in Combination, on the Oxidative Stress Caused by Diabetes in the Rat Brain

George J. Clark, Kashyap Pandya, and Cesar A. Lau-Cam

Abstract This study has compared the effects of metformin (MET) and taurine (TAU), singly and in combination, on the oxidative stress caused by diabetes in the rat brain. For this purpose, male Sprague-Dawley rats, 200–225 g in weight, assigned to groups of 6, were intraperitoneally (i.p.) treated with the diabetogen streptozotocin (STZ, 60 mg/kg, in citrate buffer pH 4.5) on day 1 and, after 14 days, orally (p.o.) with either MET, TAU or MET-TAU (each at 2.4 mM/kg, in water). Control rats received only citrate buffer pH 4.5 (2 mL) or only STZ on day 1 by the i.p. route. All the animals were sacrificed by decapitation on day 57 and their brains collected by the freeze clamp technique. Blood samples were placed in heparinized tubes and used for the assay of the plasma glucose (GLC) and blood insulin (INS) levels. Immediately thereafter, the brains were surgically removed and a portion was used to prepare a homogenate in 0.1 M PBS pH 7.4, which was used for the assay of indices of oxidative stress. Diabetes raised the plasma GLC level (+313%) but lowered that of the blood INS (−76%) compared to corresponding values from nondiabetic rats. In addition it raised the brain malondialdehyde level (+59%) but lowered the reduced/disulfide glutathione ratio (−46%), and activities of catalase (−43%), glutathione peroxidase (−48%), superoxide dismutase (−65%), glutathione reductase (−50%) and glutathione S-transferase (−51%) significantly (all at $p < 0.001$). Except for the greater decrease in GLC (+90% vs. +22%) and increase in INS (−26% vs. −52%) levels seen in rats receiving MET than in rats receiving TAU, these compounds protected the brain against oxidative stress to significant ($p \leq 0.05\%$) and rather similar extents. Furthermore, the concurrent administration of MET and TAU to the diabetic rats led to brain values of indices of oxidative stress that were lower than those attained with MET alone, although generally not to a statistically significant degree.

Keywords Diabetes • Brain • Oxidative stress • Metformin • Taurine

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Abbreviations

CAT	Catalase
GLC	Glucose
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Glutathione disulfide
GST	Glutathione S-transferase
INS	Insulin
MDA	Malondialdehyde
MET	Metformin
SOD	Superoxide dismutase
TAU	Taurine

1 Introduction

Oxidative stress is known to be at the center of the structural, neurophysiological and neuropsychological alterations associated with long-term effects of diabetes in the brain (Muriach et al. 2014). A common theory to account for the cerebral dysfunction of diabetes is a reduction of antioxidant defenses and a parallel increase in the production of free radical caused by the ensuing hyperglycemia, effects that may leave the brain vulnerable to the damaging effects of free radicals and, hence, to cellular damage and functional impairment (Baquer et al. 1990; Maritim et al. 2003).

When compared to other organs in the body, the brain is found to be particularly susceptible to the damaging effects of oxidative stress as a result of a high oxygen demand, a high rate of oxidative metabolism, an abundance of redox-active metals such as iron and copper, high levels of peroxidizable polyunsaturated fatty acids, and a relative paucity of antioxidant defenses (Wang et al. 2014; Wang and Michaelis 2010). As a result, an imbalance can develop between oxidative insults such as reactive oxygen species (ROS) and antioxidant defenses, especially antioxidant enzymes, a situation that, in turn, can favor the development of oxidative stress (Bonnefont-Rousselot 2002; Maritim et al. 2003). Specifically, in addition to peroxidative damage of brain lipids, proteins and nucleic acids (Vincent et al. 2004), there is a decrease in the activities of catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) and total antioxidant status, and an increase in glucose autoxidation, lipid peroxidation (LPO), nitrosative stress and nitric oxide (NO) formation (Acar et al. 2012; Mastrocola et al. 2005; Muriach et al. 2014). Moreover, the levels of natural antioxidants such as reduced glutathione (GSH) may be diminished as a result of an increase activity of the NADPH-requiring polyol pathway by a high glucose flux, a situation that depletes the availability of NADPH

needed for the enzymatic regeneration of GSH from its oxidized disulfide (GSSG) form (Giacco and Brownlee 2010).

From the results obtained from the brain of rats treated with the diabetogen streptozotocin (STZ) it has become evident that there is an increase in the values of malondialdehyde (MDA), ROS and NO, extent of LPO, and activity of glutathione reductase (GR), changes that are accompanied by a lower activity of brain catalase (CAT), glutathione peroxidase (GPx) and manganese SOD, an impairment of mitochondrial function, membrane potential and production of adenosine triphosphate, and a reduced ratio of GSH/GSSG relative to corresponding results from nondiabetic rats (Acar et al. 2012; Cardoso et al. 2013; Kumar and Menon 1993; Ortiz-Avila et al. 2015).

Previous studies from this laboratory have found that taurine (TAU), an endogenous and conditional β-amino acid, can protect against biochemical, functional and morphological changes caused by diabetes in the plasma, erythrocyte (Budhram et al. 2013) and kidney (Pandya et al. 2013) of rats previously treated with STZ; and that these benefits are also exerted against the oxidative stress that develops in the brain (Patel et al. 2016). Taking into account these results, the present study was specifically aimed at further investigating whether adding TAU to a treatment of diabetes with metformin (MET), a biguanide compound currently used as a first-line oral hypoglycemic for the treatment of diabetes, can lead to a greater protection of the brain than either compound alone against diabetes-related oxidative stress. To this effect, rats previously made diabetic with STZ were separately treated with MET, TAU and MET-STZ for 6 weeks and, in each case, indices of oxidative stress were measured in the brain.

2 Methods

2.1 Animals

Male Sprague-Dawley rats, 200–225 g in weight, acclimated for 2 weeks in a room maintained at a constant humidity and temperature (23 ± 1 °C) and a normal 12 h light—12 h dark cycle. The rats had free access to a commercial rodent diet (LabDiet® 5001, PMI Nutrition International, Brentwood, MO) and filtered tap water during the entire study.

2.2 Treatments and Samples

All the experimental groups consisted of 6 rats. Diabetes was induced with a single 60 mg/kg intraperitoneal (i.p.) dose of STZ in citrate buffer pH 4.5. Starting on day 15 and continuing for the next 41 days, separate groups of diabetic rats received a 2.4 mM/kg, daily dose of either MET, TAU or MET plus TAU in distilled water by

oral gavage. Nondiabetic (control) rats received only citrate buffer pH 4.5 (2 mL, i.p.) on day 1, and physiological saline (2 mL) by oral gavage from day 15 onwards. A diabetic group received no other treatment than one with STZ. All treatments were conducted for a total of 42 days. The animals were sacrificed by decapitation on day 56 and their blood samples collected without delay. A portion of blood sample was mixed with sodium heparin and processed for the plasma fraction The skulls were cut open with the help of a Friedman rongeur to expose the brains, which were immediately removed by the freeze-clamping technique of Wollenberger et al. (1960), immersed in liquid nitrogen, and stored at −80 °C until needed.

2.3 Plasma Glucose (GLC)

The GLC content was measured using a commercial colorimetric kit (Procedure No. 510 from Sigma-Aldrich, St. Louis, MO, USA). The results were expressed in mg/dL.

2.4 Blood Insulin (INS)

The concentration of INS was measured by means of a commercial assay kit (Insulin ELISA kit, Calbiotech Inc., Spring Valley, CA, USA). The results were expressed in μIU/mL.

2.5 Brain MDA

The concentration of MDA was measured as thiobarbituric acid reactive substances (TBARS) by the end point assay method of Buege and Aust (1978). The results were expressed in nM/g of tissue.

2.6 Brain GSH and GSSG

The brain levels of GSH and GSSG were measured fluorometrically by the method of Hissin and Hilf (1976), which is based on the reaction of GSH with *ortho*-phthalaldehyde (OPT) at pH 8.0 and of GSSG with OPT at pH 12.0. Prior to the measurement of GSSG, any interfering GSH was complexed with N-ethylmaleimide according to the method of Güntherberg and Rost (1966) to prevent its interfering effect on the measurement of GSSG. The concentrations of GSH and GSSG were expressed as μM/g of tissue.

2.7 Brain CAT, GPx and SOD

The CAT activity was measured according to Aebi (1984), which is based on the ability of this enzyme to degrade hydrogen peroxide (H_2O_2) to water and oxygen. The result was expressed as U/mg of protein.

The GPx activity was measured by the method of Günzler and Flohé (1985), which is based on the conversion of GSH to GSSH in the presence of an excess of NADPH and GR. The result was expressed as U/mg of protein.

The SOD activity was measured using the method of Misra (1985), which is based on the inhibitory action of this enzyme on the autoxidation of epinephrine by the superoxide radical to a pink adrenochrome. The result was expressed as U/mg of protein.

2.8 Brain GR and GST

The GR activity was measured as described by Delides et al. (1976), based on the ability of this enzyme to convert GSSG to GSH in the presence of NADPH. The decrease in absorbance at 340 nm reflected the formation of $NADP^+$ from NADPH. The GR activity was calculated using an extinction coefficient of 6.22 mM^{-1}, and was reported as μM of NADPH consumed/mg of protein.

The GST activity was measured according to the method of Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The rate of formation of CDNB-GSH conjugate, reflecting the GST activity, was followed for 5 min at 340 nm. The GST activity was calculated using an extinction coefficient of 9.6 mM^{-1}, and was reported as μM of CDNB-GSH conjugate formed/min/mg of protein.

2.9 Brain Protein

The protein content of the brain sample was measured by the Lowry method (Lowry et al. 1951), using a bovine serum albumin calibration curve covering the concentration range $1.95 \times 10^{-3} - 2 \times 10^{3}$ μM. The results were expressed in μg/mL of sample.

2.10 Statistical Analysis of Data

Experimental results are presented as mean ± SEM for n = 6. Statistical comparisons were against both normal rats and untreated diabetic rats, and were made by unpaired Student's t-test with the help of a commercial software system (JMP® 7, JMP® Statistical Discovery Software, Cary, NC, USA). Differences were considered to be statistically significant at $p \leq 0.05$.

3 Results

3.1 Effects on Circulating GLC and INS Levels

The development of type 2 diabetes was ascertained from the circulating levels of both GLC and INS shown in Table 1. Diabetic animals showed much higher levels of plasma GLC than nondiabetic ones by the end of 8 weeks (+313%, $p < 0.001$). A daily treatment of the diabetic rats with MET reduced this increase markedly (+90%, $p < 0.001$), an effect that was ~2.5-fold greater than one with TAU (+222%, $p < 0.001$). Treating the diabetic rats with MET plus TAU led only to a small increase in potency relative to MET alone (+84%, $p < 0.001$). In terms of the plasma INS, it was determined that at the end of 8 weeks diabetes had reduced the level significantly (−76%, $p < 0.001$ vs. control). This effect was significantly antagonized by both MET and TAU, with the former compound appearing twice as potent (−26%, $p < 0.01$) than the latter (−52%, $p < 0.001$). On the other hand, a treatment with MET-TAU further reduced the inhibitory action of diabetes on INS secretion (−18%, $p < 0.05$) although the effect was not significantly different from that attained with MET alone. Neither TAU or MET were found to significantly affect the basal circulating levels of both GLC and INS.

3.2 Effects on Brain MDA, GSH and GSSG Levels, and GSH/SSG Ratio

As shown in Fig. 1, the brain of diabetic rats contained higher levels of MDA (+59%, $p < 0.001$) than the brain from normal rats. This increase was reduced by about one-half by a daily treatment with either MET (+29%, $p < 0.01$) or TAU (+28%, $p < 0.01$); and still further when the two compounds were given concurrently (−10%). Neither MET nor TAU were found to raise the basal levels of MDA to a significant extent (≤5%).

Table 1 The effects of MET, TAU and MET-TAU on the plasma GLC and blood INS levels of rats made diabetic with STZ[a,b]

Group	Plasma GLC, mg/dL	Blood INS, μIU/mL
Control	$103.59 \pm 5.06^{+++}$	$44.08 \pm 2.46^{+++}$
STZ	$428.08 \pm 21.77^{***}$	$10.58 \pm 1.33^{***}$
MET	$101.54 \pm 5.57^{+++}$	$44.31 \pm 1.71^{+++}$
TAU	$102.05 \pm 3.33^{+++}$	$42.94 \pm 2.37^{+++}$
MET-STZ	$196.41 \pm 5.00^{***,+++}$	$32.50 \pm 2.35^{**,+++}$
TAU-STZ	$333.37 \pm 6.60^{***,++}$	$20.94 \pm 1.83^{***,+++}$
MET-TAU-STZ	$190.43 \pm 2.22^{***,+++}$	$36.02 \pm 1.87^{*,+++}$

[a]Values are shown as the mean ± SEM for n = 6

[b]Statistical comparisons were significantly different from Control at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; and from STZ at $^{++}p < 0.01$ and $^{+++}p < 0.001$

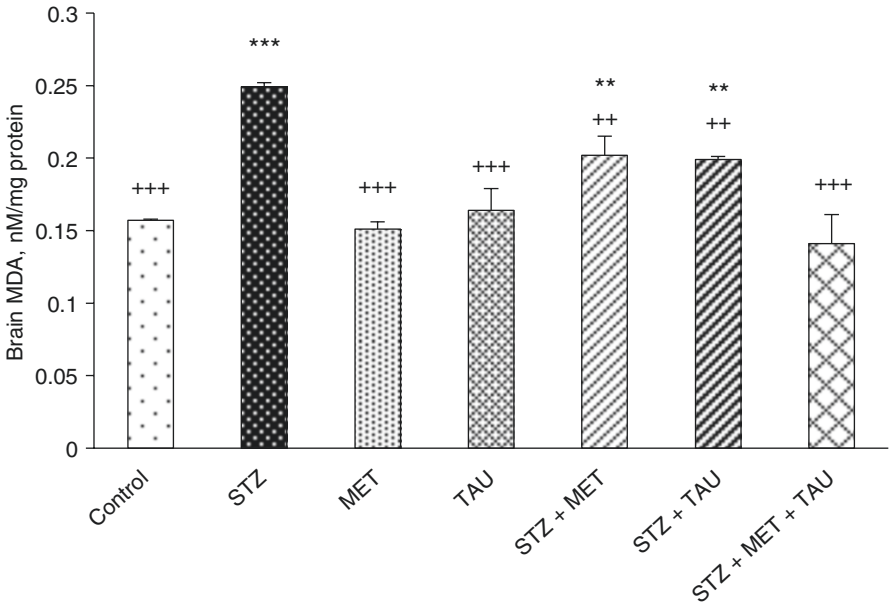

Fig. 1 The effects of a 6 weeks treatment with MET, TAU and MET-TAU on the brain MDA levels of rats made diabetic with STZ. Values are shown as the mean ± SEM for n = 6. Statistical comparisons were significantly different from Control at $^{**}p < 0.01$ and $^{***}p < 0.001$; and from STZ at $^{++}p < 0.01$ and $^{+++}p < 0.001$

From the results presented in Fig. 2, it is apparent that diabetes lowered the brain levels of GSH (−79%, $p < 0.001$) and GSSG (−62%, $p < 0.001$) to a significant extent ($p < 0.001$ with both vs. control values). Both MET (−25%, $p < 0.01$) and TAU (−28%, $p < 0.01$) were able to reduce the loss of GSH to about the same extent, an effect that was somewhat enhanced (−18%, $p < 0.05$) when these compounds were administered alongside. A similar trend of effects was observed in terms of the reduction of the GSSG levels by diabetes, with MET (−22%, $p < 0.01$) appearing slightly less effective than TAU (−18%, $p < 0.05$), and about equipotent when provided together with TAU (−25%, $p < 0.01$). On the other hand, neither compound affected the basal levels of GSSG. From these results, it was determined that diabetes lowered the GSH/GSSG ratio by 46% ($p < 0.001$ vs. control), that both MET and TAU were able to nullify this effect, and that a combined treatment with these compounds was able to reverse the effect (+9%) (Fig. 3).

3.3 Effects on Brain GR and GST Activities

The brain activity of GR was markedly decreased in diabetic rats (−50%, $p < 0.001$) relative to that in normal rats (Fig. 4). This effect was significantly attenuated by either MET (−27%, $p < 0.01$) or TAU (−29%, $p < 0.01$) and also by their

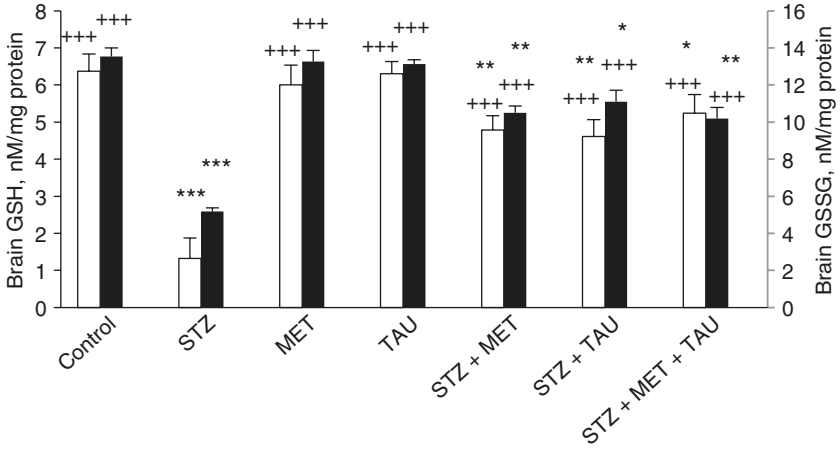

Fig. 2 The effects of a 6 weeks treatment with MET, TAU and MET-TAU on the brain GSH (*open square*) and GSSG (*filled square*) levels of rats made diabetic with STZ. Values are shown as the mean ± SEM for n = 6. Statistical comparisons were significantly different from Control at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; and from STZ at $^{+++}p < 0.001$

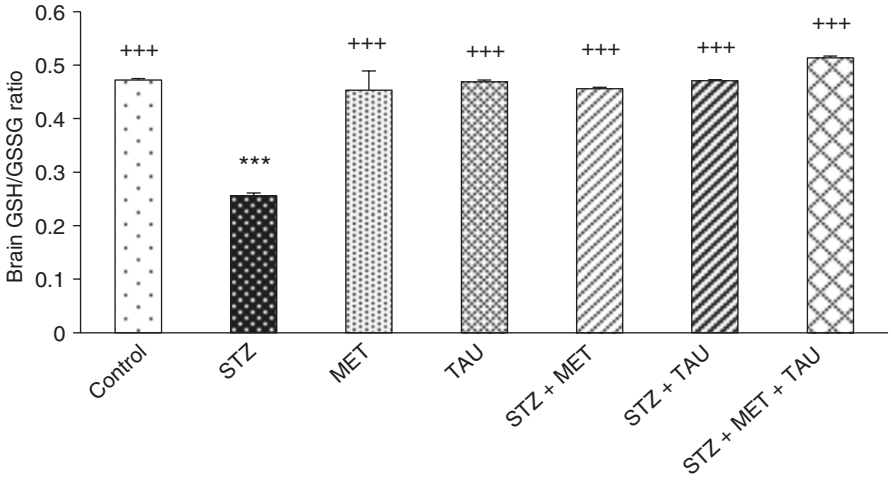

Fig. 3 The effects of a 6 weeks treatment with MET, TAU and MET-TAU on the brain GSH/GSSG ratio of rats made diabetic with STZ. Values are shown as the mean ± SEM for n = 6. Statistical comparisons were significantly different from Control at $^{***}p < 0.001$ and from STZ at $^{+++}p < 0.001$

combination (−24%, $p < 0.01$) (Fig. 4). Similarly, diabetes also reduced the brain activity of GST (−51%, $p < 0.001$), and treatments with MET (−38%, $p < 0.001$), TAU (−33%, $p < 0.001$) or MET-TAU (−30%, $p < 0.01$) were about equipotent in lowering the diabetes-induced decreases (Fig. 4). Moreover, neither MET nor TAU affected the activities of GR and GST in the brain of normal rats.

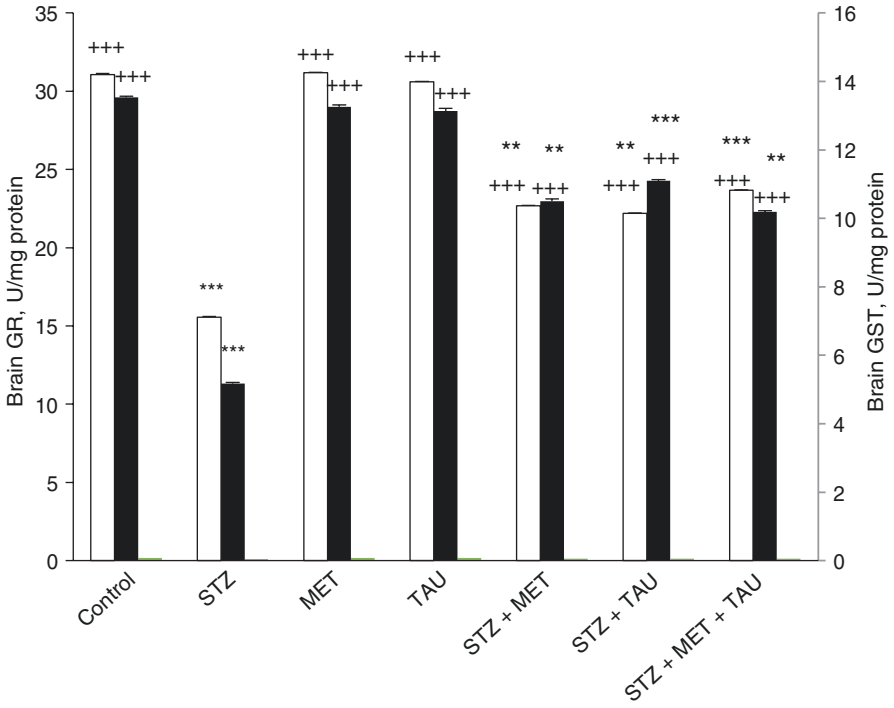

Fig. 4 The effects of a 6 weeks treatment with MET, TAU and MET-TAU on the brain GR (*open square*) and GST (*filled square*) activities of rats made diabetic with STZ. Values are shown as the mean ± SEM for n = 6. Statistical comparisons were significantly different from Control at $^{**}p < 0.01$ and $^{***}p < 0.001$; and from STZ at $^{+++}p < 0.001$

3.4 Effects on Brain CAT, GPx and SOD Activities

In diabetic rats the activities of CAT (−43%), GPx (−48%) and SOD (−65%) were markedly lower than corresponding activities for normal rats (all at $p < 0.001$) (Figs. 5, 6 and 7, respectively). A daily treatment of the diabetic rats with MET was found to effectively counteract these changes, with the effect being about equal on all three enzymes (−25%, −24% and −24%, respectively, all at $p < 0.01$ vs. control values). An identical treatment with TAU was about equipotent with MET in attenuating the changes of the CAT (−26%, $p < 0.01$, Fig. 5) and GPx (−18%, $p < 0.05$, Fig. 6) activities caused by diabetes but was slightly less potent on the change in SOD activity (−33%, $p < 0.01$, Fig. 7). However, a combined treatment of the diabetic rats with MET plus TAU led to a greater attenuation of the enzymatic changes than either compound alone (−13%, −12% and −18%, respectively, $p < 0.05$, Figs. 5, 6 and 7, respectively). Neither MET nor TAU affected the brain activities of these enzymes in normal rats to a significant extent.

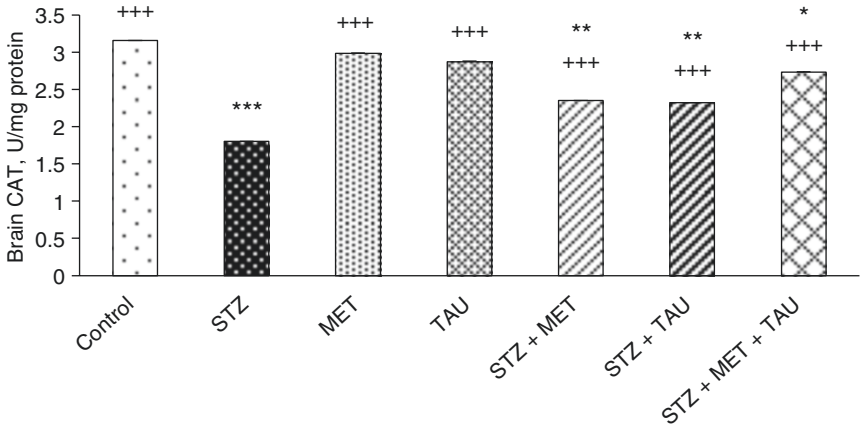

Fig. 5 The effects of a 6 weeks treatment with MET, TAU and MET-TAU on the brain CAT activity of rats made diabetic with STZ. Values are shown as the mean ± SEM for n = 6. Statistical comparisons were significantly different from Control at $^{*}p < 0.05$, $^{**}p < 001$ and $^{***}p < 0.001$; and from STZ at $^{+++}p < 0.001$

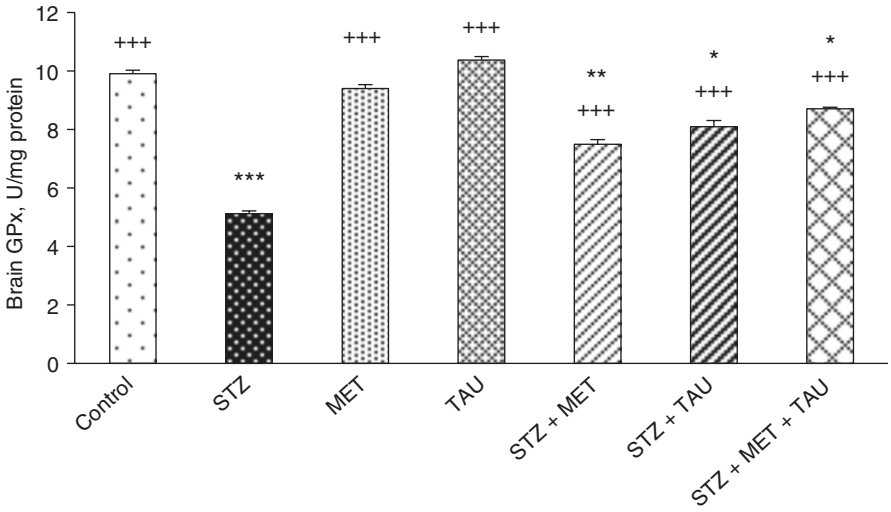

Fig. 6 The effects of a 6 weeks treatment with MET, TAU and MET-TAU on the brain GPx activity of rats made diabetic with STZ. Values are shown as the mean ± SEM for n = 6. Statistical comparisons were significantly different from Control at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; and from STZ at $^{+++}p < 0.001$

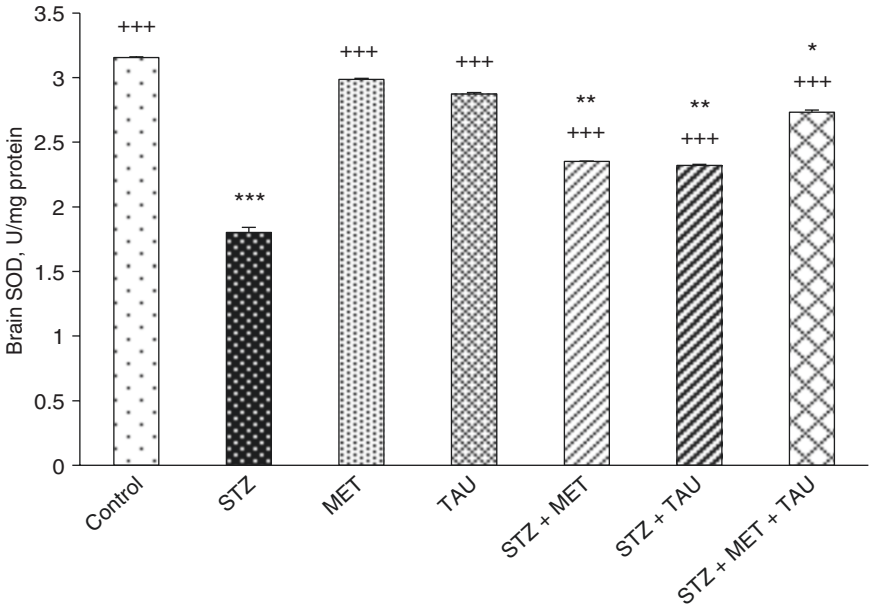

Fig. 7 The effects of a 6 weeks treatment with MET, TAU and MET-TAU on the brain SOD activity of rats made diabetic with STZ. Values are shown as the mean ± SEM for n = 6. Statistical comparisons were significantly different from Control at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; and from STZ at $^{+++}p < 0.001$

4 Discussion

Based on the results of earlier work on TAU, this conditionally essential β-amino acid has emerged as an attractive candidate for use as a protection against the biochemical and physiological alterations induced by diabetes in mammalian tissues. In addition to a lowering effect on the blood GLC with (Kaplan et al. 2004) or without (Kulakowski and Maturo 1984) an apparent effect on INS secretion, this compound has been reported to increase cell sensitivity to INS and the response of pancreatic β-cells to hyperglycemia (Ribeiro et al. 2009), to attenuate oxidative stress and accompanying INS resistance (Haber et al. 2003), and to inhibit protein glycosylation and formation of advanced glycation end products (Hansen 2001). On the other hand, there is at least one report indicating that TAU does not influence either the blood GLC or the secretion of or sensitivity to INS in humans (Brøns et al. 2004); and another one indicating that the hypoglycemic action of TAU in the rat is only observable in older but not in younger animals (Odetti et al. 2003).

In the particular case of the brain, there is ample evidence to suggest that exogenous TAU can cross the blood brain barrier and enter the brain, where it can protect against oxidative stress in spite of lacking structural features that will allow it to directly interact with H_2O_2, the superoxide radical or the hydroxyl radical (Aruoma et al. 1988). For example, a study by Gürer et al. (2001) found that rats receiving

TAU as part of the drinking water were protected against the decrease of GSH and increase of MDA in the brain of rats fed lead acetate. Furthermore, Mahalakshmi et al. (2003) showed that the consumption of TAU by rats as part of the drinking water was able to counteract the oxidative stress induced by acetonitrile in the brain by reducing LPO, and by enhancing the activities of CAT, GPx, SOD and GST and the concentrations of the antioxidant vitamins C and E. Also, while Yildirim and Kilic (2011) verified that the dosing of young rats with TAU (200 mg/kg) on a daily basis for 7 days protected the cerebellum against the formation of MDA and depletion of GSH brought about by aging, Aydin et al. (2016) reported that the addition of TAU to an animal diet was able to diminish oxidative stress, determined on the basis of MDA, protein carbonyl and GSH levels, and of SOD, GPx, GST activities and apoptosis in the brain of rats subchronically challenged with a daily subcutaneous dose of D-galactose.

This laboratory has verified that in the brain and spinal cord of rats made diabetic with STZ, TAU is able to reverse the increases in MDA and NO concentrations and to effectively protect against the decrease in GSH/GSSG ratio and the losses of CAT, GPx and SOD activities (Patel et al. 2016; Patel and Lau-Cam 2009). On the other hand, Agca et al. (2014) have indicated that TAU was able to partially reduce the serum MDA concentrations and to ameliorate diabetic neuropathy by regulating NF-κB and Nrf2/HO-1 signaling cascades but without altering body weight or blood GLC in diabetic rats, findings that led to the conclusion that in the rat brain TAU reduces oxidative stress through activation of antioxidant defense signaling pathways (Agca et al. 2014).

MET is an established hypoglycemic agent which is not only recommended for intensive glycemic control in type 2 DM but also to achieve improvement in endothelial dysfunction, hemostasis, oxidative stress, INS resistance, lipid profiles, and fat redistribution associated with this disease (Rojas and Gomes 2013). In addition, it can play a role in decreasing the risk of diabetes-related end points in overweight diabetic patients (UK Prospective Diabetes Study Group 1998). Furthermore, a study by Chakraborty et al. (2011), looking at the effects of MET on oxidative stress, nitrosative stress and biomarkers of inflammation in type 2 DM patients, found that this biguanide was able to lower ROS generation, advanced glycation products and pentosidine formation, to enhance total thiol and NO levels, and to restore C reactive protein in the plasma when compared to placebo. The role of MET in modulating oxidative stress has been examined by Esteghamati et al. (2013) in naive human patients with newly diagnosed type 2 diabetes and its effects compared with those of lifestyle modification. After a 3 months treatment, there was a significant reduction in the values of advanced oxidation protein products (AOPP) and advanced glycation end products (AGE), a significant increase in ferritin reducing ability of plasma (FRAP) and paraoxonase activity, and no changes in the value of lecithin-cholesterol acyltransferase, with the AOPP, FRAP and AGE levels changing more significantly with MET than with lifestyle modification alone. In the particular case of the brain, a 4 weeks treatment of Goto-Kakizaki rats, a model of type 2 DM, with TAU was shown to protect against the oxidative imbalance fostered by the diabetic state and manifested by higher levels of MDA, higher activities of

GP and GR but lower of MnSOD relative to corresponding values for nondiabetic Wistar rats (Correia et al. 2008).

Taking into account the reported effects of both TAU and MET on the hyperglycemia, hypoinsulinemia and brain oxidative stress brought about by diabetes, this study was aimed at investigating whether a combined treatment with these two compounds could lead to a greater protection that was possible with MET alone. Thus, diabetic rats on MET showed a greater reduction in circulating GLC levels, an effect that was ~2.5-fold greater than a treatment with TAU. On the other hand, a combined treatment with MET plus TAU enhanced the effect of MET although not to a statistically significant extent. Interestingly, the magnitude of the decrease in circulating GLC by TAU observed in the present study is of the same magnitude as that reported by Winiarska et al. (2009), and has been related, at least in part, to a decreasing effect of this amino acid on gluconeogenesis.

Similarly, both MET and TAU were able to attenuate the decrease of the plasma INS secretion caused by diabetes, with the antagonizing effect of the former being twice as much as that by the latter. In contrast, treating the diabetic rats with MET-TAU led to a smaller decrease in blood INS compared to MET alone. The present results clearly show that while MET shows a strong hypoglycemic effect as a result of positive effect on INS secretion, TAU is a much weaker hypoglycemic and stimulant of INS secretion. Consequently, TAU is found to have enhance the hypoglycemic action of MET to only a limited extent. This effect contrasts markedly with the results of a study by Kaplan et al. (2004) that found TAU to significantly decrease the blood GLC level based on the increase in circulating C-peptide level, an indicator of pancreatic INS secretion. On the other hand, it agrees with the findings of Brøns et al. (2004) in type 2 human diabetics indicating that TAU has no effect on either INS secretion or INS sensitivity. Interestingly, most studies in rodents, including one in which Otsuka Long Evans Tokushima fatty diabetic rats were fed fructose along with a diet supplemented with TAU, have shown both decreased hyperglycemia and INS resistance (Brøns et al. 2004).

MDA is a characteristic product of LPO largely produced *in vivo* through the oxidation of complex lipids, especially long-chain polyunsaturated fatty acids of cellular phospholipids, by oxygen-derived free radicals such as the hydroxyl radical (Slatter et al. 2000). In this case, LPO of cellular structures as a result of free radical activity is thought to play an important role in late complications of diabetes (Kesavulu et al. 2001, 2002). In the present study, diabetic rats showed a very high level of brain MDA at the end of 8 weeks, an effect that was reduced by one-half by both MET and TAU, and to about one-sixth by MET plus TAU.

Furthermore, the marked decrease of the accompanying brain GSH levels (~80%) by diabetes was attenuated to about one-third the control value by either MET or TAU, and to only one-fourth by MET plus TAU. In agreement with the present results, a study by Furfaro et al. (2012) found that supplementation of the drinking water of STZ-treated rats with TAU led after 6 months to attenuation of the loss of hepatic GSH and of the increase in GSSG/GSH ratio, possibly as a result of a more efficient GSH synthesis and decreased GSH loss and degradation. A further consequence of the increase in brain GSH by MET, TAU or their combination

may be responsible for the significant increase in the activity of GR, the enzyme that mediates the regeneration of GSH from its disulfide form GSSG. While these compounds almost double the brain GR activity of diabetic rats, a combined treatment did not increase the activity further over that seen with the individual treatments. However, as expected, the increase of the GSH levels by either MET or TAU was accompanied by an increase of the activity of the selenium-dependent GPx, viewed as a protection against lipid peroxide and H_2O_2 formation (Arthur 2000) as well as from hydroxyl radicals formed via iron-catalyzed Fenton-type reactions from H_2O_2 by reducing this oxidant to water (Barlow-Walden et al. 1995). In addition, while diabetes lowered the activity of GST, an enzyme conjugating GSH with a variety of electrophile and demonstrating GSH peroxidase activity (Gronwald and Plaisance 1998), by one-half of the control activity, MET, and to a slightly greater extent, TAU, were able to reduce the loss by at least 25%, an effect that was further increased when MET and TAU were given together. A previous study by Lim et al. (1998) using KK mice, a spontaneous hyperglycemic animal mode of type 2 diabetes, found that TAU was able to increase the hepatic activity of GPx but without effecting that of GST.

Furthermore, the present study found that both MET and TAU were about equipotent in almost doubling the activity of the brain GR, the enzyme that mediates the regeneration of GSH from its disulfide form, in diabetic rats, with a combined treatment providing an insignificantly better effect than either compound alone. A similar result for TAU was obtained by Winiarska et al. (2009) in the kidney of alloxan-treated rabbits consuming TAU as part of the drinking water. In general, it would appear that the effects of TAU on the activities of GR and GPx are dependent on the site examined, with the activity of the former showing a greater variability than that of the latter (Anand et al. 2011).

The activities of both CAT and SOD were markedly decreased in the brain of diabetic rats, with the decrease being greater for CAT than for SOD. A treatment with either MET or TAU led to about a one-half reduction of the decrease, an effect that was further enhanced when the two compounds were given together. A similar trend was noted in this laboratory in the brain of STZ-treated rats at 24 h after they had received an i.p. dose of TAU (Patel and Lau-Cam 2009; Patel et al. 2016). On the other hand, a reduction in these two enzyme activities in type 2 DM has been previously reported for the serum (Obi et al. 2016) and, to a lesser extent, brain (Aluwong et al. 2016) of alloxan-treated rats.

5 Conclusion

The present results indicate that although MET and TAU may differ in their intrinsic effects on the circulating GLC and INS levels of rats made diabetic with STZ, they, however, share similar effects on the accompanying oxidative stress that develops in the brain. More importantly, adding TAU to a treatment with MET will enhance the attenuating actions of MET on diabetes-induced oxidative stress in the brain.

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Investigation of the Role of a Supplementation with Taurine on the Effects of Hypoglycemic-Hypotensive Therapy Against Diabetes-Induced Nephrotoxicity in Rats

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Abstract This study has examined the role of supplementing a treatment of diabetic rats with captopril (CAP), metformin (MET) or CAP-MET with the antioxidant amino acid taurine (TAU) on biochemical indices of diabetes-induced metabolic changes, oxidative stress and nephropathy. To this end, groups of 6 male Sprague-Dawley rats (250–375 g) were made diabetic with a single, 60 mg/kg, intraperitoneal dose of streptozotocin (STZ) in 10 mM citrate buffer pH 4.5 and, after 14 days, treated daily for up to 42 days with either a single oral dose of CAP (0.15 mM/kg), MET (2.4 mM/kg) or TAU (2.4 mM/kg), or with a binary or tertiary combination of these agents. Rats receiving only 10 mM citrate buffer pH 4.5 or only STZ served as negative and positive controls, respectively. All rats were sacrificed by decapitation on day 57 and their blood and kidneys collected. In addition, a 24 h urine sample was collected starting on day 56. Compared to normal rats, untreated diabetic ones exhibited frank hyperglycemia (+313%), hypoinsulinemia (−76%) and elevation of the glycated hemoglobin value (HbA_{1c}, +207%). Also they showed increased plasma levels of Na^+ (+35%), K^+ (+56%), creatinine (+232%), urea nitrogen (+158%), total protein (−53%) and transforming growth factor-β1 (TGF-β1, 12.4-fold) values. These changes were accompanied by increases in the renal levels of malondialdehyde (MDA, +42%), by decreases in the renal glutathione redox state (−71%), and activities of catalase (−70%), glutathione peroxidase (−71%) and superoxide dismutase (−85%), and by moderate decreases of the urine Na^+ (−33%) and K^+ (−39%) values. Following monotherapy, MET generally showed a greater attenuating effect than CAP or TAU on the changes in circulating glucose, insulin and HbA_{1c} levels, urine total protein, and renal SOD activity; and CAP appeared more potent than TAU and MET, in that order, in antagonizing the changes in plasma creatinine and urea nitrogen levels. On the other hand, TAU generally provided a greater protection against changes in glutathione redox state and in CAT and GPx activities, with other actions falling in potency between those of CAP and MET. Adding TAU to a treat-

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ment with CAP, but not to one with MET, led to an increase in protective action relative to a treatment with drug alone. On the other hand, the actions of CAP-MET, which were about equipotent with those of MET, became enhanced in the presence of TAU, particularly against the changes of the glutathione redox state and activities of antioxidant enzymes. In short, the present results suggest that the addition of TAU to a treatment of diabetes with CAP or CAP-MET, and sometimes to one with MET, will lead to a gain in protective potency against changes in indices of glucose metabolism and of renal functional impairment and oxidative stress.

Keywords Diabetic nephropathy • Streptozotocin • Captopril • Metformin Taurine • Insulin • Blood and plasma biochemical parameters • Renal function tests Oxidative stress • Histological changes

Abbreviations

CAP	Captopril
CAT	Catalase
CRN	Creatinine
DM	Diabetes mellitus
DN	Diabetes nephropathy
GLC	Glucose
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Glutathione disulfide
HbA_{1c}	Glycated hemoglobin
INS	Insulin
MDA	Malondialdehyde
MET	Metformin
SOD	Superoxide dismutase
STZ	Streptozotocin
TAU	Taurine
TGF-β1	Transforming growth factor β1
UN	Urea nitrogen

1 Introduction

Diabetic nephropathy (DN) is as an important complication of diabetes mellitus (DM) affecting approximately one-third of patients with insulin-dependent diabetes and representing a major driving force of end-stage renal failure and mortality (Barnes and Viberti 1994). This functional disorder of diabetes has been well studied

in patients with types 1 and 2 DM, and it has been found characterized by microalbuminuria, renal hyperfiltration, and increased permeability to blood urea and to macromolecules, which are typically seen after 5 years of type 1 DM (Butt et al. 2010) and in about 7–10 years of type 2 DM (Barnes and Viberti 1994; Satirapoj and Adler 2014). While a persistent microalbuminuria is associated with an increased risk of developing cardiovascular disease and with progression to renal disease, values in excess of 300 mg/day of albuminuria (macroalbuminuria) are considered to represent overt nephropathy. At this point, the rate of loss of glomerular filtration rate and the development of hypertension are common to both type 1 and type 2 DM (Satirapoj and Adler 2014), and the introduction of renal replacement therapy in the form of dialysis or transplantation is not a rare event (Barnes and Viberti 1994). Also, the chronic hyperglycemic state of diabetes is responsible for the formation and accumulation of advanced glycated end products (AGEs) in the kidney and which, upon binding to AGEs receptors on mesangial cells, become major contributors to the development and progression of the glomerular and tubular structural changes that are seen in DN, including glomerular basement membrane thickening, glomerulosclerosis and tubulointerstitial fibrosis (Goh and Cooper 2008). There is also glomerular mesangial expansion as a result of initial cell proliferation and cell hypertrophy brought about at the beginning by mesangial stretch and pressure and by high glucose levels, and later by transforming growth factor-β1 (TGF-β1) and fibrosis (Butt et al. 2010). As a consequence, dysfunction of the mesangium, glomerular capillary wall, tubulointerstitium and vasculature will ensue (Satirapoj and Adler 2014). In addition, biochemical changes such as activation of the polyol pathway key enzyme aldose reductase, stress-activated signaling pathways (Evans et al. 2002) and protein kinase C (Tavafi 2013), an activator of mesangial expansion, play an important role in the development of diabetic renal complications.

Hyperglycemia is known to promote oxidative stress by contributing to the formation of reactive oxygen (ROS) and nitrogen (RNS) species, and to play a major role in the pathogenesis of DN by creating a state of oxidative stress that not only causes damage to cell membranes and to intracellular enzymes but also promotes apoptotic and necrotic cell death and alters gene expression (Allen et al. 2005). In addition, there is an increase in the levels of malondialdehyde (MDA), an index of lipid peroxidation (LPO), and a deficiency of glutathione (GSH) and of antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) in the blood and kidney of both diabetic humans (Hodgkinson et al. 2003) and animals (Kędziora-Kornatowska 1999; Kędziora-Kornatowska et al. 2000).

Drugs such as metformin (MET), a biguanide derivative that is widely used in individuals with type 2 DM and for which it represents a first line oral therapy for decreasing the plasma glucose concentration, may be useful in delaying or slowing down the development of DN (Mogensen et al. 1995) especially if it is used in conjunction with lifestyle modifications such as dietary restriction, weight control and physical activity (Viollet et al. 2012). The favorable effect of this drug on hyperglycemia has been related to an ability to augment peripheral glucose uptake through an insulin-sensitizing action without stimulating insulin secretion

(DeFronzo and Goodman 1995; Seufert et al. 2004), and to reduce glucose production through inhibition of gluconeogenesis (Seufert et al. 2004) and increased nonoxidative metabolism, including the formation of glycogen (Setter et al. 2003). The antigluconeogenic effect of MET has been related to an ability to reduce hepatic energy status (Fortes et al. 2010). MET has also been found to decrease urinary albumin excretion rates and to prevent podocyte injury in a rat model of type 2 DM (Kim et al. 2012), to reduce markers of oxidative stress, to improve renal antioxidant enzyme activity, to ameliorate apoptosis and, in general, to act as a nephroprotectant in DN (Alhaider et al. 2011; Viollet et al. 2012).

Evidence is also available in support of the benefits of therapy with angiotensin converting enzyme (ACE) inhibitors since they can be useful in impeding the progress of proteinuria and in preventing the increase of urinary albumin excretion in nonhypertensive patients with INS-dependent DM and persistent microalbuminuria (Viberti et al. 1994). In general, ACE inhibitors has been found effective in limiting macrovascular complications and in reducing the progression of retinopathy, albuminuria and nephropathy in patients with type 2 DM (King et al. 1999). As a result, drugs like CAP appear to be useful to control blood pressure in hypertensive diabetic patients with nephropathy by reducing the decline in kidney function but without impairing glucose tolerance (González-Sicilia de Llamas et al. 1991).

Taurine (TAU), the sulfonic acid analogue of β-alanine, has been extensively investigated as an antioxidant both *in vitro* and *in vivo*. In spite of lacking a readily oxidizable functional group and of having a demonstrable low free radical scavenging action, this ubiquitous nonprotein amino acid has consistently been found to protect cultured cells, organs and mammalian species against the deleterious consequences of oxidative stress fostered by a myriad of chemical agents (Das and Sil 2012; Sayed et al. 2012), biochemicals (Kalaz et al. 2013), drugs (Das et al. 2012; Hagar et al. 2006; Shao et al. 2012), toxins (Bhavasar et al. 2009; Das et al. 2012) and disease states (Ito et al. 2012; Rikimaru et al. 2012). Mechanisms such as elevation of the activities of the antioxidant enzymes CAT, SOD (Vohra and Hui 2001), GPx (Anand et al. 2011; Vohra and Hui 2001) and glutathione reductase (GR) (Anand et al. 2011), preservation of the expression and secretion of extracellular SOD (Nonaka et al. 2001), protection of GSH stores (Oudit et al. 2004) and the intracellular redox status (Acharya and Lau-Cam 2013), prevention or reduction of intracellular calcium increase (Chen et al. 2001; Yamauchi-Takihara et al. 1988) and movement (Timbrell et al. 1995), membrane stabilization (Chen et al. 2001; Timbrell et al. 1995), and direct binding to reactive aldehydes (Ogasawara et al. 1993) and to free radicals (Nakamura et al. 1993), are some of the explanations that have been mentioned to account for the seemingly unexpected beneficial actions derived from this sulfur-containing compound.

Taking into account the benefits that have been demonstrated earlier from a treatment of diabetes with MET, CAP or TAU, the present study was undertaken in rats made diabetic with the diabetogen streptozotocin to compare the effects that these compounds could have on diabetes-induced metabolic and renal function alterations when used singly or as binary or ternary combinations.

2 Materials and Methods

2.1 Treatment Compounds

The treatment agents CAP, MET and TAU were obtained from Sigma-Aldrich, St. Louis, MO, USA. Streptozotocin (STZ) was purchased from A.G. Scientific, San Diego, CA, USA. All other chemicals were from Sigma-Aldrich, St. Louis, MO, USA.

2.2 Animals

Each experimental group consisted of 6 male Sprague-Dawley rats, 200–225 g in weight, obtained from Taconic Farms, Germantown, New York, USA. During the entire study, including an acclimation period of 7 days, the rats were kept in a constant temperature (23 ± 1 °C) and humidity room and on a normal 12 h light-12 h dark cycle, and had free access to a standard rodent diet and filtered tap water.

2.3 Treatment Solutions and Treatments

STZ was dissolved in 10 mM citrate buffer pH 4.5 to provide a 0.23 mM solution. Solutions of the treatment compounds CAP, MET and TAU were freshly made each day in physiological saline. Diabetes was induced with a single, 60 mg/kg/mL, intraperitoneal dose of STZ. After 14 days, the diabetic rats started to receive a daily dose of a treatment agent (0.15 mM/kg of CAP, 2.4 mM/kg of MET or TAU, singly or as binary or ternary combinations) by oral gavage. When more than one treatment agent was involved, they were provided at 15 min intervals. Control (normal) rats received a 2 mL volume of physiological saline by the oral route in place of a treatment agent solution. Body weights and tail vein blood glucose levels were monitored on a weekly basis for a total of 56 days.

2.4 Sampling and Samples

A drop of blood was collected each week from the tail vein and used to measure the blood glucose level with the help of a commercial glucometer (TRUEtrack™ and test strips, both from Nipro Diagnostics, Fort Lauderdale, FL, USA). Only rats exhibiting a blood glucose level above 250 mg/dL were used in the study. A 24 h urine sample was collected from each rat in a metabolic cage from days 56 to 57. On day 57, the rats

were sacrificed by decapitation to collect their bloods in heparinized tubes. Immediately thereafter the kidneys were surgically excised with the help of a scalpel and kept on ice until needed for biochemical assays. A portion of each blood sample was used to measure the level of glycated hemoglobin and the other portion was centrifuged at 700 × g for 10 min to obtain the plasma fraction. A representative portion of kidney, dried with a piece of filter paper and weighing about 500 mg, was cut into small pieces with a razor blade, mixed with 10 mL of ice-cold 50 mM Tris buffer pH 7.0 containing 1 mg of phenylmethysulfonyl fluoride, and made into a fine paste with the help of a hand held electric blender (Tissue-Tearor™, Bio-Specs Products Inc., Bartlesville, OK, USA). The suspension was immediately centrifuged at 3000 rpm and 4 °C for 10 min, and the clear supernatant was kept on ice until needed for a biochemical assay.

2.5 Assay of Plasma Glucose (GLC)

The plasma glucose was measured using a commercial assay kit (Procedure No. 510, Sigma-Aldrich, St. Louis, MO, USA) based on the glucose oxidase-peroxidase colorimetric method of Raabo and Terkildsen (1960). The results were expressed in mg/dL.

2.6 Assay of Plasma Insulin (INS)

The concentration of circulating INS was measured with a commercial immunoassay kit (ELISA kit, item No. IS130D, Calbiotech Inc., Spring Valley, CA, USA) and an ELISA plate reader set at 450 nm. The results were expressed in μIU/mL.

2.7 Blood Glycated Hemoglobin (HbA_{1c})

This parameter of long term glycemic control was measured with the help of a commercial assay kit (Glycohemoglobin Test, reference No. 0350–060, Stanbio Laboratory, Boerne, TX, USA) based on an ion-exchange resin procedure and measuring both HbA_{1c} and nonglycated (HbA_1) hemoglobin. The results were expressed as a percentage of the total hemoglobin concentration.

2.8 Plasma Transforming Growth Factor-β1 (TGF-β1)

This cytokine was measured in plasma samples by a commercially available ELISA kit (Novex™ Multispecies TGF-β1, Cat. No. KAC 1688, Thermo Fisher Scientific Inc., Waltham, MA, USA). The concentration of TGF-β1 in the sample was derived from an appropriate calibration curve, and the result was expressed in pg/mL.

2.9 Plasma Creatinine (CRN)

This parameter of renal function was measured with the help of a commercial endpoint/enzymatic-colorimetric assay kit (Creatinine LiquiColor® Test, reference No. 0430–500, Stanbio Laboratory, Boerne, TX, USA) based on the Jaffe reaction according to Toora and Rajagopal (2002). The results were expressed as mg/dL.

2.10 Plasma Urea Nitrogen (UN)

This parameter of renal function was measured with a commercial assay kit (Liqui-UV® Test, reference No. 2020–430, Stanbio Laboratory, Boerne, TX, USA) using a published method based on the urease-Berthelot reagent (Tobacco et al. 1979). The results were expressed as mg/dL.

2.11 Plasma and Urine Sodium (Na^+)

The concentration of this electrolyte was measured using a commercial assay kit (Sodium Test, reference No. 0140–050, Stanbio Laboratory, Boerne, TX, USA) in which the fading of the yellow color of uranyl acetate, measured with a spectrophotometer at 420 nm, is proportional to the Na^+ content of the sample. The results were expressed as mM/L.

2.12 Plasma and Urine Potassium (K^+)

The concentration of this electrolyte was measured using a commercial turbidimetric assay kit (Potassium Test, reference No. 0160–050, Stanbio Laboratory, Boerne, TX, USA) in which K^+ is reacted with an alkaline sodium tetraphenylboron reagent, and the absorbance of the solution is read on a spectrophotometer at 580 nm. The results were expressed as mM/L.

2.13 Urine Total Protein (TP)

This biochemical parameter was measured using a commercial assay kit (Total Protein Liquicolor® Test, reference No. 0250–500, Stanbio Laboratory, Boerne, TX, USA) based on the biuret colorimetric reaction as described by Weichselbaum (1946). The results were expressed as g/dL.

2.14 Kidney Malondialdehyde (MDA)

The concentration of MDA was measured as thiobarbituric acid reactive substances (TBARS) after reaction of the test sample with a reagent containing thiobarbituric acid in an acid medium and the experimental conditions described by Buege and Aust (1978). The concentration of MDA was derived from a calibration curve of MDA generated from serial dilutions of a solution of 1,1,3,3-tetraethoxypropane (5–100 nM), and which were treated in identical manner as the test sample. The results were expressed as nM/g of tissue.

2.15 Kidney Reduced Glutathione (GSH) and Glutathione Disulfide (GSSG)

The levels of these two intracellular components were measured by the fluorometric method of Hissin and Hilf (1976), which is based on the reaction of GSH with *ortho*-phthalaldehyde at pH 8.0 and of GSSG at pH 12.0. Prior to the assay of the GSSG, any preformed GSH was removed by complexation with N-ethylmaleimide as described by Güntherberg and Rost (1966). The concentrations of both GSH and GSSG were expressed as μM/g of tissue.

2.16 Kidney Catalase (CAT) Activity

The activity of this enzyme was measured using the spectrophotometric method of Aebi (1984), which is based on the catalase-mediated degradation of hydrogen peroxide to water and oxygen. The results are reported as U/min/g of kidney sample.

2.17 Kidney Glutathione Peroxidase (GPx)

The activity of this GSH-dependent enzyme was determined by the spectrophotometric method of Flohé and Günzler (1984) and its activity in the sample was expressed as μM of NADPH converted to $NADP^+$/min/g of kidney sample.

2.18 Kidney Superoxide Dismutase (SOD) Activity

This enzyme was assayed using the spectrophotometric method of Misra (1985) and its activity in the sample was expressed as U/min/g of kidney sample.

2.19 Statistical Analyses

The experimental results are reported as mean ± SEM for groups of 6 rats each. Differences between groups were analyzed for statistical significance using unpaired Student's t-test and a commercial computer-based statistics program (GraphPad Prism® Version 4.0 from GaphPad Software, Inc., San Diego, CA, and SigmaStat® from Systat Sofware, Inc., San Jose, CA, USA), followed by one-way analysis of variance (ANOVA) and Tukey's post hoc test. Intergroup differences were considered to be statistically significant when $p \leq 0.05$).

3 Results

3.1 Plasma GLC

At the end of 8 weeks, diabetic rats demonstrated a very high (+313%, $p < 0.001$) plasma GLC level compared to normal rats (Table 1). Less marked increases were seen in diabetic rats receiving CAP (+242%), TAU (+222%) or better MET (+90%) at the end of a 6 weeks treatment (all at $p < 0.001$ vs. control). A combined treatment with CAP-MET lowered the diabetic plasma GLC to about the same extent as MET alone (+96%). Adding TAU to a treatment with CAP (+199%) and MET (+84%) led to a moderate and negligible increase, respectively, in hypoglycemic action relative to CAP and MET alone. However, a still greater effect was attained when the three compounds were made available together (+70%) but not with CAP-MET (+96%) (all comparisons vs. control were significant at $p < 0.001$, Table 1). In contrast, none of the oral treatment agents altered the control plasma GLC level to a significant extent.

Table 1 The effects of CAP, MET and TAU, singly and in combination, on the plasma GLC and INS and blood HbA_{1c} levels of diabetic rats[a,b]

Group	Plasma GLC, mg/dL	Plasma INS, μIU/mL	HbA_{1c}, %
Control	$103.59 \pm 5.06^{+++}$	$44.08 \pm 2.46^{+++}$	$6.89 \pm 0.11^{+++}$
DM	$428.08 \pm 21.77^{***}$	$10.58 \pm 1.33^{***}$	$21.14 \pm 1.19^{***}$
DM-CAP	$354.23 \pm 11.89^{***,+}$	$27.61 \pm 3.22^{***,+++}$	$4.20 \pm 1.81^{***,++}$
DM-MET	$196.41 \pm 5.00^{***,+++}$	$32.50 \pm 2.365^{**,+++}$	$7.77 \pm 0.41^{+++}$
DM-TAU	$333.27 \pm 6.60^{***,+}$	$20.94 \pm 1.83^{***,+++}$	$10.96 \pm 0.96^{***,+++}$
DM-CAP-MET	$202.62 \pm 3.51^{***,+++}$	$33.86 \pm 1.72^{**,+++}$	$8.73 \pm 0.92^{*,+++}$
DM-CAP-TAU	$310.03 \pm 5.05^{***,+}$	$38.00 \pm 1.21^{+++}$	$12.31 \pm 0.97^{***,+++}$
DM-MET-TAU	$190.43 \pm 2.22^{***,+++}$	$36.02 \pm 1.87^{*,+++}$	$7.65 \pm 1.04^{+++}$
DM-CAP-MET-TAU	$176.54 \pm 19.17^{***,+++}$	$39.09 \pm 1.93^{+++}$	$8.43 \pm 0.23^{*,+++}$

[a]Values are reported as the mean ± SEM for n = 6

[b]Statistical comparisons were vs. Control rats at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; and vs. DM rats at $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$

3.2 Plasma INS

Diabetes caused the plasma INS to markedly decrease to a value that, by the end of week 8, was markedly below the control value (−76%, $p < 0.001$) (Table 1). In contrast, a treatment with CAP, MET, TAU and their binary and ternary combinations resulted in higher plasma INS levels, with MET (−26%, $p < 0.05$) appearing much more effective than either CAP (−37%, $p < 0.01$) or TAU (−52%, $p < 0.001$). When used as binary combinations, adding TAU to a treatment with CAP or MET enhanced the effects of these drugs further, with CAP-TAU (−14%) providing an insignificantly greater effect than MET-TAU (−18%, $p < 0.05$). In contrast, while the combination CAP-MET (−23%, $p < 0.05$) was equipotent with MET alone, a treatment with MET-CAP-TAU led to a plasma INS value (−11%) that was insignificantly different from the control value (Table 1). None of the treatment agents were found to alter the basal plasma INS to a significant extent (≤3% increase).

3.3 Blood HbA_{1c}

Measurement of the HbA_{1c} level to determine the average blood glucose over a prolonged period of time is a useful way to assess the effectiveness of therapy of diabetes, with values above being 7% considered to represent poor glucose control for most human patients (American Diabetes Association 2014) and for rats of the same strain and age as those used here (Ahmadvand 2012). In the present study untreated diabetic rats demonstrated a blood HbA_{1c} that was more than 200% greater than the control value ($p < 0.001$, Table 1). Treating the diabetic rats with MET resulted in a higher attenuation of the diabetic plasma glucose (only +13%) than one with TAU (+59%, $p < 0.001$) or CAP (+106%, $p < 0.001$). Use of binary combinations led to a marked improvement in the suppression of HbA_{1c} formation, with MET-TAU (only +11%) appearing as the most effective, CAP-MET providing an intermediate potency (+27%, $p < 0.05$) and CAP-TAU exerting the lowest effect (+79%, $p < 0.001$). On the other hand, providing the diabetic rats with CAP-MET-TAU also resulted in a significant decrease of the blood HbA_{1c} level (+22%, $p < 0.05$) although not to the same extent as a treatment with MET-TAU (Table 1). When given to normal rats, CAP, MET and TAU did not alter the basal blood HbA_{1c} level.

3.4 Plasma TGF-β1

This indicator of diabetic microvascular and macrovascular complications was significantly increased in the plasma of diabetic rats (>12-fold, $p < 0.001$) relative to the control value (Table 2). A treatment of diabetic rats with CAP, MET or TAU led

Table 2 The effects of CAP, MET and TAU, singly and in combination, on the plasma TGF-β1 levels of diabetic rats[a,b]

Group	Plasma TGF-β1, pg/mL
Control	$43.64 \pm 11.22^{+++}$
DM	$543.21 \pm 47.71^{***}$
DM-CAP	$143.93 \pm 33.71^{***, +++}$
DM-MET	$167.46 \pm 15.68^{***,+++}$
DM-TAU	$168.18 \pm 21.99^{***, +++}$
DM-CAP-MET	$104.79 \pm 10.06^{***,+++}$
DM-CAP-TAU	$132.41 \pm 10.07^{***,+++}$
DM-MET-TAU	$141.75 \pm 15.85^{***,+++}$
DM-CAP-MET-TAU	$72.64 \pm 6.90^{***,+++}$

[a]Values are reported as the mean ± SEM for n = 6
[b]Statistical comparisons were vs. Control rats at $^{***}p < 0.001$; and vs. DM rats at $^{+++}p < 0.001$

to attenuation of this rise, with CAP (3.3-fold, $p < 0.001$) providing a greater attenuation than either MET or TAU (~3.85-fold increase with both, $p < 0.001$). Adding TAU to a treatment with either CAP or MET led to different effects, being negligible with CAP-TAU (threefold increase, $p < 0.001$) and to an increase with MET-TAU (~3.25-fold, $p < 0.001$) relative to a treatment without TAU. However, a greater decrease in plasma TGF-β1 levels was attained with CAP-MET (2.4-fold increase, $p < 0.001$) and especially with CAP-MET-TAU (only 1.7-fold increase, $p < 0.001$) (Table 2). When given to normal rats, these compounds were found not to alter the plasma TGF-β1 values of normal rats to a significant extent.

3.5 Plasma CRN and UN

At the end of 8 weeks, diabetic rats showed a much higher plasma CRN (+332%, $p < 0.001$) level than control rats (Table 3). A treatment of these rats with CAP (+71%), MET (+139%) or TAU (+100%) led to a significant reduction of the diabetic plasma CRN level (all at $p < 0.001$ vs. diabetes). Furthermore, a daily treatment with the combinations CAP-TAU (+77%) and MET-TAU (+148%) did not enhance the actions of either CAP or TAU further; and one with CAP-MET (+119%) was marginally better than one with MET alone; but providing the diabetic rats with the three compounds together led to a drastic gain in potency (only +39%, $p < 0.01$) over monotherapy or bitherapy (Table 3).

In parallel with an increase in plasma CRN, diabetes also a drastic (+158%, $p < 0.001$ vs. control) increase in the plasma UN at the end of a 8 weeks period (Table 3). Treating the diabetic rats with TAU (+68%) or better with CAP (+41%), was more effective than with MET (+102%, $p < 0.001$) in lowering this elevation; but a combined treatment with TAU enhanced the effect of both MET (only +23%, $p < 0.05$) and CAP (+34%, $p < 0.01$) than either treatment alone. While a combined treatment with CAP-MET (+39%, $p < 0.01$) provided an attenuating effect approximating that of CAP-TAU (+34%, $p < 0.01$), one with CAP-MET-TAU was

Table 3 The effects of CAP, MET and TAU, singly and in combination, on the plasma CRN, UN and TP levels of diabetic rats[a,b]

Group	Plasma CRN, mg/dL	Plasma UN, mg/dL	Plasma TP, g/dL
Control	$0.31 \pm 0.03^{+++}$	$11.31 \pm 0.62^{+++}$	$9.25 \pm 0.26^{+++}$
DM	$1.34 \pm 0.02^{***}$	$29.14 \pm 1.16^{***}$	$4.37 \pm 0.55^{***}$
DM-CAP	$0.53 \pm 0.06^{***,+++}$	$15.90 \pm 1.14^{***,+++}$	$6.50 \pm 0.39^{**,+++}$
DM-MET	$0.74 \pm 0.09^{***,+++}$	$22.80 \pm 0.90^{***,++}$	$5.96 \pm 0.43^{**,++}$
DM-TAU	$0.62 \pm 0.06^{***,+++}$	$18.74 \pm 1.27^{***,++}$	$7.03 \pm 0.69^{**,+++}$
DM-CAP-MET	$0.68 \pm 0.01^{***,+++}$	$15.72 \pm 1.22^{**,+++}$	$6.00 \pm 0.37^{**,++}$
DM-CAP-TAU	$0.55 \pm 0.01^{***,+++}$	$15.10 \pm 0.23^{**,+++}$	$7.11 \pm 0.31^{**,+++}$
DM-MET-TAU	$0.77 \pm 0.09^{***,+++}$	$13.89 \pm 1.53^{**,+++}$	$6.46 \pm 0.42^{**,+++}$
DM-CAP-MET-TAU	$0.43 \pm 0.08^{**,+++}$	$12.42 \pm 0.93^{+++}$	$7.11 \pm 0.11^{***,+++}$

[a]Values are reported as the mean ± SEM for n = 6

[b]Statistical comparisons were vs. Control (normal) rats at $^{**}p < 0.01$ and $^{***}p < 0.001$; and vs. diabetic (DM) rats at $^{++}p < 0.01$ and $^{+++}p < 0.001$

still more effective (+10% increase) (Table 3). None of the treatment compound were found to alter the basal plasma CRN and UN levels to a significant extent.

3.6 Plasma TP

In comparison to control rats, diabetic ones showed a large decrease in the plasma TP level (−53%, $p < 0.001$) at the end of 8 weeks (Table 3). A treatment of these rats with TAU (−22%, $p < 0.05$) reduced the loss of the plasma TP to a greater extent than one with either CAP (−30%, $p < 0.01$) or MET (−36%, $p < 0.01$). A combined treatment with CAP-TAU (−23%, $p < 0.05$) was as effective as TAU alone, but slightly better than either MET-TAU (−30%, $p < 0.01$) or CAP-MET (−35%, $p < 0.01$); and providing the diabetic rats with MET-CAP-TAU led to an equivalent effect as one with CAP-TAU (Table 3). None of the treatment agents was found not to reduce the plasma TP level of control rats by more than 10%.

3.7 Plasma and Urine Na^+

At the end of 8 weeks diabetic rats exhibited a higher level of plasma Na^+ than control rats of equivalent weight (+35%, $p < 0.01$) (Table 4). A daily treatment of the diabetic rats with CAP, MET or TAU from day 15 onwards led to much lower circulating Na^+ levels (+2% with CAP, +11% with MET and TAU), an effect that was enhanced further when TAU was co-administered with either CAP or TAU (no difference from the

Table 4 The effects of CAP, MET and TAU, singly and in combination, on the plasma and urine Na^+ levels of diabetic rats[a,b]

Group	Plasma Na^+, mM/L	Urine Na^+, mM/L	Plasma/urine Na^+ ratio
Control	$144.45 \pm 6.42^{+++}$	$19.23 \pm 3.40^{+++}$	1.21^{+++}
DM	$194.82 \pm 1.06^{**}$	$80.23 \pm 2.93^{**}$	2.43^{***}
DM-CAP	$147.60 \pm 2.76^{++}$	$99.27 \pm 1.71^{*,++}$	$1.49^{*,++}$
DM-MET	$160.36 \pm 2.84^{+}$	$101.88 \pm 3.42^{*,++}$	$1.57^{*,+}$
DM-TAU	$159.81 \pm 2.46^{+}$	$94.70 \pm 2.33^{**,+}$	$1.69^{**,+}$
DM-CAP-MET	$165.18 \pm 5.62^{*,+}$	$96.30 \pm 3.16^{*,++}$	$1.72^{**,+}$
DM-CAP-TAU	$144.79 \pm 4.62^{++}$	$103.21 \pm 7.38^{*,++}$	$1.40^{*,++}$
DM-MET-TAU	$147.40 \pm 3.41^{++}$	$98.30 \pm 4.23^{*,++}$	$1.50^{*,++}$
DM-CAP-MET-TAU	$151.38 \pm 3.71^{++}$	$10.71 \pm 2.81^{+++}$	1.37^{++}

[a]Values are reported as the mean ± SEM for n = 6

[b]Statistical comparisons were vs. Control rats at $^{*}p < 0.05$, and $^{**}p < 0.01$; and vs. DM rats at $^{+}p < 0.05$ and $^{++}p < 0.01$

control value) or as a combination with both CAP and TAU (+5%); and a treatment with CAP-MET (+2%) was about equipotent to one with CAP-MET-TAU (+5%) (Table 4).

As expected, the increase in plasma Na^+ caused by diabetes was accompanied by a significant fall in the urinary excretion of Na^+ (−39%, $p < 0.01$ vs. control) (Table 4). In contrast, treating the diabetic rats with CAP, MET or TAU led to an enhanced renal excretion of Na^+, with the effect being quantitatively rather similar (−14%, −15% and −12%, respectively); and the effectiveness of a binary treatment with CAP-TAU (−13%), MET-TAU (−18%, $p < 0.05$) or CAP-MET (−19%, $p < 0.05$) was about equal to that attained using monotherapy. In contrast, providing the diabetic rats with CAP-MET-TAU reduced the urinary loss of Na^+ to a value comparable to the value for control rats (only −7%) (Table 4). All the treatment agents and their various combinations were able to reduce the increase in plasma Na^+/urine Na^+ ratio seen in diabetic rats (+100%, $p < 0.001$ vs. control) to a significant extent, with CAP, CAP-TAU and MET-TAU providing a stronger lowering effect (only16–24% increases, $p < 0.05$) than MET, TAU or CAP-MET (40–43% increases, $p < 0.01$) on the diabetic ratio. On the other hand, CAP-MET-TAU was able to lower the ratio to a value not significantly different from the control value (+13%) (Table 4). None of the treatment compounds altered the urinary excretion of Na^+ to a significant extent.

3.8 Plasma and Urine K^+

By analogy to results for the plasma and urine Na^+ levels, those for K^+ were also increased and decreased, respectively, when compared to the corresponding control values (Table 5). In the case of the plasma K^+, diabetic rats retained a

Table 5 The effects of CAP, MET and TAU, singly and in combination, on the plasma and urine K^+ levels of diabetic rats[a,b]

Group	Plasma K^+, mM/L	Urine K^+, mM/L	Plasma/urine K^+ ratio
Control	$10.24 \pm 0.41^{++}$	$6.95 \pm 34.61^{++}$	0.18^{+++}
DM	$16.02 \pm 0.45^{***}$	$34.61 \pm 1.14^{***}$	0.46^{***}
DM-CAP	$11.40 \pm 0.39^{*,++}$	$45.74 \pm 1.25^{*,++}$	$0.25^{**,+++}$
DM-MET	$11.90 \pm 0.67^{**,+}$	$45.23 \pm 3.47^{*,++}$	$0.26^{***,+++}$
DM-TAU	$11.01 \pm 6.60^{++}$	$49.26 \pm 3.69^{+++}$	$0.22^{*,+++}$
DM-CAP-MET	$11.23 \pm 0.21^{++}$	$46.31 \pm 2.34^{*,++}$	$0.24^{**,+++}$
DM-CAP-TAU	$11.06 \pm 0.91^{++}$	$48.31 \pm 4.12^{*,++}$	$0.23^{*,+++}$
DM-MET-TAU	$11.07 \pm 0.42^{++}$	$45.31 \pm 3.68^{**,++}$	$0.24^{**,+++}$
DM-CAP-MET-TAU	$10.50 \pm 0.71^{++}$	$50.21 \pm 3.04^{+++}$	$0.21^{*,+++}$

[a]Values are reported as the mean ± SEM for n = 6

[b]Statistical comparisons were vs. Control rats at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; and vs. DM rats at $^{++}p < 0.01$ and $^{+++}p < 0.001$

greater concentration of K^+ (56%, $p < 0.001$) than normal rats. However, a daily treatment of these diabetic rats with CAP, MET or TAU lowered the plasma K^+ to a value that was comparable to the control value (+8%, +16% and +8%, respectively). On the other hand, the administration of TAU together with CAP or MET led to results identical to those seen with TAU alone (i.e., +8%), a situation that was also attained with CAP-MET (+10%). Providing the diabetic rats with the three compounds resulted in virtually normal (+2.5%) plasma K^+ values (Table 5). None of the treatment agents altered the baseline plasma K^+ values.

At variant with the increase in K^+ levels seen in the plasma of diabetic rats, the corresponding urine levels were significantly below the control value (−46%, $p < 0.001$) (Table 5). Treating the diabetic rats with CAP, MET or TAU reduced the urine K^+ by more than one-half of the diabetic value (to ~20% with both CAP and MET, $p < 0.05$; to −14% with TAU), an effect that was also seen when CAP and MET were each administered alongside TAU (−15% with CAP-TAU, −20% with MET-TAU, $p < 0.05$) or when given together (−20%, $p < 0.05$). However, administering the three compounds together led to a urine K^+ value that was not significantly different from that of control rats (−12%) (Table 5). As verified for the plasma Na^+/urine Na^+ ratio, that for plasma K^+/urine K^+ was also significantly increased in diabetic rats (+156%, $p < 0.001$ vs. control), with TAU (only +22%, $p < 0.05$) and CAP-TAU (+28%, $p < 0.5\%$) lowering the diabetic increase to a greater extent than the other treatments (+33–44%, $p < 0.01$). Providing the diabetic rats with CAP-MET-TAU led to a further, although small, reduction of the diabetic plasma K^+/urine K^+ ratio (+17%, $p < 0.05$) (Table 5) The administration of all of the test compounds to normal rats did not have a significant effect on the urine K^+ level.

Table 6 The effects of CAP, MET and TAU, alone and in combination, on the kidney MDA levels of diabetic rats[a,b]

Group	MDA, mM/g
Control	$3.94 \pm 0.07^{++}$
DM	$5.61 \pm 0.08^{***}$
DM-CAP	$4.25 \pm 0.07^{++,}$
DM-MET	$3.97 \pm 0.04^{++}$
DM-TAU	$3.99 \pm 0.07^{++}$
DM-CAP-MET	$3.77 \pm 0.05^{+++}$
DM-CAP-TAU	$3.66 \pm 0.06^{++}$
DM-MET-TAU	$3.81 \pm 0.04^{++}$
DM-CAP-MET-TAU	$3.72 \pm 0.02^{++}$

[a]Values are reported as the mean ± SEM for n = 6

[b]Statistical comparisons were vs. Control rats at $^{***}p < 0.001$; and vs. DM rats at $^{++}p < 0.01$

3.9 Kidney MDA

In comparison to normal rats, untreated diabetic ones showed a higher renal level of MDA (+41%, $p < 0.01$) (Table 6). Without exceptions, a treatment of these rats with CAP, MET or TAU brought the MDA levels to values that were similar to the baseline values (≤8%), an effect that was also achieved when CAP (−7%) or MET (−3%) were paired with TAU or with each other (−4%) or when the three compounds were given together (−6%) (Table 6). None of the treatment compounds showed an effect on the baseline kidney MDA levels.

3.10 Kidney GSH, GSSG and GSH/GSSG Ratio

Diabetic rats exhibited a moderate decrease in renal GSH (−34%, $p < 0.01$) and a very high drop in the renal GSSG level (+127%, $p < 0.001$) by the end of week 8 relative to corresponding control values (Table 7). These values were attenuated by CAP (−26%), MET (−20%) and TAU (−27%) but not to a significant extent unless CAP (−21%) or MET (−9%) were paired with TAU. or with each other (−7%). By contrast, a combined treatment with the three compounds reversed the deficit (+13%, $p < 0.01$ vs. diabetes) (Table 7). When given to normal rats, none of the test compounds were found to affect the basal GSH levels significantly.

In the case of the GSSG levels, treating the diabetic rats with either CAP (+42%), MET (+83%) or TAU (+13%) resulted in a significant ($p \leq 0.01$) reduction in the formation of GSSG caused by diabetes (Table 7); but treating the diabetic rats with either CAP-TAU (+56%) or MET-TAU (+54%) led to a lowering of the protective

Table 7 The effects of CAP, MET and TAU, alone and in combination, on the kidney GSH and GSSG levels and GSH/GSSG ratio of diabetic rats[a,b]

Group	GSH, nM/g	GSSG, nM/g	GSH/GSSG ratio
Control	$95.98 \pm 1.76^{++}$	$4.80 \pm 0.23^{+++}$	$19.99 \pm 0.08^{+++}$
DM	$63.34 \pm 0.58^{***}$	$10.88 \pm 045^{***}$	$5.82 \pm 0.03^{***}$
DM-CAP	$70.79 \pm 1.58^{**,+}$	$6.83 \pm 0.72^{***,++}$	$10.37 \pm 0.04^{***,+++}$
DM-MET	$76.33 \pm 1.55^{**,++}$	$8.81 \pm 0.25^{***,+}$	$8.66 \pm 0.04^{***,+++}$
DM-TAU	$69.59 \pm 1.08^{**,+}$	$5.43 \pm 0.21^{*,+++}$	$12.81 \pm 0.04^{**,+++}$
DM-CAP-MET	$89.17 \pm 1.92^{++}$	$8.73 \pm 0.92^{*,+++}$	$12.28 \pm 0.04^{**,+++}$
DM-CAP-TAU	$75.99 \pm 0.83^{*,+}$	$7.50 \pm 0.68^{***,++}$	$10.13 \pm 0.06^{***,+++}$
DM-MET-TAU	$87.73 \pm 2.20^{++}$	$7.39 \pm 0.61^{***,++}$	$11.87 \pm 0.06^{***,+++}$
DM-CAP-MET-TAU	$108.55 \pm 1.93^{+++}$	$5.63 \pm 0.18^{+++}$	$19.28 \pm 0.06^{+++}$

[a]Values are reported as the mean ± SEM for n = 6

[b]Statistical comparisons were vs. Control rats at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; and vs. DM rats at $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$

effect achieved with either compound alone, which was also the case when CAP and MET were given concurrently (−82%, $p < 0.001$ vs. control). In contrast, treating the diabetic rats with MET-CAP-TAU resulted in a renal GSSG value that was approximated that seen with TAU alone (+17% (Table 7). None of the treatment agents was found to affect the renal GSSG levels of normal rats to a significant extent.

Based on the renal values for GSH and GSSG, it was determined that in diabetic rats the intracellular redox state, expressed as the GSH/GSSG ratio, was drastically reduced in the kidney of diabetic rats (−71%, $p < 0.001$) relative to the control value (Table 7). This ratio was found to rise following a treatment with either MET (−57%, $p < 0.001$), CAP (−48%, $p < 0.001$) and particularly with TAU (−35%, $p < 0.01$).

Treating the diabetic rats with MET-TAU (−41%) and CAP-MET (−39%), but not with CAP-TAU (−49%), led to an improvement of the effect attained with monotherapy ($p < 0.01$ vs. control). This gain was further enhanced when the diabetic rats were fed MET-CAP-TAU, at which point the GSH/GSSG ratio became comparable to the control value (−4% decrease) (Table 7). None of the treatment agents affected the basal renal GSH/GSSG significantly.

3.11 Kidney CAT

The renal activity of CAT was grossly reduced in diabetic rats (−71%, $p < 0.001$) relative to control rats (Table 8). A 6 weeks treatment with CAT (−41%, $p < 001$), MET (−50%, $p < 0.001$) and especially TAU (−29%, $p < 0.05$) led to a significant attenuation of this effect. The addition of TAU to a treatment with CAP (−10%) or MET (−18%, $p < 0.05$) enhanced the attenuating effect of these compounds further. This effect was even greater when the treatment was with CAP-MET (−7%) or

Table 8 The effects of CAP, MET and TAU, alone and as combinations, on the kidney CAT, GPx and SOD activities of diabetic rats[a,b]

Group	CAT, U/min/g tissue	GPx U/min/g tissue	SOD U/min/g tissue
Control	$0.84 \pm 0.02^{+++}$	$0.94 \pm 0.06^{+++}$	$1.50 \pm 0.22^{+++}$
DM	$0.24 \pm 0.01^{***}$	$0.28 \pm 0.02^{***}$	$0.23 \pm 0.06^{***}$
DM-CAP	$0.52 \pm 11.89^{**,+++}$	$0.69 \pm 0.03^{**,+++}$	$0.88 \pm 0.05^{***,+++}$
DM-MET	$0.42 \pm 0.03^{***,+++}$	$0.75 \pm 0.06^{**,+++}$	$1.09 \pm 0.06^{**,+++}$
DM-TAU	$0.60 \pm 0.04^{**,+++}$	$0.83 \pm 0.03^{+++}$	$0.97 \pm 0.01^{**,+++}$
DM-CAP-MET	$0.78 \pm 0.05^{+++}$	$0.84 \pm 0.01^{**,+++}$	$1.02 \pm 0.08^{**,+++}$
DM-CAP-TAU	$0.76 \pm 0.05^{+++}$	$0.77 \pm 0.02^{*,+++}$	$1.04 \pm 0.05^{**,+++}$
DM-MET-TAU	$0.69 \pm 0.01^{*,+++}$	$0.84 \pm 0.01^{**,+++}$	$1.00 \pm 0.04^{**,+++}$
DM-CAP-MET-TAU	$0.99 \pm 0.06^{*,+++}$	$0.90 \pm 0.03^{+++}$	$1.38 \pm 0.03^{+++}$

[a]Values are reported as the mean ± SEM for n = 6

[b]Statistical comparisons were vs. Control (normal) rats at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; and vs. Diabetic (DM) rats at $^{+++}p < 0.001$

better when CAP-MET and TAU were made available together, in which case the effect of diabetes on the renal CAT activity was totally reversed (+18%, $p < 005$) (Table 8). On the other hand, none of the treatment compounds was found to affect the basal renal CAT activity to a significant extent.

3.12 Kidney GPx

In comparison to normal rats, diabetic ones showed a profound decrease (−70%, $p < 0.001$) in renal GPx activity (Table 8). A daily treatment of the diabetic rats with MET (−20%, $p < 0.05$), CAP (−27%, $p < 0.05$) and especially TAU (−12%) resulted in a strong attenuation of the diabetic effect. The protective effect was enhanced by adding TAU to a treatment with either CAP (−18%, $p < 0.05$) or MET (−11%) or by a combined treatment with CAP-MET (−11%). An additional gain in potency was achieved by treating the diabetic rats with these three compounds, in which case an almost complete reversal of the diabetic effect was achieved (only 4% decrease) (Table 8). None of the treatment agents had a significant effect on the basal kidney GPx activity.

3.13 Kidney SOD

By analogy to the effect of diabetes on other antioxidant enzymes, after 8 weeks the renal activity of SOD in diabetic rats was also significantly reduced (−85%, $p < 0.001$) compared to the control group (Table 8). A 5 weeks treatment of these rats with CAP (−44%, $p < 0.001$), MET (−27%, $p < 0.05$) or TAU (−35%, $p < 0.01$)

reduced this effect significantly. A combined treatment with CAP-TAU (−31%, $p < 0.01$), CAP-MET (−32%) or MET-TAU (−33%, $p < 0.01$) led to an effect approximating that seen with TAU alone; and treating the diabetic rats with these three compounds led to an almost complete restoration of the basal SOD activity (−8%) (Table 8). The administration of the treatment compounds to normal rats resulted in different degrees of elevation, being insignificant with MET +11%) and TAU (+13%) and significant with CAP (+20%, $p < 0.05$).

4 Discussion

The present study has examined the effects of a 6 weeks treatment with either CAP, MET or TAU and their binary and ternary combinations on biochemical indices of GLC metabolism and kidney function and oxidative stress associated with STZ-induced DM. The dose of STZ used in the study was one found to cause a persistent glucose level that exceeded 350 mg/dL soon after its administration (Gajdošik et al. 1999).

Untreated diabetic rats showed a plasma GLC level that was more than fourfold higher than that of normal rats. Although treatment with a single agent was able to reduce the diabetic GLC levels, MET was at least 2.5-times more effective than either TAU or CAP in that order, an effect that was enhanced when CAP and MET were given along with TAU, more with CAP than with MET. While the hypoglycemic action of MET has been related to an increase in peripheral INS sensitivity and, hence, to GLC utilization, to inhibition of hepatic gluconeogenesis, and to a reduction of gastrointestinal GLC absorption, but without stimulating INS secretion (Davidson and Peters 1997; Viollet et al. 2012), less is known on how CAP negatively influences the circulating GLC levels. One possibility may be through an increase in peripheral INS sensitivity without an effect on INS secretion (Pollare et al. 1989; Rett et al. 1986); and another is through an ability to promote the uptake GLC by the skeletal muscle for eventual metabolic disposal (Kodama et al. 1990; Rett et al. 1986). Regardless of the exact mechanism underlying the hypoglycemic action of CAP, this issue is still unsettled since another study found CAP to neither influence GLC tolerance nor INS sensitivity in hypertensive patients with type 2 DM (Yin et al. 1994). TAU is amino acid reported to exert a hypoglycemic effect by increasing INS availability, by promoting hepatic GLC accumulation as glycogen (Gavrovskaya et al. 2008), and by lowering the rate of renal gluconeogenesis (Koh et al. 2014) in animal models of diabetes. In spite of the numerous studies describing the beneficial effects of TAU on the blood GLC and INS secretion (de Oliveira et al. 2011), resistance (Kim et al. 2012) or sensitivity (Brøns et al. 2004; Nakaya et al. 2000), there are also reports indicating a lack thereof (Brøns et al. 2004; Goodman and Shihabi 1990). The present study determined that although TAU lowered the plasma GLC of diabetic rats to a greater extent than CAP, this effect was less than one-half of that shown by MET. Furthermore, when co-administered with CAP or MET this amino acid was found to enhance the effect of CAP, but not of MET, mildly. In any case, while both treatment combinations were slightly more

effective in lowering the plasma GLC of diabetic rats than MET-CAP, a combined treatment with all three compounds was the only one to reduce the diabetic plasma GLC to within control values.

While diabetes reduced the plasma INS to about one-fourth the normal level, a treatment with either CAP, MET or TAU was found to counteract this effect to a significant but different extents. Thus, MET was found to produce the highest increase of the plasma INS in diabetic animals, followed by CAP, with TAU appearing as the least potent. The effect of MET on INS secretion may reflect an ability to reestablish the INS secretory function of the pancreas that became impaired by a chronic exposure to the high levels of free fatty acids and GLC typically seen in diabetes (Patané et al. 2000). This effect, has been described as a desensitization of pancreatic islets as a result of a prolonged exposure to high concentrations of GLC (Lupi et al. 1999). The present finding for CAP contrasts markedly with those reported by Jasik et al. (1996) and indicating that the hypoglycemic action of this ACE inhibitor is exerted through an improvement of INS sensitivity rather than of any influence on INS secretion by pancreatic β-cells. In contrast, an elevating effect on the plasma INS by an ACE inhibitor has been previously reported by Roysommuti et al. (2013). In parallel with CAP, the effect of TAU on INS secretion is also surrounded by controversy, with some studies describing an increasing effect (L'Amoreaux et al. 2010; Ribeiro et al. 2009), some an inhibitory effect (Wang et al. 2008), and still some reporting no effect on either secretion or sensitivity to the hormone (Brøns et al. 2004). Regardless of the exact mechanism by which TAU lowers the circulating GLC, the present study found that even though TAU was able to raise the plasma INS it was, however, much weaker than either MET or CAP. However, when used alongside MET or CAP it was able to enhance the actions of these compounds, more so with CAP than with MET. Although a treatment with CAP-MET was not as potent as one with CAP-TAU or MET-TAU, when available along with TAU, the ternary combination raised the plasma INS to a value not significantly different from the normal value.

The measurement of the HbA_{1c} level is considered to be a useful approach to monitoring long term glycemic control in diabetic patients since it can serve as an indicator of adverse outcome risks. In general, an agreement has been established in human patients between the circulating levels of GLC and those of HbA_{1c} in human patients and with renal function. Indeed, very high levels of blood HbA_{1c} have been closely associated with a rapid decline of renal function and with an increased risk of mortality irrespective of the type of diabetes (Rossing et al. 2004. In the present study, an increase of the plasma GLC in diabetic rats was accompanied by a marked increase of the blood HbA_{1c} relative to the same values for normal rats. Such an increase was lowered by MET to a level insignificantly different from the normal one; but one with either CAP or TAU reduced the diabetic blood HbA_{1c} to a level was much still higher than normal (by twofold and 1.6-fold, respectively). A combined treatment of these compounds with TAU led to an improvement of the effect of CAP (~1.8-fold increase) but not of MET; and using the three compounds together resulted in a HbA_{1c} level that was intermediate to those seen with TAU-CAP and TAU-MET. The present results with MET contrast markedly with those

attained in humans with type 2 DM and in which the success of monotherapy in bringing the HbA_{1c} to a desirable level is much lower than that reported here, especially since drug effectiveness decreases with increasing patient age and its attainment may require a combination therapy (Nosadini 2002; Turner et al. 1999).

TGF-β1 is a fibrogenic cytokine that in addition to being increased in the glomeruli of type 2 diabetic patients (Genc et al. 2010) and in DM-induced diabetic rats it is stimulated by the diabetic process to play a key role in the pathogenesis of DN (Gilbert et al. 1998; Wolf et al. 2005). Specifically, TGF-β1 causes an increase in mesangial matrix deposition, glomerular basement membrane (GBM) thickening, podocyte apoptosis or detachment, and adhesion of the bare GMB to the Bowman's capsule to initiate the process of glomerulosclerosis (Wolf et al. 2005). Conversely, inhibition of the expression of this protein in diabetic *db/db* mice with a neutralizing anti-TGF-β1 antibody has resulted in the prevention of diabetic renal hypertrophy, mesangial matrix expansion and the development of renal insufficiency but without affecting the accompanying albuminuria (Ziyadeh et al. 2000). In the present study, all treatment compounds were able to attenuate the more than 12-fold rise of the plasma TGF-β1 level, caused by diabetes with CAP providing a greater decreasing effect than either MET and TAU. When CAP and MET were given together with TAU, the potency of MET-TAU increased to a somewhat greater extent than that of CAP-TAU (threefold increase) relative to MET and CAP alone. However, the greatest reduction in plasma TGF-β1 was observed in diabetic rats treated with a combination of the three test compounds, in which case the TGF-β1 level was only ~1.7-fold above the control value. Since in subjects with type 2 DM an elevated level of TGF-β1 indicates a tendency for renal damage, the use of TAU along with standard hypoglycemic therapy could be of value in preventing renal diabetic complications.

The decreasing effect of CAP on the plasma TGF-β1 has been previously reported by Sharma et al. (1999) and confirmed *in vitro* by Noh et al. (2005) using cultures of human peritoneal mesothelial cells. Protection by MET against diabetic nephropathy, including fibrosis, has been related to an ability to inhibit the binding of TGF-β1 to its receptor at target sites, thus preventing downstream signal transduction leading to fibrosis (Xiao et al. 2016). In the case of TAU, there is evidence to indicate that the daily consumption of this amino acid by rats with DM-induced diabetes as part of the drinking water (1%, w/v), and started at 4 months after the induction of diabetes, can lower TGF-β1 expression in the renal glomerulus and stabilize the urinary excretion of proteins, effects that have been ascribed, at least in part, to an antioxidant action (Higo et al. 2008). Overall, it has been stated that elevations of the circulating levels of TFG-β1 levels in patients with type 2 DM may indicate a tendency for renal cell damage, and that a lack of changes in the levels of this cytokine after therapy could reflect inadequate therapy duration with an antidiabetic drug such as MET (Yener et al. 2008). Although it is likely that an improvement in the glycemic control of diabetes by MET reduces TGF-β1 levels and, thus, partly contributes to renoprotection (Vinagre et al. 2014), in the case of TAU other factors are probably involved since TAU is intrinsically a much weaker hypoglycemic than MET.

To check the renal function of diabetic rats, the plasma levels of CRN, UN, and TP and the plasma and urine levels of Na^+ and K^+ of diabetic rats were measured at the end of 56 days. CRN and UN are two metabolic waste products that are normally removed from the body by renal excretion. In kidney diseases the renal excretion of these two substances is impaired and, as a result, they tend to accumulate in the circulation. In this work the plasma CRN level of diabetic rats was at least fourfold higher than that of normal rats. Treating the diabetic rats with either CAP, MET or TAU led to reductions of the CRN caused by diabetes, with CAP providing the greatest protection and MET the least. Pairing CAP or MET with TAU resulted in no appreciable gain in potency relative to a treatment with each compound alone, and adding CAP to MET led to an effect intermediate to those of the individual compounds. On the other hand, a combined treatment with MET-CAP-TAU was more potent than any single or binary treatment. While the present findings on the effects of CAP on the serum CRN agree with those described by Akbar et al. (2013) for rats with diabetic nephropathy, they differ from those by Katoh et al. (2000), who found CAP to lower blood pressure and to inhibit urinary albumin excretion but to fail to inhibit renal hypertrophy and elevation of the CRN clearance in STZ-treated rats. In contrast, the present results for TAU confirm those of a previous study that found that daily consumption of this sulfur-containing compound as part of the drinking water reduced the plasma levels of both CRN and UN in diabetic rats (Wang et al. 2008).

In parallel to the effects of CAP, MET and TAU on the diabetic plasma CRN, those on the plasma UN followed a similar trend, with CAP exerting a greater effect than either TAU or MET in that order. While diabetes raised the plasma UN by about 2.6-fold, in the presence of CAP this increase was only 1.4-fold above the control value; and about twofold in the presence of MET. Again TAU showed an intermediate potency (~1.7-fold increase). However, in contrast to the findings for the serum CRN, pairing TAU with either CAP or MET led, in both cases, to a gain in potency in reducing the plasma UN, more with MET-TAU (1.2-fold increase) than with CAP-TAU (1.3-fold increase). As shown for the serum CRN, a treatment of the diabetic rats with CAP-MET led to an potency intermediate to those of the individual compounds, and one with CAP-MET-TAU was able to reduce the plasma UN to a level only 10% above the control value. A study looking at the relationship between an increase in serum GLC with an increase in the circulating levels of CRN and urea in diabetic patients with progressive renal damage determined that it was more consistent with an increase of the latter than of the former compound (Shrestha et al. 2008). Furthermore, over a period of more than 15 years from the time of diagnosis of a case of type 2 diabetes in human patients, further increases in circulating CRN have been associated with the occurrence of nephropathy and macroalbuminuria and with an increased risk of cardiovascular death among those not requiring renal replacement therapy (Adler et al. 2003); although people who lived more than 25 years without any signs of kidney failure may have a decrease risk of developing it (Dabla 2010).

In diabetic rats, the plasma TP level was reduced by about 50% of the control value. This effect was reduced by TAU by more than one-half, and to a lesser,

although still significant extent, by CAP and MET. While a treatment with CAP-MET did not change the potency of MET, one with MET-TAU was marginally better than one with MET alone, and one with CAP-TAU was equipotent with TAU. Since attenuation of the TP loss caused by diabetes by a combined treatment with CAP-MET-TAU was identical to that seen with TAU alone, it is safe to conclude that TAU is the main protective factor. Furthermore, the present results confirm the view that since the progression of diabetic nephropathy is not only dependent on the activity of ACE, renoprotection should be widen to accommodate therapeutic agents with other biological actions (Bernadet-Monrozies et al. 2002). Therapy with CAP has been found to significantly impede progression to clinical proteinuria and to prevent the increase in albumin excretion rate in normotensive patients with INS-dependent diabetes mellitus and persistent microalbuminuria (Viberti et al. 1994); and MET has demonstrated the ability to significantly decrease albuminuria in patients with type 2 DM while ameliorating tubular cell injury (Nasri et al. 2013). The beneficial effects of MET on the kidney of diabetic rats, including albuminuria, could be a consequence of an ability to reduce ROS, to preserve the viability of podocytes or to promote the activation of the enzyme adenosine monophosphate-activated protein kinase, a major regulator of basal and INS-stimulated glucose uptake, lipid and protein synthesis and an inhibitor of complex I of the respiratory chain in the mitochondrion (Kim et al. 2012; Liu et al. 2008). Adding TAU to the drinking water of DM-treated diabetic rats has been reported to reduce total proteinuria and abuminuria by almost 50%, to normalize the renal cortical MDA content and to decrease the formation of advanced glycoxidation products (Trachtman et al. 1995). In common with MET, supplementation of a rat diet with TAU has been reported to suppress the progression of DN through a reduction of renal oxidant injury, LPO, renal accumulation of AGEs (Trachtman et al. 1995) and the development of fibrosis (Koh et al. 2014).

The diabetic rats were found to retain both K^+ and Na^+ in their plasma, with the increase of the former exceeding that of the latter by more than 20%. Treating these rats with CAT, MET and/or TAU was found effective in reducing these accumulations which, in general, amounted to values not exceeding the control plasma K^+ and Na^+ levels by more than 19% and 11%, respectively, with the individual compounds and becoming virtually normal when given as a binary or ternary treatment combination. Relative to the diabetic plasma levels of K^+ and Na^+, those in the urine were significantly lower than corresponding control values, with the extent of the reductions being rather similar for both K^+ and Na^+. As was the case with the diabetic plasma levels of Na^+ and K^+, CAP, MET and/or TAU were also found to effectively counteract the urinary decreases of K^+ and Na^+, which roughly was reduced by at least one-half of those seen in diabetic rats. In general, the potency differences among the various treatment compounds were both narrow and rather equivalent, with CAP and MET appearing slightly more potent than TAU on the urine $Na^{+,}$ and the reverse been the case on the urine K^+. While these actions were enhanced by pairing CAP and MET with each other or individually with TAU and by treating the diabetic rats with a ternary combination, the gain in potency relative to monotherapy was negligible. Even though the use of an ACE inhibitor like CAT can lead to

an increase in the plasma and a corresponding decrease in the urine of the K^+ level, the present study verified the opposite trend, possibly because of only an incipient renal dysfunction (Riebel et al. 2010). The improved urinary excretion of K^+ and Na^+ by MET probably reflects the enhancing effect of this drug on the glomerular filtration rate (Dorella et al. 1996) which, in turn, may reflect a renoprotective ability against GLC-induced nephropathy, including an antioxidant and antiapoptotic effect at the renal tubular level (Nasri et al. 2013). A similar argument has been advanced to explain the benefits of TAU on diabetic nephropathy (Abebe and Mozaffarin 2011), to which one can add the preservation of the normal renal anatomy and decreased tubular fibrosis (Ito et al. 2012).

Localized tissue oxidative stress is regarded as a key component in the development and progress of DN. A key initiating factor seems to be chronic hyperglycemia, and mitochondrial dysfunction seems to play a key role. Oxidative stress leading to the production of ROS is known to contribute to the oxidation of important biomolecules, including proteins, lipids, carbohydrates and DNA and, hence, to diabetic complications including nephropathy (Forbes et al. 2008). In addition, oxidative stress is regarded as an accelerating factor for the formation of MDA, a LPO end product that can be detected by immunohistochemistry in diabetic renal glomerular lesions and trough chemical tests in the plasma and urine of diabetic patients with glomerulosclerosis and mesangial expansion (Chang et al. 2005). In the present study MDA was clearly elevated in the kidney of diabetic rats, an effect that was effectively reduced to almost basal levels by CAP, MET and TAU and by their combinations. The present results on CAP confirm the results of a previous study indicating that ACE inhibitors like CAP and enalapril can be useful in lowering LPO, measured as MDA, in the diabetic kidney (Kęziora-Kornatowska 1999; Kędziora-Kornatowska et al. 2000). Furthermore, it has been reported that the administration of MET to rats made diabetic with STZ has a profound decreasing effect (almost 50%) on the renal MDA level (Erejuwa et al. 2011). However, these results are in clear contrast with those of a study in newly diagnosed type 2 diabetic patients and in which a 12 weeks treatment with this biguanide did not have a clear effect on the plasma MDA (Gupta et al. 2010). On the other hand, rats made diabetic with SZT and consuming TAU (1%) as part of the drinking water showed a marked reduction of the renal MDA (Wang et al. 2008), and the administration of this amino acid by oral gavage to Otsuka Long-Evans Tokushima Fatty rats resulted in a reduction of the urine MDA (Koh et al. 2014). Although the present study has verified that a treatment of diabetic rats with either CAP, MET or TAU and their various combinations can virtually normalize the renal MDA levels, they are, in all likelihood, operating by different mechanisms, especially since they differ quite widely in their quantitative effects on the circulating levels of GLC and HbA_{1c}. Hence, an earlier proposal suggesting that the occurrence of oxidative stress leading to a higher than normal LPO is closely dependent on the prevailing circulating levels of both GLC and HbA_{1c} (Bandeira et al. 2012) may need a revision, at least in the case of TAU, to include other mechanisms of antioxidant protection. In agreement with the results of the present work, which finds TAU to lower the renal levels of MDA, there is evidence to indicate a difference in response between different animal models of

diabetes since a 1-week consumption of this amino acid as part of the drinking water by KK mice, a genetically hyperglycemic animal model of type 2 diabetes, was able to lower the liver and pancreatic islet cell levels of MDA; but the same treatment approach failed to reproduce this effect in the liver and pancreas of alloxan-treated ICR mice, an animal model of type 1 DM (Lim et al. 1998). These results are illustrative of the variability of the effect of TAU on LPO which, in addition to the type of animal model of diabetes used, is also dependent on the area of the kidney examined (Trachtman et al. 1995).

The renal GSH concentration was moderately decreased (~35%) and that of GSSG was markedly increased (>125%) in diabetic rats. While some investigators have reported decreases of the GSH in the diabetic kidney (Anjaneyulu and Chopra 2004), others have verified no changes in either the GSH (Baştar et al. 1998) or the GSH plus GSSG contents (Bräunlich et al. 1994) in the same animal model of diabetes used here. In the present study CAP, MET and TAU were found to reduce the loss of the renal GSH, an effect that was, however, only significant with MET. On the other hand, combining MET or CAT with TAU raised the ability of these compounds to preserve the renal GSH stores, and providing the diabetic rats with MET-CAP or MET-CAP-TAU led to only a negligible loss of the renal GSH. In the case of GSSG, however, all the three test compounds were able to lower the accumulation of this index of oxidative stress in the diabetic kidney, with TAU appearing much more effective than MET and CAP in that order. A combined treatment with binary combinations of CAP and MET either with TAU or with each other led to a reduction of the diabetic GSSG concentration to level insignificantly different from the control value. On the other hand, a concurrent treatment with the three compounds reduced the diabetic GSSG value to a nearly normal value. Based on these results and on the effects of the test compounds on the redox ratio (GSH/GSSG), it can be inferred that all the test compounds used in the present study can significantly antagonize the lowering effect of diabetes on the ratio. In this case, TAU appeared as the most protective, MET as the least and CAP providing an intermediate protection. In addition, the protective effect of MET was found to increase not only in the presence of TAU but also of CAP.

Another contributing factor to diabetic microvascular disease appears to be a deficiency of antioxidant enzymes such as CAT and SOD, which are known to participate in the respective detoxification of harmful hydrogen peroxide and superoxide anion radical (Sindhu et al. 2004); and of the selenoprotein GPx, which simultaneously reduces organic hydroperoxides to the corresponding alcohols and hydrogen peroxide to water, with GSH serving as a co-substrate (Hamanishi et al. 2004). A review of the scientific literature has disclosed that a wide variation exists in the reported activities of these enzymes in the diabetic kidney, with some studies finding higher than normal activities of GPx and SOD along with a lower of CAT activity (Erejuwa et al. 2011), a decreased CAT and SOD activity plus an increased GPx activity (Wohaieb and Godin 1987), a generalized decrease in the activity of all three enzymes (Sadi et al. 2012) or even no changes (Elmali et al. 2004). Regardless of the trend of the changes in the activities of these enzymatic antioxidant defenses, their deficiencies emerge as an important factor in the etiology of diabetic renal

complications since in diabetes there is an increase in the production of both hydrogen peroxide and lipid peroxides by renal cells and a balance between the production of oxidants, notably superoxide anion and hydrogen peroxide, and the status of antioxidant defenses in the form of antioxidant enzymes determines the extent of the oxidative stress and the occurrence of renal injury (Asaba et al. 2005). In the present study the activities of all three antioxidant enzyme were markedly decreased in the diabetic kidney, by ~70% in the case of CAT and GPx and by more than 80% in the case of SOD, compared to corresponding activities in normal kidneys. Treating the diabetic rats with CAP, MET or TAU raised these values by at least one-half of the diabetic value, effects that were improved to when these compounds were used together, more as a ternary combination than as a binary combination with TAU.

5 Conclusions

This study has verified that in rats made diabetic with STZ, an oral treatment with CAP, MET or TAU and their binary and tertiary combinations can lead to a marked improvement of the metabolic, biochemical and functional alterations associated with the diabetic state, with the differences in potency among the treatment compounds varying according to parameter being evaluated and the treatment approach used, being usually greater when TAU was combined with either MET or CAP or, better, with both compounds. When used on naive rats, the test compounds were usually without significant effects on the parameter evaluated in the present study.

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Part III
Taurine for Energy Production and Muscle

Increased *N*-Acetyltaurine in the Skeletal Muscle After Endurance Exercise in Rat

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Abstract Taurine is metabolized to a novel metabolite, *N*-acetyltaurine (NAT), through N-acetylation with acetate. Furthermore, NAT production increases when the endogenous production of acetate is elevated in some situations, such as alcohol catabolism and endurance exercise. We have previously reported that both the serum concentration and urinary excretion of NAT from humans were increased after endurance exercise, and that NAT was secreted by cultured skeletal muscle cells exposed to both acetate and taurine. The present study evaluated the hypothesis that NAT is synthesized in the skeletal muscle after endurance exercise. Normal rats were loaded to a transient treadmill running until exhaustion. Serum, skeletal muscle, and liver were collected immediately after the exercise. The NAT concentration in the plasma and in the soleus muscle from the exercised rats was significantly increased compared to that in the samples from the sedentary control rats. There was a significant positive correlation in the NAT concentration between the plasma and soleus muscle. The NAT concentration in the liver was unchanged after the endurance exercise. These results confirm that the significantly increased NAT in both the serum and urine after endurance exercise is derived from NAT synthesis in the skeletal muscle.

Keywords Treadmill running • *N*-Acetylation • Acetate • Acetyl-CoA • Energy production

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Abbreviations

ACS2	Acetyl-CoA synthetase 2
EX group	Exercise group
IS	Internal standard
NAT	*N*-acetyltaurine
PDH	Pyruvate dehydrogenase
SED group	Sedentary group
TCA	Trichloroacetic acid

1 Introduction

Taurine (2-aminoethanesulfonic acid) is the most abundant free amino acid-like compound found in various mammalian tissues, including skeletal muscle (Awapara 1956; Jacobsen and Smith 1968; Huxtable 1980). Many physiological and pharmacological actions of taurine have previously been established, such as stabilization of the cellular plasma membrane, osmoregulation, anti-oxidant effects, and detoxification (Pasantes et al. 1998; Huxtable 1992; Miyazaki and Matsuzaki 2014; Nakamura et al. 1993; Miyazaki 2010; Nieminen et al. 1988). The most established activity of taurine is the conjugation with bile acids in the liver to increase the excretion of bile acid into the bile through enhancement of the solubility of hydrophobic bile acids, which reduces the toxicity of hydrophobic bile acid (Danielsson 1963; Sjovall 1959). Based on the conjugation with bile acids, taurine has the potential to react with many substances.

In 2012, Shi et al. reported that taurine was metabolized to *N*-acetyltaurine (NAT) in the liver by acetylation with acetate (Shi et al. 2012), which is a metabolite in ethanol detoxification (Buckley and Williamson 1977). NAT is transported from the liver to the circulation, and then, is excreted into the urine. Because acetate produced through alcoholic catabolism is finally metabolized to H_2O and CO_2 in extrahepatic tissues, mainly in skeletal muscle (Zakhari 2006), it is suggested that taurine might enhance the excretion of acetate in urine before metabolism to H_2O and CO_2.

Acetate is also produced through hepatic lipid oxidation when energy production is in high demand in the skeletal muscle, such as during endurance exercise. We have previously reported that both the serum concentration and urinary excretion of NAT were significantly increased after endurance running by humans (Miyazaki et al. 2015). In addition, we have confirmed that NAT was excreted into the culture medium from cultured skeletal muscle cells incubated with both acetate and taurine (Miyazaki et al. 2015). Thus, our previous studies indirectly suggested that the increased NAT in both serum and urine after endurance

exercise might be derived from the skeletal muscle. The present study aimed to evaluate the NAT concentration in the skeletal muscle *in vivo* after endurance exercise.

2 Methods

2.1 Experimental Animal and Exercise

Male Fischer 344 rats (Japan SLC, Shizuoka, Japan), 10 weeks of age, were randomly divided into two groups after 1 week acclimatization: a sedentary group (SED: n = 7, body weight 195 ± 4 g; mean ± SD) and an exercise group (EX: n = 8, 189 ± 4 g). Rats in the EX group were habituated to treadmill running for 5 days by gradual increases in both the running speed and duration for up to 45 m/min and 10 min, respectively. After 1 day following the habituation period, rats in the EX group were fasted for 3 h, and, put on the treadmill for 15 min. Thereafter, the rats ran on the treadmill at 40 m/min until exhaustion based on the justification point previously reported (Dohm et al. 1980; Miyazaki et al. 2004). We found that the running time until exhaustion was 40.1 ± 3.0 min. Immediately after the exercise, the rats were euthanized by cervical dislocation under anesthesia with pentobarbital (*i.p.* 64.8 mg/kg body weight). In the SED group, the rats were euthanized following placement on the treadmill for 15 min without exercise. Plasma, liver, and soleus muscle that contain abundant taurine, were collected, and kept at −80 °C until analysis.

2.2 Taurine and NAT Analyses

For taurine quantification, muscle and hepatic tissues were homogenized with a 15-fold volume (*w/v*) of 5% trichloroacetic acid (TCA) solution, and centrifuged at 6200 × *g*, 4 °C for 20 min. In addition, 100 μL of plasma was mixed with the TCA solution, and centrifuged at 12000 × *g*, 4 °C for 15 min. Thereafter, the supernates were used for taurine quantification using an automatic amino acid analyzer. The NAT concentration was quantified by an HPLC-MS/MS system according to our previously reported method. Standard and internal standard (IS) NATS were synthesized from taurine and 2-aminoethane-d4 sulfonic acid (taurine-d4), respectively, by reaction with acetic anhydride. Muscle and hepatic tissues were homogenized with a tenfold volume (*w/v*) of 1% formic solution. After centrifugation at 3500 × *g* at 4 °C for 10 min, the supernate was collected for analysis. Five μL of plasma and of the supernate of the homogenized tissue were mixed with 1 ng NAT-d4 as an IS

in 50 μL of acetonitrile-water (19:1, *v/v*), and centrifuged at 2000 × *g* for 1 min. The supernate was evaporated to dryness at 80 °C under a nitrogen stream. The residue was redissolved in 60 μL of 1% formic acid, and an aliquot (5 μL) was analyzed by HPLC-MS/MS.

2.3 Statistic Analysis

Statistical significance was determined by the unpaired Student's *t*-test. Each value was expressed as the mean ± SEM. Differences were considered as statistically significant when the calculated *P*-value was less than 0.05. Correlations between plasma and tissue levels were analyzed by the Pearson's correlation coefficient.

3 Results

3.1 Taurine Concentrations in Plasma, Skeletal Muscle, and Liver From Rats after Transient Endurance Running Exercise

After the endurance treadmill exercise, the plasma taurine concentration in the EX group was significantly increased compared to that in the SED group (Fig. 1). On the other hand, the taurine concentration in the soleus muscle was significantly

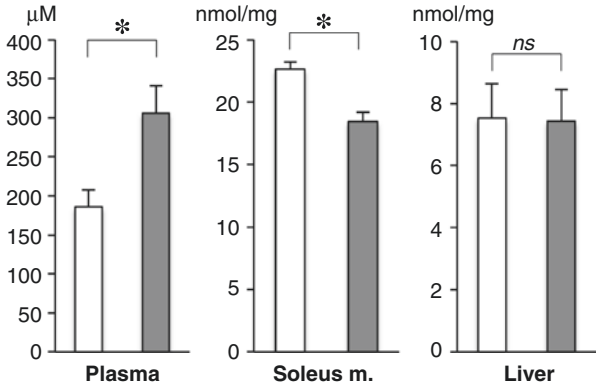

Fig. 1 Taurine concentration in plasma, skeletal muscle, and liver after a transient endurance running exercise until exhaustion. Opened and closed columns show the sedentary (n = 7) and exercise (n = 8) groups, respectively. Tissue taurine concentration is expressed as per tissue wet weight. Data are the mean ± SEM. **P* < 0.05 by unpaired Student's *t*-test. *Soleus m.* soleus muscle, *ns* no significant difference

lower in the EX group than that in the SED group. There was no significant difference in the hepatic taurine concentration between the two groups.

3.2 *NAT Concentration in Plasma, Skeletal Muscle, and Liver From Rats after Transient Endurance Running Exercise*

Similar to the taurine concentration, the NAT concentration in plasma was significantly higher in the EX group than that in the SED group (Fig. 2). Furthermore, the NAT concentration in the soleus muscle in the EX group was also significantly increased compared to that in the SED group. In the liver, there was no significant difference in the NAT concentration between the two groups.

This study found that there was a significant positive correlation in the NAT concentration between the plasma and skeletal muscle ($y = 4.1\times + 43.7$, $R^2 = 0.2437$, $P < 0.05$).

4 Discussion

In the present study, the NAT concentration in both plasma and skeletal muscle of rats was significantly increased after transient endurance treadmill running with a significant positive correlation to the exercise. Previously, we reported in humans that serum NAT concentration was significantly increased after an endurance exercise (Miyazaki et al. 2015). We also confirmed in the cell culture study that NAT

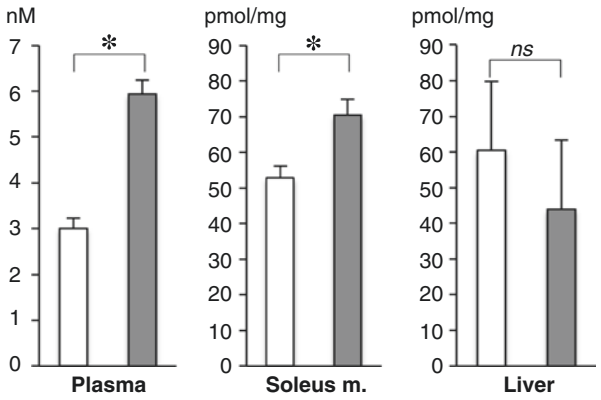

Fig. 2 NAT concentration in plasma, skeletal muscle, and liver after a transient endurance running exercise until exhaustion. Opened and closed columns show the sedentary (n = 7) and exercise (n = 8) groups, respectively. Tissue NAT concentration is expressed as per tissue wet weight. Data are the mean ± SEM. *$P < 0.05$ by unpaired Student's *t*-test. *NAT N*-acetyltaurine, *Soleus m.* soleus muscle, *ns* no significant difference

was synthesized in skeletal muscle exposed to both acetate and taurine in the media. Our previous and present results in human, rat, and cell culture suggest that NAT is synthesized from both acetate and taurine in the skeletal muscle and is excreted into the circulation with endurance exercise.

We have reported a significant decrease in the taurine concentration in rat skeletal muscle dependent on the exercise duration up to exhaustion (Matsuzaki et al. 2002). In the present study, the taurine concentration was significantly decreased in the soleus muscle in which the NAT concentration was significantly decreased after endurance exercise. It is suggested from these results that the significant decrease in muscular taurine concentration after endurance exercise might be partly due to utilization of taurine for NAT synthesis.

During endurance exercise, lipid metabolism for energy production is activated dependently on exercise duration and/or intensity. With β-oxidation of fatty acids in the liver, acetyl-CoA is metabolized to ketone bodies that are an energy source for extrahepatic tissues, mainly skeletal muscle and brain. In the ketogenic conditions during endurance exercise, acetate is also metabolized from the acetyl-CoA in the liver by the acetyl-CoA hydrolase, and then, it is reconverted to acetyl-CoA by the acetyl-CoA synthetase 2 (ACS2) in the mitochondria of the skeletal muscle for energy production (Fig. 3) (Fukao et al. 2004; Luong et al. 2000; Sakakibara et al. 2009). Because the metabolism from acetate of acetyl-CoA by the ACS2 is carried

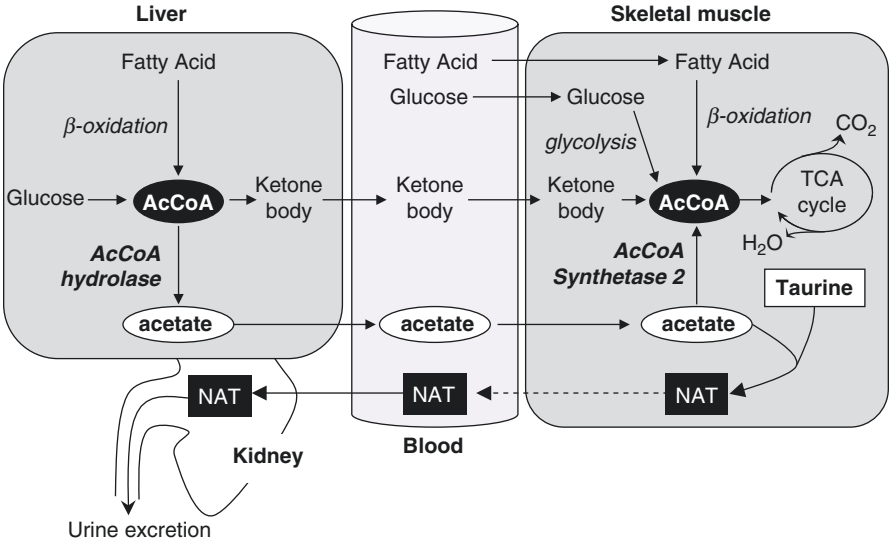

Fig. 3 Putative metabolic pathways of acetate and NAT during/after endurance exercise between the liver and skeletal muscle. In endurance exercise, NAT is likely to be synthesized from taurine and acetate in the skeletal muscle. By synthesis of NAT, taurine may play a role to prevent from acetate-induced acetyl-CoA accumulation in the mitochondrial, which is a possible cause of metabolic abnormality or delayed metabolic normalization in the recovery period after endurance exercise. *AcCoA* acetyl-CoA, *NAT N*-acetyltaurine

out in one reaction, acetate is easier to convert to energy when compared with other nutrients, including carbohydrates, fatty acids, ketone bodies, and amino acids, which require multiple metabolic reactions to form acetyl-CoA in skeletal muscle.

Although the energy expenditure declines after endurance exercise, acetate needs to be continuously broken down into H_2O and CO_2 via acetyl-CoA in the skeletal muscle. These findings suggest that acetate could cause excess acetyl-CoA accumulation in the skeletal muscle during the recovery period after endurance exercise. Excess acetyl-CoA accumulation in the mitochondria of the skeletal muscle leads to a metabolic imbalance. Mitochondrial acetyl-CoA accumulation inhibits negative feedback of the activity of the pyruvate dehydrogenase (PDH) complex that catalyzes an irreversible reaction from pyruvate to acetyl-CoA, through upregulation of the pyruvate dehydrogenase kinase 4 gene (Kerbey et al. 1976; Sugden and Holness 2003; Wu et al. 1998). The reduction of PDH activity induces a delayed flux through glycolytic reactions, and consequently, causes insulin resistance (Hoy et al. 2009; Fueger et al. 2007; Furler et al. 1991; Furler et al. 1997; Katz et al. 1991). Indeed, our previous study confirmed that the excess acetyl-CoA accumulation in the mitochondria induced by exogenous treatment of acetyl-carnitine that is the end product of fatty acid β-oxidation significantly decreased insulin-dependent glucose uptake in cultured C2C12 myotubes (Miyamoto et al. 2016). Therefore, it is necessary to eliminate the acetate from the skeletal muscle as soon as possible after the endurance exercise.

Furthermore, Takahashi et al. have reported a mouse study where taurine administration immediately after a transient endurance treadmill test significantly facilitated the recovery of muscle glycogen concentration at 120 min after the exercise (Takahashi et al. 2014). The effect of taurine administration on glycogen recovery might be due to the acceleration of glucose uptake in the skeletal muscle, because post exercise taurine administration significantly inhibited the elevation of the blood glucose concentration following oral glucose ingestion during the recovery period (Takahashi et al. 2014). The acceleration of glycogen recovery and glucose uptake in the skeletal muscle by taurine administration after the endurance exercise likely prevents excess mitochondrial acetyl-CoA accumulation induced by acetate through the conversion of taurine to NAT. Thus, taurine might play a role in eliminating acetate through NAT synthesis in the skeletal muscle to prevent mitochondrial acetyl-CoA accumulation and to normalize muscular energy metabolism after the exercise.

5 Conclusion

In summary, the present study shows that the NAT concentration in rat skeletal muscle was significantly increased by transient endurance exercise and showed a significant positive correlation with the plasma NAT concentration. These findings suggest that taurine in skeletal muscle might react with the acetate that is supplied from hepatic tissue during exercise, and thus the metabolite NAT would be excreted into the urine in order to prevent intramuscular excess accumulation of acetyl-CoA that might induce a metabolic imbalance after exercise.

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Gestational Protein Restriction in Wistar Rats; Effect of Taurine Supplementation on Properties of Newborn Skeletal Muscle

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Abstract Taurine ameliorates changes occurring in newborn skeletal muscle as a result of gestational protein restriction in C57BL/6 mice, but taurine supplementation effects may be exaggerated in C57BL/6 mice due to their inherent excessive taurinuria.

We examined if maternal taurine supplementation could ameliorate changes in gene expression levels, properties of mitochondria, myogenesis, and nutrient transport and sensing, in male newborn skeletal muscle caused by a maternal low protein (LP) diet in Wistar rats.

LP diet resulted in an 11% non-significant decrease in birth weight, which was not rescued by taurine supplementation (LP-Tau). LP-Tau offspring had significantly lower birth weight compared to controls. Gene expression profiling revealed 895 significantly changed genes, mainly an LP-induced down-regulation of genes involved in protein translation. Taurine fully or partially rescued 32% of these changes, but with no distinct pattern as to which genes were rescued.

Skeletal muscle taurine content in LP-Tau offspring was increased, but no changes in mRNA levels of the taurine synthesis pathway were observed. Taurine transporter mRNA levels, but not protein levels, were increased by LP diet.

Nutrient sensing signaling pathways were largely unaffected in LP or LP-Tau groups, although taurine supplementation caused a decrease in total Akt and AMPK protein levels. PAT4 amino acid transporter mRNA was increased by LP, and normalized by taurine supplementation.

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In conclusion, gestational protein restriction in rats decreased genes involved in protein translation in newborn skeletal muscle and led to changes in nutrient transporters. Taurine partly rescued these changes, hence underscoring the importance of taurine in development.

Keywords Maternal low protein • Taurine • Low birth weight • Skeletal muscle

Abbreviations

ACTB	Beta-actin
ADO	Cysteamine dioxygenase
ATF4	Activating transcription factor 4
CDO	Cysteine dioxygenase
CK	Creatine kinase
CON	Control
CS	Citrate synthase
CSAD	Cysteinesulfinic acid decarboxylase
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
gDNA	Genomic DNA
GLUT	Glucose transporter
IUGR	Intrauterine growth restriction
LP	Low protein
LP-TAU	Low protein diet with taurine supplementation
MEF	Myocyte enhancer factor
MHC	Myosin heavy chain
mtDNA	Mitochondrial DNA
Myf5	Myogenic factor 5
Myo	Myosin
MyoD	Myogenic differentiation 1
Ndufb6	NADH Dehydrogenase (Ubiquinone) 1 beta subcomplex 6
Ndufs1	NADH Dehydrogenase (Ubiquinone) Fe–S Protein 1
PDK1	Phosphoenolpyruvate carboxykinase 1
PDK4	Pyruvate dehydrogenase kinase 4
PGC-1α	Peroxisome proliferator-activated receptor γ, coactivator-1α
PGC-1β	Peroxisome proliferator-activated receptor γ, coactivator-1β
PPARα	Peroxisome proliferator activated receptor α
PPARγ	Peroxisome proliferator activated receptor γ
REDD1	DNA-Damage-Inducible Transcript 4 (DDIT4)
S6K1	Ribosomal Protein S6 Kinase
TauT	Taurine transporter
TFAM	Transcriptional factor A, mitochondrial

1 Introduction

Low birth weight is a hallmark of adverse developmental programming (Hales and Barker 1992) and is associated with a higher risk of developing metabolic disease in later life, including increased risk of impaired insulin secretion (Snoeck et al. 1990; Petrik et al. 1999), and insulin resistance in skeletal muscle (Ozanne et al. 2005). A clear association between low birth weight and insulin resistance has been observed, clearly not with a classical genetic causality, as demonstrated in twin studies (Poulsen et al. 1997). However, the exact mechanism by which low birth weight, as a result of intrauterine growth restriction (IUGR), leads to an increased risk of type 2 diabetes and metabolic syndrome later in life, is still largely unknown.

Skeletal muscle is known to play a key role in whole body metabolism, glucose clearance, and insulin sensitivity. Therefore, a reduction in skeletal muscle mass, and thereby altered muscle myogenesis and function as observed after IUGR, may be causally related to the increased risk of developing metabolic disease in later life (Abdul-Ghani and DeFronzo 2010).

Several animal models have been used to mimic impaired fetal growth (Armitage et al. 2004) including the most studied one, gestational protein restriction. In rats, this model displays a low birth weight of the offspring, impaired glucose homeostasis and insulin resistance (Ozanne et al. 1996, 2005), as well as β-cell dysfunction later in life (Boujendar et al. 2002; Reusens et al. 2008).

Taurine is a semi-essential amino sulphonic acid, which is not a protein component. Taurine is known to be involved in physiological functions such as bile acid formation, intracellular volume regulation, heart function, mitochondrial tRNA conjugation, and reproduction (Lambert et al. 2015).

Human IUGR fetuses have decreased plasma taurine levels (Economides et al. 1989; Cetin et al. 1990), which has also been observed in animal models of IUGR (Reusens et al. 1995; Wu et al. 1998). Taurine deficiency during development and in adult life results in several pathological conditions, including dysfunction of skeletal- and heart muscle (Sturman 1993). Furthermore, the taurine transporter (TauT) knockout mice have revealed the importance of maintaining normal taurine concentrations in tissues, especially in skeletal muscle and heart. In these mice, taurine concentration in skeletal muscle and heart are decreased by 98% compared to wild type mice (Warskulat et al. 2004; Ito et al. 2008, 2014a, b) and the mice exhibit lower body weight, decreased skeletal muscle growth, and lower exercise capacity (Heller-Stilb et al. 2002; Warskulat et al. 2004; Ito et al. 2008). These observations indicate that taurine is very important during development and that taurine deficiency during gestation may lead to adverse developmental programming in skeletal muscle of the offspring.

Maternal taurine supplementation during gestation is, at least partially, able to prevent the effects induced by low birth weight in several animal models of low birth weight and can also prevent the adverse offspring phenotype seen as a consequence of maternal obesity (Li et al. 2013) as well as a maternal high fructose

intake (Li et al. 2015). Maternal taurine supplementation has, in the context of gestational protein restriction, been shown to stabilize proliferation and vascularization of the pancreas (Boujendar et al. 2002, 2003) as well as prevent all changes in mRNA expression levels in the pancreas of newborn Wistar rats (Reusens et al. 2008). Likewise, maternal taurine supplementation rescued a large portion of the changes in mRNA expression levels caused by gestational protein restriction in both skeletal muscle and liver (Mortensen et al. 2010a) in newborn C57BL/6J mice. However, the C57BL/6J mouse strain has a defect in taurine renal reabsorption (Harris and Searle 1953) that leads to excessive taurinuria (Chesney et al. 1976). Therefore, the effects of taurine supplementation may be exaggerated when using this animal model.

In the present study, we used gene expression microarrays to examine the effect of maternal taurine supplementation on changes in overall gene expression levels, in newborn Wistar rat hind leg skeletal muscle subjected to gestational protein restriction. Furthermore, we examined if taurine supplementation could ameliorate maternal low protein induced changes in taurine biosynthesis, mitochondrial properties, myogenesis, amino acid transport, and nutrient sensing.

2 Materials and Methods

2.1 Animals and Chemicals

All experimental procedures were approved by The Danish Animal Experiments Inspectorate (license: 2008/561-1515) and were performed according to the ARRIVE (Animal Research: Reporting In vivo Experiments) guidelines. Female Wistar rats (N = 38, 8 weeks of age) were purchased from Taconic Europe A/S (Ejby, Denmark) on day 1 of pregnancy. The animals were housed with a 12 h light/dark cycle, constant temperature (23 °C), and constant humidity (42%) at the University of Copenhagen, Faculty of Health and Medical Sciences, Panum Institute. Unless otherwise stated, chemicals were from Sigma-Aldrich (St. Louis, MO, USA) and were of analytical grade or better.

2.2 Animal Study Design and Diet

The pregnant Wistar dams were randomly assigned to either a low-protein diet (LP, N = 15) with a protein content of 5% or a matching control diet containing 20% protein (CON, N = 15) or an LP diet supplemented with 2% taurine in the drinking water (LP-TAU, N = 8) from day 1 of gestation. Dams were weighed on day 1 of gestation and on day 20 (the day before giving birth). The animals had ad libitum access to food and water. All diets were isocaloric and obtained from Hope Farms, catalog #4400.12 and #4400.00 for 5% and 20% protein, respectively

(Woerden, NL). Ten rats were not pregnant or gave birth to very small litters (N of pups <2) and were removed from the study resulting in CON N = 10, LP N = 10, LP-TAU N = 6.

2.3 Animal Sample Collection

At birth, hind leg skeletal muscle was collected from the pups by cutting off the hind legs followed by dissection using a stereomicroscope; the skin was peeled off and the skeletal muscle tissue dissected free of skin and bone after which it was snap-frozen in liquid nitrogen and stored at −80 °C until further analysis. The hind leg skeletal muscle from three male pups from the same dam was pooled resulting in an N of one per dam. Gender was determined visually before dissection by comparing the distance between the anus and the genital papilla (Whishaw and Kolb 2004).

2.4 RNA Purification and Quantitative Real-Time PCR

RNA purification and quantitative real-time PCR were performed as previously described (Larsen et al. 2013). GAPDH mRNA levels were used for normalization between samples. The primers were designed using the National Center for Biotechnology Information "primer design tool" online using Rattus Norvegius (organism 10116) Refseq RNA database and were designed according to the MIQE guidelines. Primer sequences (gene symbol, genbank acession number: Forward & reverse primer 5′-3′): 4f2hc, NM_019283.3: GCTGACATTGTAGCCACCAAA & GCAATCAAAAGCCTGTCCTCA. ADO, NM_001107626.2: CCGGTCACT TACATGCACATC & CGTACAGCACCTTGAGCATAC. ATF4, NM_024403.2: TCAGAATGGCTGGCTATGGAT & AAGCTCATCTGGCATGGTTTC. Cat2a, NM_022619.3: CCTTATCAACAAGTCTTCTGGGTT & GGGTGAC TGCCTCTTACTCA. Cat2b, NM_001134686.2: TTCCCAATGCGTCGTGTAATC & GCACCCGATGACAAAGTAGC. CDO, NM_052809.1: GCCTTCACTTGTACA GTCCAC & CTCCAGTGAACCTGAAGTTGTAAAT. CS, NM_130755.1: CACTGTGGA CATGATGTACGG & ATACTATAGCCTCGGAAGCGG. CSAD, NM_001134454.1: TGGTCATGGAGCCCAAGTTC & CATCATGGTTCCCTTCTTCACC. GLUT1, NM_138827.1: GTGTATCCTGTTGCCCTTCTG & GCTTTTTCAGCACAC TCTTGG. GLUT4, NM_012751.1: ATGGGTTTCCAGTATGTTGCG & TAAGAGAG AAGGTGTCCGTCG. LAT1, NM_017353.1: GGGGAACATTGTGTTGGCATT & TGGGCAAGGAGATGATGATGG. Mef2a, NM_001014035.1: CATAAAATCGC ACCTGGCTTG & AACTCCCTGGGTTAGTGTACG. MHCIb, NM_017240.2: CGTTCTGTCAATGACCTCACC & TTTCCTCCTCGTACTGTTCCC. MHCIIa, NM_001135157.1: AACCATTCAGAGCAAAGACGC & GCTCCTGCTTCAG TTTTCACA. MHCIIb, NM_019325.1: ATCAGTGTTTGTGGTGGATGC & TGCACGGTTGCTTTCACATAG. MHCIIx, NM_001135158.1: CTTCAAGT

TCAGACCCACGG & GGAGAGTCTGCCTTTAGGGATG. MHCemb, NM_012604.1: CCACAGTCAGAGGTCCCATAG & ATGCCAAACACTTCCATCTCG. MHCper, NM_001100485.1: GTCCTATGTGAAGAGCGTCATAC & TTAACGGTCACT TTCCCTCCT. Myf5, NM_001106783.1: TCAAACGCATGTGCTTCAGATAA & AGATAAGTCTGGAACTGGAGGAC. Myo5a, NM_022178.1: TCCTGA AGAAAAGAGGTGACGA & GGTCAAAATTGGTGAGGCAGT. Myo5b, NM_017083.1: CAGACAAACAGCCAGACTGAG & AGCCGAGTTCTCCATCTTCAT. MyoD1, NM_176079.1: GCCTGAGCAAAGTGAACGA & CAGACCTTCAA TGTAGCGGATG. MyoG, NM_017115.2: AGGAAGTCTGTGTCTGTGGAC & GCTCAATGTACTGGATGGCAC. Ndufb6, NM_001106646.1: TGGCACGAA GCCCAGAATA & TCTCCAGTCTCCAGAATTGTATC. Ndufs1, NM_001005550.1: GTGGGAAGTAACATTGTGGTTAG & TGCCGTTTCAGTCCATCATAG. PAT4, NM_001108127.1: CTGTGAGAGGAGAAGTGTGGA & CTATCGGAA GGTTGTGGGGAT. PDK1, NM_053826.2: TTGACTGTGAAGATGAGTGACC & GCCAATCCGTAACCAAATCCA. PDK4, NM_053551.1: ACACATAC TCCACTGCTCCAA & TAACCAAAACCAGCCAAAGGG. Pgc1a, NM_031347.1: GAGTACAACAATGAGCCCGC & CAATCCGTCTTCATCCACCG. Pgc1b, NM_176075.2: AAGTAAGAGAGGCCAGAAGCA & ATTGCGTTT TCTCAGGGTAGC. PPARa, NM_013196.1: GCGGACTACCAGTACTTAGGG & GGAGAGAGGGTGTCTGTGATG. PPARg, NM_013124.3: AGAGCCTTCAAA CTCCCTCAT & CTTCAATCGGATGGTTCTTCGG. REDD1, NM_080906.2: CATCAGTTCGCTCACCCTTC & GGACGAGAAACGATCCCAAAG. Sirt1, XM_003751934.1: TTCAGAACCACCAAAGCGG & CCCACAGGAAA CAGAAACCC. Sirt3, NM_001106313.2: TCTGGTATCCCTGCCTCAAAG & ATCACGTCAGCCCGTATGTC. SNAT1, NM_138832.1: AACTCAAAGACGGTGTACGCT & TGCTCTGGTACTTGTGAAGGA. SNAT2, NM_181090.2: CGAAGGAGGGTCTTTATTATACGAAC & TGACAATGGGAGAATGAGCAC. TauT, NM_017206.1: TGGACAGCCAG TTTGTTGAAG & GCAATGAAGATTTCCCGACGA. TFAM, NM_031326.1: TGACCTTTTGAGCCTTGACAG & CGCACAGTCTTGATTCCAGTT.

2.5 *Affymetrix Gene Expression Microarray*

For gene expression profiling using GeneChip Rat Gene 1.0 ST Arrays (Affymetrix Inc., Santa Clara, CA, USA), four RNA samples, isolated using Qiazol from hindleg skeletal muscle as described above, were chosen at random from each group and analyzed as previously described (Mortensen et al. 2014) followed by analysis using R 3.2.2 (open source: http://www.r-project.org) and Bioconductor 3.1 (open source: http://www.bioconductor.org) with all subsequent calculations carried out on log2 transformed gene expression values. Raw CEL files were normalized using the Robust Multi-array Average method. The expression level threshold (detection limit) was set to the median of the antigenomic (negative controls) probeset expression levels and calculated for each sample and further analyzed as described in the

GEO dataset (see below) resulting in 1224 genes changed by the LP diet, of which 895 were known genes. Out of the 895 changed genes, the changes to 88 genes were fully rescued by taurine and the changes to 195 genes were partially rescued by taurine (for calculations and data, see the GEO dataset).

Overrepresentation of gene sets were estimated using DAVID v6.7 (Huang et al. 2009) with probe IDs for the significantly changed genes as input (either all, down-regulated or up-regulated by LP diet) compared against the list of detected probes examining the categories: GOTERM_BP_FAT, GOTERM_CC_FAT, GOTERM_MF_FAT, PANTHER_BP_ALL, PANTHER_MF_ALL, KEGG_PATHWAY, and PANTHER_PATHWAY.

The raw CEL files as well as the analysis results have been deposited in the NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) with the GEO accession number GSE89054.

2.6 Biochemical Assays

Biochemical assays measuring citrate synthase (CS) activity, creatine kinase (CK) activity as well as the taurine content of skeletal muscle tissues were carried out as described previously (Mortensen et al. 2006, 2010b; Larsen et al. 2015)

2.7 Western Blot Analysis

Western blot analysis of skeletal muscle tissue was carried out as previously described (Larsen et al. 2013) using antibodies purchased from Cell Signaling Technology: total and phosphorylated S6K1 (Thr389) (cat #9234 and #2708); and phosphorylated AMPKα (Thr172) (cat #2603 and #2535); total and phosphorylated Akt (Thr308 and Ser473) (cat #4691, #2965, and #4060). Protein expression of β-actin (ACTB, cat #Sc-47778) and TauT (#166640) were obtained from Santa Cruz Biotechnology. The antibody REDD1 (cat #10638-1-AP) was obtained from Proteintech, OXPHOS obtained from Abcam (cat#ab-110413). Beta-actin (ACTB) protein levels were used for normalization between samples.

2.8 mtDNA

The mtDNA:gDNA ratio was determined as previously described (Gam et al. 2014) using primers specific for either mitochondrial ND1 (mtDNA) or genomic GAPDH (gDNA). The mtDNA:gDNA ratio was calculated as $2*2^{-(C_t\text{Dloop} - C_t\text{GAPDH})}$.

2.9 Statistics

All data were analyzed using ANOVA followed by post-hoc tests correcting for multiple testing using Tukey-Kramer using SAS 9.4. All mRNA data were log-transformed before statistical analysis in order to obtain a normal distribution. Data are presented as means ± s.e.m. A p-value less than 0.05 was considered significant. NS denotes non-significance, $p > 0.1$. A p-value between 0.05 and 0.1 was considered a trend.

3 Results

3.1 Birth Weight, Litter Size, Body Weight, and Food Intake

The LP diet group exhibited an 11% decrease in birth weight, but this was not significantly different from CON. However, LP-TAU was significantly lighter at birth compared to CON. Litter size was similar between groups, but LP dams ate significantly less and also gained 17% less weight during pregnancy compared to CON (Table 1).

3.2 Effect of LP Diet and Taurine Supplementation on Taurine Content, Transport, and Synthesis

Taurine content in newborn skeletal muscle increased in the LP-TAU group compared to both CON and LP with 43% and 40% respectively, as seen in Fig. 1a. Changes in taurine content may alter the expression levels of the enzymes involved

Table 1 Birth weight, litter size, dam body weight, and dam food intake

	CON	LP	LP-TAU	ANOVA
Birth weight (g)	5.6 ± 0.14	5.0 ± 0.21	4.7 ± 0.29[a]	0.01
Litter size	11.8 ± 0.7	10.9 ± 0.6	12.8 ± 1.0	NS
Dam start weight (g)	203.2 ± 3.1	202.1 ± 3.3	205.3 ± 3.2	NS
Dam end weight (g)	325.4 ± 4.6	269.8 ± 4.5[a]	279.7 ± 3.1[a]	<.0001
Dam weight gain	122.2 ± 4.0	67.7 ± 2.8[a]	74.3 ± 4.8[a]	<.0001
Dam food intake (g)	410 ± 9.5	362.9 ± 7.2[a]	380 ± 11.5	0.003
Dam food intake (KJ)	5505.0 ± 127.0	4869.7 ± 97.3[a]	5102.3 ± 153.8	0.003

Numbers are mean ± SEM

[a]Significantly different from CON ($p < 0.05$)

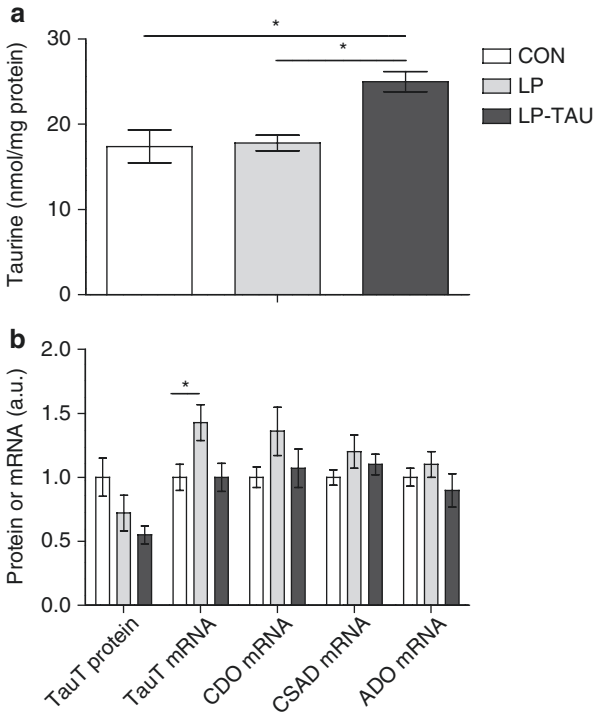

Fig. 1 Taurine concentration, transport and biosynthesis in skeletal muscle of newborn male rats subjected to maternal LP diet with 2% taurine in drinking water. (**a**) Taurine content in newborn skeletal muscle (ANOVA $p = 0.012$). (**b**) Taurine transport and synthesis: Protein levels of TauT (ANOVA NS) and mRNA levels of TauT (ANOVA $p = 0.03$), CDO (ANOVA NS), CSAD (ANOVA NS), ADO (ANOVA NS). Numbers are mean ± SEM. a.u. (arbitrary units). *Denotes a significant difference in the Tukey-Kramer post hoc test between the indicated groups

in taurine biosynthesis (cysteinesulfinic acid decarboxylase (CSAD), cysteine dioxygenase (CDO), and cysteamine dioxygenase (ADO)). However, we found no difference in the mRNA levels of these genes between groups (Fig. 1b). Although the TauT mRNA levels increased 42% in LP compared to CON, the protein levels of TauT were similar between groups (Fig. 1b).

3.3 Mitochondrial Properties

The activity of CS tended to increase in LP-TAU compared to CON as shown in Fig. 2a. Transcriptional factor A, mitochondrial (TFAM) mRNA levels decreased 20% in LP-TAU compared to CON (Fig. 2b). LP diet decreased NADH Dehydrogenase (Ubiquinone) Fe–S Protein 1 (Ndufs1) mRNA levels with 19%

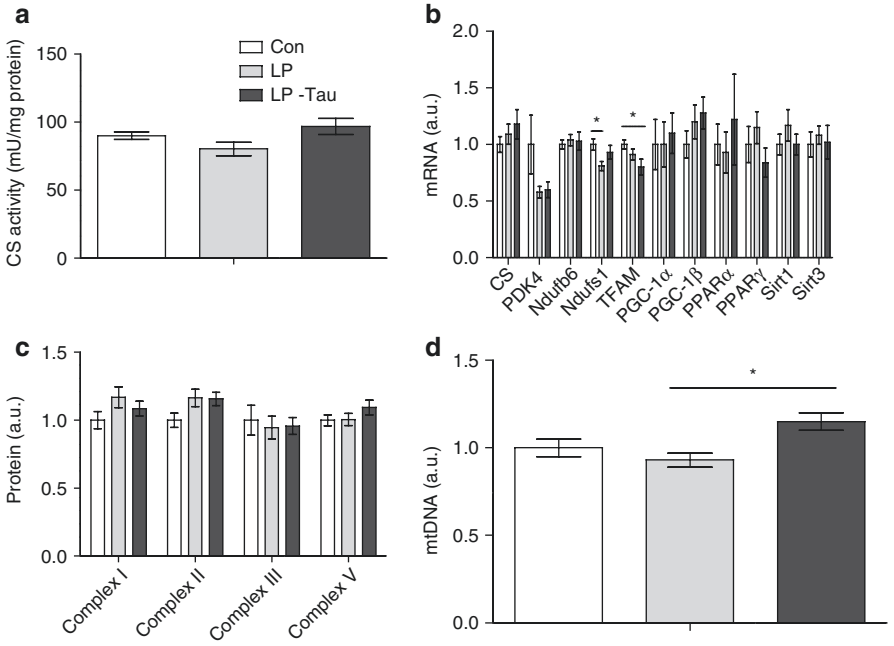

Fig. 2 Mitochondrial properties in skeletal muscle of newborn male rats subjected to maternal LP diet with 2% taurine in drinking water. (**a**) CS activity (ANOVA NS) in newborn skeletal muscle. (**b**) mRNA levels of CS (ANOVA NS), PDK4 (ANOVA NS), NNDUFb6 (ANOVA NS), NDUFs1 (ANOVA $p = 0.02$) and TFAM (ANOVA $p = 0.03$), of PGC-1α (ANOVA NS), PGC-1β (ANOVA NS), PPARα (ANOVA NS), PPARγ (ANOVA NS), Sirt1 (ANOVA NS), Sirt3 (ANOVA NS). (**c**) Protein levels of mitochondrial complex I, II, III and V (ANOVA NS for all). (**d**) mtDNA:gDNA levels (ANOVA $p = 0.02$). Numbers are mean ± SEM. a.u. (arbitrary units). *Denotes a significant difference in the Tukey-Kramer post hoc test between the indicated groups

compared to CON, with no effect on NADH Dehydrogenase (Ubiquinone) 1 beta subcomplex, 6 (Ndufb6) as shown in Fig. 2b. In contrast, CS, pyruvate dehydrogenase kinase, isozyme 4 (PDK4), peroxisome proliferator-activated receptor gamma, coactivator-1α (PGC-1α), PGC-coactivator-1β (PGC-1β), peroxisome proliferator activated receptor α (PPARα) and γ (PPARγ) mRNA levels were similar between groups (Fig. 2b).

We also measured the protein levels of the complexes in the respiratory chain, but did not see any significant differences between groups (Fig. 2c).

mtDNA (ND1) copy number relative to gDNA were significantly increased by taurine supplementation compared to LP alone—thereby indicating a possible rescue effect of taurine on mtDNA content (Fig. 2d).

3.4 Gene Expression Profiling

The LP diet caused changes in gene expression levels in 895 known genes, with 225 being down-regulated and 670 being up-regulated (Fig. 3a). Taurine supplementation resulted in a partial or full rescue of the LP-induced changes in gene expression levels of 283 known genes (88 fully rescued, 195 partially rescued), corresponding to a 32% rescue (Fig. 3b).

When we examined gene-set over-representation in all the genes changed by LP diet (e.g. both up- and down-regulated by LP) we found that mainly genes involved in protein synthesis were changed (Table 2). When we only examined the genes that were down-regulated by the LP diet, we found that besides changes in genes involved in protein synthesis, there were also changes in genes involved in the response to glucose stimulus (Table 3). Interestingly, when we only examined the genes up-regulated by the LP diet we found no over-representation of genes involved in protein synthesis, but instead we observed changes in cell-communication (Table 4). We found no evidence of taurine rescuing any specific gene sets.

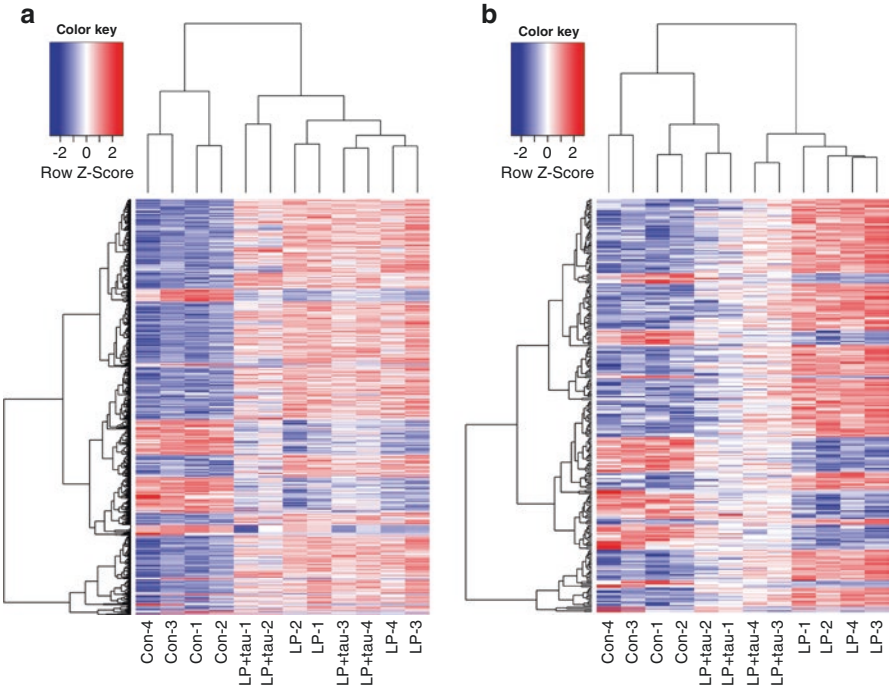

Fig. 3 Skeletal muscle gene expression profiling of newborn male rats subjected to maternal LP diet with 2% taurine in drinking water. Hierarchical clustering of genes. (**a**) Genes changed by maternal LP diet (895 known genes). (**b**) Genes rescued by taurine (283 genes, 88 fully rescued and 195 partially rescued)

Table 2 Gene set over-representation analysis of genes changed by the LP diet in newborn skeletal muscle

Category	Term	Ease score	FDR	Fold enrichment
PANTHER_MF_ALL	MF00075:Ribosomal protein	8.19E−09	1.01E−05	2.66
PANTHER_BP_ALL	BP00061:Protein biosynthesis	1.30E−06	1.61E−03	2.11
GOTERM_CC_FAT	GO:0005840~ribosome	1.50E−06	2.08E−03	2.64
GOTERM_MF_FAT	GO:0003735~structural constituent of ribosome	1.48E−05	2.22E−02	2.56

Genes changed by maternal LP diet were examined for over representation of specific gene sets as described in materials and methods. The significantly over-represented gene sets are shown (Ease score) a modified Fisher exact test describing the probability of gene set enrichment (FDR) False discovery rate multiple testing corrected Ease Score (Fold enrichment) a ratio describing the proportional increase in the number of significant genes present in the gene set compared to number of genes present in the gene set in the background

Table 3 Gene set over-representation analysis of genes down-regulated by the LP diet in newborn skeletal muscle

Category	Term	Ease score	FDR	Fold enrichment
PANTHER_MF_ALL	MF00075:Ribosomal protein	5.67E−−28	6.19E−25	10.09
PANTHER_BP_ALL	BP00061:Protein biosynthesis	3.94E−24	4.38E−21	7.35
GOTERM_CC_FAT	GO:0005840~ribosome	7.93E−18	9.48E−15	8.83
GOTERM_MF_FAT	GO:0003735~structural constituent of ribosome	3.74E−17	4.80E−14	9.55
GOTERM_MF_FAT	GO:0005198~structural molecule activity	3.49E−15	4.41E−12	5.53
PANTHER_MF_ALL	MF00042:Nucleic acid binding	2.72E−14	2.97E−11	2.45
GOTERM_BP_FAT	GO:0006412~translation	1.59E−13	2.59E−10	6.02
GOTERM_CC_FAT	GO:0030529~ribonucleoprotein complex	1.57E−12	1.87E−09	5.13
GOTERM_BP_FAT	GO:0006414~translational elongation	4.29E−10	6.97E−07	10.60
KEGG_PATHWAY	rno03010:Ribosome	6.29E−10	6.37E−07	11.09
GOTERM_CC_FAT	GO:0043228~non-membrane-bounded organelle	1.97E−07	2.36E−04	2.11
GOTERM_CC_FAT	GO:0043232~intracellular non-membrane bounded organelle	1.97E−07	2.36E−04	2.11
GOTERM_BP_FAT	GO:0009749~response to glucose stimulus	2.74E−05	4.44E−02	8.88

Genes down-regulated maternal LP diet were examined for over representation of specific gene sets as described in materials and methods. The significantly over-represented gene sets are shown. Ease Score, FDR and Fold Enrichment, see Table 3

Table 4 Gene set over-representation analysis of genes up-regulated by the LP diet in newborn skeletal muscle

Category	Term	Ease score	FDR	Fold enrichment
PANTHER_BP_ALL	BP00274:Cell communication	1.35E−06	1.66E−03	1.90
GOTERM_CC_FAT	GO:0042995~cell projection	8.47E−06	1.16E−02	1.95

Genes up-regulated maternal LP diet were examined for over representation of specific gene sets as described in materials and methods. The significantly over-represented gene sets are shown. Ease Score, FDR and Fold Enrichment, see Table 3

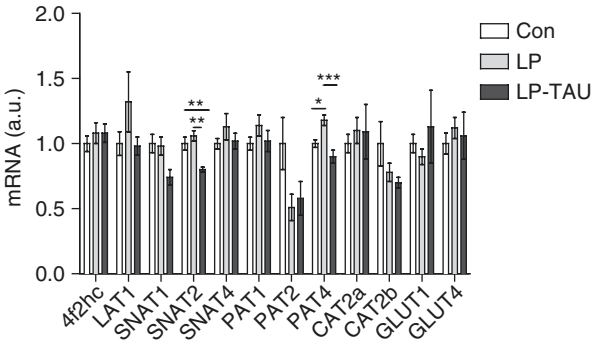

Fig. 4 Nutrient transporters in skeletal muscle of newborn male rats subjected to maternal LP diet with 2% taurine in drinking water. The graph shows mRNA levels of amino acid and glucose transporters: System L; 4F2HC (ANOVA NS), LAT1 (ANOVA NS). System A; SNAT1 (ANOVA p = 0.06), SNAT2 (ANOVA p = 0.002), SNAT4 (ANOVA NS). PAT; PAT1 (ANOVA NS), PAT2 (ANOVA p = 0.07), PAT4 (ANOVA p = 0.0004). CAT; CAT2a (ANOVA NS), CAT2b (ANOVA NS). Glucose transporters; GLUT1 (ANOVA NS), GLUT4 (ANOVA NS). Numbers are mean ± SEM. a.u. (arbitrary units). *Denotes a significant difference in the Tukey-Kramer post hoc test between the indicated groups

3.5 Changes in System A and PAT Amino Acid Transporters Induced by LP and Taurine

As the gene expression profiling pointed towards major changes in protein translation in newborn skeletal muscle, as an effect of the maternal LP diet, we wondered if the LP diet might have affected amino acid- and glucose transporters in newborn skeletal muscle and measured mRNA levels of several amino acid and glucose transporter systems (System A—sodium-coupled neutral amino acid transporters (SNAT), System L—large neutral amino acid transporters (LAT), PAT—proton-assisted amino acid transporters, CAT—cationic amino acid transporters, GLUT1, and GLUT4—glucose transporters) (Fig. 4).

In system A, SNAT1 tended to decrease with LP-TAU compared to CON. SNAT2 mRNA levels were significantly lower in the LP-TAU group compared to both LP and CON. SNAT4 mRNA levels were similar between groups. In system L, 4f2hc

and LAT1 mRNA levels between groups were similar. LAT2 mRNA levels were not detectable by qPCR. PAT1 mRNA levels were similar between groups. PAT2 mRNA levels tended to decrease with LP diet. PAT4 mRNA levels increased 18% when comparing LP to CON, whereas taurine ameliorated this effect—indicating a rescue effect of taurine. However, Cat2a and Cat2b mRNA levels were similar between groups, whereas Cat1 mRNA could not be detected by qPCR. Furthermore, both GLUT1 and GLUT4 mRNA levels were similar between groups (Fig. 4).

3.6 Taurine Supplementation Decreased Both Total AKT and Total AMPK Protein Levels

Due to the changes in both protein translation and amino acid transporters we hypothesized that these changes may be associated with changes in some of the major nutrient sensing signaling pathways.

Phosphoenolpyruvate carboxykinase 1 (PDK1) mRNA and activating transcription factor 4 (ATF4) levels were similar between groups (Fig. 5a). Phosphorylation of AKT p-Thr308 was also similar between groups both when normalized to β-actin and total AKT. Phosphorylation of AKT p-Ser473 tended to increase with LP diet compared to CON when normalized to β-actin and total AKT. Surprisingly, total AKT protein levels decreased in LP-TAU compared to both LP and CON (Fig. 5b).

As shown in Fig. 5b, AMPK p-Thr172 phosphorylation, when normalized to β-actin or total AMPK, was similar between groups. However, total AMPK protein levels also decreased significantly in LP-TAU compared to both LP and CON.

Both DNA-Damage-Inducible Transcript 4 (REDD1) mRNA levels and protein levels were similar between groups (Fig. 5a). Ribosomal Protein S6 Kinase (S6 K1) p-Thr389 phosphorylation tended to increase in LP-TAU compared to LP, when normalized to both β-actin and total S6k1. Total S6k1 protein levels were similar between groups (Fig. 5b).

3.7 Markers of Myogenesis were Unaffected by LP Diet or Taurine Supplementation

The previously discovered LP-induced changes, as well as the observed amelioration by taurine, may have affected changes in skeletal muscle growth and differentiation, therefore, we investigated different markers of myogenesis.

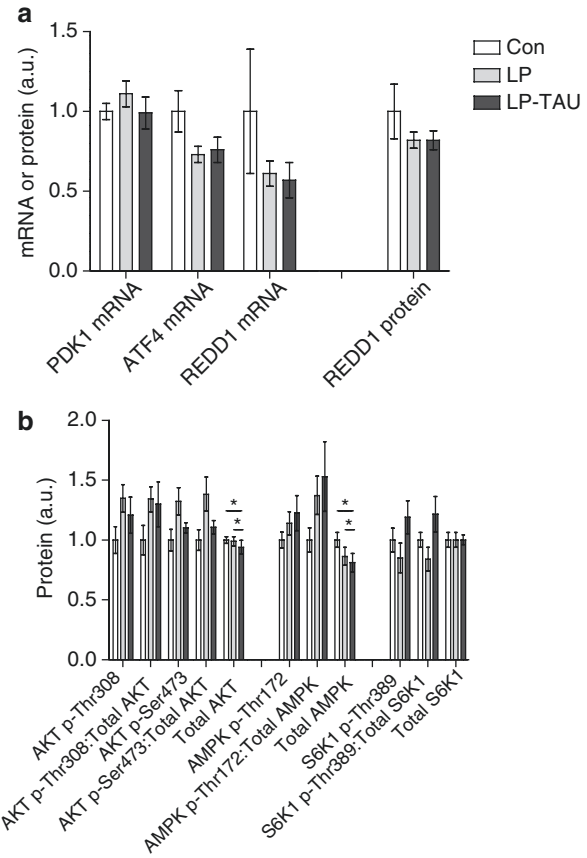

Fig. 5 Nutrient-sensing pathways in skeletal muscle of newborn male rats subjected to maternal LP diet with 2% taurine in drinking water. (**a**) PDK1, ATF4, and REDD1 mRNA levels and REDD1 protein levels (ANOVA NS for all). (**b**) Protein levels of AKT p-Thr308 (ANOVA NS) and p-Ser473 NS (ANOVA $p = 0.07$) and total AKT (ANOVA $p = 0.02$), AMPK p-Thr172 (ANOVA NS), total AMPK (ANOVA $p = 0.02$), S6K1 p-Thr389 (ANOVA NS) and total S6K1 (ANOVA NS). Numbers are mean ± SEM. a.u. (arbitrary units). *Denotes a significant difference in the Tukey-Kramer post hoc test between the indicated groups

The activity of CK was similar between groups as shown in Fig. 6a. Different markers were measured by qPCR and Myogenin, Myosin heavy chain (MHC) IIX, MHCemb, MHCper, MHCIb, MHCIIa, MHCIIb, Myogenic factor 5 (Myf5), Myosin (Myo5a + 5b), and Myocyte enhancer factor (Mef) 2a mRNA levels were all similar between groups (Fig. 6b). However, Myogenic differentiation 1 (MyoD) mRNA levels tended to decrease with LP diet when taurine was supplemented—indicating some effect of taurine. Mef2d mRNA levels tended to increase with LP diet compared to CON with no effect of taurine (Fig. 6b).

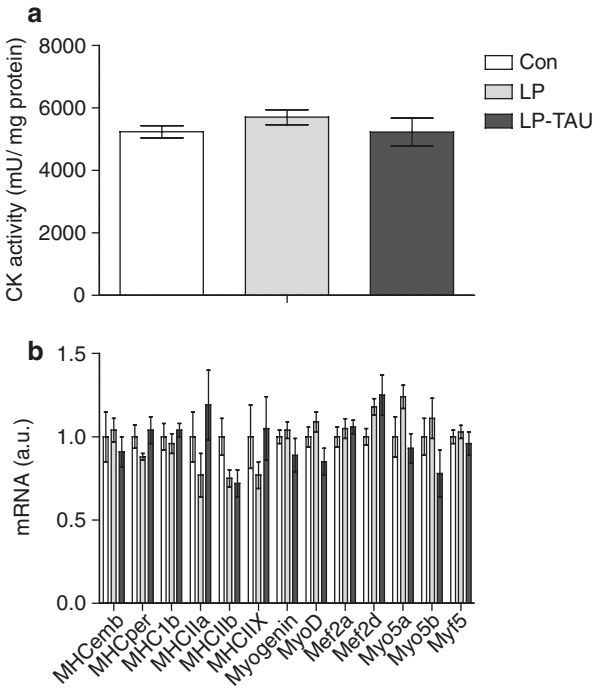

Fig. 6 Myogenesis in skeletal muscle of newborn male rats subjected to maternal LP diet with 2% taurine in drinking water. (**a**) Creatine kinase enzymatic activity (ANOVA NS). (**b**) mRNA levels of markers of myogenesis; MHCemb, MHCper, MHCIb, MHCIIa, MHCIIb, MHCIIX, myogenin, MyoD (ANOVA $p = 0.07$), Mef2a, Mef2d (ANOVA $p = 0.04$), Myo5a, Myo5b, Myf5 (ANOVA NS unless stated otherwise). Numbers are mean ± SEM. a.u. (arbitrary units)

4 Discussion

The aim of this study was to evaluate the effects of taurine supplementation during maternal protein restriction on skeletal muscle of male newborn offspring, with respect to overall gene expression changes, taurine transport and biosynthesis, mitochondrial properties, nutrient transporters and signaling sensing pathways, and fetal skeletal muscle growth and differentiation.

We found that LP-Tau had a lower birth weight compared to CON. Yet maternal protein restriction alone (LP vs. CON) did not lead to a significant decrease in birth weight in the male offspring (Table 1).

Low birth weight is a hallmark feature of developmental programming (Hales and Barker 1992) and maternal protein restriction induced low birth weight has been demonstrated in several animal models (Boujendar et al. 2002; Merezak et al. 2004; Mortensen et al. 2010a). In the present study, however, the low birth weight phenotype was worsened by taurine supplementation. In C57BL/6J mice, maternal low protein diet resulted in a 40% lower birth weight compared to control offspring, and taurine prevented half of the decrease (Mortensen et al. 2010a). The effect of taurine on birth weight in the present study might be caused by differences in taurine homeostasis between species. It is well known, as mentioned, that the mouse strain C57BL/6J has a defect in taurine renal reabsorption (Harris and Searle 1953; Chesney et al. 1976). The rescue effect of taurine in C57BL/6 mice could possibly

be interpreted as compensation for this renal absorption defect or as a reflection of species differences.

In the present study, taurine content increased significantly in skeletal muscles of the taurine supplemented group compared to control (Fig. 1c). Furthermore, earlier studies have shown that taurine concentration in plasma decreases with intrauterine growth restriction (Economides et al. 1989; Cetin et al. 1990; Reusens et al. 1995; Wu et al. 1998). However, we saw no effect on taurine content of intrauterine protein restriction in skeletal muscle. To our knowledge, this is the first study to examine the taurine level in newborn skeletal muscle, whereas other studies examined the taurine levels in newborn plasma. Alterations in taurine biosynthesis and transport have been observed with different maternal diet insults (Bagley and Stipanuk 1994; Bella et al. 1999; Tsuboyama-Kasaoka et al. 2006; Stipanuk et al. 2009). In the present study, we saw no changes in mRNA levels of enzymes from the taurine synthesis pathway. Although TauT mRNA levels were increased by gestational protein restriction, the protein levels were unaffected.

Microarray gene expression analysis of newborn skeletal muscle revealed significant changes in 895 genes (Fig. 3a). Taurine fully or partially rescued 32% of these changes, but with no distinct pattern as to which genes (pathways) were rescued (Fig. 3b). In mice, we have previously demonstrated that taurine supplementation rescued a large portion of the changes in mRNA expression levels in both skeletal muscle and liver caused by maternal protein restriction (Mortensen et al. 2010a). In newborn skeletal muscle, a large fraction of genes involved in amino acid metabolism, protein synthesis, TCA cycle, and energy metabolism genes involved in both Complex I–IV and ATP synthesis showed decreased expression in mice offspring subjected to gestational protein restriction (Mortensen et al. 2010a). In the present study, low protein diet also decreased the mRNA levels Ndufs1 in Complex I and microarray gene expression revealed a decrease in genes involved in protein synthesis, protein translation and genes involved in the response to glucose stimulus (Fig. 2 and Tables 2 and 3).

Interestingly, Reusens et al., showed that taurine supplementation prevented all changes in mRNA expression levels of tricarboxylic acid (TCA) cycle and ATP production in the pancreas in newborn rats caused by gestational protein restriction (Reusens et al. 2008).

Taurine is a constituent of mitochondrial tRNA (Suzuki et al. 2002) and might be essential for normal mitochondrial function. Thus, studies of fetal gene expression profile in pancreas, skeletal muscle and liver suggest that there may be a mitochondrial component in the rescue effect of taurine, due to the distinct pattern of genes that were rescued by taurine supplementation (Reusens et al. 2008; Mortensen et al. 2010a). In the present study, we found no evidence of taurine rescuing in any specific gene sets and we did not see major changes in mitochondrial gene expression as an effect of the LP diet. However, we did observe an increase in mtDNA in offspring of taurine supplemented rats with gestational protein restriction, compared to gestational protein restriction alone, with no difference between CON and LP-TAU. Furthermore, we saw no difference between groups in CS activity, which can be considered a measure of mitochondrial mass (Larsen et al. 2012). In contrast,

Park et al., reported a decrease in skeletal muscle mtDNA in gestational protein restricted 5-week old rats (Park et al. 2004).

The concept that intrauterine growth restriction may impair myogenesis has been demonstrated many years ago and has been studied in a variety of species. Effects such as impaired or limited myoblast cell cycle activity, reduced number of myonuclei, and reduced amount of myofibers in the fetus have been reported (Wilson et al. 1988; Dwyer and Stickland 1992; Prakash et al. 1993; Dwyer et al. 1995; Greenwood et al. 1999; Osgerby et al. 2002; Bayol et al. 2004; Costello et al. 2008). In contrast, in the present study, protein restriction throughout gestation had no significant impact on the different markers of myogenesis we measured, other than a trend in MyoD mRNA level towards a more mature myogenesis with taurine supplementation. Furthermore, nutrient sensing pathways were largely unaffected by both gestational protein restriction and taurine supplementation, despite changes seen in system A and PAT amino acid transporters. Taurine supplementation, however, did cause an unexpected decrease in total Akt and AMPK protein levels indicating a possible effect of taurine on growth, insulin signaling, and nutrient sensing.

5 Conclusion

In conclusion, gestational protein restriction in Wistar rats decreased expression of genes involved in protein translation in newborn skeletal muscle and led to a decrease in nutrient transporters in system A and PAT. Taurine was able to rescue parts of these effects, but not as systematic as in C57BL/6J mice. However, the present study further solidifies the importance of taurine availability in fetal development.

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Impaired Energy Production Contributes to Development of Failure in Taurine Deficient Heart

Stephen Schaffer, Chian Ju Jong, Aza Shetewy, KC Ramila, and Takashi Ito

Abstract Taurine forms a conjugate in the mitochondria with a uridine residue in the wobble position of $tRNA^{Leu(UUR)}$. The resulting product, 5-taurinomethyluridine $tRNA^{Leu(UUR)}$, increases the interaction between the UUG codon and AAU anticodon of $tRNA^{Leu(UUR)}$, thereby improving the decoding of the UUG codon. We have shown that the protein most affected by the taurine conjugation product is ND6, which is a subunit of complex I of the respiratory chain. Thus, taurine deficiency exhibits reduced respiratory chain function. Based on these findings, we proposed that the taurine deficient heart is energy deficient. To test this idea, hearts were perfused with buffer containing acetate and glucose as substrates. The utilization of both substrates, as well as the utilization of endogenous lipids, was significantly reduced in the taurine deficient heart. This led to a 25% decrease in ATP production, an effect primarily caused by diminished aerobic metabolism and respiratory function. In addition, inefficient oxidative phosphorylation causes a further decrease in ATP generation. The data support the idea that reductions in energy metabolism, including oxidative phosphorylation, ATP generation and high energy phosphate content, contribute to the severity of the cardiomyopathy. The findings are also consistent with the hypothesis that taurine deficiency and reduced myocardial energy content increases mortality of the taurine deficient, failing heart. The clinical implications of these findings are addressed.

Keywords Mitochondrial ATP production • Cardiac work-oxidative phosphorylation relationship • Metabolic shift in heart failure • Glucose oxidation • Taurine-mediated regulation of complex I activity

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1 Introduction

Taurine is ubiquitous β-amino acid, which is required for normal cellular function. Among the defects observed in taurine deficiency are cardiomyopathy, retinopathy, immune deregulation, muscle weakness, osmotic imbalances, renal insufficiency, hypertension, atherosclerosis, obesity, neurological abnormalities, hearing loss and accelerated aging (Rascher et al. 2004; Huang et al. 2006; Sergeeva et al. 2007; Ito et al. 2008, 2014a, b; Kaesler et al. 2012; Schaffer et al. 2014).

The susceptibility of different species to taurine deficiency varies widely. Taurine is considered nonessential for rodents, essential for cats and fox, and conditionally essential for humans (Gaull 1986). These species differences are attributed to variations in hepatic taurine biosynthesis and the rate of taurine loss from the body. In humans, the low rate of hepatic taurine biosynthesis is balanced by enhanced retention of taurine by the body, rendering humans fairly resistant to taurine deficiency. Rodents are also resistant to taurine deficiency, but largely because of the rapid rate of hepatic taurine biosynthesis. By contrast, cats and fox require a nutritional source of taurine to avoid signs of taurine deficiency. Nonetheless, taurine deficiency will develop in all species when treated with a taurine transport inhibitor or if the species undergoes genetic modification of the taurine transporter, as occurs in the taurine transporter knockout mouse TauTKO) (Ito et al. 2008). Thus, the four common experimental animal models of taurine deficiency are (1) cats fed a taurine deficient diet (Novotny et al. 1991), (2) rodents treated with a taurine transport inhibitor (Allo et al. 1997), (3) TauTKO mice (Ito et al. 2008) and (4) genetic models that knockout taurine biosynthesis (Park et al. 2014; Jurkowska et al. 2016).

A characteristic feature of taurine deficiency is development of dilated cardiomyopathy exhibiting impaired systolic and diastolic function (Novotny et al. 1991; Ito et al. 2008). In the taurine deficient cat, cardiac function is restored by normalization of tissue taurine content, but taurine treatment has no effect on the cardiomyopathy of the TauTKO mouse, as the transporter is required for restoration of myocardial taurine levels (Pion et al. 1991; Ito et al. 2008). Although the cardiomyopathy in cats is reversible, the most severe cases of cardiomyopathy lead to premature death of the animal (Ito et al. 2014a). Among the factors thought to influence the severity of the cardiomyopathy are impaired calcium handling (Ramila et al. 2015), alterations in response to neurohumoral factors (Schaffer et al. 2003), oxidative stress (Jong et al. 2012) and impaired autophagy (Jong et al. 2015). However, a recent study revealed that the energy state of the diseased heart is one of the most important determinants of mortality among patients with congestive heart failure (Neubauer et al. 1997). Because taurine deficiency is associated with respiratory chain dysfunction, we hypothesized that taurine deficiency might also worsen the severity of the taurine deficient cardiomyopathy by diminishing the energy state of the heart. Hence, the effect of ATP generation by glycolysis, glucose oxidation, the citric acid cycle and fatty acid metabolism was compared in control and taurine deficient hearts.

2 Methods

2.1 Animal Model of Taurine Deficiency

To produce the animal model of taurine deficiency, male Wistar rats were maintained for 3 weeks on tap water containing 3% β-alanine. This model reduced myocardial taurine content from 98.5 to 59.3 μmol/g dry wt. Hearts were removed from taurine deficient and control rats and then perfused with Krebs-Henseleit buffer containing 5 mM glucose, 5 mM acetate and 2.5 U/L insulin. The hearts were paced at 300 beats/min throughout the experiment. Oxygen consumption was monitored with a Clark oxygen electrode. Total O_2 consumption was calculated from coronary flow and the amount of oxygen extracted from the perfusate. The rate of glucose utilization and oxidation were determined from the rates of [3-^{3}H]-glucose conversion to ^3H_2O, along with lactate and pyruvate production, the latter assayed in coronary effluent samples. The rate of acetate oxidation was determined from the conversion of [2-^{14}C]-acetate to $^{14}CO_2$. The formation of NADH and $FADH_2$ by the citric acid cycle was calculated from the oxidation rates of acetate, glucose and endogenous lipids. ATP and creatine phosphate content were measured from extracts of freeze-clamped hearts.

2.2 Calculation of Metabolic Rates of Taurine Deficient and Control Hearts

Acetate oxidation was calculated from the generation of $^{14}CO_2$ from [2-^{14}C]-acetate. Endogenous lipid oxidation, which we call palmitate oxidation, refers to O_2 consumption not related to glucose and acetate oxidation. Glucose oxidation was calculated from glucose utilization, lactate generation and pyruvate production (Schaffer et al. 2016). The ratios of ATP produced to O_2 consumed, which is used in the calculation of ATP synthesis are: glucose, 3.17; acetate, 2.5; and palmitate, 2.8. The net yield of ATP from lactate output was assumed to be 2 μmol ATP/μmol of glucose, whereas pyruvate output was assumed to be 8 μmol/μmol of glucose (two for glycolysis and six for oxidation of cytoplasmic NADH).

2.2.1 Statistical Analysis

All results are reported as means ± S.E.M. Statistical significance was determined using the Student's t-test for comparison within groups or ANOVA followed by Newman-Keuls test for comparison between groups. Values of $p < 0.05$ were considered statistically significant.

3 Results

3.1 Major Site of Taurine Action

Jong et al. (2012) have previously found that respiratory chain function and oxygen consumption of taurine deficient cardiomyocytes are diminished, an effect reversed by restoration of the intracellular taurine pools. Two lines of evidence suggest that the major site of taurine action in the heart is complex I of the respiratory chain. First, NADH dehydrogenase activity is diminished in taurine deficient mitochondria, suggesting that the handling of reducing equivalents by complex I is defective (Jong et al. 2012). Second, taurine deficient mitochondria respire normally in the presence of the complex II substrate, succinate, but not in the presence of the complex I substrates, malate/glutamate, implying that electron transfer of taurine deficient mitochondria is normal between complexes II-V but not between complex I-II (Shetewy et al. 2016). As a result of impaired complex I (NADH dehydrogenase) activity, less NADH enters the respiratory chain and the rate of ATP generation declines.

3.2 Diminished Energy State of the Taurine Deficient Heart

A common chemical marker of energy deficiency in the heart is the creatine phosphate/ATP ratio (Neubauer et al. 1997). Although both creatine phosphate and ATP are considered high-energy phosphate compounds, ATP is the predominant high-energy phosphate providing the cell a readily available source of energy while creatine phosphate is a high-energy phosphate reserve that is utilized by creatine phosphokinase to form ATP from ADP. Therefore, a reduction in the creatine phosphate/ATP ratio serves as a marker of low energy and an imbalance between ATP generation and ATP demand. Accordingly, the taurine deficient heart is energy deficient, as the creatine phosphate/ATP ratio of the normal heart is 30% greater than that of the taurine deficient heart (Fig. 1).

Regulatory events within the energy deprived heart act to restore energy balance. One of the common events is the stimulation of glycolysis. To determine if glycolytic flux is enhanced in the taurine deficient heart, control and taurine deficient hearts were perfused with buffer containing 5 mM ^{3}H–glucose, 5 mM acetate and 2.5 U/L insulin; glucose utilization was determined from the rate of $^{3}H_2O$ generation from ^{3}H–glucose. The rate of glycolysis was calculated from the rates of glucose utilization and glycogenolysis, the latter which was zero. Figure 2 shows that the rate of glycolysis is 17% greater in the taurine deficient heart than in the control heart. Although a small amount of ATP is generated from glycolysis by pyruvate kinase and phosphoglycerate kinase, with additional ATP generated from NADH (glyceraldehyde-3-phosphate dehydrogenase), the major source of ATP from glucose utilization is glucose oxidation, which is defined as the rate of pyruvate

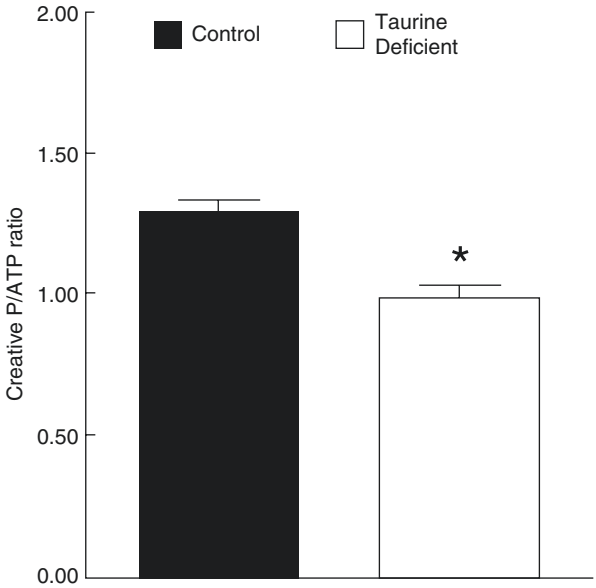

Fig. 1 Effect of taurine deficiency on myocardial high-energy phosphate status. Hearts from taurine deficient and control hearts were perfused with buffer containing 5 mM glucose, 5 mM acetate and 2.5 U/L insulin. After 30 min of perfusion, the hearts were frozen with tongs frozen in liquid nitrogen. The levels of ATP and creatine phosphate were measured from extracts of the frozen tissue. Values shown represent means ± S.E.M. of six hearts. The asterisk denotes a significant difference between control and taurine deficient hearts ($p < 0.05$)

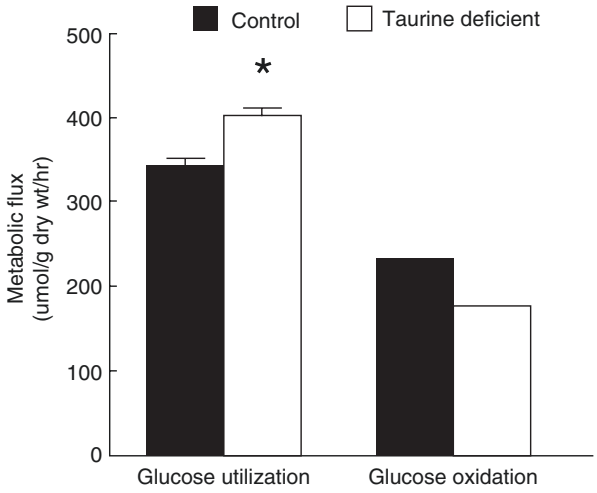

Fig. 2 Effect of taurine deficiency on the rates of glycolysis and glucose oxidation. Hearts from taurine deficient and control hearts were perfused with buffer containing 5 mM ^{3}H–glucose, 5 mM acetate and 1 U/L insulin. The rate of glycolysis was determined from the rate of ^3H_2O generation from ^{3}H–glucose. To calculate the rate of glucose oxidation, the rates of lactate and pyruvate production were subtracted from the rate of ^3H_2O generation from ^{3}H–glucose. Values shown represent means ± S.E.M. of five hearts. The asterisk denotes a significant difference between the control and taurine deficient hearts ($p < 0.05$)

utilization by the citric acid cycle. Unfortunately, for the taurine deficient heart, glucose oxidation is significantly depressed (Fig. 2), an effect indirectly mediated by inhibition of NADH oxidation by NADH dehydrogenase (complex I). In the taurine deficient heart, the levels of mitochondrial NADH increase, which in turn inhibits the oxidation of pyruvate by pyruvate dehydrogenase (Schaffer et al. 2016). High mitochondrial NADH content also leads to an elevation in cytosolic NADH, which is used by lactate dehydrogenase to generate lactate from pyruvate; in the taurine deficient heart, lactate formation increases 2.5 fold (Schaffer et al. 2016). Because of the large increase in lactate production, less NADH is available for ATP biosynthesis. Moreover, glucose oxidation is reduced by 25% (Schaffer et al. 2016). Thus, despite the increase in ATP generation from glycolysis, the amount of ATP generated from glucose metabolism is 25% less in the taurine deficient than in the control heart, an effect largely attributed to reduced glucose oxidation (Fig. 3).

Most of the ATP generated by glucose oxidation is derived from NADH and $FADH_2$ produced by the citric acid cycle. Because β-oxidation of fatty acids, like pyruvate oxidation, generates acetyl CoA for use by the citric acid cycle, most of the ATP generated by fatty acid metabolism depends upon the formation of reducing equivalents by the citric acid cycle. Thus, the activity of the citric acid cycle was examined in control and taurine deficient hearts perfused with buffer containing 5 mM glucose, 5 mM ^{14}C–acetate and 5 U/L insulin. Because acetate is converted directly to acetyl CoA, the oxidation of ^{14}C–acetate to $^{14}CO_2$ serves as a direct measure of citric acid cycle activity. Based on the formation of $^{14}CO_2$ from the heart, we found that citric acid cycle activity of the taurine deficient heart is depressed 23% relative to that of the control heart. From the amount of NADH and $FADH_2$

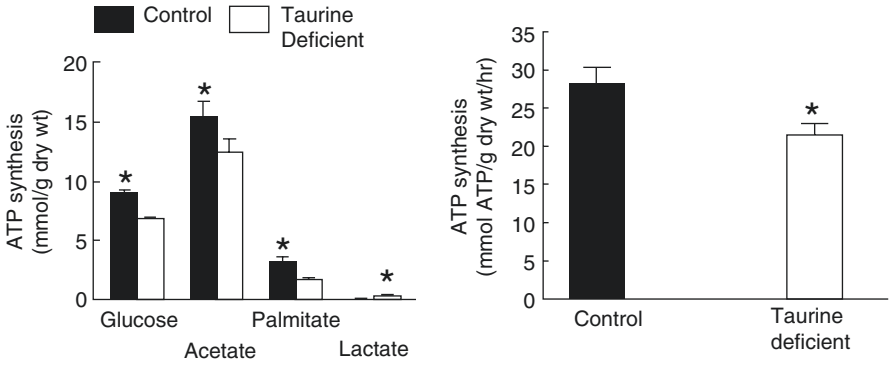

Fig. 3 Effect of taurine deficiency on the rates of ATP biosynthesis from glucose, acetate, palmitate and lactate. *Left panel*: the contribution of glucose metabolism, acetate utilization, endogenous fatty acid metabolism and lactate metabolism toward total ATP biosynthesis of taurine deficient and control hearts perfused with buffer containing 5 mM glucose, 5 mM acetate and 2.5 U/L insulin. *Right panel*: total ATP biosynthesis of control and taurine deficient hearts perfused with buffer containing 5 mM glucose, 5 mM acetate and 2.5 U/L insulin. Values shown represent means ± S.E.M. of six different hearts per group. Asterisks denote a significant difference between the control and taurine deficient hearts ($p < 0.05$)

produced from each mole of acetate oxidized by the citric acid cycle, we calculated that 12.45 ± 1.11 and 15.52 ± 1.15 mmol ATP/g dry wt/h are generated from acetate metabolism by the taurine deficient heart and control heart, respectively (Fig. 3).

The final source of ATP production in the perfused heart is endogenous lipid metabolism, which we call palmitate metabolism. To calculate the contribution of palmitate metabolism toward total ATP generation, we made the assumption that palmitate oxidation is the amount of O_2 consumption not related to glucose and acetate oxidation. In the taurine deficient heart and control heart, the rate of O_2 consumption from all three substrates was found to be 3.87 ± 0.05 and 5.14 ± 0.11 mmol O_2/g dry wt/h, respectively. In calculating the contribution of glucose metabolism toward the total rate of O_2 consumption, we assumed that O_2 equivalents (mol/mol) for glucose is six (four for the citric acid cycle, one at pyruvate dehydrogenase and one in the oxidation of glycolytic NADH) while the contribution of acetate toward total O_2 consumption is derived from the rate of the production of NADH and $FADH_2$ by the citric acid cycle (from $^{14}CO_2$ generation). Finally, the ratio of ATP produced for each mole of O_2 consumed was assumed to be 2.8 for endogenous lipid metabolism. Hence, we found that the rate of ATP synthesis from palmitate is 1.74 ± 0.12 and 3.35 ± 0.29 mmol/g dry wt/h for taurine deficient and control hearts, respectively (Fig. 3).

Based on the contributions of glucose, acetate and endogenous lipids, the mean rate of total ATP generation by the taurine deficient and control hearts is 21.45 ± 1.11 and 28.17 ± 2.11 mmol/g dry wt/h, respectively (Fig. 3). Although the rate of ATP generation from each of the substrates is decreased in the taurine deficient heart relative to that of the control heart, ATP generation from palmitate metabolism was most severely depressed in the taurine deficient heart.

4 Discussion

The present study supports the hypothesis that the taurine deficient heart is energy starved and that the energy deficient state contributes to impaired contractile function. The reduction in ATP generation by the taurine deficient heart is not surprising because taurine deficiency causes a decrease in respiratory chain (NADH dehydrogenase) activity, leading to a decline in respiration and an elevation in myocardial NADH content (Jong et al. 2012; Schaffer et al. 2016; Shetewy et al. 2016). A similar decrease in O_2 consumption and elevation of NADH is observed in the hypoxic heart, but in hypoxia the respiratory chain is incapable of oxidizing NADH because the activity of complex IV (cytochrome c oxidase) is reduced by the decline in its substrate, O_2. Nonetheless, in both conditions a bottleneck in the respiratory chain develops that leads to an elevation in NADH content, a decrease in ATP generation and impaired contractile function.

In both hypoxia and taurine deficiency, glycolysis is stimulated in response to the energy deficient state of the heart. While anaerobic glycolysis provides a fresh source of ATP, the amount of ATP generated by the taurine deficient heart via

anaerobic metabolism is small and is easily overwhelmed by the suppression of oxidative metabolism (including glucose oxidation, fatty acid oxidation and citric acid cycle flux). Two factors contribute to the suppression of oxidative metabolism in the taurine deficient heart. First, the NADH dehydrogenase (complex I) activity is reduced, which limits the capacity of the respiratory chain of the taurine deficient heart to utilize NADH generated by aerobic metabolism. Second, the elevation in NADH content of the taurine deficient heart restricts further NADH production by the oxidative metabolic pathways of the heart. Among the enzymes inhibited by a significant rise in the NADH/NAD^+ ratio are the key regulators of oxidative metabolism, namely, glyceraldehyde-3-phosphate dehydrogenase, pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, isocitrate dehydrogenase and fatty acyl CoA dehydrogenase (Neely and Morgan, 1974; Stanley et al. 2005). Hence, both the production and utilization of NADH by the taurine deficient heart are impaired. As a result, ATP generation declines, leading to a net decrease in the energy state of the heart.

In the beating heart, the primary use of ATP is the maintenance of contractile function. Not only is ATP cleaved during the contraction-relaxation cycle by a myosin ATPase, but large amounts of ATP are used by transporters that ensure the appropriate distribution of key cations during excitation-contraction coupling. Among the largest energy-consuming ATPases in the heart are SERCA2a (the sarcoplasmic reticular Ca^{2+} ATPase) and the Na^+–K^+ ATPase, enzymes that play central roles in excitation-contraction coupling (Coutu and Metzger 2005). Moreover, ATP serves as a substrate for protein kinases that regulate the activity of multiple Ca^{2+}-handling proteins, including SERCA2a (Luo and Anderson 2013). Nonetheless, for many years a role for ATP in the regulation of contractile function of the failing heart was largely dismissed, as the ATP concentration of the failing heart, despite declining, remains well above its Km for multiple ATP-dependent proteins.

One of the characteristic features of the failing heart is impaired sarcoplasmic reticular Ca^{2+} uptake (Luo and Anderson 2013). This decrease in SERCA2a activity has been attributed in part to the dephosphorylation of phospholamban, a regulatory phosphoprotein that is a potent inhibitor of SERCA2a in its dephosphorylated state. Sarcoplasmic reticular Ca^{2+} ATPase activity is also diminished in the heart of the TauTKO heart, with the effect attributed to the combination of phospholamban dephosphorylation by protein phosphatase 1 and reduced phosphorylation of the phosphoprotein by calcium calmodulin kinase II (Ramila et al. 2015). These changes in SERCA2a activity, likely contribute to prolongation of the Ca^{2+} transient and development of diastolic dysfunction of the TauTKO heart (Schaffer et al. 2014). However, it remains to be determined if changes in ATP biosynthesis also contribute to the observed decline in SERCA2a activity.

The heart consumes ~1 mM ATP/s, which means that the entire high-energy phosphate pool of the heart must be renewed every 20 s to maintain normal high-energy phosphate levels. Thus, a strictly linear relationship exists between cardiac performance (energy utilization) and O_2 consumption (energy production) (Ventura-Clapier et al. 2011). However, the factor that couples energy utilization with energy production remains an area of active research. While increases in $[Ca^{2+}]_i$ dramati-

cally stimulate contractile function, the activities of only a few enzymes (isocitrate dehydrogenase and α-ketoglutarate dehydrogenase) in the citric acid cycle are elevated by an increase in $[Ca^{2+}]_i$ (Luo and Anderson 2013). Although activation of the Ca^{2+} sensitive dehydrogenases by increased $[Ca^{2+}]_i$ stimulates both the generation of reducing equivalents and cardiac work it does not lead to an actual elevation in the NADH/NAD^+ ratio (Korzeniewski et al. 2005). Moreover, Ca^{2+} excess can initiate changes in the mitochondria that injure or kill the cell. Thus, the cardiomyocyte must maintain an appropriate Ca^{2+} gradient across the mitochondria, a process that costs the cell considerable amounts of ATP. Together, these data suggest that an increase in $[Ca^{2+}]_i$ stimulates both contractile function and NADH generation, but a change in $[Ca^{2+}]_i$ does not directly couple contractile function to energy production.

ADP, which is rapidly generated by ATPases during muscle contraction, is a much better coupling agent than $[Ca^{2+}]_i$ for signaling both increased ATP utilization and demand. When added to isolated mitochondria, ADP stimulates O_2 consumption until the added ADP is converted to ATP. Theoretically, the oxidation of one mole of NADH and $FADH_2$ via the respiratory chain results in the consumption of one mole of O_2 and the generation of three moles and two moles of ATP, respectively. However, the presence of uncoupling proteins and energy–requiring transporters, combined with the loss of O_2 in reactions, such as reactive oxygen species generation, significantly reduces the theoretical ratio for ATP synthesized/O_2 consumed (P/O ratio). In the taurine deficient heart, the generation of superoxide by the respiratory chain is a major cause for the reduction in the P/O ratio (Jong et al. 2012; Shetewy et al. 2016). This contention is supported by the substrate dependency of the effect. For complex I substrates, such as malate and glutamate, taurine deficiency significantly reduces the P/O ratio, however, taurine deficiency has no effect on the P/O ratio when taurine deficient mitochondria oxidize the complex II substrate, succinate (Shetewy et al. 2016).

The observation that oxidative phosphorylation is highly dependent on the intensity of cardiac work proves that the two processes are tightly coupled *in vivo*. It is relevant that when cardiac work is low, only ADP activates oxidative phosphorylation while at elevated cardiac work, oxidative phosphorylation depends upon both ADP and NADH (Korzeniewski et al. 2005). Usually, at low cardiac work, the rate of oxidative phosphorylation proceeds slowly and the content of NADH rises, as the production of NADH exceeds NADH utilization. Significantly, the low cardiac work state of the taurine deficient heart is associated with elevations in both NADH and ADP. Nonetheless, NADH content increases in the taurine deficient heart, not because of the low cardiac work state, but because NADH dehydrogenase (complex I) activity is inhibited. Moreover, oxidative phosphorylation must be activated within the heart for the contractile machinery to reach a high work state. However, the taurine deficient heart is relegated to a slow rate of oxidative phosphorylation, which alone precludes the taurine deficient heart from achieving a high cardiac work load (Korzeniewski et al. 2005).

The heart utilizes fatty acids, carbohydrate, ketone bodies, lactate and amino acids as sources of energy, with fatty acids serving as the preferred substrate

under normal conditions. However, in the advanced stages of human heart failure and in the taurine deficient heart, fatty acid oxidation is downregulated (Stanley et al. 2005). The shift away from fatty acid metabolism in both conditions has been attributed to the downregulation of peroxisome proliferator-activated receptor-α (PPAR-α), a transcription factor that increases the expression of proteins involved in fatty acid β-oxidation and long chain fatty acid transport (Stanley et al. 2005; Campbell et al. 2002; Schaffer et al. 2016). In response to the decline in fatty acid metabolism, the taurine deficient heart and failing heart increase glucose metabolism (Fillmore et al. 2013). However, the increase in glycolysis is coupled with a decrease in glucose oxidation, resulting from the increase in NADH levels that inhibit the metabolism of pyruvate via pyruvate dehydrogenase and result in the diversion of pyruvate to lactate. These changes in glucose metabolism lead to a decrease in cardiac efficiency and function, as the levels of protons increase, cation transport is impaired and ATP utilization is focused on improving Ca^{2+} transport rather than maintaining contractile function (Fillmore et al. 2013). Genetic mouse models of heart failure have also implicated impaired glucose and fatty acid oxidation in the development of heart failure (Abel et al. 1999; Hansson et al. 2004; King et al. 2007). Using a pressure overload model of heart failure, Doenst et al. (2010) found a close relationship between decreased oxidative metabolism and the development of heart failure. Together, these data support the notion that contractile function of the taurine deficient heart is depressed, in large part because of the reduced rates of oxidative metabolism and oxidative phosphorylation.

5 Conclusion

Taurine deficient heart is energy deprived, largely because of impaired complex I activity, which reduces the utilization of NADH by the respiratory chain. As result of the defect in respiratory chain function, the generation of NADH by all of the major metabolic pathways is diminished. Also decreasing ATP generation is a defect in oxidative phosphorylation as evidenced by the decrease in the P/O ratio. The major defect arising from decreased ATP production is impaired contractile function. However, the resulting decrease in the energy state of the heart may also be associated with an elevation in the mortality of the TauTKO mouse from heart failure. These results have clinical implications as large amounts of taurine are lost from the heart during a heart attack. When these patients develop congestive heart failure, they are at risk of death from impaired mitochondrial function and reduced energy levels. Moreover, the formation of 5-taurinomethyluridine $tRNA^{Leu(UUR)}$ is defective in patients suffering from the mitochondrial disease, MELAS. These patients often develop cardiomyopathies, whose severity is likely enhanced by the energy deficient state of the heart.

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Part IV
Taurine Deficiency and Knockout of Taurine

A Novel Cysteine Sulfinic Acid Decarboxylase Knock-Out Mouse: Immune Function (II)

Eunkyue Park, Seung Yong Park, In Soo Cho, Bo Sook Kim, and Georgia Schuller-Levis

Abstract Taurine deficient mice lacking cysteine sulfinic acid decarboxylase (CSAD KO) were developed for investigating the various physiological roles of taurine including the development of the brain and eye as well as immune function. Due to severe abnormalities of immune function in a taurine deficient cat, the immune function including adoptive and innate immunity in taurine-deficient mice have been studied. Previously we demonstrated that B cell function in CSAD KO was reduced in both females and males. However, T cell function was significantly reduced only in females. In this study, we have examined innate immunity using macrophage activation with LPS or/and IFN-γ and polymorphonuclear leukocytes (PMN) activation with phorbol myristate acetate (PMA). Pro- and anti-inflammatory cytokines including IL-6, TNF-α and IL-10 as well as nitric oxide (NO) were determined using ELISA and Griess reagent, respectively. Peritoneal macrophages were activated with 1 μg/mL of lipopolysaccharide (LPS) and/or 50 U/mL of IFN-γ. In addition, superoxide anion was measured using peritoneal PMN activated with PMA in the presence and absence of superoxide dismutase. Superoxide anion production in activated PMN from CSAD KO homozygotes (HO) was not significantly different from wild-type (WT) with and without 25 mM taurine. IL-10 and TNF-α production in both female and male CSAD KO were not significantly different. IL-6 and NO were significantly lower only in females as previously observed in Con A-activated cellular proliferation of splenocytes. Cytokine production with 10 mM

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of taurine was not different, indicating the reduction of NO and IL-6 in females may be due to the absence of the CSAD gene, not due to low taurine concentrations.

These data indicate that some measures of innate immunity were altered in female CSAD mice.

Keywords CSAD KO • Taurine • Innate immunity • Macrophages • PMN • Taurine deficiency

Abbreviations

Con A	Concanavalin A
CSAD KO	Cysteine sulfinic acid decarboxylase knockout mice
CSAD	Cysteine sulfinic acid decarboxylase
HO	Homozygotic mice ($CSAD^{-/-}$)
LPS	Lipopolysaccharide
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear leukocytes
WT	Wild-type ($CSAD^{+/+}$)

1 Introduction

Taurine is an essential amino acid for feline and a conditional indispensable amino acid for humans and nonhuman primates although it does not incorporate into protein synthesis (Sturman 1993). Taurine is dependent on dietary supplementation in cats as well as human and nonhuman primates. Taurine may play a critical role in the regulation of immune response (Schuller-Levis and Park 2006; Sturman 1993; Gordon et al. 1992) in taurine-deficient cats and taurine-supplemented rodents since taurine is the most abundant single amino acid in leukocytes (20–50 mM) (Fudaka et al. 1982). A significant leukopenia, a shift in the percentage of polymorphonuclear leukocytes and mononuclear leukocytes, an increase in the leukocyte count, and a change in the sedimentation characteristics of leukocytes were found in taurine-deficient cats (Schuller-Levis et al. 1990; Schuller-Levis and Sturman 1992). In addition, a significant decrease in the respiratory burst and a decrease in phagocytosis of polymorphonuclear leukocytes (PMN) from taurine-deficient cats were observed. Serum gamma globulin was significantly increased, and histologic changes in lymph nodes, and the spleen in taurine-deficient cats were demonstrated.

In several vivo models of oxidant-induced damage in rodents and hamster, supplementation of taurine protects against subsequent inflammation. Hamsters did not show typical pathology associated with nitrogen dioxide damage when animals

were pretreated with supplemental taurine and exposed to nitrogen dioxide (Gordon et al. 1992). The inflammation and fibrosis resulting from bleomycin treatment in an animal model were reduced with treatment of taurine and/or niacin (Schuller-Levis et al. 2009; Wang et al. 1991). Pretreatment of rodents with 5% taurine for 10 days prior to ozone exposure decreased ozone-induced lung inflammation (Schuller-Levis et al. 1995; Gordon et al. 1998). The reduction of number of inflammatory cells and hydroxyproline, markers for inflammation and fibrosis, were significantly observed in taurine-treated rats compared to untreated rats exposed to ozone. Thus, maintenance of tissue taurine levels is critical for the prevention of oxidant-induced injury in several animal models. Recently, we developed taurine-deficient mice, an absence of cysteine sulfinic acid decarboxylase (CSAD KO) to understand the role of taurine in reproduction as well as immune function (Park et al. 2014). While B lymphocyte function was decreased in the male of CSAD KO, not T lymphocyte function, immune function of both T and B lymphocyte was reduced in the female of CSAD KO (Park et al. 2015b).

In various laboratories, taurine and its derivative, taurine chloramine (Tau-Cl), have been extensively investigated to understand the regulation of immune function. Since leukocytes are capable of generating hypochlorous acid (HOCI) from hydrogen peroxide and chloride via the myeloperoxidase pathway and have intracellular high taurine concentrations (Schuller-Levis and Park 2003, 2006), less toxic and long-lived oxidant, Tau-Cl is produced under physiological condition. Tau-Cl, a stable oxidant and a metabolite of taurine, down-regulates the production of proinflammatory cytokines leading to a significant reduction in the immune response (Park et al. 1993, 1995; Marcinkiewicz et al. 1995; Quinn et al. 1996). Specifically, Tau-Cl reduces production of proinflammatory mediators such as nitric oxide, tumor necrosis factor alpha (TNF-α), and prostaglandin E_2 in lipopolysaccharide (LPS)-activated rodent cells. Tau-Cl suppressed superoxide anion, interleukin-6 (IL-6), and interleukin-8 (IL-8) production in activated human polymorphonuclear leukocytes (Park et al. 1998). Tau-Cl inhibits the production of IL-6, IL-1β, and IL-8 in LPS-activated human adherent monocytes by (Park et al. 2002). These data demonstrate that the ability of Tau-Cl to down-regulate the immune response is involved not only in rodent but also in humans. Tau-Cl also reduces IL-6 and IL-8 produced by fibroblasts-like synoviocytes isolated from patients with rheumatoid arthritis (Chorazy-Massalska et al. 2004; Kontny et al. 2006). Tau-Cl diminished the activity of NF-kB and to a lesser extent that of AP-1 transcription factor (Barua et al. 2002). Overall, the presence of high concentrations of taurine in leukocytes and the ability to form Tau-Cl in the presence of neutrophils coupled with effects on regulating non-adherent and adherent human leukocytes suggest a central role for taurine and its chloramine in regulating the immune response.

To understand the role of taurine in innate immunity in taurine-deficient CSAD KO, proinflammatory and anti-inflammatory cytokines including IL-6, TNF-α, and IL-10 as well as nitric oxide (NO) from LPS-activated macrophages were determined. Superoxide production in phorbol myristate acetate (PMA)-activated polymorphonuclear leukocytes (PMN) was also examined in CSAD KO.

2 Materials and Methods

2.1 Reagents

LPS (*Escherichia coli* (0111:B4) and thioglycollate were purchased from BD Biosciences (Sparks, MD). RPMI 1640, IFN-γ, penicillin and streptomycin were obtained from Invitrogen (Carlsbad, CA). Heat inactivated fetal calf serum (FCS) was purchased from Gemini Products (Woodland, CA). Taurine, nitrite, sulfanilamide, naphthylethylene diamine dihydrochloride, phosphoric acid and PMA were obtained from Sigma Chemical (St. Louis, MO). Duoset ELISA kits for measuring murine TNF-α, IL-6, and IL-10, were purchased from R&D System (Minneapolis, MN). All reagents used for cell activation including IFN-γ and complete medium containing FCS were tested for endotoxicity using a Limulus assay (Assoc. of Cape Cod, East Falmouth MA. Endotoxin was not detectable (EU < 0.25 EU/mL).

2.2 CSAD KO Mice

CSAD KO mice were produced previously described from our laboratory (Park et al. 2014). All mice were kept under 12-h day/night with free access to food and water. For optimum reproductive performance, one or two females were mated to a single male. Animals were weaned at 3–4 weeks of age. Generation 3 $CSAD^{-/-}$ (G3 $CSAD^{-/-}$, G3 HO) used in this study were born from G2 HO. G1 HO were born from heterozygotes (HT, $CSAD^{+/-}$). All procedures involving live animals were approved by the Institutional Animal Care and Use Committee of IBR.

2.3 Preparation of Peritoneal Macrophages and PMN

Peritoneal PMN and macrophages were prepared 1 day or 4 days after peritoneal injection of 4% thioglycollate, respectively (Cho et al. 2013). Differential staining showed greater than 98% purity of macrophages. Differential staining showed greater than 98% purity of PMN.

2.4 Superoxide Production

Superoxide anion production was determined (Kim et al. 1996) in PMN incubated for 30 min at 37 °C with 400 μg of ferricytochrome *c* and 100 ng of PMA in 200 μL of HBSS without phenol red. Superoxide dismutase (SOD) was added to parallel samples to measure SOD-inhibitable values. Cytochrome *c* reduction was measured

at 550 nm in a microplate reader (Bio-Tek Instrument, Winooski, VT) with 490 nm reference filter. Superoxide anion production was calculated using an mM extinction coefficient of 21.1.

2.5 Nitrite Determination

Nitrite was measured by the method described previously (Park et al. 1993). The calibration curve was prepared using sodium nitrite as a standard.

2.6 Measurement of TNF-α, Il-10 and IL-6 Using ELISA

Secretion of TNF-α, IL-10 and IL-6 was measured using Duoset ELISA kits by a modified method described previously (Cho et al. 2013). The concentrations of TNF-α, IL-10 or IL-6 released were calculated using rTNF-α, rIL-10 or rIL-6 as a standard.

2.7 High-Performance Liquid Chromatography (HPLC)

Peritoneal macrophages were obtained from WT and HO 4 days after mice were injected *ip* with thioglycollate. Taurine concentrations were determined using HPLC (Waters, Milford, MA) (Park et al. 2015a, b). Taurine concentrations were determined by comparison to a standard.

2.8 Statistical Analysis

Data are presented as mean ± SE. Statistical significance was determined using Statistica 13 (StatSoft, Tulsa, OK). Significant differences between experimental and control groups were determined as $p < 0.05$ using LSD or Tukey HSD in posthoc under one-way ANOVA.

3 Results

Previously we demonstrated that adaptive immunity in CSAD KO was reduced as measured by cellular proliferation of B and T lymphocytes activated with LPS and Con A, respectively (Park et al. 2015a, b). In addition to reduced adaptive immunity in CSAD KO, innate immunity using peritoneal PMNs and macrophages was

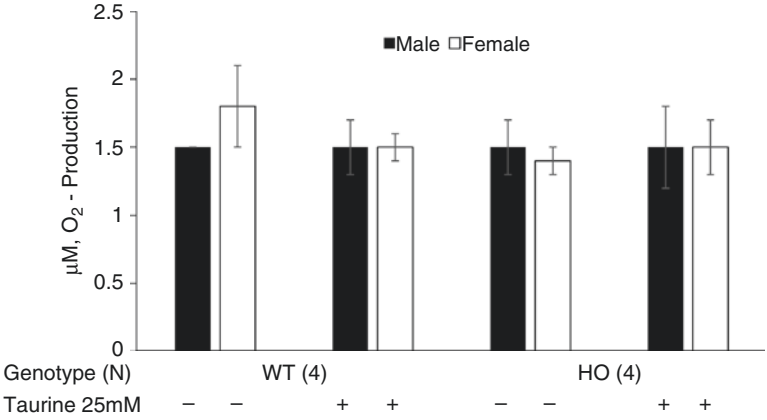

Fig. 1 Superoxide anion production in PMA-activated PMNs from WT and HO of CSAD KO. Four females and four males at the age of 8–12 weeks were used in this study. One additional experiment demonstrated similar results. HO is not significantly different from WT with and without 25 mM taurine. Data are expressed as mean ± SE (µM)

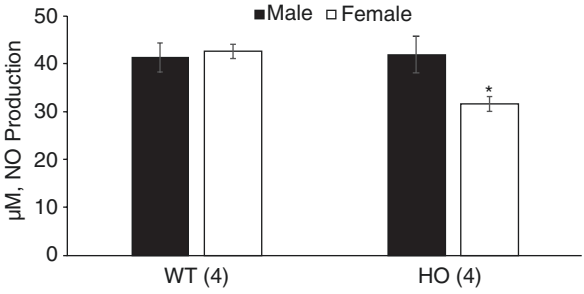

Fig. 2 NO production in LPS plus IFN-γ activated peritoneal macrophages from WT and HO of CSAD KO. Four females and four males at the age of 8–12 weeks were used in this study. One additional experiment demonstrated similar results. Female HO (*) is significantly different compared to WT, not male. Data are expressed as mean ± SE (µM)

examined. Innate immunity was determined using activation of peritoneal PMNs with PMA and peritoneal macrophages with LPS and/or interferon-γ (IFN-γ). Superoxide anion produced from activated PMNs was measured in the presence of absence of superoxide dismutase using cytochrome *c*. Superoxide production in activated PMNs from HO of CSAD KO was not significantly different from WT (Fig. 1).

In the presence of 25 mM taurine, superoxide production was without effect in both strains including HO and WT. Cytokines including IL-6, IL-10, and TNF-α as well as nitric oxide (NO) produced by activated peritoneal macrophages were measured using ELISA or Griess reagent, respectively. IL-10 and TNF-α production in both female (Figs. 3 and 5) and male CSAD KO were not significantly different. IL-6 (58%) and NO (26%) were significantly lower only in females of CSAD KO (Figs. 2 and 4) as similarly found in T and B lymphocyte proliferation. IL-6 and NO in males of CSAD KO were not significantly different from WT.

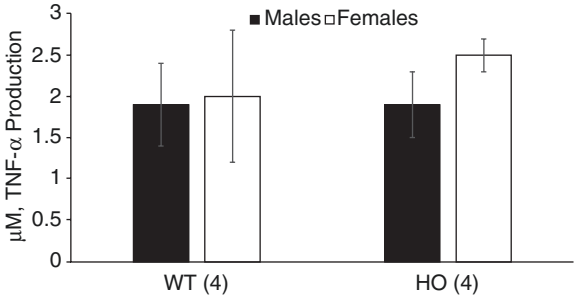

Fig. 3 TNF-α production in LPS-activated peritoneal macrophages from WT and HO of CSAD KO. Four females and four males at the age of 8–12 weeks were used in this study. One additional experiment demonstrated similar results. HO in both females and males is not significantly different from WT. Data are expressed as mean ± SE (μM)

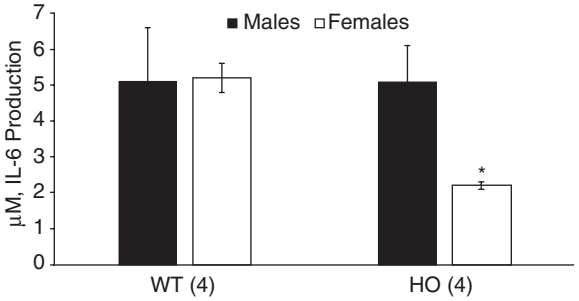

Fig. 4 IL-6 production in LPS-activated peritoneal macrophages from WT and HO of CSAD KO. Four females and four males at the age of 8–12 weeks were used in this study. One additional experiment demonstrated similar results. Female HO (*) is significantly different compared to WT, not male. Data are expressed as mean ± SE (μM)

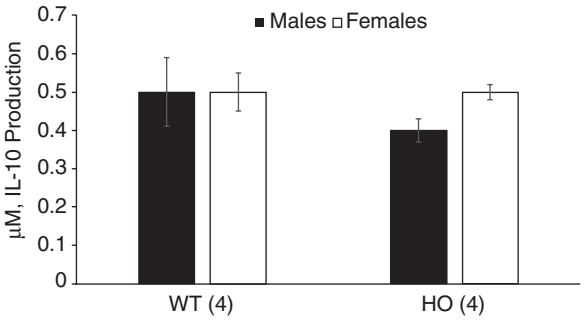

Fig. 5 IL-10 production in LPS-activated peritoneal macrophages from WT and HO of CSAD KO. Four females and four males at the age of 8–12 weeks were used in this study. One additional experiment demonstrated similar results. HO in both females and males is not significantly different from WT. Data are expressed as mean ± SE (μM)

Table 1 Production of cytokines and NO[a] in activated macrophages from HO of CSAD KO with and without taurine

	Taurine (mM)	Males	Females
NO	0	41.9 ± 3.8[b]	31.6 ± 1.5
	10	36.4 ± 3.0	26.3 ± 2.7
IL-6	0	5.2 ± 1.0	2.2 ± 0.1
	10	5.1 ± 1.1	2.1 ± 0.4
TNF-α	0	1.9 ± 0.3	2.5 ± 0.2
	10	2.1 ± 0.3	2.4 ± 0.4
IL-10	0	0.4 ± 0.03	0.5 ± 0.02
	10	0.4 ± 0.03	0.4 ± 0.05

[a]Cytokines and NO were produced in peritoneal macrophages activated with 1 μg/mL LPS or 1 μg/mL LPS plus 50 U/mL IFN-γ, respectively
[b]Data were mean ± SE (μM) from four mice per group
[c]Similar results were obtained from an additional experiment

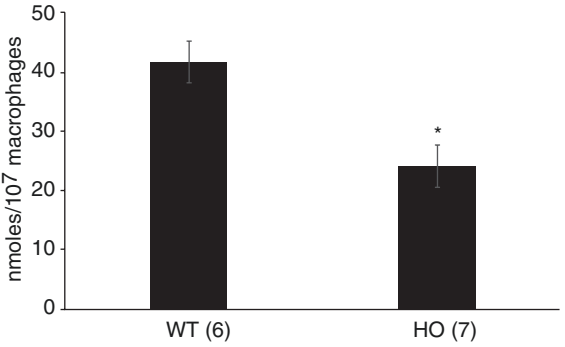

Fig. 6 Taurine concentrations in peritoneal macrophages from WT and HO of CSAD KO. A parenthesis is a number of mice. Asterisk (*) in HO is significantly different compared to WT ($p < 0.05$). Data are expressed as mean ± SE

Production of all cytokines and NO in the presence of 10 mM taurine were not different from those in the absence of taurine, similarly observed in reduced cellular proliferation in CSAD KO (Table 1).

Taurine concentrations in peritoneal macrophages from CSAD KO were significantly reduced 42%, compared to wild type (Fig. 6). Taurine concentrations in macrophages were obtained from both females and males from WT and HO because females and males were not significantly different as previously described (Park et al. 2015a).

4 Discussion

The taurine deficient cat showed severe immune dysfunction (Schuller-Levis and Park 2003). However, the limitations of immunologic technologies applied to the cat have led to the development of a murine model to understand the role of taurine in immune function. A novel taurine-deficient CSAD KO was developed in our laboratory (Park et al. 2014). Taurine supplementation in this model prior to and during pregnancy

rescues neonatal mortality. Some genes including antioxidant enzymes and metabolic enzymes for taurine biosynthesis were restored using taurine supplementation. Taurine deficient CSAD KO is a good model to examine immune function including the production of cytokines and NO and cellular proliferation. (Schuller-Levis et al. 1990; Schuller-Levis and Sturman 1992). Previously, we demonstrated that adaptive immune function in CSAD KO, including B and T lymphocytes, was significantly reduced in taurine-deficient CSAD KO compared to WT although the spleen index compared to WT was unaffected. LPS- and Con A-activated lymphocyte proliferation was significantly decreased, especially in females. Con A-induced lymphocyte transformation in males differed from females. Although LPS-induced lymphocyte proliferation in males from HO was lower than WT, Con A-induced lymphocyte proliferation was increased but without significance (Park et al. 2015b).

In this study, IL-6 and NO in female CSAD KO were altered compared to controls. However, superoxide anion, IL-10 and TNF-α in both females and males (Figs. 1, 2, 3, 4, and 5) were not changed. Similarly, the decrease of IL-6 and NO production was not restored to WT in the presence of 10 mM taurine in culture medium (Table 1). These data indicate reduced immune function which may be attributed to the lack of a CSAD gene instead of low taurine concentrations. These data also suggested that the CSAD gene may be involved in regulation of genes related to lymphocyte proliferation and cytokine production. Since alteration of immune functions was detected in females of CSAD KO, not in male, sex hormone including estrogen or/and testosterone may be involved in regulation of immune function. Recently, Ma et al. demonstrated that 17β-estradiol decreased taurine concentrations by reducing CSAD and cysteine dioxygenase (CDO) in the liver (Ma et al. 2015). Initially, they found that taurine levels was lower during estrus than diestrus and found that estrogen receptor-α (ERα) expression was much higher than estrogen receptor-β in the liver. Reduction of taurine levels in the serum is less in ERα knockout mice, indicating the decrease of taurine was mediated through ERα. Sex hormone are implicated in the immune response, with estrogens at least in the humoral immunity and androgens and progesterone as natural immunosuppressors (Cutolo et al. 2006). In particular, the enhancing role of estrogens on immune/inflammatory response is exerted by activating the NFκB complex pathway. A local increase of estrogens activates cellular proliferation and increases cellular markers in synovial tissue in rheumatoid arthritis or skin in systemic lupus erythematosus, suggesting the role of estrogen in autoimmunity. Therefore, our data indicate that alteration of hormone in female CSAD KO should be considered to explain the down-regulation of immune function. Overall, our results demonstrate that alterations of immune function in CSAD KO were less severe compared to taurine deficient cats as demonstrated by Schuller et al. (Park et al. 2015b; Schuller-Levis et al. 1990).

Taurine is actively transported into macrophages and splenocytes through taurine transporter because of the absence of CSAD in these cells (Uchida et al. 1992; Grillo et al. 2008). Taurine concentrations in the splenocytes and macrophages from CSAD KO are the most abundant amino acid and are significantly higher compared to those in the liver and plasma from CSAD KO (Fig. 6) (Fudaka et al. 1982; Park et al. 2015a), indicating taurine is required for critical antioxidant and/or anti-inflammatory functions. Several laboratories demonstrated taurine supplemen-

tation protects against inflammatory injury in various organs under oxidative stress in animal models (Gordon et al. 1992; Wang et al. 1991; Schuller-Levis et al. 1995, 2009; Gordon et al. 1998). This protection may be mediated by either taurine itself or its metabolite, Tau-Cl. We demonstrated that Tau-Cl inhibits LPS- and Con A-induced human lymphocyte proliferation and cytokine production in activated macrophages and PMNs (Park et al. 2002; Schuller-Levis and Park 2004). These data indicated that taurine and/or its metabolite, Tau-Cl, play a critical role in the regulation of immune function and inflammatory responses importantly in both humans and animals. However, the selective reduction of cytokines in female HO of CSAD in this study may not be mediated by Tau-Cl because Tau-Cl inhibits all four mediators including NO, TNF-α, Il-6 and IL-10 in activated murine and human macrophages (Kim et al. 1996; Park et al. 2002; Schuller-Levis and Park 2004). Hormonal regulation may be involved in the selective decrease of NO and IL-6 in female HO because taurine is lower in both male and female HO compared to WT (Ma et al. 2015; Cutolo et al. 2006). Since previous experiments for the role of taurine in the protection of oxidative injury were performed using rats and mice with high taurine concentrations, taurine deficient CSAD KO provides a model to better define the role of taurine in immune function and the inflammatory response with or without taurine supplementation. Therefore, a novel taurine-deficient CSAD KO will be an indispensable animal model for further studies on the role of taurine and the CSAD gene in immune function and the inflammatory response.

5 Conclusion

Innate immune function including IL-6 and NO in taurine-deficient female CSAD KO was significantly reduced compared to WT but not in male CSAD KO. Production of IL-6 and NO from LPS-activated peritoneal macrophages in female CSAD KO was not restored by taurine supplementation in culture medium *in vitro,* compared to WT. Our data suggested that taurine and the CSAD gene is required for normal immune function. Hormonal regulation of immune function in CSAD KO is also implicated because abnormalities were observed in females, not males.

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A Novel Cysteine Sulfinic Acid Decarboxylase Knock-Out Mouse: Taurine Distribution in Various Tissues With and Without Taurine Supplementation

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Abstract Taurine, a sulfur containing amino acid, has various physiological functions including development of the eye and brain, immune function, reproduction, osmo-regulatory function as well as anti-oxidant and anti-inflammatory activities. In order to understand the physiological role, we developed taurine deficient mice deleting a rate-liming enzyme, cysteine sulfinic acid decarboxylase (CSAD) for biosynthesis of taurine. Taurine was measured in various tissues including the liver, brain, lung, spleen, thymus, pancreas, heart, muscle and kidney as well as plasma from CSAD knock-out mice (CSAD KO) with and without treatment of taurine in the drinking water at the age of 2 months (2 M). Taurine was determined using HPLC as a phenylisothiocyanate derivative of taurine at 254 nm. Taurine concentrations in the liver and kidney from homozygotes of CSAD KO (HO), in which CSAD level is high, were 90% and 70% lower than WT, respectively. Taurine concentrations in the brain, spleen and lung, where CSAD level is low, were 21%, 20% and 28% lower than WT, respectively. At 2 M, 1% taurine treatment of HO restored taurine concentrations in all tissues compared to that of WT. To select an appropriate taurine treatment, HO were treated with various concentrations (0.05, 0.2, 1%) of taurine for 4 months (4 M). Restoration of taurine in all tissues except the liver, kidney and lung requires 0.05% taurine to be restored to that of WT. The liver and kidney restore taurine back to WT with 0.2% taurine. To examine which enzymes influence taurine concentrations in various tissues from

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WT and HO at 2 M, expression of five taurine-related enzymes, two antioxidant enzymes as well as lactoferrin (Lft) and prolactin receptor (Prlr) was determined using RT^2 qPCR. The expression of taurine transporter in the liver, brain, muscle and kidney from HO was increased except in the lung. Our data showed expression of glutamate decarboxylase-like 1(Gadl-1) was increased in the brain and muscle in HO, compared to WT, indicating taurine in the brain and muscle from HO was replenished through taurine transporter and increased biosynthesis of taurine by up-regulated Gadl-1. The expression of glutathione peroxidase 3 was increased in the brain and peroxireductase 2 was increased in the liver and lung, suggesting taurine has anti-oxidant activity. In contrast to newborn and 1 month CSAD KO, Ltf and Prlr in the liver from CSAD KO at 2 M were increased more than two times and 52%, respectively, indicating these two proteins may be required for pregnancy of CSAD KO. Ltf in HOT1.0 was restored to WT, while Prlr in HOT1.0 was increased more than HO, explaining improvement of neonatal survival with taurine supplementation.

These data are essential for investigating the role of taurine in development of the brain and eye, immune function, reproduction and glucose tolerance.

Keywords CSAD KO • Taurine distribution of various tissues • Gene expression • CSAD KO with taurine treatment

Abbreviations

2 M and 4 M	The age of 2 months and 4 months
ADO	Cysteamine (2-aminoenthanethiol) dioxygenase
CDO KO	Cyteine dioxygenase knockout mice
CSAD KO	Cysteine sulfinic acid decarboxylase knockout mice
CDO	Cysteine dioxygenase
CSAD	Cysteine sulfinic acid decarboxylase
Gpx 3	Glutathione peroxidase 3
HO	Homozygotic mice ($CSAD^{-/-}$)
HOT	Homozygotic mice treated with 1% taurine
HOT0.05, HOT0.2 and HOT1.0	Homozygotic mice treated with 0.05%, 0.2% or 1% taurine, respectively
Ltf	Lactoferrin
Prdx 2	Peroxireductase 2
Prlr	Prolactin receptor
TauT KO	Taurine transporter knockout mice
TauT	Taurine transporter
WT	Wild type ($CSAD^{+/+}$)

1 Introduction

Taurine is considered a conditionally essential amino acid in humans and primates and is required during their development. The essential physiological functions of taurine in development of the brain and eye, in reproduction, endocrine regulation, kidney and cardiovascular function, membrane stabilization, osmotic regulation as well as immune function have been well documented (Schuller-Levis and Park 2006; Sturman 1993). Taurine deficient animals are useful in investigations of taurine's physiological functions because it is possible to study both taurine deficiency itself and the effect of supplementation with taurine in the food and drinking water. Cats and rodents have been used as animal models for taurine studies because their taurine levels could be easily manipulated (Sturman 1993; Sturman and Messing 1991, 1992). Cats have been used for taurine studies because they produce only low levels of cycteine dioxygenase (CDO) and cysteine sulfinic acid decarboxylase (CSAD) leading to a dependence on dietary sources of taurine. However, the cat model has limitations including a long gestation period, a heterogeneous genetic background and a relatively large maintenance expense and the absence of genetic, molecular and immunological reagents. Rodents have high levels of CSAD (Schuller-Levis and Park 2006; Sturman 1993; Huxtable 2000) and taurine is not essential to their diet. Due to high concentrations of taurine in rodents, competitive inhibitors of taurine transport including guanidinoethanesulfonate (GES) or β-alanine have been used to produce taurine deficiency (Bonhaous et al. 1985; Dela Rosa and Stipanuk 1984; Jong et al. 2010). However, these chemicals have toxic side effects.

Five gene products are critical to taurine homeostasis: cysteine dioxygenase (CDO; EC 1.13.11) oxidizes cysteine to cysteine sulfinic acid which is converted to hypotaurine which is then oxidized to taurine (Stipanuk 2004; Bella et al. 2000; Hosokawa et al. 1990); cysteamine dioxygenase ADO (EC1.13.11.19) which converts cysteamine to hypotaurine (Dominy et al. 2007); the taurine transporter (TauT) (Uchida et al. 1992); and cysteine sulfinic acid decarboxylase, CSAD (EC 4.1.1.29), which is the enzyme that converts cysteine sulfinic acid to hypotaurine (Park et al. 2002); Glutamate decarboxylase-like 1 (GADL-1) which produces taurine from CSAD and aspartate (Liu et al. 2012; Winge et al. 2015). Recently four genetically modified mice have been developed to model taurine deficiency using gene targeting methods. These taurine-deficient knock-out mice include two taurine transporter knockout mice (TauT KO), cysteine dioxygenase knockout mice (CDO KO) and cysteine sulfinic acid decarboxylase (CSAD KO). Two TauT KO mouse models show reduced levels of taurine in various tissues including heart, brain, muscle, kidney and liver (Heller-Stilb et al. 2002; Warsulat et al. 2007; Ito et al. 2008). These TauT KO models demonstrate developmental effects in various organs including the retina, liver, brain, muscle and heart. A cysteine dioxygenase deficient (CDO KO) model was produced by deleting CDO, thereby disabling the production of cysteine sulfinic acid, a substrate for CSAD, from cysteine (Ueki et al. 2011, 2012; Roman et al. 2013). These mice have severe taurine deficiency and increased

catabolism of cysteine to hydrogen sulfide, which leads to pulmonary and pancreatic toxicity. Our laboratory produced a cysteine sulfinic acid decarboxylase knockout mouse (CSAD KO) as a novel mouse model of taurine deficiency (Park et al. 2014). We demonstrated high neonatal mortality in the third and fourth generation of $CSAD^{-/-}$ homozygotes (G3 HO and G4 HO) and restoration of neonatal survival by the addition of 0.05% taurine added to the drinking water. Compared to wild type (WT), taurine concentrations in the liver and brains of newborn pups are significantly lower except in G1 HO, born from $CSAD^{+/-}$ heterozygous (HT) dams which have near normal levels of serum taurine. Low taurine concentrations in the liver and brain in HOs are significantly restored by supplementation of taurine in the drinking water of the dam. Gene expression of prolactin receptor (Prlr) and lactoferrin (Ltf) is decreased but gene expression of glutathione peroxidase 3 (Gpx 3) and peroxireductase (Prx 2) increased, suggesting oxidative stress may be involved in neonatal mortality. Subsequently newborn pups and mice at 1 M after weanling were compared for changes in taurine distribution in the brain and liver and gene expression (Park et al. 2015). Data demonstrated that a decrease in taurine concentrations in the liver from weanling mice are more profound than those in the brain. Surviving CSAD KO after weaning indicated that taurine is redistributed on the basis of need, regardless of its origin, which suggests that the requirement for taurine for homeostasis and survival may vary from organ to organ.

In order to confirm this finding, we examined nine tissues including the liver, brain, kidney, pancreas, thymus, spleen, heart, lung and muscle as wells as blood at 2 M with and without 1% taurine in the drinking water. Taurine concentrations at 2 and 4 M were compared in nine tissues and plasma. Various taurine amounts including 0.05, 0.2 and 1.0% at 4 M were examined for appropriate amount of taurine supplementation for restoring the taurine concentrations in various tissues to WT. Gene expression correlated to different taurine concentrations in various tissues were also examined in this study.

2 Materials and Methods

2.1 Materials

Chemicals used in this study were purchased from Sigma Chemicals (St. Louis, MO) if not otherwise noted. Oligonucleotide primers for PCR for genotype were obtained from Eurofins MWG Operon (Huntsville, AL). Primers were designed by Primer Designer 4 (Scientific and Educational Software, Cary, NC). Taq polymerase and deoxynucleotides were purchased from New England Biolabs (Ipswich, MA). Agarose was obtained from Lonza Group Ltd. (Rockland, ME). Trizol and RNeasy kit for RNA extraction were obtained from Invitrogen and Qiagen (Valencia, CA), respectively. The SYBR master mix and primers used in RT^2 qPCR were purchased from Qiagen.

2.2 CSAD KO Mice

CSAD KO mice were produced as previously described from our laboratory (Park et al. 2014). Experimental mice were fed taurine-free chow (LabDiet^{R}, PMI Nutrition International, St. Louis, MO). Taurine concentrations in commercial food were confirmed by HPLC. All mice were kept under 12-h day/night with free access to food and water. For optimum reproductive performance, one or two females were mated to a single male. Both females and males used for mating in the taurine-treated groups were supplemented with various concentrations of taurine including 0.05, 0.2 and 1.0% in the drinking water as indicated in Results and Discussion and offspring used in this study were treated with same taurine concentrations after weaning. All mice at the age of 2 and 4 months (2 M and 4 M) used in this study were separated from their dam 3–4 week after birth. All procedures involving live animals were approved by the Institutional Animal Care and Use Committee of IBR.

2.3 High Performance Liquid Chromatography (HPLC)

All tissues including the liver, brain, kidney, spleen, thymus, heart, lung, muscle and pancreas as well as plasma were obtained from all groups including WT, HO and HO treated with various taurine (HOT) after mice were sacrificed with *ip* injection of avertin (250 mg/Kg). Taurine concentrations were determined using HPLC (Waters, Milford, MA) (Battaglia et al. 1999). Briefly, tissues and plasma were homogenized using 5% TCA and centrifuged for removal of proteins. After samples were dried using a Speedvac (Savant, Holbrook, NY), they were derivatized using phenylisothiocyanate (PITC) and separated using a C18 column with a gradient of acetate buffer containing 2.5% acetonitril (pH 6.5) and 45% acetonitril solution containing 15% methanol at 45 °C. The flow rate was 1 mL/min. Taurine concentrations were determined by comparison to a standard.

2.4 RT^2 qPCR Ananlysis

Total RNA was extracted using RNeasy kit (Qiagen) from various tissues including the liver, brain, kidney, muscle and lung from WT, HO and HO treated with 1% taurine (HOT1.0) was reverse-transcribed using cDNA kit according to the manufacturer's instruction (Qiagen). Quantitative real time PCR with 10 ng of cDNA were carried out in duplicate in a 7300 real-time PCR system (Effendorff, Hauppauge, NY) using the SYBR master mix (Qiagen) and the following cycles: 2 min at 50 °C, 10 min at 95 °C and 40 cycles each at 95 °C for 15 s and 60 °C for 60 s (19). RT^2 qPCR analysis was also carried out according to manufacturer's

manual using β-actin as a control. All primers were purchased from Qiagen. For data analysis the Ct method was used; for each gene fold-changes were calculated as difference in gene expression of HO and HOT1.0, compared to that in WT. ΔCt was calculated by subtraction of Ct of β-actin from Ct of the interesting gene. ΔΔCt was calculated by subtraction of ΔCt of WT from ΔCt of HO or HOT. Fold change was determined by $2^{\wedge\Delta\Delta Ct}$. More than one indicates gene up-regulation and less than one indicates gene down-regulation.

2.5 Statistical Analysis

Data are presented as mean ± SE. Statistical significance was determined using Statistica 13 (StatSoft, Tulsa, OK). Significant differences between groups were determined as $p < 0.05$ using LSD or Tukey HSD in post-hoc under one way ANOVA.

3 Results

3.1 Taurine Concentrations in Various Tissues

Since the decrease of taurine concentrations in the liver and brain from HO were remarkably different compared to WT at the age of 1 M, we determined taurine concentrations in 9 tissues including the liver, brain, kidney, pancreas, spleen, thymus, lung, heart and muscle as well as plasma from WT, HO and HO treated with 1% taurine in the drinking water (HOT1.0) at 2 M. Taurine concentrations in various tissues are variable (Figs. 1 and 2).

The liver and kidney in HO showed the lowest taurine concentrations. Both the liver and kidney produce taurine due to high levels of CSAD in WT and decreased taurine concentrations 90% and 70% in CSAD KO, respectively (Fig. 2). Meanwhile,

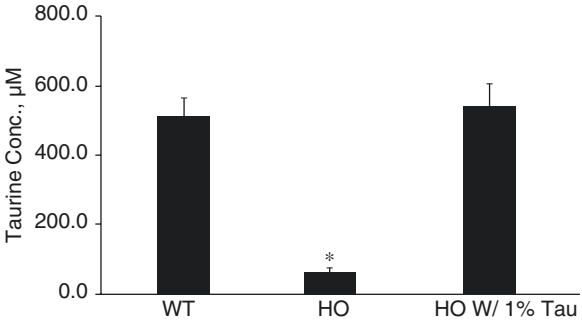

Fig. 1 Plasma taurine concentrations in WT, HO and HO with 1% taurine treatment at the age of 2 months. Eight mice were used in each group. Data are expressed mean ± SE. *$p < 0.05$ statistically significant, compared to WT

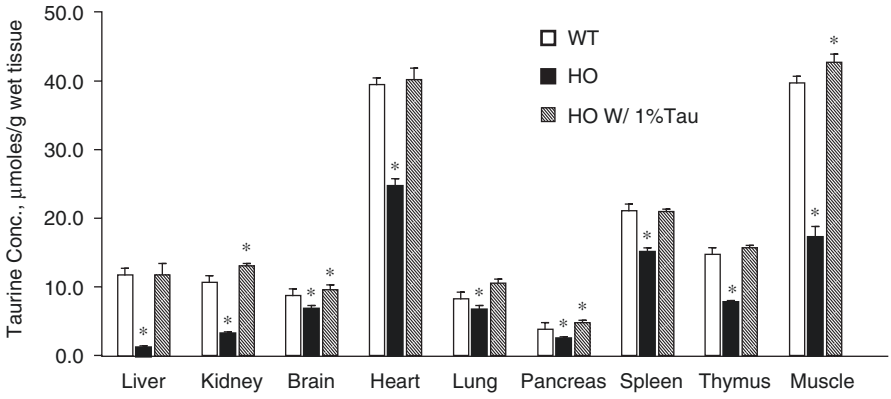

Fig. 2 Taurine concentrations in various tissues from WT, HO and HO with 1% taurine treatment at the age of 2 months. Eight mice were used in each group. Data are expressed mean ± SE. *p < 0.05 statistically significant, compared to WT

the brain, spleen and lung, which have low CSAD levels and transport taurine through TauT, were decreased 21%, 21% and 28% lower than WT, respectively. At 2 M, HOT1.0 restored taurine concentrations in all tissues to those of WT (Fig. 2). Taurine concentraions in some tissues including the kidney, brain, pancreas and muscle from HOT1.0 are significantly increased. Plasma taurine concentrations in HO CSAD KO were 88% lower than WT and restored completely to WT when HO were treated with 1% taurine (Fig. 1). Previously we demonstrated that taurine concentrations in the liver and brains from females and males in all groups were not significantly different although taurine concentrations in CDO KO females were higher than in males (Park et al. 2015; Ueki et al. 2011). Taurine concentrations in this study were obtained from both females and males.

3.2 Taurine Treatment of Various Taurine Treatment Including 0.05, 0.2 and 0.1% in the Drinking Water

Four month old mice to 2 M old mice were compared to determine how much taurine concentrations is changed in WT and HO (Table 1). All tissues at 4 M except the heart, muscle and lung from HO were not significantly different, compared to 2 M. However, taurine concentrations in the heart, muscle and lung from HO at 4 M were significantly increased although the heart and muscle were in WT of CSAD KO not significantly different at both ages. Interestingly, taurine concentrations in the lung from WT and HO at 4 M was increased 3.2 times and 2.5 times compared to in both WT and HO at 2 M, respectively. Lower taurine treatment including 0.05 and 0.2% were compared to 1% taurine treatment in the drinking water at 4 M. 0.05% taurine treatment in HO restored taurine concentrations in all tissues except the liver and kidney. Taurine concentrations in the liver and kidney were restored to

Table 1 Taurine concentrations in various tissues from WT and HO at the age of 2 and 4 months

	WT		HO	
	2 M (8)[c]	4 M (8)	2 M (8)	4 M (8)
Plasma[a]	512 ± 53.3	319.7 ± 29.7	65.0 ± 11.1	77.7 ± 10.2
Liver[b]	11.8 ± 0.9	9.0 ± 0.9	1.2 ± 0.1	1.8 ± 0.2
Kidney	10.7 ± 0.6	12.6 ± 0.2	3.2 ± 0.2	5.5 ± 0.4
Brain	8.8 ± 0.6	10.5 ± 1.0	6.9 ± 0.5	6.8 ± 0.6
Heart	39.4 ± 3.6	38.6 ± 0.6	24.7 ± 1.0	32.4 ± 0.7[d]
Lung	8.3 ± 0.3	26.2 ± 4.4[d]	6.7 ± 0.6	17.2 ± 2.9[d]
Pancreas	3.9 ± 0.2	4.5 ± 0.4	2.6 ± 0.2	3.2 ± 0.3
Spleen	21.0 ± 0.2	20.7 ± 0.5	15.1 ± 0.6	15.8 ± 0.6
Thymus	14.7 ± 1.0	15.7 ± 1.1	7.7 ± 0.3	11.2 ± 0.5
Muscle	39.6 ± 1.0	44.2 ± 1.2	17.4 ± 1.4	31.5 ± 1.2[d]

[a]Data from plasma are expressed as mean ± SE (μM)
[b]Data from various tissues are expressed as mean ± SE (μmoles/g wet tissue)
[c]Number in parentheses is number of mice used in each group
[d]Significant difference statistically between the age of 2 and 4 months in WT and HO, respectively, $p < 0.05$. All HO in both ages are significantly different, compared to WT, <0.05

Table 2 Taurine concentrations in various tissues from WT, HO, HO with 0.05, 0.2 and 1.0% taurine

	WT[c]	HO	HOT0.05	HOT0.2	HOT1.0
Plasma	319.7 ± 29.7[a]	77.7 ± 10.2[d]	422.8 ± 90.1	385.8 ± 82.6	540.4 ± 65.2[d]
Liver	9.0 ± 0.9[b]	1.8 ± 0.2[d]	5.9 ± 1.1	7.1 ± 1.2	10.7 ± 0.7
Kidney	12.6 ± 0.2	5.5 ± 0.4[d]	8.6 ± 0.5	12 ± 0.9	15.2 ± 1.0[d]
Brain	10.5 ± 1.0	6.8 ± 0.6[d]	10.7 ± 0.5	10.6 ± 0.8	13.7 ± 0.9[d]
Spleen	20.7 ± 0.5	15.8 ± 0.6[d]	20.8 ± 1.0	22.3 ± 0.6	21.7 ± 0.4
Thymus	15.7 ± 1.1	11.2 ± 0.5[d]	14.8 ± 1.1	17.1 ± 0.4	18.7 ± 0.8
Pancreas	4.5 ± 0.4	3.2 ± 0.3[d]	4.8 ± 0.6	5.3 ± 0.2	5.0 ± 0.2
Heart	38.6 ± 0.6	32.4 ± 0.7[d]	37.6 ± 0.9	39.2 ± 1.3	43.2 ± 1.8[d]
Muscle	44.2 ± 1.2	31.5 ± 1.2[d]	45.5 ± 1.5	44.4 ± 2.3	49.0 ± 1.4[d]
Lung	26.2 ± 4.4	17.2 ± 2.9	18.7 ± 3.3	18.3 ± 4.3	21.7 ± 3.9

[a]Data from plasma are expressed as mean ± SE (μM)
[b]Data from various tissues are expressed as mean ± SE (μmoles/g wet tissue)
[c]Eight mice were used in each group
[d]Significant difference statistically between WT to HO, HOT0.05, HOT0.2 and HOT1.0, $p < 0.05$

WT when HO were treated with 0.2% taurine solution (Table 2). Taurine concentrations in four tissues including the kidney, brain, heart and muscle as well as plasma were increased significantly when HO were treated with 1% taurine at 4 M. The taurine concentration in the lung from WT and HO as well as HO treated with various taurine in the drinking water were not significantly different at 4 M, not consistent with results from 2 M (Fig. 2). Taurine concentrations in the lung from WT, HO and HOT were not statistically different because the data from the lung are remarkably variable in individual mice.

3.3 Gene Expression Measured Using RT² qPCR

Taurine tissue distribution was remarkably different in various tissues (Fig. 2 and Table 1). In order to examine correlation of taurine tissue concentrations as well as various taurine biosynthetic enzymes and taurine transporter, gene expression of four enzymes including Csad, Cdo, Ado and Gadl-1 as well as TauT was determined using five tissues from females of WT, HO and HOT1.0 at 2 M (Table 3). Gene expression of five proteins in five tissues including the liver, brain, lung, muscle and kidney from HO was compared to WT. The taurine transporter was increased more than 1.5 times in four tissues including the liver, brain, kidney and muscle from HO compared to WT. TauT in the lung from HO were not significantly different compared to WT. TauT in all tissues from HOT1.0 was restored to WT. CSAD KO in all tissues from HO and HOT1.0 was extremely low, confirming the absence of CSAD. Cdo was increased in the brain from HO and Ado was increased in the brain and muscle compared to WT, while Gadl-1 was increased in the brain and muscle compared to WT. Cdo in the brain from HOT1.0 was even increased compared to HO. Meanwhile, Ado in both tissues was restored to WT. Gadl-1 in the brain from HOT was recovered to WT but that in the muscle was even increased compared to HO. Gadl-1 was not detected in the liver and kidney which is expressed previously described by Liu et al. (Liu et al. 2012). In addition to taurine-related genes, antioxidant-related genes including Gpx3 and Prdx2 were determined as measured

Table 3 Fold changes[a] in various tissues from WT, HO and HO with 1% taurine treatment

Tissue	Genotype	Csad	Cdo	Ado	Gadl-1	TauT
Liver	WT	1.00[b]	1.00	1.00	ND	1.00
	HO	<0.01	0.80	1.20	ND	1.70
	HOT1.0	<0.01	1.20	1.30	ND	1.40
Brain	WT	1.00	1.00	1.00	1.00	1.00
	HO	0.05	1.70	1.87	3.10	2.30
	HOT1.0	0.05	2.20	1.32	1.10	1.40
Lung	WT	1.00	1.00	1.00	ND[c]	1.00
	HO	0.01	0.88	0.72	ND	1.05
	HOT1.0	0.02	1.04	1.05	ND	0.08
Muscle	WT	1.00	1.00	1.00	1.00	1.00
	HO	0.02	0.56	1.91	1.98	1.77
	HOT1.0	0.02	1.06	1.39	2.68	1.23
Kidney	WT	1.00	1.00	1.00	ND	1.00
	HO	0.01	1.23	1.23	ND	1.62
	HOT1.0	0.02	0.86	0.86	ND	0.91

[a]Fold change was determined by $2^{\wedge\Delta\Delta Ct}$

[b]Data are expressed as average of two mice. Similar results were obtained from one additional experiment

[c]ND means "not detected"

Table 4 Fold change[a] in various tissues from WT, HO and HO with 1% taurine treatment

Tissue	Genotype	Gpx3	Prdx2
Liver	WT	1.00[b]	1.00
	HO	1.26	1.52
	HOT1.0	1.07	1.42
Brain	WT	1.00	1.00
	HO	1.98	1.10
	HOT1.0	1.90	0.74
Lung	WT	1.00	1.00
	HO	1.14	1.58
	HOT1.0	1.45	1.41
Muscle	WT	1.00	NT[c]
	HO	0.97	NT
	HOT1.0	0.82	NT
Kidney	WT	1.00	1.00
	HO	1.38	1.38
	HOT1.0	1.06	1.06

[a]Fold change was determined by $2^{\wedge\Delta\Delta Ct}$

[b]Data are expressed as average of two mice. Similar results were obtained from one additional experiment

[c]NT means "not tested"

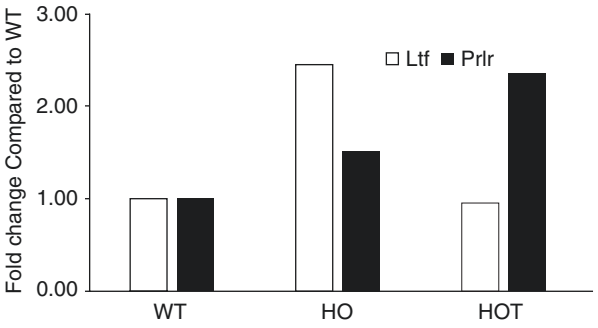

Fig. 3 Fold changes of Prlr and Ltf in HO and HOT compared to those in WT at the age of 2 months. HOT mice were treated with 1% taurine in the drinking water. Data are expressed as mean of two mice. Similar results were obtained from one additional experiment

previously in newborn pubs and 1 month old mice (1 M) (Table 4). Gpx3 was increased in the brain from HO and HOT1.0, while Prdx2 was increased in the liver and lung. Prolactin receptor (Prlr) and lactoferrin (Ltf), which were significantly decreased at newborn pups and weanling mice in previous studies, were included in this study to compare 2 M to newborn pups and 1 M (Fig. 3) (Park et al. 2015). In contrast to newborn pubs and weanling mice, both genes were increased in HO compared to WT at 2 M. However, Lft in HOT were recovered to WT, while Prlr in HOT1.0 was increased compared to HO.

4 Discussion

Taurine tissue distribution in WT, HO and HOT was examined because taurine concentrations in the liver and brain are remarkably different as previously described (Park et al. 2015). Taurine concentration in the liver from HO at the age of 2 months was decreased much more than those in the brain from HO which has low CSAD levels although taurine concentrations in both of the liver and brain from pups of HO were low. (Park et al. 2014, 2015). Taurine concentrations in nine tissues including the liver, kidney, brain, lung, heart, spleen, thymus, pancreas, and muscle as well as plasma from HO and HOT1.0 were compared to WT. Data demonstrated that the liver and kidney from HO, which has high CSAD levels, showed the greatest decrease, compared to WT (Fig. 2). The spleen, brain and lung were the least decrease, compared to WT. The heart and muscle were less decrease than the liver and kidney. These data indicated that taurine concentration was replenished through TauT and/or by biosynthesis of taurine *in situ* using up-regulated Ado and/or Gadl-1 depending on tissue (Table 3). All three proteins were up-regulated in the brain to maintain relatively high taurine HO compared to WT. Even Cdo in the brain from HO was up-regulated to produce more CSA which is a substrate of Gadl-1. Since TauT in the brain in HOT1.0 was restored to WT, Cdo in HOT1.0 in the brain was increased more than HO to produce CSA for restoring taurine to WT. Due to lack of Gadl-1 and CSAD in the liver and kidney in HO, taurine is low in the liver and kidney from HO although TauT was up-regulated by taurine deficiency (Fig. 2 and Table 3). These results demonstrated that taurine deficiency by deleting the CSAD was involved in the dynamic regulation of taurine-related genes to fulfill requirement of taurine for homeostasis. (Figs. 1 and 2, Table 3). The lung is a unique tissue in which any genes examined are not regulated in HO and HOT1.0 compared to WT.

Taurine concentrations in the lung from WT and HO at 4 M were remarkably increase compared to 2 M (Table 1). Taurine concentrations in the lung from WT, HO and three HOTs including HO0.05, HO0.2 and HO1.0 were not significantly different with various amounts of taurine (Table 2). These data indicated that the lung may not be regulated expression of taurine-related genes and TauT in both WT and HO by taurine-deficiency and/or deletion of CSAD gene. Up-regulation of Gpx3 in the lung from HO may partially explain anti-oxidant activity of taurine (Table 4). Our results with Gadl-1 enzyme, which produce taurine from CSA and aspartate, was not consistent from previous results of Liu et al. and Winge et al. (Liu et al. 2012; Winge et al. 2015). Although expression of Gadl-1 was detected in the kidney, muscle and brain previously, Gadl-1 was expressed in the brain and muscle but not in the kidney in our study (Table 3). It is of interest that taurine in the lung, muscle and heart were remarkably increased in HO at 4 M, compared to 2 M, indiciating taurine may be required more for maintaining their physiological functions. In contrast to CSAD KO, TauT KO shows low taurine concentrations in all tissues including the heart, muscle, eye, brain, kidney and plasma (Heller-Stilb et al. 2002; Ito et al. 2008) due to the absence of Tau T. Taurine concentrations in the liver from CDO KO are significantly low, similarly to CSAD KO (Ueki et al. 2011). CSAD gene expression was absent in both HO and HOT in this study, confirming deletion of CSAD (Table 3).

The antioxidant enzyme, Gpx 3 expression in the liver was not changed at 2 M although Gpx 3 expression were increased in both newborn pups and weanling mice (Table 4) (Park et al. 2014, 2015; Schaffer et al. 2009; Brigelius-Flohe and Maiorino 2013; Lubos et al. 2011). However, Gpx3 expression in the brain was increased. Prdx2 expression in the liver and lung was increased at 2 M. Gpx 3 expression in the brain was restored in HOT1.0. Alteration of these genes may indicate taurine deficiency may induce oxidative stress. Since Ltf and Prlr in the liver from newborn pups and weanling mice were decreased in HO, we also examined these gene expression at 2 M. Ltf has innate immune function to protect newborn offspring from infection and is elevated in colostrum (Ward and Conneely 2004; Legrand and Mazurier 2010; Legrand 2012). Ltf is widely present with a high affinity for iron in fluids such as milk and colostrum. Ltf, an indispensable component of the innate immune system, has bacteriostasis and required for optimal neutrophil function. Prolactin, a lactogenic hormone, regulates the output of insulin-like growth factor −1. Genetic ablation of Prlr results in mice which show multiple defects in reproduction leading to infertility, altered maternal behavior and reduced bone development (Brooks 2012; Binart et al. 2010; Bole-Feysot et al. 1998). While Ltf and Prlr are decreased significantly in liver from HO at newborn and 1 M, both genes were increased at 2 M (Park et al. 2014, 2015). In contrast to newborn and weanling mice, Prlr in HOT1.0 at 2 M was increased even more than HO. These data suggest that mature mice may require taurine to maintain a stable pregnancy. Antibacterial lactoferrin is high in colostrum same as taurine and Ig A (Sturman 1993; Sanchez et al. 1992; Weaver et al. 1991). An increase in lactoferrin in HO may compensate for taurine deficiency because lactoferrin expression is recovered to WT in HOT0.1. In contrast to lactoferrin, an increase of prolactin receptor expression in HO and HOT1.0 may be required for increased survival of offspring as demonstrated previously in our laboratory. The survival rate in HOT0.05 was remarkably high, 92%, compared to HO, 13%. These results supported improvement of neonatal survival with taurine supplementation. Alteration of gene expression was observed in CDO KO as in CSAD KO. Expression of Csad gene in the liver from CDO KO was increased significantly compared to WT and expression of Ado gene in CDO KO was without effect. However, Ado in the brain in CSAD KO was increased at 2 M but not in the liver. Regulation of TauT in CSAD KO is consistent to that in CDO KO. TauT expression in CDO KO is increased and restored to WT in taurine-treated CDO KO (Roman et al. 2013). These data indicated that taurine deficiency as well as deletion of taurine biosynthetic enzymes may be involved in gene regulation of various genes.

5 Conclusion

Taurine concentrations in the liver and kidney which have high levels of CSAD were decreased more severely than the other tissues, compared to WT. Taurine centrations in the brain in CSAD KO were decreased much less by increasing Cdo,

Ado, Gadl-1and TauT, compared to the liver. These data indicated that taurine concentrations in HO of CSAD KO was replenished through an increase of TauT and/or taurine-bioxsynthetic enzymes according to necessity of taurine in various tissues. Redistribution of taurine and regulation of gene expression in various tissues from HO of CSAD KO are important for understanding the role of taurine in development of the brain, immune function, reproduction and glucose tolerance.

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Identification of Taurine-Responsive Genes in Murine Liver Using the *Cdo1*-Null Mouse Model

Martha H. Stipanuk, Halina Jurkowska, Julie Niewiadomski, Kevin M. Mazor, Heather B. Roman, and Lawrence L. Hirschberger

Abstract The cysteine dioxygenase (*Cdo1*)-null mouse is unable to synthesize hypotaurine and taurine by the cysteine/cysteine sulfinate pathway and has very low taurine levels in all tissues. The lack of taurine is associated with a lack of taurine conjugation of bile acids, a dramatic increase in the total and unconjugated hepatic bile acid pools, and an increase in betaine and other molecules that serve as organic osmolytes. We used the *Cdo1*-mouse model to determine the effects of taurine deficiency on expression of proteins involved in sulfur amino acid and bile acid metabolism. We identified cysteine sulfinic acid decarboxylase (*Csad*), betaine:homocysteine methytransferase (*Bhmt*), cholesterol 7α-hydroxylase (*Cyp7a1*), and cytochrome P450 3A11 (*Cyp3a11*) as genes whose hepatic expression is strongly regulated in response to taurine depletion in the *Cdo1*-null mouse. Dietary taurine supplementation of *Cdo1*-null mice restored hepatic levels of these four proteins and their respective mRNAs to wild-type levels, whereas dietary taurine supplementation had no effect on abundance of these proteins or mRNAs in wild-type mice.

Keywords Betaine:homocysteine methytransferase (BHMT) • Cysteine dioxygenase (CDO) • Cysteine sulfinic acid decarboxylase (CSAD) • Cytochrome P450, family 3, subfamily A, member 11 (CYP3A11) • Cholesterol 7α-hydroxylase (cytochrome P450, family 7, subfamily A, member 1) (CYP7A1)

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Abbreviations

ABCB11	ATP-binding cassette, subfamily B, member 11
BHMT	Betaine:homocysteine methytransferase
CDO	Cysteine dioxygenase
CSAD	Cysteine sulfinic acid decarboxylase
CYP27A1	Sterol 27-hydroxylase (cytochrome P450, family 27, subfamily A, member 1)
CYP3A11	Cytochrome P450, family 3, subfamily A, member 11
CYP7A1	Cholesterol 7α-hydroxylase (cytochrome P450, family 7, subfamily A, member 1)
FXR	Farnesoid X receptor
LRH1	Liver receptor homolog 1
OSTα-OSTβ	Organic solute and steroid transporter
SHP	Small heterodimer partner (also known as NR0B2)
SLC6A6	Sodium- and chloride-dependent taurine transporter (also known as TAUT)

1 Introduction

Taurine, or 2-aminoethanesulfonic acid, is an abundant organic compound in mammalian cells, being found in the range of 10–20 μmol per gram in murine tissues (Roman et al. 2013; Wójcik et al. 2010). Taurine has diverse biological roles and is involved in the processes of bile acid conjugation, cell volume regulation, modification of mitochondrial tRNAs, nervous and visual system development, antioxidation, and immune defense (Huxtable 1992; Ripps and Shen 2012). In general, taurine has protective effects on cells that are manifest under various types of injury as a result of its homeostatic effects.

Cysteine dioxygenase (CDO), which is encoded by the *Cdo1* gene, catalyzes the addition of oxygen to cysteine to form cysteine sulfinate. Cysteine sulfinate undergoes further metabolism, either by transamination to yield pyruvate and sulfite or by decarboxylation to hypotaurine and its further oxidation to taurine. The CDO-dependent pathway of taurine biosynthesis is the major route for taurine synthesis in mammals, and hence the *Cdo1* knockout mouse has a severely limited capacity to synthesize taurine and very low tissue taurine levels if fed a taurine-free diet.

A taurine-responsive decrease in cysteine sulfinic acid decarboxylase (CSAD) protein abundance has been reported previously for the *Cdo1* knockout mouse (Roman et al. 2013; Ueki et al. 2011), and we recently reported that BHMT mRNA and protein levels were downregulated in liver of the *Cdo1* knockout mouse (Jurkowska et al. 2016). Because of the dramatic effects of taurine of the hepatic bile acid pool and because of recent reports that CSAD and CYP7A1 (cholesterol

7α-hydroxylase) may be regulated in a common fashion by bile acids (Kerr et al. 2013), we decided to use the *Cdo1*-null mouse model to identify other hepatic proteins that are expressed in a taurine-responsive manner.

2 Methods

2.1 Animals, Diets, and Liver Collection

Cdo1$^{-/-}$ and *Cdo1*$^{+/+}$ mice for this study were generated by crossing C57BL/6 *Cdo1*$^{+/-}$ male and female mice as described previously (Ueki et al. 2011; Roman et al. 2013). Animal studies were conducted with the approval of the Cornell University Institutional Animal Care and Use Committee (#2009-0138). Mice were housed at 23 °C and 45–50% humidity with light from 6:00 to 20:00 daily. Pups, as well as their dams, had free access to the standard semipurified taurine-free diet available in their cages from birth to postnatal day 21. Pups were weaned at day 21 and assigned to either a basal (taurine-free) diet based on the AIN93G semi-purified diet for growing mice or to the same diet supplemented with 5 g taurine/kg. Six mice were assigned to each sex/genotype/diet group (48 mice total).

After weaning, mice assigned to the same diet were housed together with two to four mice per cage. All experimental mice were fed the basal diet from weaning through postnatal day 37. From postnatal day 38 until postnatal day 62–68 when liver was collected, mice were fed the assigned treatment diet, which was either the same taurine-free diet or that diet supplemented with taurine. To obtain liver, mice were euthanized between 10:00 and 14:00 h with an overdose of isoflurane. Liver was removed, immediately frozen in liquid nitrogen, and stored at −80 °C until samples were analyzed.

2.2 Determination of Hepatic Protein and Taurine Levels and Western Blotting to Measure the Relative Abundance of Proteins

Frozen liver samples were homogenized in four volumes of lysis buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA and 0.5% Nonidet P-40] containing 1× Complete Protease Inhibitor Cocktail (Roche) and 1× PhosSTOP phosphatase inhibitor (Roche). The supernatant fraction was obtained by centrifuging the homogenates at 18,000 × *g* for 20 min at 4 °C and used for determination of total soluble protein, taurine levels, and the relative abundances of selected mRNAs and proteins.

Total cellular protein in hepatocyte lysates and soluble protein in the supernatant fractions from liver homogenates were determined using the BCA Protein Assay Kit (Thermo Scientific/Pierce) using bovine serum albumin (BSA) as the standard.

For measurement of taurine and hypotaurine, the cell lysate or the liver supernatant fraction was mixed with one volume of 5% (wt/vol) sulfosalicylic acid, and the mixture was centrifuged at 15,000 × *g* for 15 min at 4 °C to obtain the acid supernatant. Taurine and hypotaurine were measured by HPLC as described previously (Ueki et al. 2011). Samples were derivatized with *o*-phthaldialdehyde (OPA) and separated on a C18 column by gradient elution using 0.05 M potassium phosphate buffer (pH 7.0) with 3.5% (vol/vol) tetrahydrofuran mobile phase without or with 40% (vol/vol) acetonitrile. Detection of OPA-derivatized compounds was performed using excitation and emission peaks at 360 and 455 nm, respectively.

For measurement of relative protein abundance, aliquots of liver supernatant equivalent to 30 μg of total protein were separated by SDS-PAGE (12%, w/v, polyacrylamide). Protein bands were transferred onto a 0.45-μm Immobilon-FL PVDF membrane (Millipore Corp.). Immunoblotting was performed by first exposing membranes to blocking buffer for near infrared fluorescent westerns (LI-COR Biosciences) and then blotting for immunoreactive proteins. The primary antibodies included anti-CSAD at a 1:8000 dilution (gift from Dr. Marcel Tappaz, INSERM, France), anti-CYP7A1 at a 1:500 dilution (GeneTex), anti-CYP3A11/4 at a 1:1000 dilution (Cell Signaling), anti-CYP27A1 at a 1:1000 dilution (Thermo Fisher Scientific), anti-OSTβ at a 1: 400 dilution (Bioss), anti- BHMT at a 1:1000 dilution (Thermo Fisher Scientific), anti-β-tubulin at a 1:500 dilution (Santa Cruz Biotechnology), and anti-β-actin at a 1:1000 dilution (Proteintech Group). Immunoreactive bands were detected and quantified using an infrared fluorescent dye-labeled secondary antibody (IRDye, LI-COR Biosciences) and the Odyssey direct infrared imaging system and software (LI-COR Biosciences). Protein abundances were divided by β-tubulin or β-actin abundance to normalize the values, which were then expressed as fold the value for wild-type mice of the same sex fed the basal taurine-free diet.

2.3 PCR Measurement of mRNA Relative Abundance

The relative abundance of mRNA was measured by PCR. The RNeasy mini kit (Qiagen) was used to isolate RNA isolated from the liver samples, and complementary DNA was reverse transcribed using Applied Biosystems High Capacity cDNA kit (Applied Biosystems). Quantification of mRNA relative abundance was done using Power Sybr Green (Applied Biosystems) and a Roche 480 Lightcycler (Roche Diagnostics). The forward and reverse primer sequences were: ABCB11 forward 5′-ACTGAACTTGGAAAGGGGTGT-3′ reverse 5′-TCACTCAACAACCCT ACAGATG-3′; BHMT forward 5′-CGGCTTCAGAAAAACATGG-3′ reverse 5′-TCTGCCAGATTCCTTTCTGG-3′; CYP3A11 forward 5′-GAAGCATTGAGGAGGATCACA-3′ reverse 5′-GGTCCATCCCT GCTGTTT-3′; CYP7A1 forward 5′-CACCATTCCTGCAACCTTCT-3′ reverse 5′-TTGGCCAGCACTCTGTAATG-3′; CYP27A1, forward 5′-GTGGACAACCTCCTTTGGGAC-3′ reverse 5′-CCCTCCTGTCTC ATCACTTGC-3′; CSAD forward 5′-CCAGTGCCTCTGAGAAGGTC-3′

reverse 5′-TGACACTGTAGTGAATCACAGTCC-3′; OSTβ forward 5′-TGACAAGCATGTTCCTCCTG-3′ reverse 5′-TGGAGTCATCAAGATGCAGGT-3′; and SLC6A6 forward 5′-CTGCCTGGATTTGGAAGG-3′ reverse 5′-GCCACTGAAGACAGGTGAGG-3′.

Values for ABCB11, BHMT, CYP7A1, CYP27A1, CYP3A11, CSAD, OSTβ, and SLC6A6 mRNAs were normalized to values for β-actin mRNA, and the normalized values were used to calculate the fold differences relative to the average value for wild-type mice of the same sex fed the basal diet.

2.4 LC/MS Analysis of Liver Metabolites

Samples were prepared for LC/MS analysis by homogenizing a weight aliquot of frozen liver in ice-cold 80% methanol/water (200 μL per 5 mg liver) and then diluting the homogenate with an additional volume of ice-cold 80% methanol/water (200 μL per 5 mg liver). The homogenate was then vortexed, allowed to sit on ice for 10 min, and centrifuged (20,000 × *g* at 4 °C for 10 min) to obtain supernatant. A 200 μL aliquot of the supernatant was transferred to a microcentrifuge tube, dried in a SpeedVac (Thermo Scientific), and stored at −80 °C until the LC-MS analysis was done.

For LC-MS, dried supernatant samples were reconstituted with 30 μL water, diluted with an additional 30 μL acetonitrile/methanol (1:1, v/v), and centrifuged (20,000 × *g* at 4 °C for 3 min) to obtain the final supernatant used for LC/MS.

A 4 μL-aliquot of the final supernatant was injected into the LC-MS system, which was an Ultimate 3000 UHPLC (Dionex) coupled to a Q Exactive-Mass spectromer (QE-MS, Thermo Scientific). LC was done at room temperature. For analysis of bile acids and related compounds, reversed phase LC was performed using a Luna C18 column (100 × 2.0 mm i.d., 3 μm; Phenomenex) with a gradient mobile phase system (A: 5 mM ammonium acetate in water; B: methanol). The percentage of mobile phase B was 2% B between 0 and 1.5 min, was linearly increased to 15% B between 1.5 and 3 min and to 95% between 5.5 and 14.5 min, was held at 95% between 14.5 and 15 min before being returned to 2% B between 15 and 20 min. For analysis of other polar metabolites, hydrophilic interaction LC (HILIC) was run with an Xbridge amide column (100 × 2.1 mm i.d., 3.5 μm; Waters). Details of the LC/MS analysis were published previously by Liu et al. (2014).

2.5 Statistical Analysis

Results of measurements are expressed as means ± SEM for six mice. Statistical analysis was run as a full factorial least squares model using JMP version 10 (SAS, Cary, NC). Results for male and female mice were analyzed separately. Differences were accepted as significant at $p \leq 0.05$ for main effects (genotype, treatment) and at $p \leq 0.1$ for interactions. Post-hoc individual pairwise comparisons of least squares

means by Tukey's procedure were considered significant at $p < 0.05$. Data for hepatic mRNA and protein abundances of CSAD, CYP3A11, and OSTβ were square root transformed prior to statistical analysis. Data for taurine and hypotaurine levels, the hepatic abundances of BHMT mRNA and protein, and metabolite differences were log-transformed prior to statistical analyses.

3 Results

3.1 Taurine and Metabolite Concentration Differences

Cdo1-null mice fed a standard semi-purified rodent diet, which contained no taurine, had very low taurine levels as reported previously (Roman et al. 2013; Ueki et al. 2011). Hepatic taurine concentrations in the *Cdo1*-null mice fed a taurine-free diet were less than 3% of wild-type levels (Fig. 1). Supplementation of the diet with taurine restored the hepatic taurine level. Hepatic taurine levels in *Cdo1*-null and wild-type mice fed the taurine supplemented diet were similar to each other as well as similar to the level in wild-type mice fed the basal taurine-free diet.

To further explore the changes in liver metabolites in the taurine-deficient *Cdo1*-null mice and to assess the extent to which any alterations were due to taurine deficiency, metabolomic profiles were run on liver samples from male rats in each genotype-diet group. Taurine and bile acid levels exhibited the largest fold differences between the two genotypes when mice fed taurine-free diets were compared. *Cdo1*-null mice fed the standard taurine-free semipurified diet had very low hepatic taurine levels (Table 1), which was consistent with the dramatic reductions based on

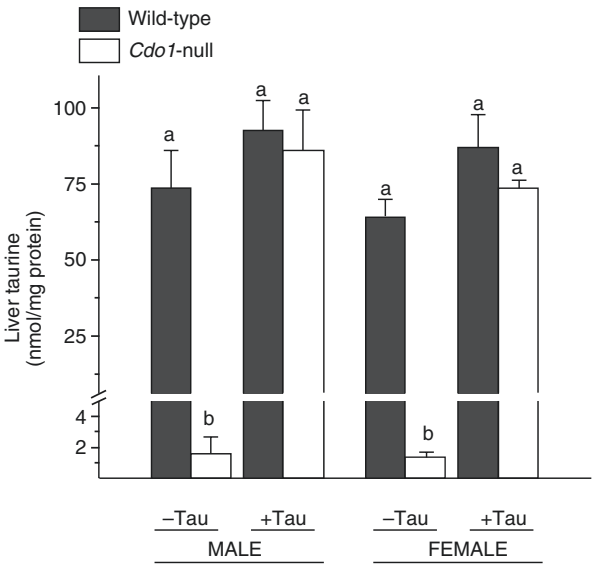

Fig. 1 Taurine concentration in the liver of male and female *Cdo1*-null and wild-type mice fed either a basal (−Tau) or taurine-supplemented (+Tau) diet. Values shown in bar graphs are means ± SEM for six mice. Bars not denoted by the same letter are significantly different from other values for mice of the same sex ($P < 0.05$)

Table 1 Fold differences in taurine, bile acids and organic osmolytes in liver of male wild-type and *Cdo1*-null mice fed a basal taurine-free or a taurine-supplemented diet

Metabolites	Basal taurine-free diet		Taurine-supplemented diet	
	Wild-type	*Cdo1*-null	Wild-type	*Cdo1*-null
Taurine	$1 \pm 0.10^{a*,\dagger}$	0.048 ± 0.01^{b}	0.97 ± 0.18^{a}	1.16 ± 0.06^{a}
Betaine	1 ± 0.09^{b}	2.29 ± 0.34^{a}	0.91 ± 0.09^{b}	1.19 ± 0.22^{b}
Choline	1 ± 0.05^{b}	1.57 ± 0.04^{a}	0.96 ± 0.11^{b}	0.80 ± 0.32^{b}
sn-Glycerol-3-phosphocholine	1 ± 0.23^{b}	1.82 ± 0.27^{a}	0.92 ± 0.10^{b}	0.81 ± 0.12^{b}
Carnosine	1 ± 0.11^{b}	2.12 ± 0.14^{a}	0.87 ± 0.11^{b}	0.76 ± 0.06^{b}
myo-Inositol	1 ± 0.04	1.42 ± 0.16	1.12 ± 0.06	1.20 ± 0.09
Taurocholate/tauromuricholate	1 ± 0.26^{a}	0.23 ± 0.04^{b}	0.96 ± 0.11^{a}	1.11 ± 0.32^{a}
Taurodeoxycholate/ taurochenodeoxycholate/ tauroursodeoxycholate	1 ± 0.23^{a}	0.18 ± 0.03^{b}	2.40 ± 0.10^{a}	1.02 ± 0.21^{a}
Taurolithocholate	1 ± 0.18^{a}	0.15 ± 0.04^{b}	0.92 ± 0.11^{a}	0.84 ± 0.11^{a}
Glycocholate/glycomuricholate	1 ± 0.64^{b}	110 ± 38^{a}	0.88 ± 0.27^{b}	1.45 ± 0.70^{b}
Glycodeoxycholate/ glycochenodeoxycholate/ glycoursodeoxycholate	1 ± 0.44^{b}	49.6 ± 7.6^{a}	0.89 ± 0.36^{b}	0.67 ± 0.28^{b}
Cholate/muricholate	1 ± 0.27^{b}	86 ± 12^{a}	3.20 ± 2.29^{b}	0.36 ± 1.51^{b}
Deoxycholate/chenodeoxycholate/ ursodeoxycholate	1 ± 0.13^{b}	39.7 ± 4.7^{a}	2.26 ± 1.24^{b}	1.13 ± 0.21^{b}
Lithocholate	1 ± 0.15^{b}	14.0 ± 1.3^{a}	1.22 ± 0.09^{b}	1.14 ± 0.27^{b}
3α-7α-12α-Trihydroxy-5β-cholestanoate	1 ± 0.14^{b}	3.40 ± 0.63^{a}	1.03 ± 0.10^{b}	0.72 ± 0.12^{b}
3α-7α-Dihydroxy-5β-cholestanoate	1 ± 0.23^{b}	2.27 ± 0.43^{a}	0.90 ± 0.13^{bc}	0.65 ± 0.17^{c}
7α-Hydroxy-3-oxo-4-cholestenoate	1 ± 0.20^{b}	2.70 ± 0.68^{a}	0.71 ± 0.06^{b}	1.04 ± 0.22^{b}

*All values for metabolite concentrations are expressed relative to the average value for the wild-type/basal diet group, which was set as 1.0. Data are expressed as means ± SEM

†All values were transformed to $\log_{10}$ values prior to statistical analysis by a general linear model for the two categorical variables and their interaction using JMP, version 11 (SAS, Cary, NC). Values not followed by the same superscript letter are significantly different at $p < 0.05$ as determined by Tukey's post-hoc comparison test

HPLC analysis reported in Fig. 1. The hepatic levels of taurine-conjugated bile acids in taurine-deficient *Cdo1*-null mice were low (15–23% of wild-type for mice fed the basal diet). Levels of unconjugated bile acids and glycine-conjugated bile acids were elevated in *Cdo1*-null mice fed the basal diet, and the abundances of several other molecules that can function as organic osmolytes (betaine, choline, glycerophosphocholine, and carnosine) were significantly higher in the *Cdo1*-null mice fed the basal diet (Table 1). In addition, the concentration of 7α-hydroxycholest-4-en-3one, an early metabolite of cholesterol in the CYP7A1-initiated neutral pathway of bile acid synthesis; 3α,7α,12α-trihydroxy-5β-cholestanoate, a subsequent intermediate in the pathway for cholate synthesis; and 3α,7α,dihydroxy-5β-cholestanoate, a subsequent intermediate in the pathway for chenodeoxycholate/muricholate synthesis were all significantly elevated (to ~2.3 to 3.4-fold wild-type levels) in liver of *Cdo1*-null mice fed the taurine-free basal diet. Notably, all of these

differences disappeared when taurine was added to the diet, indicating that the lack of taurine biosynthesis in the *Cdo1*-null mouse was responsible for the changes in levels of both bile acid metabolites and organic osmolytes.

3.2 *Effect of Taurine Status on Gene Expression*

Hepatic levels of CSAD mRNA and protein have been reported by several investigators to be strongly influenced by taurine status and were examined to verify the usefulness of the *Cdo1*-null mouse model for detecting taurine-responsive genes. CSAD mRNA abundance was increased to 12.8-fold control in male and 7.5-fold control in female *Cdo1*-null mice fed the taurine-free basal diet, and this was reversed when the *Cdo1*-null mice were given the taurine-supplemented diet (Fig. 2). Similarly, CSAD protein abundance was increased to 10.5-times control levels in male and to 8.8-times control levels in female *Cdo1*-null mice fed the taurine-free diet.

The mRNA abundance for the sodium- and chloride-dependent taurine transporter SLC6A6 was also significantly elevated by 50–60% in liver of taurine-deficient *Cdo1*-null mice, but not in the liver of taurine-supplemented *Cdo1*-null mice (Fig. 3). We were unsuccessful in detecting SLC6A6 protein in liver due to nonspecificity of the antibody we used. Expression of *Slc6a6* (*Taut*) has been shown to be sensitive to hypertonic stress and to be transcriptionally regulated by the tonicity-responsive element (TonE) and the TonE-binding protein (TonEBP) in

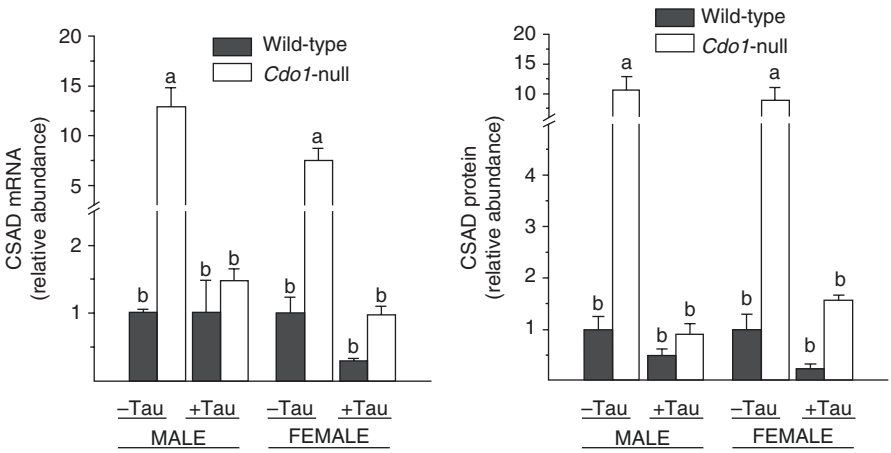

Fig. 2 CSAD mRNA and protein abundance in liver of male and female *Cdo1*-null and wild-type mice fed either a basal (−Tau) or taurine-supplemented (+Tau) diet. Values shown in bar graphs are means ± SEM for six mice and are expressed as fold the value for wild-type mice of the same sex fed the basal diet; bars not denoted by the same letter are significantly different from other values for mice of the same sex ($P < 0.05$)

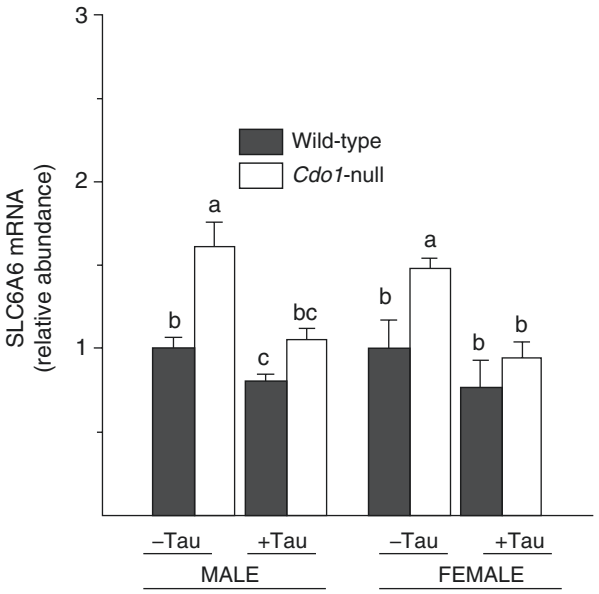

Fig. 3 SLC6A6 mRNA abundance in liver of male and female *Cdo1*-null and wild-type mice fed either a basal (−Tau) or taurine-supplemented (+Tau) diet. Values shown in bar graphs are means ± SEM for six mice and are expressed as fold the value for wild-type mice of the same sex fed the basal diet; bars not denoted by the same letter are significantly different from other values for mice of the same sex ($P < 0.05$)

HepG2 cells (Ito et al. 2004). Both the mRNA and protein abundance for SLC6A6 increased in HepG2 cells cultured in hypertonic medium (Ito et al. 2004; Satsu et al. 2003). In addition, culturing HepG2 cells in taurine-rich medium resulted in down-regulation of both SLC6A6 mRNA and protein abundance (Satsu et al. 2003). The metabolomics results shown in Table 1 show increases in other organic osmolytes, especially betaine, in the *Cdo1*-null mouse liver, which supports the likelihood of osmotic stress (cell shrinkage) in the taurine-deficient *Cdo1*-null mice. Whether the upregulation of SLC6A6 mRNA abundance was due to hypertonicity or low taurine levels, or both, cannot be distinguished, but taurine supplementation of the diet returned the hepatic SLC6A6 mRNA level to that of wild-type taurine-sufficient mice.

A third taurine-responsive gene, *Bhmt*, was recently identified by our laboratory (Jurkowska et al. 2016). BHMT encodes the betaine: homocysteine methyltransferase, which consumes betaine as a source of methyl groups for remethylation of homocysteine in the liver. As shown in Fig. 4, BHMT mRNA and protein abundances were suppressed in the *Cdo1*-null mice fed the taurine-free basal diet, with mRNA levels being reduced to 37% of control in males and 42% of control in females and protein levels being reduced to 48% of control in both male and female mice. Taurine supplementation reversed the low BHMT mRNA and protein levels in *Cdo1*-null mice. It is notable that the decrease in BHMT mRNA and protein levels in *Cdo1*-null mice fed the basal taurine-free diet was associated with elevated hepatic betaine levels that were 2.3-times those of wild-type mice fed the same diet (Table 1).

Because of the dramatic changes in the hepatic bile acid metabolite profile in liver of *Cdo1*-null mice fed the taurine-free diet (Table 1) and because Kerr et al. (2013)

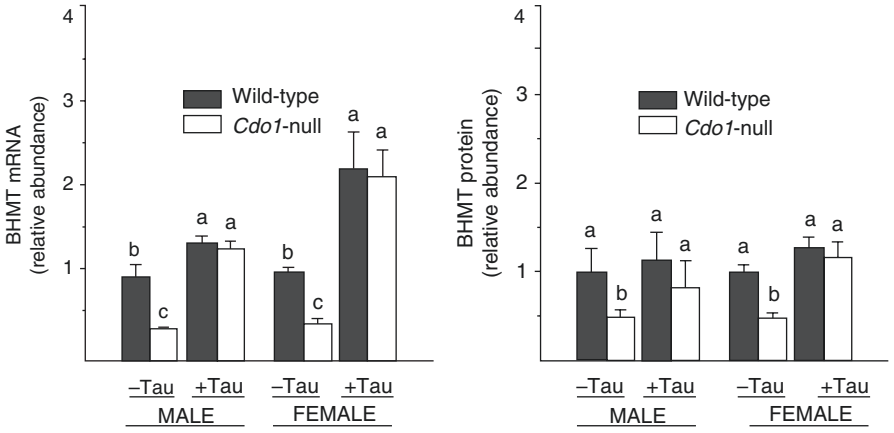

Fig. 4 BHMT mRNA and protein abundance in liver of male and female *Cdo1*-null and wild-type mice fed either a basal (−Tau) or taurine-supplemented (+Tau) diet. Values shown in bar graphs are means ± SEM for six mice and are expressed as fold the value for wild-type mice of the same sex fed the basal diet; bars not denoted by the same letter are significantly different from other values for mice of the same sex ($P < 0.05$)

reported similarities between the mechanisms of *Csad* and *Cyp7a1* induction via farnesoid X receptor (FXR) and small heterodimer partner (SHP)-dependent signaling, we decided to examine the expression of several proteins involved in bile acid synthesis and transport. [SHP is the same as nuclear receptor subfamily 0, group B, member 2 (NR0B2).] First, we looked at the expression of several cytochrome P450 (CYP) enzymes involved in bile acid synthesis, CYP7A1, CYP27A1, and CYP3A11. CYP7A1 (cholesterol 7α-hydroxylase) catalyzes the initial step in the major pathway for bile acid synthesis from cholesterol, whereas CYP27A1 (sterol 27-hydroxylase) catalyzes the initial step in an alternate pathway for bile acid biosynthesis. The major bile acids synthesized by human liver are cholic acid and chenodeoxycholic acid, but in mice chenodeoxycholic acid is readily further converted to muricholic acid by CYP3A11. In rodent liver, CYP3A11 hydroxylates chenodeoxycholate at the 6β-position to form the more hydrophilic α-muricholate, which can be further converted to β-muricholate by epimerization of its 7α-OH to 7β-OH (Martignoni et al. 2006; Gardès et al. 2013). In *Cdo1*-null mice, CYP7A1 mRNA was 3.0–3.4-times that in liver of wild-type mice and CYP7A1 protein abundance was 2-times that in liver of wild-type mice (Fig. 5). Supplementation of *Cdo1*-null mice with taurine returned CYP7A1 to wild-type levels. Results were identical for male and female mice. CYP27A1 mRNA and protein abundances were not affected by the *Cdo1* genotype or by taurine supplementation, indicating the absence of regulation of the alternative pathway of bile acid synthesis by taurine (Fig. 6). CYP3A11 mRNA and protein abundances were markedly increased in male *Cdo1*-null mice, with CYP3A11 mRNA and protein being 6-times and 15-times wild-type levels, respectively (Fig. 7). In female mice,

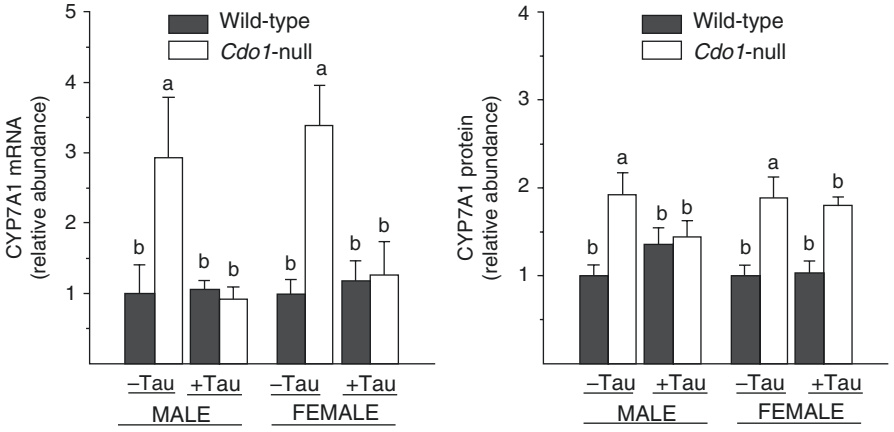

Fig. 5 CYP7A1 mRNA and protein abundance in liver of male and female *Cdo1*-null and wild-type mice fed either a basal (−Tau) or taurine-supplemented (+Tau) diet. Values shown in bar graphs are means ± SEM for six mice and are expressed as fold the value for wild-type mice of the same sex fed the basal diet; bars not denoted by the same letter are significantly different from other values for mice of the same sex ($P < 0.05$)

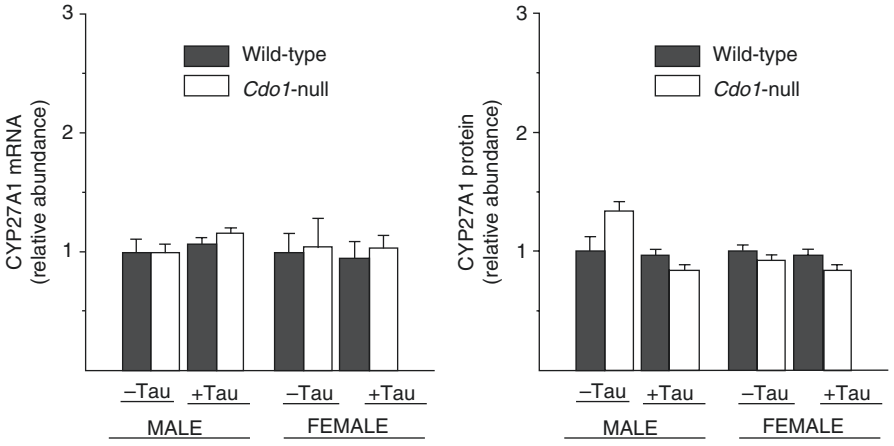

Fig. 6 CYP27A1 mRNA and protein abundance in liver of male and female *Cdo1*-null and wild-type mice fed either a basal (−Tau) or taurine-supplemented (+Tau) diet. Values shown in bar graphs are means ± SEM for six mice and are expressed as fold the value for wild-type mice of the same sex fed the basal diet; bars not denoted by the same letter are significantly different from other values for mice of the same sex ($P < 0.05$)

CYP3A11 mRNA and protein expression were both 3-times those in wild-type mice. Supplementation of *Cdo1*-null mice with taurine returned CYP3A11 mRNA and protein abundances to the lower wild-type levels. Thus, both CYP7A1 and CYP3A11 are sensitive to taurine status, being elevated in the taurine-deficient

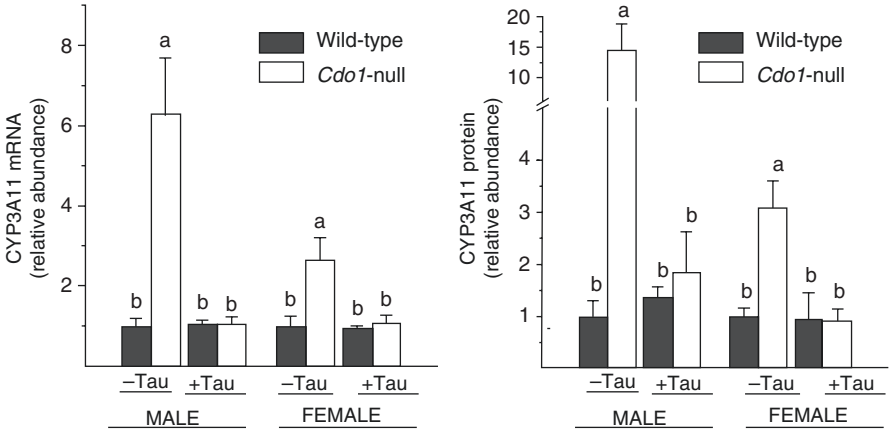

Fig. 7 CYP3A11 mRNA and protein abundance in liver of male and female *Cdo1*-null and wild-type mice fed either a basal (−Tau) or taurine-supplemented (+Tau) diet. Values shown in bar graphs are means ± SEM for six mice and are expressed as fold the value for wild-type mice of the same sex fed the basal diet; bars not denoted by the same letter are significantly different from other values for mice of the same sex ($P < 0.05$)

Cdo1-null mice but reduced to control levels by taurine supplementation. The greater expression of these two enzymes involved in bile acid synthesis is consistent with the fold increases in abundance of cholestanoate and cholestenoate intermediates in bile acid synthesis (Table 1). These responses may be seen as an attempt of the liver to increase bile acid synthesis when taurine conjugation of bile acids is deficient.

Finally, we looked at the expression of two bile acid transporters, OSTβ and ABCB11. OSTβ is a subunit of the heteromeric organic solute and steroid transporter (OSTα-OSTβ), which transports bile acids, conjugated steroids and structurally-related molecules according to their electrochemical gradients. Hepatic expression of OSTβ subunit mRNA and protein in *Cdo1*-null male mice was 3-times that of wild-type male mice, but taurine-supplementation did not reverse this effect, suggesting it might be due to some aspect of the *Cdo1*-null genotype other than taurine depletion (Fig. 8). In female *Cdo1*-null mice, OSTβ mRNA and protein abundances were not significantly greater than those of wild-type mice, regardless of diet. Overall these results suggest a more complicated regulation of OSTβ expression that is not solely responsive to taurine, although taurine appears to have some affect. For *Abcb11* expression, we only looked at mRNA levels, and these were not affected by *Cdo1* genotype or taurine supplementation (Fig. 9). The *Abcb11* gene encodes the ATP-binding cassette, subfamily B, member 11, which is also known as the bile salt export pump (BSEP) because of its role in the transport of taurocholate and other bile acids from hepatocytes into the bile.

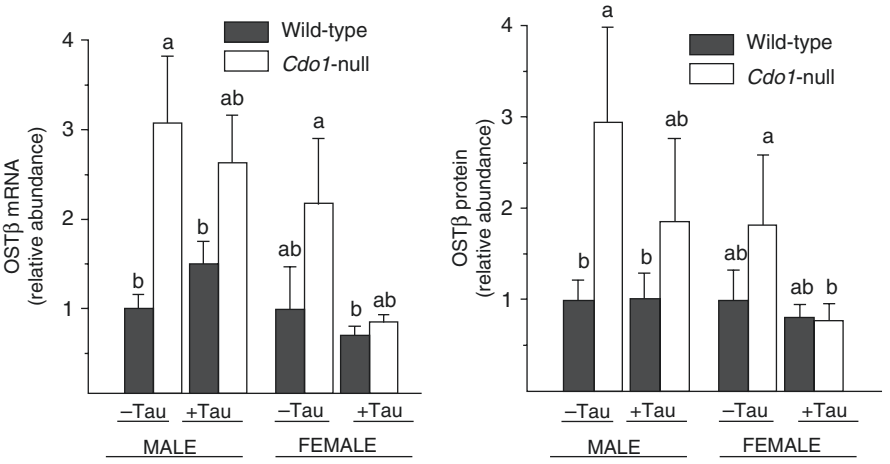

Fig. 8 OSTβ mRNA and protein abundance in liver of male and female *Cdo1*-null and wild-type mice fed either a basal (−Tau) or taurine-supplemented (+Tau) diet. Values shown in bar graphs are means ± SEM for six mice and are expressed as fold the value for wild-type mice of the same sex fed the basal diet; bars not denoted by the same letter are significantly different from other values for mice of the same sex ($P < 0.05$)

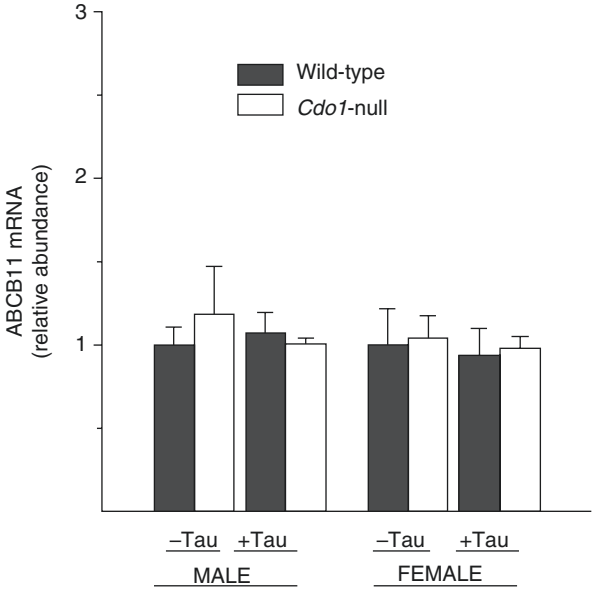

Fig. 9 ABCB11 mRNA in liver of male and female *Cdo1*-null and wild-type mice fed either a basal (−Tau) or taurine-supplemented (+Tau) diet. Values shown in bar graphs are means ± SEM for six mice and are expressed as fold the value for wild-type mice of the same sex fed the basal diet; bars not denoted by the same letter are significantly different from other values for mice of the same sex ($P < 0.05$)

4 Discussion

Unconjugated bile acids accumulate in liver of Cdo1-null mice as a result of a lack of taurine for bile acid conjugation. Murine bile acids undergo conjugation with taurine in the liver prior to their secretion in the bile. Mice differ from humans and rats in that they conjugate bile acids almost solely with taurine instead of with either glycine or taurine. This is due to the specificity of bile acid CoA:amino acid *N*-acyltransferase in different species of mammals, with murine bile acid CoA:amino acid *N*-acyltransferase almost exclusively conjugating bile acids with taurine (Falany et al. 1997). Both newly synthesized bile acids and deconjugated bile acids returning to the liver via the enterohepatic circulation are normally conjugated with taurine prior to secretion into the bile.

Many studies have demonstrated that the percentage of the hepatic or biliary bile acid pools present as taurine-conjugates reflects hepatic taurine concentrations (Stephan et al. 1981; Stipanuk et al. 1984; De la Rosa and Stipanuk 1985). Similarly, in *Cdo1*-null mice in this study, in which hepatic taurine was depleted to 3–5% of wild-type levels, there was a dramatic decrease in the hepatic concentration of taurine-conjugated bile acids and there were large fold elevations in unconjugated and glycine-conjugated bile acids. These changes were clearly related to taurine deficiency because the hepatic bile acid pools of *Cdo1*-null mice fed the taurine-supplemented diet were similar to those of wild-type mice.

Although bile acid concentrations were not directly measured in our study, we estimated the bile acid pool size in *Cdo1*-null mouse liver using the fold differences we observed along with the molar concentrations reported by Alnouti et al. (2008) and García-Cañaveras et al. (2012). Compared to their observations of ~97–98% taurine-conjugated, ~0.1% glycine-conjugated, and ~2–3% unconjugated bile acids in murine liver, we estimate that the total hepatic bile acid pool in the *Cdo1*-null mice was 30–40% higher than in wild-type mice and comprised about 3.9% taurine-conjugated, 0.7% glycine-conjugated, and 95.4% unconjugated bile acids. Clearly, the absolute concentrations of unconjugated bile acids were elevated many-fold, with unconjugated bile acids replacing taurine-conjugated bile acids as the dominant species, due to both the dramatically lower formation of taurine conjugates and the somewhat larger total bile acid pool. Although glycine-conjugated bile acids were elevated in *Cdo1*-null mice, they accounted for little of the total murine bile acid pool due to the mouse's lack of glycine conjugating activity.

Either an increase in synthesis or a decrease in biliary secretion, or both, could account for the 30–40% increase in the hepatic bile acid pool. Because we observed approximately twofold increases in key intermediates in the pathway for bile acid synthesis from cholesterol (i.e., 7α-hydroxycholest-4-en-3one; 3α,7α,12α-trihydroxy-5β-cholestanoate and 3α,7α,dihydroxy-5β-cholestanoate) as well as an 80% increase in CYP7A1 abundance, the rate of bile acid synthesis was almost certainly elevated in liver of *Cdo1*-null mice fed taurine-free diets. The observed upregulation of bile acid synthesis and the hepatic bile acid pool size in response to taurine depletion has not been reported previously and it is not clear whether this

response might be unique to mice. Although a diminished ability of the liver to secrete bile acids in the bile cannot be ruled out, bile acid secretion was sufficient for essentially complete fat digestion and absorption because no fat (triglyceride assay) was detectable in the colonic fecal pellets of either *Cdo1*-null or wild-type mice (data not shown). In addition, beyond the modest increase in the hepatic bile acid pool, there was no evidence for cholestasis in the *Cdo1*-null mice.

Csad, Cyp7a1, Cyp3a11, and Bhmt are genes whose expression appears to be regulated by taurine status. We have identified four genes whose hepatic expression appears to be strongly regulated by taurine status in murine liver (Fig. 10). These include *Csad*, which was highly upregulated (>6-fold) by taurine deficiency; *Cyp3A11*, which was upregulated to 2- to 6-times basal levels by taurine deficiency; *Cyp7A1*, which was upregulated to two- to threefold basal levels by taurine deficiency; and *Bhmt*, which was downregulated to less than 50% of control (~2-fold difference) by taurine deficiency. Whether *Slc6A6*, which was only mildly upregulated by about 50% by taurine deficiency, and *Ostβ*, which was variably affected by *Cdo1* genotype and taurine status in male and female mice, should be included in the list of taurine-responsive genes in mouse liver requires further study.

Although the mechanisms by which taurine deficiency regulates the expression of these genes is not known, it is clear that they are not related to tissue hypotaurine pools, as dietary taurine increases hepatic levels of taurine but not of hypotaurine (Roman et al. 2013). Mechanisms related to hepatic levels of taurine, hepatic levels of various bile acids or bile acid metabolites, or the effects of taurine depletion on cell volume are possibly involved.

Studies in several models (3T3-L1 adipocytes, MEFs, HepG2 cells, rat kidney, and rat brain) have shown that *Slc6A6* (*Taut*) expression is upregulated by hyperto-

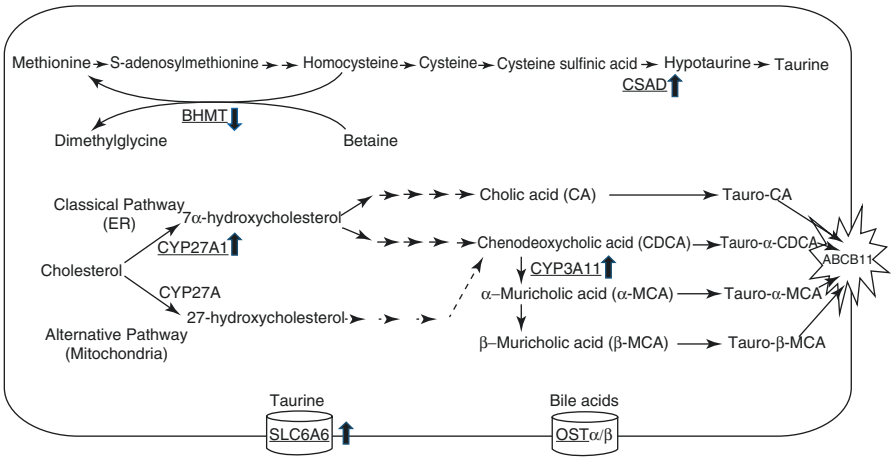

Fig. 10 Diagram illustrating functions of BHMT, CSDAD, CYP7A1, CYP27A, AYP3A11, SLC6A6, OSTα/β, and ABCB11. Those proteins whose expression was clearly responsive to taurine status are indicated by an arrow that also shows the direction of change in response to taurine deficiency

nicity and/or downregulated by taurine-rich conditions (Takasaki et al. 2004; Oh et al. 2006; Schaffer et al. 2000; Satsu et al. 2003; Bitoun et al. 2001; Bitoun and Tappaz 2000). In one study, SLC6A6 (TAUT) mRNA was shown to be upregulated by cholate infusion in both wild-type and *Fxr*-null mice, suggesting FXR is not required for bile acid-mediated upregulation of *Slc6a6* expression (Miyata et al. 2005). Although CSAD was not upregulated in rat kidney or rat brain in response to salt loading (Bitoun et al. 2001; Bitoun and Tappaz 2000), substantial increases in hepatic CSAD abundance in response to taurine deficiency were observed in mice and other species (Roman et al. 2013; Rentschler et al. 1986; De la Rosa and Stipanuk 1985). Recently, both CSAD mRNA and CYP7A1 mRNA levels were shown to be downregulated in mice given dietary cholate, upregulated in mice that received cholestyramine, downregulated in mice dosed with FXR agonist GW4064, and upregulated in *Shp*-null mice compared to wild-type mice (Kerr et al. 2013). These observations suggested that the FXR/SHP signaling may regulate taurine biosynthesis as well as bile acid synthesis. The possibility that *Bhmt* expression may also be regulated by FXR/SHP signaling pathways was suggested by recent reports that *Bhmt* expression was lower in mice supplemented with dietary cholate but higher in mice that received cholestyramine (Tsuchiya et al. 2015). Additionally, hepatic *Bhmt* expression in liver of *Shp*-null mice was 2.8-fold the wild-type level, and *Shp* re-expression in the *Shp*-null mice lowered the BHMT mRNA abundance back to wild-type levels (Tsuchiya et al. 2015). Overall, these studies suggest that *Csad*, *Cyp7a1*, and *Bhmt* expression could be regulated by a common mechanism involving FXR/SHP, with excess bile acid activating ligands for FXR leading to downregulation of expression of all three genes.

In *Cdo1*-null mice fed a taurine-free diet, we observed a robust increase in the abundance of both CSAD and CYP7A1, but a decrease in the abundance of BHMT. Several observations are immediately obvious compared to the suggested regulation of these genes by FXR/SHP. First, *Bmht* expression was regulated in a different direction than *Csad* and *Cyp7a1*. Second, based on our estimation that the total bile acid pool was increased in taurine-deficient *Cdo1*-null mice, we would have expected the expression of all three genes to be downregulated, but only expression of *Bhmt* was repressed. It is not known whether the conjugation state of bile acids affects their ability to regulate expression of *Cyp7a1* or other genes, but *Cdo1*-null murine liver was dramatically depleted of taurine-conjugated bile acids even though the total bile acid concentration was not reduced. Sayin et al. (2013) recently reported that taurine conjugation is essential for the antagonistic activity of α- and β-muricholate in FXR activation assays. Whether taurine conjugation affects the agonistic activity of any of the bile acids has not been studied in any detail. Even if the taurine-conjugation status of bile acids affects their interaction with FXR, the fact that *Bhmt* expression was regulated in the opposite direction than *Csad* and *Cyp7a1* by taurine status suggests that a mechanism other than the FXR/SHP signaling pathway may be responsible for responses to taurine deficiency.

Of the genes studied, the expression of *Csad* was the most highly regulated in response to taurine status. Unfortunately, almost nothing is known about the tran-

scriptional regulation of the *Csad* gene. *Csad* also is the only one of the four genes for which a dose-response effect related to taurine status, albeit indirectly, has been reported (Stipanuk et al. 2002). Hepatic CSAD mRNA and protein abundances responded in a dose-responsive manner to changes in dietary protein intake which were in turn associated with changes in hepatic taurine concentration.

Cyp7a1 expression is well-known to be inhibited by bile acids (e.g., cholate, chenodeoxycholate), providing a feedback mechanism to control the rate of bile acid production (Chiang 2009; Gardès et al. 2013; Davis et al. 2002). Consistent with this, Kerr et al. (2013) suggested that bile acid binding to FXR was responsible for the repression of *Cp7a1* expression in mice given cholate or an FXR agonist, acting through FXR-induced expression of *Shp*, with SHP in turn negatively interacting with LRH-1 or other nuclear receptors that are known to regulate *Cyp7a1* expression. Clearly, this does not explain observations in the *Cdo1*-null mouse in which elevated hepatic bile acid pools were associated with upregulation, rather than downregulation, of *Cyp7a1* expression, and in which taurine-deficiency presumably was the change that promoted upregulation of bile acid synthesis, perhaps in response to a lack of taurine-conjugated bile acids.

We have no clear hypothesis for the mechanism underlying the elevation of hepatic CYP7A1 abundance in taurine-deficient *Cdo1*-null mice. In general, the regulation of bile acid synthesis is complex, being regulated by hormones, oxysterols, bile acids, drugs, and other factors including factors secreted by the intestine. The fact that muricholic acid, which comprises about half of the murine bile acid pool (Alnouti et al. 2008; García-Cañaveras et al. 2012), is an FXR-antagonistic bile acid (Hu et al. 2014) and the observation that taurine conjugation is essential for the antagonistic activity of α- and β-muricholic acid in FXR activation assays (Sayin et al. 2013) suggest that a lack of antagonism of FXR by tauromuricholic acid is a possible explanation. Because hepatic bile acid metabolism also depends upon intestinal FXR signaling (Sayin et al. 2013; Kuribayashi et al. 2012; Miyata et al. 2013; Li et al. 2013), any effects of taurine deficiency on the secretion of bile acids in the bile, their metabolism by gut microbiota, or their reabsorption by the enterohepatic circulation could also impact bile acid-mediated regulation of gene expression.

CYP3A11, which is homologous to CYP3A4 in humans, is responsible for the conversion of chenodeoxycholic acid to muricholic acid in mice and is also responsible for metabolism of many xenobiotics. The *Cyp3a11* gene contains a pregnane X receptor (PXR), which can be activated by bile acid metabolites and a variety of other compounds. Increased abundance and activity of CYP3A11 protein and activity has been reported in mice given cholic acid (Hrycay et al. 2014), and increased expression of *Cyp3a11* was observed in response to cholic acid or ursodeoxycholic acid in mice in an FXR-independent manner (Zollner et al. 2006).

BHMT mRNA and protein levels previously have been reported to be responsive to osmotic conditions. Although not present in as high a concentration as taurine, betaine is a relatively abundant organic osmolyte in cells (Jurkowska et al. 2016; Hoffmann et al. 2013; Mong et al. 2011). In H4IIE rat hepatoma cells, *Bhmt* expres-

sion was suppressed by hyperosmotic conditions that caused cell shrinkage but enhanced by hypoosmotic conditions which also led to a decrease in intracellular betaine (Schäfer et al. 2007). This study along with our observations for liver of intact mice suggest that betaine likely functions as an organic osmolyte in cells and that its hepatic abundance can be altered by regulating the expression of BHMT.

It is interesting that bile acid status and SHP (NR0B2) have been resported, independently, to have effects on both *Csad* and *Bhmt* expression. A link with bile acid metabolism is suggested by the observations that *Bhmt* expression and *Csad* expression were lower in mice supplemented with dietary cholate but higher in mice that received cholestyramine (Tsuchiya et al. 2015; Kerr et al. 2013). A link with SHP is suggested by observations of increased hepatic CSAD mRNA and hypotaurine levels (Kerr et al. 2013) and increased hepatic BHMT mRNA and protein abundances and an elevated betaine concentration (Tsuchiya et al. 2015) in liver of *Shp* (*Nr0b2*)-null mice. However, despite this suggestion that there might be a common mechanism, acting through SHP, for regulation of *Bhmt* and *Csad* expression in response to changes in bile acid metabolism, this mechanism would not account for our observations because *Csad* and *Bhmt* expression in liver of *Cdo1*-null mice changed in opposite directions in response to taurine deficiency and taurine supplementation rather than in the same direction as the SHP mechanism would predict.

5 Conclusion

In conclusion, we identified cysteine sulfinic acid decarboxylase (*Csad*), betaine:homocysteine methytransferase (*Bhmt*), cholesterol 7α-hydroxylase (*Cyp7a1*), and cytochrome P450 3A11 (*Cyp3a11*) as genes whose hepatic expression is strongly regulated in response to taurine depletion in the *Cdo1*-null mouse. it seems that there are a group of genes whose expression, at the level of mRNA and protein, is sensitive to taurine status. Further exploration of the ability of taurine status to regulate gene expression is needed to discern the mechanism by which taurine exerts these effects and whether it is a direct response to taurine. Exploration of the taurine sensitivity of *Csad* expression in nonhepatic tissues might be helpful, particularly because it would remove the potentially confounding effects of dramatic changes in bile acid conjugation. Given the widespread effects of taurine on diverse physiological processes, an effect of taurine on gene transcription would seem to potentially be an important avenue by which taurine exerts those effects.

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Beta-Catenin and SMAD3 Are Associated with Skeletal Muscle Aging in the Taurine Transpoeter Knockout Mouse

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Abstract Tissue taurine depletion mediated by knocking out the taurine transporter causes several skeletal muscle abnormalities, including acceleration of cellular aging. In the present study, we investigated the signaling pathway involved in the acceleration of skeletal muscle aging by tissue taurine depletion using the bioinformatic approach of transcriptome data. We previously performed transcriptome analysis on skeletal muscle of taurine transporter knockout (TauTKO) mice using DNA microarray. Bioinformatic analysis of transcriptome data predicted the activation of SMAD3 and β-catenin as upstream signaling molecules of cyclin-dependent kinase inhibitor 2A (CDKN2A, also called p16INK4A), which is a biomarker gene of cellular senescence. The activation of SMAD3 and β-catenin in old TauTKO muscle was verified by western blot analysis. These data indicate that SMAD3- and β-catenin-dependent induction occurs in the TauTKO mouse.

Keywords Taurine transporter • Knockout mouse • Skeletal muscle • Aging • Cyclin kinase inhibitor p16

Abbreviations

TauT	Taurine transporter
TauTKO	Taurine transporter knockout
WT	Wild type controls

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1 Introduction

Taurine is one of the most abundant free amino acids in mammalian tissues; its high tissue content is dependent on the biosynthesis from methionine and its intake from food. Taurine deficiency in cats causes several tissue disorders, such as blindness and cardiomyopathy (Pion et al. 1987; Hayes and Carey 1975). While the pathological role of taurine deficiency in humans remains to be clarified, the amount of taurine intake is negatively correlated with the rate of ischemic heart disease according to a worldwide epidemiological study (Yamori et al. 2001). Moreover, taurine administration in human clinical studies is effective against various aging-related diseases, including chronic heart failure, diabetes, endothelial dysfunction (Azuma et al. 1992; Ito et al. 2012; Fennessy et al. 2003). These findings imply that taurine deficiency may accelerate aging.

An aging-dependent decrease in skeletal muscle mass and function (sarcopenia) is a major concern in old adults. Administration of taurine with branched amino acids diminishes muscle damage after exercise in humans, suggesting a beneficial role of taurine in skeletal muscle (Ra et al. 2013). The tissue taurine-depleted mouse (taurine transporter-knockout mouse (TauTKO)) exhibits a loss in muscle mass and a decrease in exercise endurance (Ito et al. 2008, 2014a). Moreover, several aging-related features, including the accumulation of muscle fibers containing centrally localized nuclei and a decrease in mitochondrial complex I activity, which are commonly observed with aging, were detected in TauTKO at an early age (Ito et al. 2014b). These data suggest a role of taurine deficiency in sarcopenia.

We have previously analyzed the transcriptome profile of skeletal muscle of young and old TauTKO mice (Ito et al. 2014b). We have detected the induction of chaperone proteins and signal molecules which are responsible for the unfolded protein response, including Hsp78 and XBP1, suggesting that these genes may be associated with a protein folding disorder in taurine deficient skeletal muscle. Subsequent bioinformatic analysis predicted the activation of several signaling pathways. However, the signal pathway involved in the acceleration of muscle aging by taurine depletion had not been clarified. Therefore, in the present study we investigated the signaling pathway associated with the acceleration of skeletal muscle aging using the bioinformatic approach of transcriptome data.

2 Methods

2.1 Pathway Analysis of Microarray Data

Ingenuity pathway analysis (IPA) software (Agilent technology, Santa Clara, CA) was used for pathway analysis of transcriptome data deposited in Gene Expression Omnibus; GSE57373. The genes which are differentially expressed by more than 1.8-fold between old TauTKO mice and old WT mice ($n = 3$, $p < 0.05$, Student's t-test) were analyzed by IPA.

2.2 Animal Care

The experimental procedures were approved by the Institutional Animal Care and Use Committee of Hyogo University of Health Sciences. Male TauTKO and littermate mice were housed in SPF environment, fed standard chow (MF, Oriental Yeast, Japan), had access to water ad libitum and maintained on a 12-h light/dark cycle. Eighteen- to 22-month-old mice were used as old mice.

2.3 Western Blot of Nuclear Extract from Skeletal Muscle

Nuclear samples were isolated from tibial anterior skeletal muscle by using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents according to manufacturer's protocol (ThermoFisher Scientific, MA, USA). After protein concentration was determined by the bicinconic acid assay method (Pierce BCA assay kit, ThermoFisher Scientific), protein samples were subjected to Western blot as previously described (Ito et al. 2014b). Anti-SMAD3 and β-catenin antibodies (Cell Signaling) were used as first antibodies.

3 Results

3.1 Activation of SMAD3- and β-Catenin-p16INK4a Axis in Aged TauTKO Muscle

Cyclin-dependent kinase inhibitor 2A (CDKN2A, also called p16INK4A) is a biomarker of cellular senescence and plays a critical role in cellular aging (Krishnamurthy et al. 2004). For example, elimination of senescent cells, which express CDKN2A, prevents some age-associated diseases and extends lifespan in progeria mice and normally aged mice (Baker et al. 2011, 2016). Elimination of senescent cells attenuates the loss of muscle mass and reductions in exercise performance in progeria mice (Baker et al. 2011), suggesting a contribution of senescent cells in the development of sarcopenia. We have reported that the level of the CDKN2A gene is markedly higher in skeletal muscle of old TauTKO mice than in muscle of age-matched control wild-type mice (Ito et al. 2014b). In the present study, the gene set of differentially expressed genes between old TauTKO muscle and old WT muscle was analyzed using IPA to predict the signaling pathway involved in CDKN2A transcription. As shown in Fig. 1, TGF-β, SMAD3 and β-catenin were predicted to be activated in TauTKO muscle and to be involved in transcriptional activation of CDKN2A as well as in other elevated genes, such as CDKN2B, MMP3, CXCL10 etc.

Subsequent Western blot analysis revealed that both SMAD3 and β-catenin proteins β-catenin in nuclear extract isolated from old TauTKO muscle (Fig. 2), indicating that SMAD3 and β-catenin are activated in old TauTKO muscle.

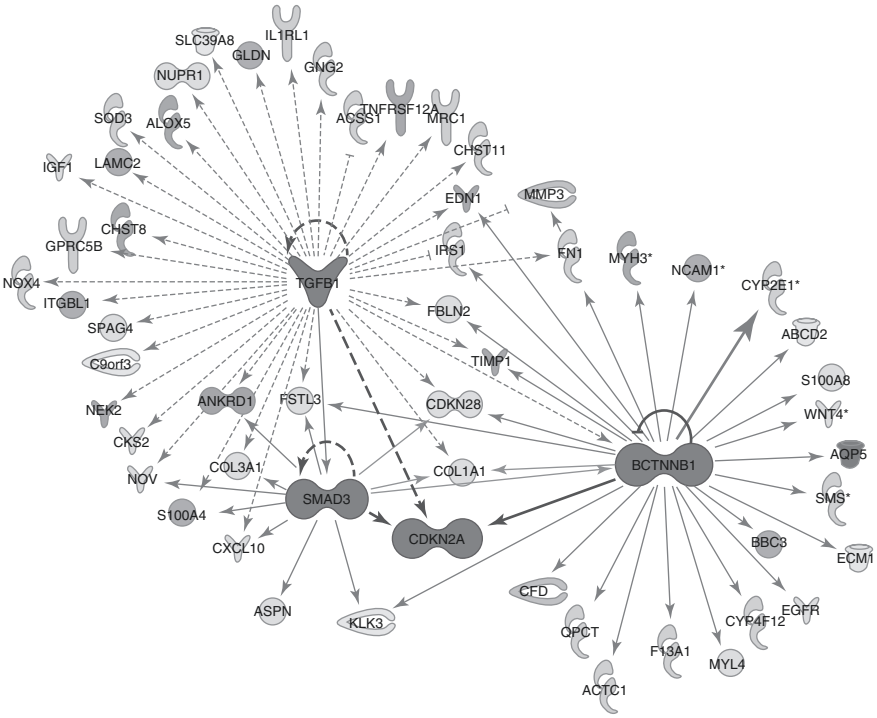

Fig. 1 Putative signaling pathway predicted by bioinformatic approach. Upstream analysis using Ingenuity pathway analysis was performed. The light gray shapes indicate the genes (65 genes, such as CDKN2A, CDKN2B, MMP3, CXCL10 etc.) included in the gene set which change by more than 1.8-fold in 18-month-old TauTKO muscle compared to littermate WT muscle according to previous transcriptome analysis. The dark gray-indicated shapes (SMAD3, CTNNB1, TGFB1) are upstream molecules of light gray-indicated genes

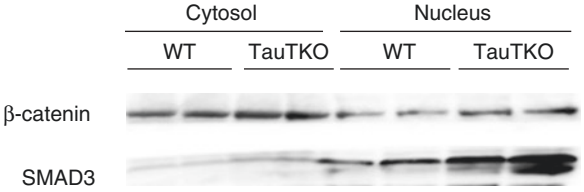

Fig. 2 Increases in β-catenin and SMAD3 in nuclei of old TauTKO muscle. Nuclear and cytosolic CTNNB1 of skeletal muscle isolated from 18-month-old TauTKO and WT mice were analyzed by western blot. Both nuclear β-catenin and SMAD3 were higher in TauTKO muscle compared to WT muscle

4 Discussion

The present study uncovered a signaling pathway associated with acceleration of skeletal muscle aging in TauTKO mice using pathway analysis based on the transcriptome profile of skeletal muscle of old TauTKO and WT mice. The pathway analysis suggests that SMAD3 and/or β-catenin are involved in CDKN2A induction. We further demonstrated that SMAD3 and β-catenin accumulate in nuclei of old TauTKO skeletal muscle more than in WT skeletal muscle. It has been reported that phosphorylated SMAD3 induces cyclin-dependent kinase inhibitors, including p16, in muscle satellite cells, while phosphorylated SMAD3 is increased in satellite cells of old mice compared to young mice (Carlson et al. 2008). These findings suggest that SMAD3 contributes to cellular senescence in satellite cells and interferes with regenerating capacity. Moreover, attenuation of SMAD3 by Fst promotes mTOR/S6 kinase signaling, which enhances protein synthesis (Winbanks et al. 2012). Therefore, SMAD3 activation may also contribute to age-associated reductions of muscle mass. Meanwhile, activation of β-catenin in aged muscle progenitor cells has also been reported previously (Brack et al. 2007). Additionally, administration of Wnt1, a β-catenin activator, to regenerating skeletal muscle (in vivo) delays the regeneration of muscle tubes, while inhibition of Wnt1 signaling enhances regeneration in old mice. Finally, β-catenin blockade by siRNA prevents serum-induced CDKN2A elevation in old mice, as well as p53 and p21 proteins in mesenchymal stem cells (Zhang et al. 2013), indicating that β-catenin may contribute to the induction of CDKN2A based on the transcriptome data during advanced aging.

TGF-β has been reported to be higher in old skeletal muscle than in young skeletal muscle (Carlson et al. 2008). However, neutralization of TGF-β1 failed to inhibit SMAD3 phosphorylation and muscle loss in old mice, indicating that the other TGF-β family proteins contribute to SMAD3 activation in old skeletal muscle (Carlson et al. 2008). Similarly, based on the transcriptome data, we found no difference in the TGF-β genes (TGF-β1,2,3) between TauTKO and WT muscle (data not shown). Therefore, future studies are required to identify the regulatory factor that activates SMAD3 in old skeletal muscle, which in turn initiates the SMAD3-dependent cellular senescence mechanism.

5 Conclusion

In conclusion, acceleration of skeletal muscle aging in taurine depleted mice may occur through SMAD3 and β-catenin signaling. The role of taurine on these signaling pathways should be further investigated to clarify the anti-aging property of taurine.

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Glucose Homeostasis and Retinal Histopathology in CSAD KO Mice

Francoise Sidime, Greg Phillips, Nicole LaMassa, Eunkyue Park, and Abdeslem El Idrissi

Abstract In this study we examined glucose homeostasis and retinal histology in homozygous knockout mice lacking CSAD (CSAD-KO). Two-month-old male mice were used including wild type (WT), homozygotes with without supplementation of taurine in the drinking water (1% w/v). Mice were sacrificed and the eyes processed for histology and immunohistochemistry. Additional mice were subjected to a glucose tolerance test (7.5 mg/kg BW) after 12 h fasting. We found that CSAD-KO and CSAD-KO treated with taurine were slightly hypoglycemic prior to glucose injection and showed a significantly reduced plasma glucose at 30, 60 and 120 min post-glucose injection, compared to WT. While glucose homeostasis in CSAD-KO was significantly different compared to WT, CSAD-KO supplemented with taurine was without effect. Analysis of retinas by electron microscopy showed that CSAD-KO without taurine supplementation exhibited substantial retinal degeneration. Remaining photoreceptor outer and inner segments were disorganized. Retinal nuclear and synaptic layers were largely absent and there was apparent reorganization of the pigmented epithelial cells. The choroid and sclera were intact. These histological aberrations were largely rectified by taurine supplementation in the drinking water.

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These data indicate that taurine deficiency alters glucose homeostasis and retinal structure and taurine supplementation improves these retinal abnormalities, but not in hypoglycemia.

Keywords Retina • Pancreas • Glucose • Islets of Langerhans • CSAD • Taurine

Abbreviations

CSAD	Cysteine sulfonic acid decarboxylase
HO	Homozygote CSAD knock out
Tau	Taurine
WT	Wild type controls

1 Introduction

Taurine (2-aminoethanesulfonic acid) is a sulfur-containing amino acid very abundant in excitable tissues, such as the eyes, brain, skeletal and cardiac muscles. Taurine has been shown to have many physiological actions, including a role as an antioxidant, a membrane stabilizer, an osmoregulator and a modulator of intracellular calcium homeostasis (Lambardini 1985; Solis et al. 1988; Foos and Wu 2002; Saransaari and Oja 2000; Schaffer et al. 2000). The latter function is important for preventing glutamate-induced excitotoxicity (Militante and Lombardini 1998; El Idrissi and Trenkner 1999, 2004). Because of the structural similarities with GABA, taurine has been shown to activate $GABA_A$ receptors and play a role in modulating GABA-mediated signaling pathways (El Idrissi and Trenkner 1999). Retinal cells contain the highest concentration of taurine compared to other organs and taurine has been shown to play a major role in retinal development (Huxtable 1992). Similarly, taurine plays an equally important role in pancreatic development and health (Dahri et al. 1991; Cherif et al. 1996; Merezak et al. 2001; Boujendar et al. 2002). Taurine delayed the onset of diabetes in NOD mice (Arany et al. 2004); and induced tolerance to glucose challenges in mice (Merezak et al. 2001). Moreover, the role of taurine in pancreatic function extends into adulthood where it has been shown to play a role in glucose metabolism (Hansen 2001; Franconi et al. 2006).

Pancreatic islets undergo postnatal remodeling where a balance between apoptosis and newly formed cells leads to the formation of functional islets. Many factors have been involved in this process of remodeling and taurine has been shown to tilt the balance in favor of new cell formation probably because of the potent antioxidant effect of taurine. Increase cell proliferation in response to taurine supplementation has been shown by increased number of cells positive for proliferating cell nuclear antigen (PCNA). Furthermore, taurine induced an increase in IGF-II

expression a survival factor and a mitogen for β cells (Scaglia et al. 1997; Petrik et al. 1998; Arany et al. 2004). Furthermore, taurine inhibited apoptosis in β cells, through inhibition of nitric oxide synthase (Liu et al. 1998).

Consistent with previous data on the role of taurine on pancreatic function, we have shown that taurine supplementation to mice led to an increase in the size and number of the islets. Furthermore, mice supplemented with taurine showed a significant tolerance to glucose challenges. We found that taurine fed mice have increased immunoreactivity to insulin in β cells of the islets and increased plasma insulin levels in response to glucose injection. The functional significance of increased insulin production and secretion in response to plasma glucose elevations in taurine fed mice may first activate IGF-II receptors expressed in the islets, further enhancing the survival of β cells. Additionally, the elevated levels of insulin in taurine fed mice activate IRs on neurons and cause hyperexcitability. Interestingly, taurine fed mice show hyper-excitability.

In this study we examined glucose homeostasis and retinal histology in homozygous knockout mice lacking Cysteine sulfonic acid decarboxylase (CSAD-KO) since taurine has been shown to play a role in the histogenesis and functional maturity of these two organs. (CSAD; EC 4.1.1.29) was identified first in the liver and is thought to be the rate limiting enzyme in taurine biosynthesis and has been found to be expressed in the brain.

2 Methods

2.1 Animals

All mice used in this study were two-month-old FVB/NJ males. For taurine-fed mice, taurine was dissolved in water at 1%, and this solution was made available to the mice in place of drinking water for 4 weeks beginning at 4 weeks of age. All mice were housed in groups of three in a pathogen-free room maintained on a 12 h light/dark cycle and given food and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee of the College of Staten Island/CUNY, and were in conformity with National Institutes of Health Guidelines. The number of mice used in these studies was sufficient to provide statistically reliable results.

2.2 Intraperitoneal Glucose Tolerance Test

Glucose test was performed as previously reported (El Idrissi et al. 2009). Briefly, mice were fasted overnight (12 h) and then injected intraperitoneally with 0.02 mL/g of body weight D-glucose (7.5% stock solution in saline). Blood samples were taken by tail venesection at the indicated times.

2.3 Electron Microscopy

Mouse eyes fixed in 4% glutaraldehyde, 100 mM cacodylate buffer, 1 mM $CaCl_2$. Eyes were then rinsed extensively in 100 mM cacodylate buffer and treated with 1% osmium tetroxide, 1.5% potassium ferricyanide, in 100 mM cacodylate for 3 h. Eyes were rinsed in buffer, dehydrated though an ethanol series followed by propylene oxide, and then infiltrated with a 1:1 ratio of EMbed 812: propylene oxide for 24 h. Eyes were then further infiltrated with several changes of pure resin for an additional 24 h and embedded in the cap end of a BEEM capsule. Eyes were sectioned with a glass knife and retinas were identified in thick sections by staining with methylene blue. Retinas were trimmed, ultrathin sections cut with a diamond knife, mounted on Formvar coated slot grids and contrasted with uranyl acetate and lead citrate. Ultrathin sections were imaged on a Fei Tecnai Spirit transmission electron microscope.

2.4 Quantification of Size and Number of Pancreatic Islets

Two months old mice were perfused with 4% paraformaldehyde and pancreas were isolated attached the pyloric region of the stomach and the duodenum. The initial part of the duodenum served to orient the pancreas for the sectional plane. Tissue was cryoprotected with 30% sucrose and cryosectioned at a thickness of 15 μm. Sections were stained with hematoxylin and eosin. Microscopy was performed by histologist unaware of the treatment conditions.

2.5 High Performance Liquid Chromatography (HPLC)

The pancreas was obtained from all groups including WT, HO and HO treated with 1% taurine in the drinking water after mice were sacrificed with *ip* injection of avertin (250 mg/Kg). Taurine concentrations were determined using HPLC (Waters, Milford, MA) (Park et al. 2015). Briefly, tissues were homogenized using 5% TCA and centrifuged for removal of proteins. After samples were dried using a Speedvac (Savant, Holbrook, NY), they were derivatized using phenylisothiocyanate (PITC) and separated using a C18 column with a gradient of acetate buffer containing 2.5% acetonitril (pH 6.5) and 45% acetonitril solution containing 15% methanol at 45 °C. The flow rate was 1 mL/min. Taurine concentrations were determined by comparison to a standard.

2.6 Statistical Analysis

Statistical significance was determined by Student's t-test. Each value was expressed as the mean ± SEM. Differences were considered statistically significant when the calculated P value was less than 0.05.

3 Results

3.1 Taurine-Fed Mice Exhibit Hyperinsulinemia and Glucose Tolerance

We have previously reported that taurine-supplemented mice have increase islets size and number. These changes has functional significance on glucose handling. Control mice showed a drastic increase in plasma glucose concentration 30 min after challenge with a gradual decrease through 120 min. By the end of the experiment, mice regained their baseline glucose levels (Fig. 1). In contrast, HO mice with or without taurine supplementation, showed a significantly different response to glucose challenge and showed a significant tolerance to glucose injection. Baseline plasma glucose levels indicated that these mice were significantly hypoglycemic compared to controls. However, the response to glucose injection was drastically reduced ($p < 0.001$) at 30 min compared to controls. At 60 and 120 min following the challenge, the plasma glucose level in HO mice rose remained low. This very atypical response to glucose challenges in HO mice even when supplemented with taurine indicates that taurine depletion in HO mice alters the biological mechanisms that regulate glucose homeostasis.

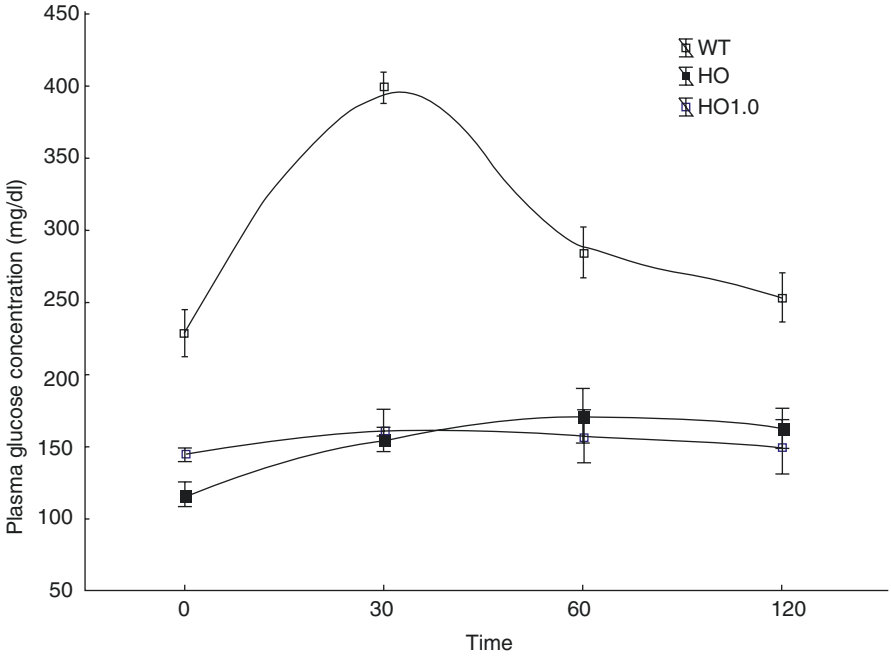

Fig. 1 Glucose homeostasis in HO mice. Intraperitoneal glucose tolerance test on overnight fasted control mice (n = 12), HO (n = 6) and HO1 (n = 6). Values are expressed as means ± S.E.M obtained from three experiments. Plasma glucose values in HO and HO1 mice were significantly ($p < 0.01$) different from control values but not significantly different from each other

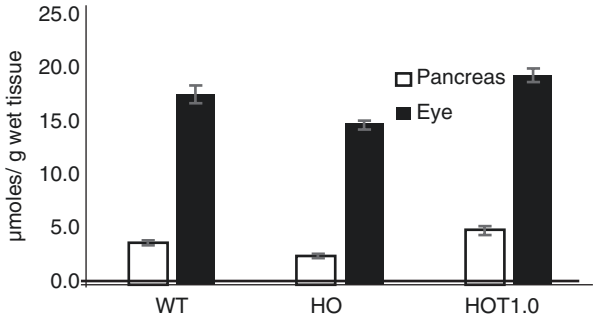

Fig. 2 Taurine levels in eyes and pancreas. HO mice showed a reduction in taurine content in both organs examined. Taurine supplementation caused an increase in taurine content in both the eyes and pancreas. Pancreas (n = 8 for each group), eyes (n = 8 WT, n = 10 HO, n = 6 HO1.0)

3.2 Taurine Levels in the Pancreas and Eyes

To further investigate the functional significance of silencing CSAD gene we measured taurine levels in the eyes and pancreas (Fig. 2). Consistent with previous findings, the eyes contain elevated levels of taurine. The levels of taurine in the pancreas were significantly lower than in the retina. HO mice showed a drop in taurine content in both eyes and pancreas which was reversed when supplemented with taurine (1%) in the drinking water (Fig. 2). These data indicate that taurine content is highly regulated in the eyes and pancreas and potentially other organs.

3.3 Taurine Depletion Induces Histopathologies in the Retina and the Pancreas That Were Reverse by Taurine Supplementation

Taurine is found in very high concentrations in the retina (Pasantes-Morales et al. 1972) and lateral geniculate nuclei (Guidotti et al. 1972), suggesting a role of taurine in vision. Taurine has been investigated with regards to its roles in osmoregulation, as a potent antioxidant, and as a neurotransmitter or neuromodulator. Throughout life, taurine appears to play an integral role in the function and health of the retina. Therefore, we investigated the role CSAD plays in retinal physiology. Using electron microscopy, we found that in the HO mice have severe alterations to the histology of the photoreceptors (Fig. 3). The histology of the outer segments was typified by broken photoreceptors lacking the stack organization of the photo disks. Several vesicular structures were seen in these retinas presumably phagoxomes (Fig. 3). The onset of these histopathologies in HO mice was prevented with supplementation of taurine in the drinking water.

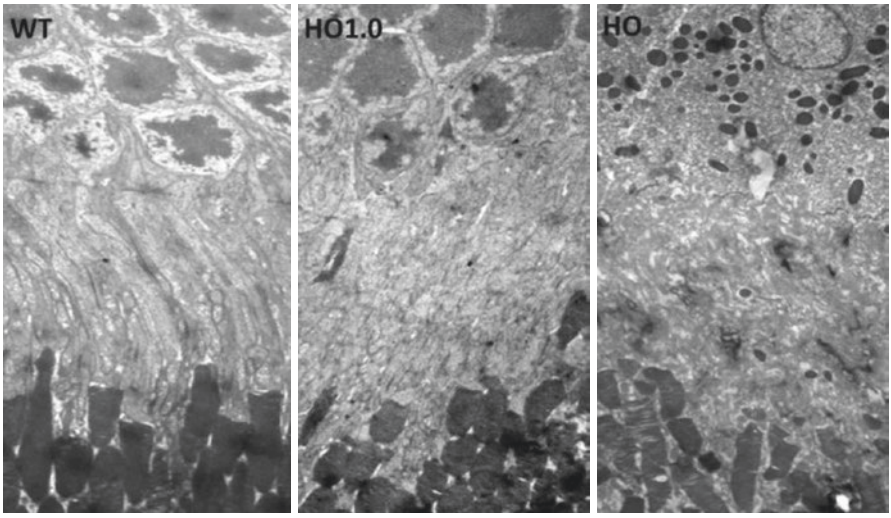

Fig. 3 HO mice have severe alterations to the histology of the photoreceptors. Representative EM images showing disruption of the outer segment of the photoreceptors of HO mice. Presumptive phagosomes are also apparent in the retina of these mice. Supplementation with taurine (HO1.0) significantly reversed these histological alteration

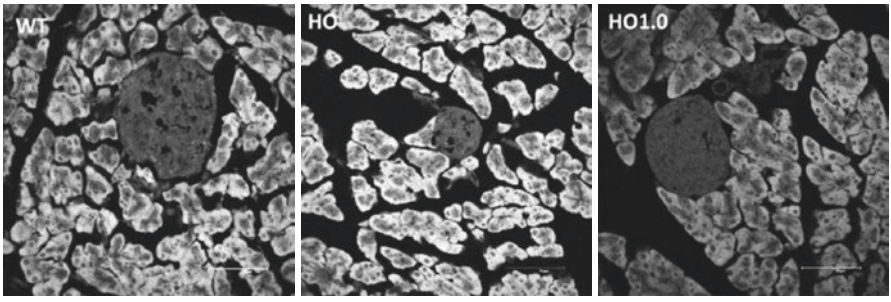

Fig. 4 Sections of the pancreas stained with propidium iodide showing the serous acini and islets of Langerhans. Representative images of an islet from WT, HO, HO1.0 pancreas. The relative size of an islet from an HO mouse is significantly smaller that WT. Supplementation with taurine in drinking water let to an increase in the size of the islets. Images taking at 40X

We also examined the histology of the pancreas as this organ plays a critical role in the regulation of glucose homeostasis and taurine is important for the development, survival and function of the pancreas. We found that HO mice have a significant reduction in the size of the islets of Langerhans compared to WT controls. When HO mice were supplemented with taurine in their drinking water, the islet size was significantly increased to reach levels comparable to controls (Fig. 4).

4 Discussion

These data show that CSAD KO mice have several functional alterations. These mice have altered histology of the retinas and pancreas. We found that HO mice were hypoglycemic at base line and responded significantly different to glucose challenges compared to controls. Supplementation of taurine in the drinking water to HO mice did not affect how these mice handled the glucose challenge. The response of HO1.0 mice was indistinguishable from HO mice and significantly different that WT controls (Fig. 1). Interestingly, taurine supplementation to HO mice corrected the histology of pancreas but the response to glucose remained unchanged (Fig. 4). On the other hand, knocking CSAD gene had a significant effect on retinal histology. When we examined the levels of taurine in the retina and eyes, we found that the eyes contain approximately threefolds higher taurine that the pancreas, confirming the role of taurine in retinal physiology and vision. HO mice had reduced taurine content in both the retina and pancreas. Supplementation of taurine to drinking water brought the levels of taurine in these organs to WT control levels. Furthermore, there was a significant alteration of retinal histology and a significant reduction in the islets size in the pancreas in HO mice. EM images from HO mice retinas revealed a complete disruption of the retinal histology. Outer segments of the photoreceptors were fractured and disorganized with the presence of several vacuoles, presumable phagosomes. These retinal histopathologies were corrected when HO mice were supplemented with taurine in their drinking water (Fig. 3).

These data suggest that taurine plays an important role in both the visual system and in the pancreas. Eliminating CSAD, the rate-limiting enzyme in taurine biosynthesis led to altered histology and function of these organs. The endocrine pancreas and retina undergo significant modification during neonatal life. Dysregulation of this remodeling process during this period of time when a fine balance between cell replication and cell death are occurring, determines the development of the retina and islets of Langerhans in the pancreas and has important effects on the development and function of these organs.

5 Conclusion

In summary, the histological changes observed after taurine supplementation on the pancreas and retina are indicative of the functional importance of taurine in the visual physiology and pancreatic function and regulation of glucose homeostasis. CSAD plays an important role in making taurine bio-available to maintain the function of pancreas and retina and other organs.

Acknowledgments This work was supported by OPWDD; PSC-CUNY and CSI.

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Part V
Biology and Chemistry of Taurine Derivatives

Comparative Analysis of Microbicidal and Anti-inflammatory Properties of Novel Taurine Bromamine Derivatives and Bromamine T

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Abstract Taurine, the most abundant free amino acid in leukocyte cytosol traps hypohalous acids (HOCl and HOBr) to produce N-chlorotaurine (taurine chloramine, NCT and N-bromotaurine (taurine bromamine, Tau-NHBr,) respectively. Both haloamines show anti-inflammatory and antimicrobial properties. However, the therapeutic applicability of Tau-NHBr is limited due to its relatively poor stability. To overcome this disadvantage, we have synthesized the stable N-bromotaurine compounds N-monobromo-2,2-dimethyltaurine (Br-612) and N-dibromo-2,2-dimethyltaurine (Br-422). The aim of this study was to compare anti-inflammatory and microbicidal properties of Br-612 and Br-422 with that of Tau-NHBr and bromamine T (BAT). We have shown that all the tested compounds show similar anti-inflammatory properties. Importantly, the stable N-bromotaurine compounds exerted even stronger microbicidal activity than Tau-NHBr. Finally, for the purpose of topical application of these compounds we have developed a carbomer-based bioadhesive solid dosage form of BAT and Br-612, featuring sustained release of the active substance.

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Keywords Taurine • Taurine bromamine • N-bromotaurine • Taurine derivatives • Inflammatory diseases • Infectious diseases • Biocompatibility index • Antiseptic • Anti-inflammatory • Antimicrobial

Abbreviations

BAT	Bromamine T
C. albicans	*Candida albicans*
CFU	Colony forming units
HOBr	Hypobromous acid
HOCl	Hypochlorous acid
LCL	Luminol dependent chemiluminescence
LPS	Lipopolysaccharide
MRI	Magnetic Resonance Imaging
NCT	N-chlorotaurine, taurine chloramine
OZ	Opsonized zymosan
P. acnes	*Propioniobacterium acnes*
P. aeruginosa	*Pseudomonas aeruginosa*
PGE_2	Prostaglandins
ROS	Reactive oxygen species
S. aureus	*Staphylococcus aureus*
Tau-NHBr	N-bromotaurine, taurine bromamine

1 Introduction

Taurine, a non-protein sulphur amino acid, is present at extremely high concentration in neutrophil cytosol (Schuller-Levis and Park 2003). In activated neutrophils, taurine fulfils its cytoprotective and antioxidant properties through neutralization of highly toxic hypohalous acids (HOCl and HOBr), the products of the myeloperoxidase (MPO)—halide system (Klebanoff 2005; Weiss et al. 1982). The reaction of taurine with HOCl and HOBr results in generation of N-chlorotaurine (taurine chloramine, NCT) and N-bromotaurine (taurine bromamine, Tau-NHBr), respectively (Thomas et al. 1985, 1995). Both haloamines exert anti-inflammatory and microbicidal properties (Gaut et al. 2001; Marcinkiewicz et al. 2005; Nagl et al. 2000). Their bactericidal, fungicidal, antiviral and antiparasitic activity *in vitro* have been demonstrated in a number of papers (Nagl et al. 2000, 2003; Marcinkiewicz et al. 2000, 2005; Yazdanbakhsh et al. 1987). Moreover, well documented outstanding tolerability of NCT allows the use of NCT at a high concentration (1% aqueous solution) as a local antiseptic (Gottardi and Nagl 2010). Importantly, Tau-NHBr

shows its microbicidal properties even at very low physiological concentrations (Koprowski and Marcinkiewicz 2002; Marcinkiewicz et al. 2006). Furthermore, it has been demonstrated that Tau-NHBr exerts anti-inflammatory properties by suppressing the production of such mediators as nitric oxide, PGE_2, TNFα, IL6, IL8, IL12p40 and chemokines in both rodent and human leukocytes (Marcinkiewicz et al. 2005, 2006; Olszanecki et al. 2008; Park et al. 1997). In addition, Tau-NHBr can induce generation of heme oxygenase-1 (HO-1), a stress-inducible enzyme, which also has anti-oxidative and anti-inflammatory capacity (Olszanecki and Marcinkiewicz 2004).

These unique anti-inflammatory and antiseptic properties of NCT and Tau-NHBr were tested in a number of clinical studies. It has been shown that NCT is effective in the local treatment of inflammatory diseases such as otitis externa, chronic rhinosinusitis (Neher et al. 2004; Gstöttner et al. 2003), or chronic leg ulcers (Nagl et al. 2003) and Tau-NHBr in *acne vulgaris* (Marcinkiewicz et al. 2008). Moreover, only recently we have reported that Tau-NHBr may be of potential benefit as adjunctive local therapy in periodontal diseases and killing of oral biofilm pathogens (Pasich et al. 2015). Also *herpes zoster* may be a promising indication (Kyriakopoulos et al. 2016).

However, Tau-NHBr therapeutic effectiveness in infectious and inflammatory diseases was limited due to its poor stability. To overcome this disadvantage of Tau-NHBr, we have synthesized the stable N-bromotaurine compounds, namely, N-monobromo-dimethyltaurine (Br-612), and N-dibromo-dimethyltaurine (Br-422).

The aim of this study was to compare anti-inflammatory and microbicidal properties of Br-612 and Br-422 with that of Tau-NHBr and bromamine T (BAT), to check whether modified forms of Tau-NHBr retained their antiseptic potential. Moreover, for the purpose of topical application of these compounds we have conducted a preliminary study on the best drug formulation containing the stable bromamine compounds and evaluation of bioadhesive solid dosage form with carbomer.

2 Methods

2.1 Tested Agents

N-monobromo-dimethyltaurine (Br-612), N-dibromo-dimethyltaurine (Br-422), bromamine T (N-bromo-N-sodio-p-toluenesulfonamide, BAT), N-bromotaurine (taurine bromamine, Tau-NHBr). Dimethyltaurine was kindly provided by D. Debabov and R. Najafi (NovaBay Pharmaceuticals, Inc., Emeryville, CA, USA) and brominated to Br-612 and Br-422. BAT was synthesized from dibromamine T as published (Nair et al. 1978).

2.1.1 Tau-NHBr (Taurine Bromamine) Preparation

Tau-NHBr was prepared in a two-step procedure (Marcinkiewicz et al. 2006). First, NaOBr was synthesized in reaction between equimolar amounts of NaOCl and NaBr (*POCH*) in the phosphate-buffered solution (PBS). In such conditions virtually all the OCl^- present reacts with Br^- to form OBr^- and Cl^-.The presence and concentration of OBr^- was confirmed by UV spectra (λ = 200 to 400 nm). In the second step, 20 mM NaOBr was added dropwise to equal volume of 400 mM taurine. UV absorption spectrum was checked to exclude the formation of taurine dibromamine or chloramines and to estimate the concentration of Tau-NHBr (molar extinction coefficient—430 M^{-1} cm^{-1} at A_{288}). Stock solution of Tau-NHBr was kept at 4 °C for a maximum period of 3 days before use.

2.2 Cell Culture

J774.A1 murine macrophages (Mϕ) were cultured in 24-well flat-bottom cell culture plates at 5 × 10^5/cells/well in DMEM medium supplemented with 5% fetal bovine serum (FBS), at 37 °C in an atmosphere of 5% CO_2. In all experimental models Mϕ were pre-incubated with the tested agents at concentrations (100–300 μM) in DMEM without FBS, after 1.5 h medium was removed and fresh DMEM +5% FBS with LPS (100 ng/mL) was added for additional 24 h. After 24 h culture supernatants and/or cell lysates were collected for further analysis.

2.3 Cell Viability

Cell respiration, an indicator of cell viability, was assessed by mitochondrial-dependent reduction of MTT to formazan.

2.4 Determination of PGE_2 and Cytokines (IL6, IL12p40, TNFα)

2.4.1 PGE_2

PGE_2 concentration in supernatants was determined by using a monoclonal antibody/enzyme immunoassay kit from Cayman Chemical Co, according to the manufacturer's instruction.

2.4.2 Cytokines

Cytokine levels in cell culture supernatants were measured by sandwich ELISA. Microtiter plates (Costar EIA/RIA plates, Corning Inc.) were coated with a cytokine-specific antibody. Expression levels of IL6, and IL12p40 were measured according to the manufacturer's instructions (OptEIA Sets, BD Biosciences). TNFα level was measured according to the manufacturer's instructions (ELISA Ready-Set-Go, eBioscience). In all cases, 10% FBS in PBS was used as a blocking solution. Tetramethylbenzidine (TMB) substrate solution (BioLegend) was used to develop a colorimetric reaction, which was stopped with 2 M sulfuric acid. Optical density was measured at 450 (570) nm using a microtiter plate reader (PowerWaveX, Bio-Tek Instruments).

2.5 Western Blot Analysis of Hemeoxygenase-1 (HO-1) and Cyclooxygenase-2 (COX-2) Expression

Twenty-four hours after *in vitro* stimulation of Mϕ, expression levels of HO-1 and COX-2, proteins in cell cytosol were determined by Western blot analysis. After incubation, cells were lysed in lysis buffer (1% Triton X-100, 0.1% SDS in PBS) containing protease inhibitor cocktail (Sigma–Aldrich). Protein concentrations in lysates were determined by using a bicinchoninic acid protein assay kit (Sigma–Aldrich). Samples containing equal amounts of total protein were mixed with gel loading buffer (0.125 M Tris, 4% SDS, 20% glycerol, 0.2 M dithiothreitol, 0.02% bromophenol blue) at a 2:1 ratio (v/v) and boiled for 4 min. Samples of 20 μg of total protein per lane were separated on 10% SDS-polyacrylamide gels (Mighty Small II, Amersham Biosciences) using the Laemmli buffer system. Proteins were transferred to nitrocellulose membranes (Bio-Rad). Nonspecific binding sites were blocked overnight at 4 °C with 3% nonfat dried milk. Membranes were incubated for 2 h at room temperature (RT) with rabbit polyclonal antibodies to COX-2 (1:1000, Cayman), or mouse monoclonal antibodies to HO-1 (1:2000, Enzo Life Sciences). Bands were detected with alkaline phosphatase-conjugated secondary goat antibody to the rabbit IgG whole molecule (1 h, RT, 1:3000, Sigma–Aldrich) or alkaline phosphatase-conjugated secondary goat antibody to the mouse IgG whole molecule (1 h, RT, 1:3000, Sigma–Aldrich) and developed with BCIP/NBT alkaline phosphatase substrate (Sigma–Aldrich). Membranes were re-probed with monoclonal mouse anti β-actin antibody (clone AC-15, 1 h, RT, 1:3000, Sigma–Aldrich). Pre-stained SDS-PAGE standards (low and high range; Bio-Rad) were used for molecular weight determinations. Protein bands were scanned and analyzed with the Scion Image freeware (Scion Corp.). Data were normalized to the constitutive expression level of β-actin protein.

2.6 Nitrite (NO_2^-) Determination

Nitric oxide, quantified by the accumulation of nitrite as a stable end product, was determined by a microplate assay (Ding et al. 1988). Briefly, 100 μL of sample supernatants were incubated with an equal volume of Griess reagent [1% sulphanilamide in 2 M HCl (Sigma-Aldrich) and 0.1%N-1-naphthylenediamine dihydrochloride in deionized water (POCH) at room temperature for 10 min. The absorbance at 550 nm was measured with a microplate reader. Nitrite concentration was calculated from a sodium nitrite standard curve.

2.7 Reactive Oxygen Species Generation

2.7.1 Animals

To evaluate reactive oxygen species generation, we used CBA male/female mice, between 6 and 8 weeks of age, from the breeding unit, Department of Immunology UJ CM, Krakow. The mice were fed a commercial, granulated food and water *ad libitum*. The study protocol was approved by the Local Ethics Committee in Krakow (No 91/2011).

2.7.2 Cells

Neutrophils (PMNs) isolated from CBA mice were induced by intraperitoneal injection of 1.0 mL of thioglycolate. The cells were collected 18 h later by washing out the peritoneal cavity with 5 mL of PBS containing 5 U heparin/mL. Then the cells were centrifuged and red blood cells were lysed by osmotic shock using distilled water; osmolarity was restored by addition of 2× concentrated PBS.

2.7.3 Luminol-Dependent Chemiluminescence Assay

Effect of the tested agents on the generation of reactive oxygen species (ROS) by PMNs was evaluated *in vitro* using luminol-dependent chemiluminescence (LCL). LCL was counted at 37 °C in temperature-stabilized luminometer Lucy 1 (Anthos). Briefly, 18 h neutrophils (5×10^5/cells/well), were mixed with luminol (0.8 mg/mL) at 1:1 volume ratio (both Sigma-Aldrich) and incubated at 37 °C for 30 min, then the cells were incubated with tested agents in Hank's balanced salt solution (10 min at 37 °C in an atmosphere of 5% CO_2) on a 96-well flat-bottom black plate (Nunc). After incubation, the cells were immediately stimulated with opsonized zymosan

0.2 mg/mL (Sigma-Aldrich). Photon emission over 75 min with 3 min intervals was measured. Results are expressed as relative light units (RLU) where photons were counted every 5 s. Each type of experiment was performed in duplicate.

2.8 Bacterial Strains

To test microbicidal activity of the tested agents we used four different strains of the microbes: *Candida albicans ATCC 90029*, *Staphylococcus aureus ATCC 25923*, *Pseudomonas aeruginosa ATCC 27853* and *Propionibacterium acnes ATCC 11827*. *C. albicans* was grown on Sabouraud agar and incubated for 48 h at 37 °C (bioMérieux). *S. aureus* and *P. aeruginosa* were grown on Mueller-Hinton agar (bioMérieux) and incubated at 37 °C for 48 h. *P. acnes* was grown on Schaedler agar (bioMérieux) and incubated at 37 °C for 5 days.

2.9 Microbicidal Activity of the Tested Compounds

The following two protocols were used to assess the microbicidal activity of the tested agents (Tau-NHBr, Br-422, Br-612 and BAT).

(1) Microbes (*S. aureus, P. aeruginosa, C. albicans*) were diluted to the concentration of 10^5 CFU/mL or 10^8 CFU/mL in 0.9% NaCl (pH 7.4) and then were incubated with the agents at 37 °C for 1 h. The tested agents were used at concentration range of 10^{-5}–10^1 mM. Immediately after the incubation, aliquots of 100 μL were removed and diluted 100-fold. Aliquots (100 μL) of this dilution were spread on growing agar plates, incubated at 37 °C and CFU were counted after 48 h. A detection limit was 100 CFU/mL taking into account both plates and the dilution. Control samples (microbes diluted in the buffers only), were treated the same way. The minimal concentration of the test agents that led to complete killing of bacteria or fungi to the detection limit (zero CFU on the agar plates) was determined as minimal bactericidal concentration (MBC).

(2) For quantitative killing assays, the test compounds were dissolved in 0.1 M phosphate buffer (pH 7.1) to the desired concentration. Controls were performed in buffer without additives. Bacterial suspensions (40 μL) were added to 4 mL of the test and control solutions. After different incubation times at room temperature, aliquots of 100 μL were removed and diluted tenfold or 100-fold in 0.6% sodium thiosulfate solution to inactivate the test compound. Aliquots (50 μL) of these dilutions were spread in duplicate on tryptic soy agar plates with an automatic spiral plater (model WASP 2, Don Whitley Scientific, Shipley, UK), allowing a detection limit of 100 CFU mL^{-1} taking into account both plates and the dilution. The plates were incubated at 37 °C, and CFU were counted after 24 and 48 h. Inactivation

controls where bacteria were added to mixtures of the test substances with their neutraliser sodium thiosulphate did not show reduction of bacteria in the concentration range used in this study.

2.10 Formulation and Evaluation of Tablets Containing the Stable Bromamine Compounds

2.10.1 Materials

Carbomer (Carbopol® 940 Caesar & Loretz GmbH) and synthetic inorganic substances were used as tablet excipients. All other materials were of analytical grade.

2.10.2 Preparation of Bioadhesive Tablets

The tablets containing BAT (20%) were prepared by direct compression in Korsch EK0 eccentric tablet machine (Emil Korsch Maschinenfabrik, Berlin, Germany) equipped with 6 mm in diameter multi-tips flat-faced beveled edge punch. Before tableting, the active substance and excipients were mixed using mortar and pestle.

2.10.3 Dissolution Testing

The dissolution studies were carried out using USP apparatus four. The special tablet holder was designed to simulate the method of tablet application and to reduce its release surface (holder excluded hydration of one side of the tablet). The holder was manufactured with ABS (acrylonitryle butadiene styrene) thermoplastic polymer in Fused Filament Fabrication 3D printer. The flat surface of the tablet was mounted to the holder by cyanoacrylic glue (Fig. 1), and the holder was fitted by two silicon o-rings in 22.6 mm flow through cell, of DFZ 60 (Erweka) apparatus, connected to piston pump HPK 60 (Erweka).

The dissolution medium (100 mL of phosphate buffer—pH 6.8) was circulated with the minimum piston pump circulation rate—4.5 mL/min. in the closed loop. The temperature in the dissolution cell was maintained at 37 °C ± 0.5 °C. Ten milliliters of aliquots were withdrawn at 30 and 60 min time intervals and subsequent aliquots of 20 mL were taken every hour up to 4 h. At each time point, the same volume of fresh dissolution medium was replaced. The amounts of released BAT were assayed at 224 nm with UV-VIS spectrophotometer Jasco V-530 and calculated using a calibration curve of the drug (molar attenuation coefficient $\varepsilon_{224} = 11.0\ 2 \times 10^3\ \mathrm{L} \times \mathrm{mol}^{-1} \times \mathrm{cm}^{-1}$). Drug dissolution tests were conducted in triplicate and the results were reported as the mean ± SD.

Fig. 1 Image of 3D printed holder with mounted tablet containing BAT

2.10.4 Magnetic Resonance Imaging (MRI)

Swelling of the tablets was studied using MRI. This method was previously applied to compare oral drug delivery systems based on polycarbophil-cysteine polymer *in vivo* (Albrecht et al. 2006). In the current study MRI was carried out for the first time for evaluation of the mucoadhesive tablets *in vitro*. The ultra-short echo time pulse sequence (UTE) was previously applied for evaluation of enteric coated tablets (Dorozynski et al. 2013).

The tablets were mounted inside the holder the same way as in dissolution studies. The holder was put into the polycarbonate chamber filled with 30 mL of dissolution medium and placed in a 9.4 T Bruker Biospin magnet. The studies were carried out at room temperature (~25 °C). The images were taken after 1, 2, 3 and 4 h. For the study purpose 3D ultra-short echo time (3D UTE) sequence was used. The following parameters of the sequence were applied number of scans (NS) = 1, echo time (TE) = 20 μs, repetition time (TR) = 8 ms, field of view (FOV) = 2.63 × 2.63 × 2.63 cm, matrix size 256 × 256 × 256. MR images were processed in Image Jv.1.44 (http://rsb.info.nih.gov/ij/)—image processing software.

2.11 Statistics

Statistical significance between two groups were tested using Student's t test. For more groups, comparison one-way ANOVA and Tukey post hoc test were used. Results are expressed as a mean +/− SEM or SD (if indicated in the legend) values. A p value <0.05 was considered statistically significant. Analysis was performed using GraphPad Prism version 5.01 program (GraphPad Software, USA).

3 Results and Discussion

3.1 Cytotoxic and Microbicidal Capacities of Tau-NHBr, Br-612, Br-422, BAT

The first aim of this study was to test cytotoxic properties of Br-612 and Br-422, the stable derivatives of Tau-NHBr. Cytotoxicity of the tested agents was assessed by mitochondrial-dependent reduction of MTT to formazan as described before (Olszanecki et al. 2008). Importantly, the stable forms of Tau-NHBr when used at concentration of up to 300 μM were non-cytotoxic against J774.A1 macrophages, the representative inflammatory cells (Fig. 2).

The next step of our study was to test microbicidal capacity of the bromine compounds against major pathogens associated with wound infections and skin inflam-

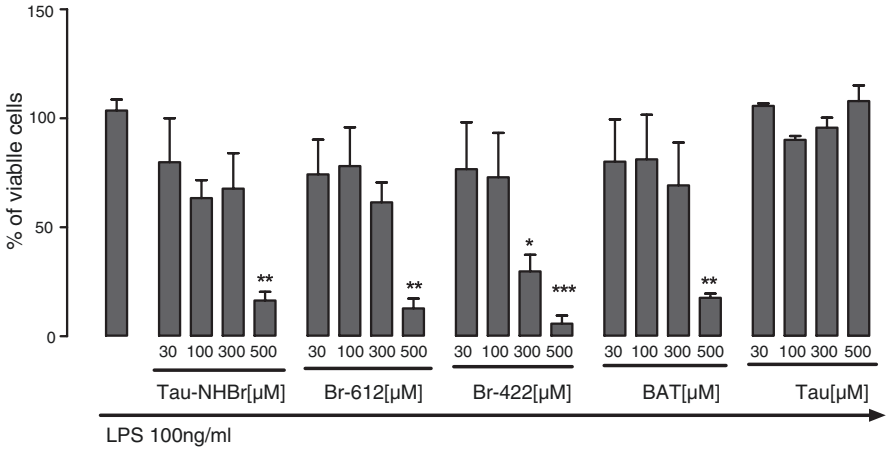

Fig. 2 Influence of Tau-NHBr, Br-612, Br-422, BAT and taurine on the cell viability. J774.A1 macrophages were pre-incubated with the tested agents in DMEM without FBS. After 1.5 h the medium was removed and fresh DMEM +5% FBS with LPS was added for additional 24 h. Cell viability was tested using MTT. Data were calculated from three separate experiments,*$p < 0.05$; **$p < 0.005$; ***$p < 0.001$ *versus* LPS stimulated cells

matory diseases, such as *S. aureus*, *P. aeruginosa*, *P. acnes* and *C. albicans*. Previously, we have demonstrated that Tau-NHBr is a stronger antiseptic agent than NCT and kills a wide range of pathogens at non-cytotoxic concentrations (Marcinkiewicz et al. 2005, 2006). As expected, killing planktonic forms of bacteria prevented the formation of biofilm (Marcinkiewicz et al. 2013). However, Tau-NHBr did not kill sessile bacteria hidden in biofilm matrix due to the low concentration tested, at which consumption of oxidation capacity by organic matter may have taken place (Gottardi et al. 2014).

In our experimental set-up, the microbicidal activity of Tau-NHBr, Br-612, Br-422 and BAT at concentrations ranging from 10^{-5} to 10^{1} mM was tested. In a control group, bacteria were diluted in the buffer only. All the tested agents showed similarly strong bactericidal properties at concentrations below their cytotoxic activity. However, Tau-NHBr at micromolar concentrations, in contrast to its stable derivatives, did not kill *C. albicans* strain ATCC 90029 and eliminated 10^{8} CFU/mL of *S. aureus* not below 700 μM (Table 1). In conclusion, these results suggest that Br-422 is the strongest antiseptic agent among the tested Tau-NHBr compounds, explained by its two oxidizing bromine atoms.

To determine the effective time to achieve microbicidal effect of the stable forms of Tau-NHBr, the agents were incubated with bacteria for various periods of time. At low micromolar concentrations in buffer solution, slightly above the MBC, Br-612 and Br-422 showed significant bactericidal activity against *S. aureus* within a few min already at room temperature (Fig. 3). The killing curves for *P. aeruginosa* were similar (data not shown). This was in accordance to BAT and Tau-NHBr, but also HOBr, dibromo-isocyanuric acid, and bromantine, where the bactericidal activity of all these compounds appeared similar (Gottardi et al. 2014), At higher micromolar concentrations, all bromamines killed bacteria immediately within seconds in buffer solutions, while 10 min were necessary at a concentration of approximately 500 μM for reduction of *C. albicans* strain ATCC 10231 by >3 $\log_{10}$. Such effective killing of microbes as demonstrated for all tested N-bromotaurine derivatives is desired upon clinical application of antiseptics, particularly in early stages of infec-

Table 1 MBC of Tau-NHBr, Br-612, Br-422 and BAT tested at 37 °C for 1 h

Microbe	CFU/mL	Tau/NHBr	BAT	Br-612	Br-422
		MBC[μM]			
C. albicans	10^{5}	2500	15	7	1.5
	10^{8}	10000	30	30	7
S. aureus	10^{5}	70	7	1,5	0.7
	10^{8}	700	7	7	1.5
P. aeruginosa	10^{5}	17	15	3	0.7
	10^{8}	70	70	15	3
P. acnes	10^{5}	0.7	0.7	0.15	0.07
	10^{8}	3	3	0.7	0.3

MBC was defined as the minimal concentration of the antiseptic in μM that reduced the CFU count to the detection limit of 2.7 $\log_{10}$

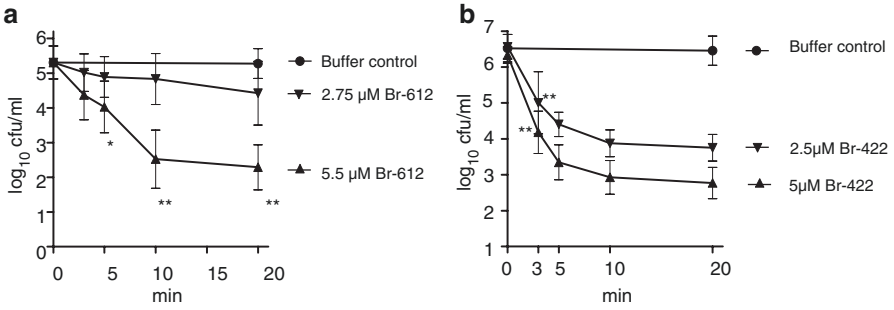

Fig. 3 Bactericidal activity of Br-612 (**a**) and Br-422 (**b**) against *S. aureus* ATCC 25923. Quantitative killing assays at RT and pH 7.1. Detection limit 2 $\log_{10}$ cfu/mL. Mean values ± SD of five independent experiments. *p < 0.05; **p < 0.01 *versus* control

tions, and protects from biofilm formation in addition to the curative effect (Marcinkiewicz et al. 2013). Because of their higher consumption of oxidation capacity compared to low-reactive chloramines in the presence of protein load, bromamines seem to be particularly suited for application on the skin or mucous membranes with low exudate, while NCT appears to be most favourable in delicate body regions or in the presence of high organic load (Gottardi et al. 2013, 2014).

For comparison of tolerability of antiseptics and their microbicidal activity, the biocompatibility index has been suggested (Müller and Kramer 2008). Thereby, the minimal concentration exerting in-vitro cytotoxicity in cell culture is divided by the minimal one exerting in-vitro killing activity against bacteria. An index of 1 or slightly >1 has been found for antiseptics from the class of tensides (Müller and Kramer 2008). Although it is more difficult to evaluate for oxidants because of dependence on the test media, consumption effects etc., for NCT (which has extraordinary tolerability (Gottardi and Nagl 2010) it can be estimated around one, too, if buffer solution without contents susceptible to oxidation is used. Astonishingly, for bromamines the index ranges approximately between one and even 100, comparing Fig. 2 and Table 1. This may indicate a very good tolerability by human tissue, as confirmed by the study in acne (Marcinkiewicz et al. 2008) and the application in herpes zoster (Kyriakopoulos et al. 2016) where a millimolar concentration of Tau-NHBr was very well tolerated.

3.2 Anti-inflammatory Properties of Tau-NHBr, Br-612, Br-422, BAT

It has been demonstrated in a number of studies that taurine haloamines, NCT and Tau-NHBr, at non-cytotoxic concentrations exert both antimicrobial and anti-inflammatory properties (Marcinkiewicz et al. 2005, 2006, 2008; Pasich et al. 2015; Strus et al. 2015). Such unique capacities of Tau-NHBr, in spite of its relatively short life-span, are responsible for Tau-NHBr effectiveness in the treatment of inflammatory diseases associated with bacterial infections (Marcinkiewicz et al.

2008; Marcinkiewicz 2009; Marcinkiewicz 2010). Therefore, it was reasonable to test whether Tau-NHBr modification to achieve stable derivatives retained its important anti-inflammatory properties. These properties were evaluated by comparing their effect on activation of J774.A1 macrophages. In our experimental model, we observed that at a concentration of 300 μM all the tested agents significantly inhibited the production of TNFα (Fig. 4b) IL12p40 (Fig. 4c) and PGE_2 (Fig. 5a). At the concentration of 300 μM, only Br-612 did not inhibit the production of NO_2^- (Fig. 5b). IL-6 was significantly decreased by 300 μM Br-422, while this tendency did not reach a level of significance for Br-612 and BAT (Fig. 4a).

Moreover, all agents at concentration of 300 μM enhanced the expression of HO-1, the crucial stress inducible enzyme. This effect was associated with a slight reduction of COX-2 expression (Fig. 4c, d). Importantly, taurine alone did not affect activity of macrophages in our experimental model.

To determine the effect of the tested agents on ROS production by neutrophils we used luminol-dependent chemiluminescence (LCL) (Schleupner and Glasgow 1978). Upon addition of opsonized zymosan, activated neutrophils increased LCL light emission due to generation of ROS. When the testes agents were added to the reaction mixture, a dose dependent decrease in LCL was observed. At a concentration of 300 μM, Tau-NHBr, Br-612 and BAT significantly reduced generation/enhanced neutralization of ROS as shown by the inhibition of LCL (>90%) (Fig. 6).

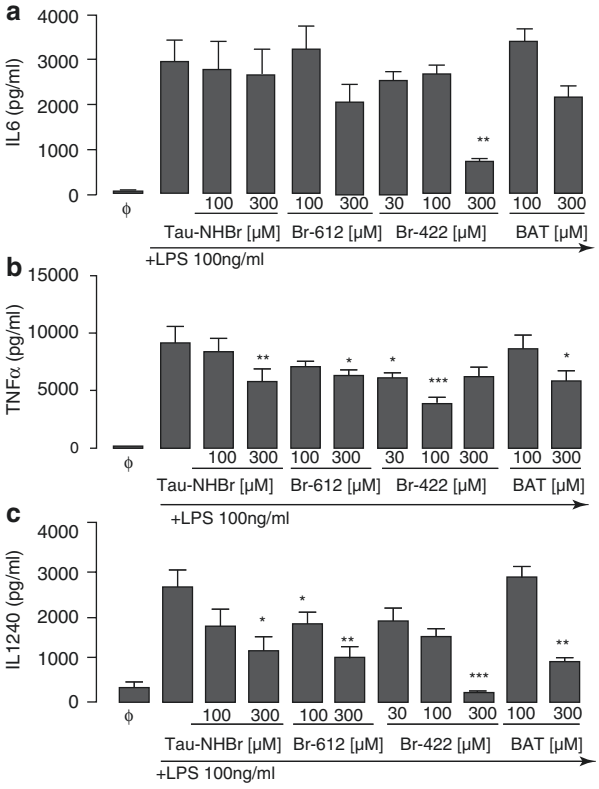

Fig. 4 The effect of Tau-NHBr, Br-612, Br-422 and BAT on the production of IL6 (**a**), TNFα (**b**), IL12p40 (**c**). Macrophages were pre-incubated with the tested agents in DMEM without FBS. After 1.5 h the medium was removed and fresh DMEM +5% FBS with LPS was added for additional 24 h. Medium content of cytokines was measured by ELISA. Data were calculated from three to five separate experiments.*$p < 0.05$; **$p < 0.005$; ***$p < 0.001$ *versus* LPS stimulated cells

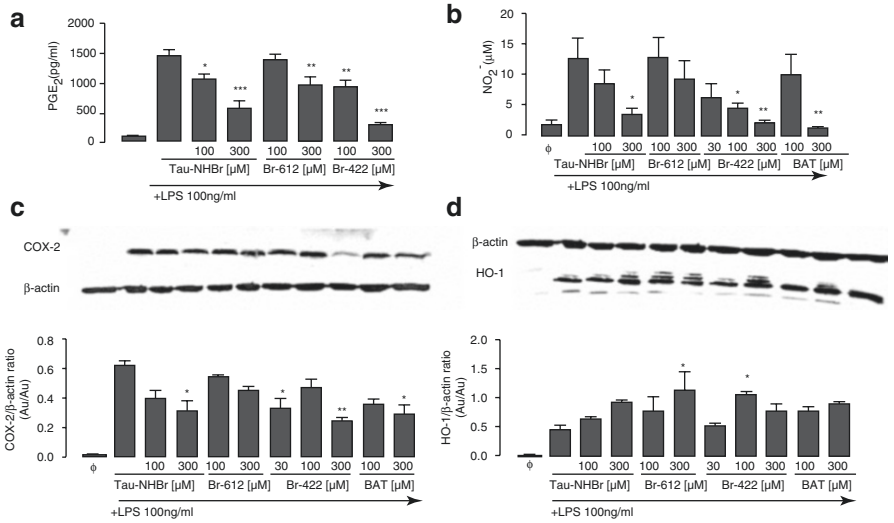

Fig. 5 Effect of Tau-NHBr, Br-612, Br-422, and BAT on the production of PGE2 (**a**) and NO_2^- (**b**) and expression of COX-2 (**c**), HO-1(**d**). Macrophages were pre-incubated with the tested agents in DMEM without FBS. After 1.5 h the medium was removed and fresh DMEM +5% FBS with LPS was added for additional 24 h. After 24 h supernatants and cell lysates were collected. The production of PGE_2 (**a**) and NO_2^- (**b**) was determined in culture supernatants (Data were calculated from three separate experiments.*$p < 0.05$; **$p < 0.005$; ***$p < 0.001$ versus LPS stimulated cells). In cell lysates, COX-2 (**c**) and HO-1 (**d**) expression was analyzed using western blot (Densytometric analysis of bands from two experiments *$p < 0.05$ *versus* LPS stimulated cells)

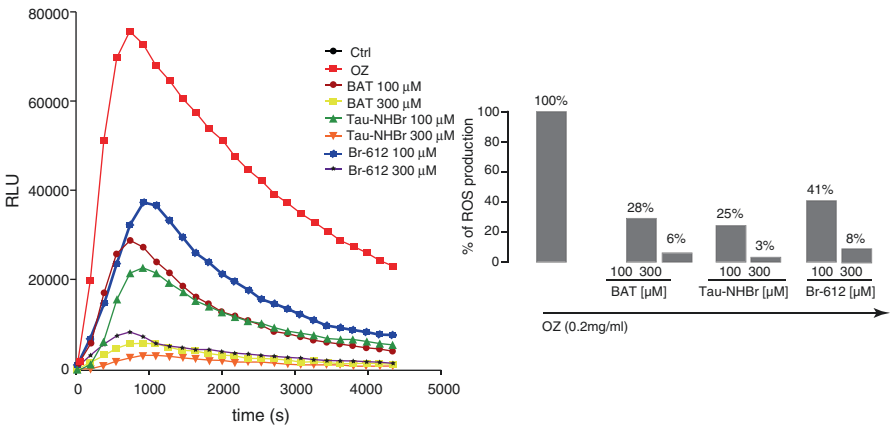

Fig. 6 Effect of Tau-NHBr, Br-612 and BAT on ROS production by PMNs stimulated with opsonized zymosan. PMNs (5×10^5/cells/well), were preincubated with the tested agents, for 10 min. Then, LCL was performed and measured as described in methods. Ctrl—non-stimulated cells. OZ—stimulated cells, positive control LCL = 100%. The figure shows one representative experiment

3.3 Evaluation of Delivery Systems with Bromamine Compounds

The aim of the solid dosage form development was to achieve a matrix tablet with sustained release characteristics, containing stable bromamine forms. For example, in treatment of periodontal diseases such sustained release delivery systems with bioadhesive properties may be used in the oral cavity to retain high concentrations of substances with microbicidal and anti-inflammatory activities.

Development of drug formulation was based on preliminary studies evaluating the influence of excipients on the stability of Tau-NHBr and the properties of the formulation. Polymers widely used in pharmaceutical technology including (methyl cellulose, hydroxypropyl methyl cellulose, sodium alginate, poloxamer) were taken into account as thermosensitive gelling drug delivery carriers of unstable Tau-NHBr in liquid form. The properties of gelling systems and their mixtures with Carbopole®, a swellable mucoadhesion enhancer, and the influence of the excipients on the stability of the Tau-NHBr were evaluated.

Nevertheless, the stability of Tau-NHBR in the presence of matrix polymers was insufficient. However, Carbopole® did not affect the stability of the substance (Jamróz et al. 2014). The results of the study led to the development of carbomer-based, bioadhesive solid dosage form, featuring sustained release of the active substance, which can be used as a carrier of stable Tau-NHBr forms Br-422 and Br-612 (Fig. 7). Limited number of stable taurine derivatives and analytical considerations entail BAT as a model, solid state, stable bromamine compound with oxidizing properties similar to taurine derivatives.

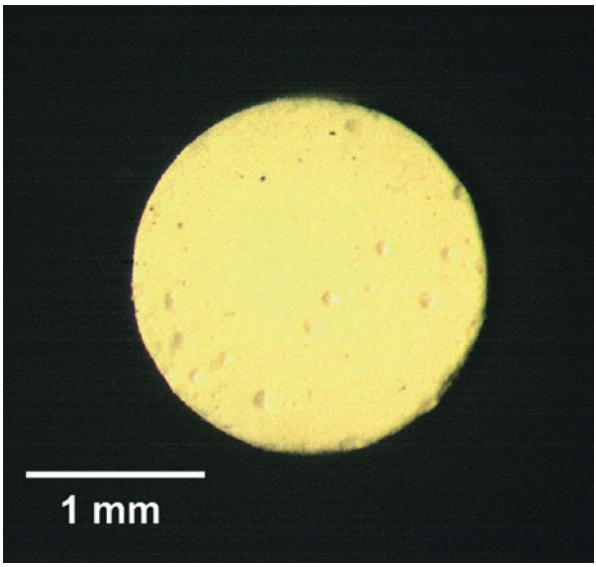

Fig. 7 Image of mintablet containing 612-Br

Mixtures of BAT and Carbopole® had poor flow properties and adhered to punches during tableting. Addition of other excipients was necessary to improve technological properties of tablet mass. The inorganic excipients with low reactivity were chosen to minimize the influence on stability of the active bromamine compounds.

Different amounts of excipients were used in preliminary studies in order to prepare blend with required properties for direct compression. As a result of formulation studies, white tablets 6 mm in diameter and 3 mm in thickness were obtained. Average mass of the tablets was 51.3 mg (±2.5 SD).

3.3.1 MRI Studies and Dissolution Profiles of BAT

The MRI images show cross section of the tablet (Fig. 8). The white dotted line marks the border of the hydrogel. The hydration of the tablet resulted in expansion of the swollen hydrogel. The core of the tablet characterized by lower hydration was retained during the whole experiment. The core is visible in Fig. 8 as dark, purple colors inside the tablet. During 4 h of the study, changes in size and hydration of tablet matrix were observed and the unhydrated areas spread through the swollen hydrogel matrix.

The dissolution profile of BAT is shown in Fig. 9 over a period of 4 h. The sample volume collected every hour during dissolution studies corresponded to saliva secretion in healthy individuals (Ekberg 2012; Fejerskov and Kidd 2008). Before the studies, tablets were mounted to the holder to reduce active dissolution surface and to mimic the release of active substance from the tablet adhered to the biologically impermeable surface. During 4 h dissolution studies, changes in size, color and transparency of the tablets were observed. After contact with the dissolution medium, the tablet core changed its color to yellow. The expanding hydrogel was white and opaque. After 4 h, the hydrogel almost filled the dissolution chamber and it was difficult to state the presence and the size of the dry core which can be observed using MRI. During the observation period, BAT was gradually released from the swollen matrix. After 4 h, 50.1% of BAT was released from tablets. These data were used to evaluate the kinetics of drug release by *KinetDS* software (Mendyk et al. 2012). Considering the determination coefficient (R^2), the dissolution profile was best described by the zero-order model (Table 2).

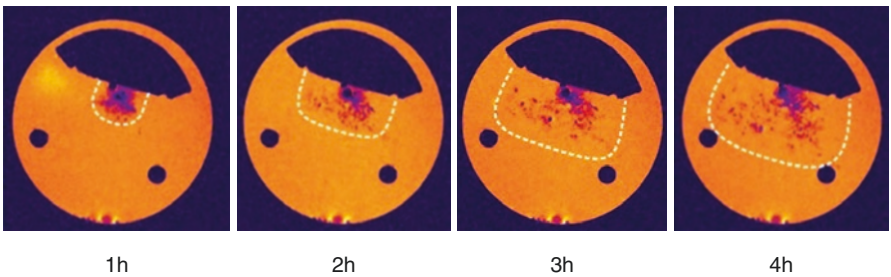

Fig. 8 MRI images of swollen BAT tablet

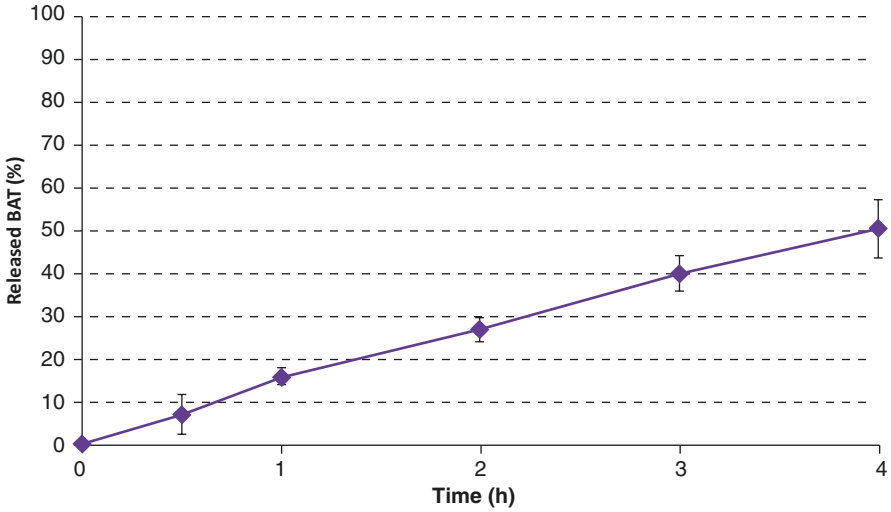

Fig. 9 Dissolution profile of BAT from tablet

Table 2 Fitting of experimental release data to kinetic models

Model	Dissolution constant (a)	Intercept (b)	Determination coefficient (R^2)
Zero-order	12.266	1.9728	0.9956
First-order	0.5332	1.9837	0.8935
Second-order	−0.031	0.1244	0.6902
Third-order	−0.0048	0.0159	0.5321

In conclusion, the results of dissolution studies showed that 50% of BAT was released after 4 h of the study, no burst effect was observed and active substances was released according to zero order kinetics model what suggest that the release process takes place at a constant rate, independent of drug concentration. These desirable properties of dosage form keep the concentration of active substance on constant level what is important in the case of microbicidal substances. The results of MRI studies showed the distribution of water in the hydrogel matrix and indicate the area of anhydrated polymer which could be the reservoir of undissolved BAT.

4 Conclusions

The present study clearly indicates that N-monobromo-2,2-dimethyltaurine (Br-612), N-dibromo-2,2-dimethyltaurine (Br-422), and bromamine T (BAT) are better candidates than Tau-NHBr (taurine bromamine) for a treatment of infectious diseases associated with inflammatory response. This opinion is supported by the following data:

- Br-422, Br-612, and BAT in contrast to Tau-NHBr, show long-term stability.
- They are non-cytotoxic at concentrations up to 300 μM.
- Br-422, Br-612, and BAT retained anti-inflammatory properties of Tau-NHBr. At non-cytotoxic concentrations, they significantly inhibited the production of pro-inflammatory cytokines and nitric oxide. Moreover, they markedly enhanced the expression of HO-1, the crucial stress-inducible enzyme.
- Br-422, Br-612, and BAT exert stronger microbicidal activity than Tau-NHBr against all the tested bacteria and particularly yeasts. Of note, Br-422 has higher activity in most in-vitro tests because it is a di-bromo compound.
- The biocompatibility index of bromamines appears to be particularly high (1–100) compared to other antiseptics, which indicates very good tolerability.
- Finally, successful drug formulation using Carbopol®, a swellable polymer with good bioadhesive properties, allows the preparation of matrix tablets containing stable bromamine forms with sustained release.

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HPLC Determination of Bioactive Sulfur Compounds, Amino Acids and Biogenic Amines in Biological Specimens

Antonio Francioso, Sergio Fanelli, Daniele Vigli, Laura Ricceri, Rosaria A. Cavallaro, Alessia Baseggio Conrado, Mario Fontana, Maria D'Erme, and Luciana Mosca

Abstract There is an increasing interest for analytical methods aimed to detect biological sulfur-containing amines, because of their involvement in human diseases and metabolic disorders. This work describes an improved HPLC method for the determination of sulfur containing amino acids and amines from different biological matrices. We optimized a pre-column derivatization procedure using dabsyl chloride, in which dabsylated products can be monitored spectrophotometrically at 460 nm. This method allows the simultaneous analysis of biogenic amines, amino acids and sulfo-amino compounds including carnosine, dopamine, epinephrine, glutathione, cysteine, taurine, lanthionine, and cystathionine in brain specimens, urines, plasma, and cell lysates. Moreover, the method is suitable for the study of physiological and non-physiological derivatives of taurine and glutathione such as hypotaurine, homotaurine, homocysteic acid and S-acetylglutathione. The present method displays good efficiency of derivatization, having the advantage to give rise to stable products compared to other derivatizing agents such as *o*-phthalaldehyde and dansyl chloride.

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With this method, we provide a tool to study sulfur cycle from a metabolic point of view in relation to the pattern of biological amino-compounds, allowing researchers to get a complete scenario of organic sulfur and amino metabolism in tissues and cells.

Keywords • High pressure/performance liquid chromatography • Sulfur-containing bioactive compounds • Dabsyl chloride

Abbreviations

DABS	4-N,N-Dimethylaminoazobenzene-4′-sulfonyl chloride
DANS	5-Dimethylaminonaphthalene-1-sulfonyl chloride
HPLC	High performance liquid chromatography
OPA	*o*-phthalaldehyde
TCA	Trichloroacetic acid
TDGA	Thiodiglycolic acid

1 Introduction

Analysis of biogenic and sulfur-containing amines is becoming increasingly important in clinical and biochemical research (Curran et al. 2016). Taurine is a sulfur-containing β-amino acid present in different human body areas and is one of the most important and studied sulfurous organic bioactive molecules involved in human health (Jacobsen and Smith 1968). It is one of the end-products of cysteine metabolism and is excreted in urines. Taurine concentration in humans ranges from high millimolar in plasma, heart and brain, to micromolar amounts in tissue and body fluids such as urines (Schuller-Levis and Park 2003). Apart from its important physiological role as a neuroactive molecule (Wade et al. 1988), it displays a wide range of pharmacological effects including membrane stabilization, cytoprotective effects, antioxidant and anti-inflammatory action (Chaturvedi et al. 2015; Abdel-Moneim et al. 2015). In addition taurine is able to restore muscle function and performance in different pathological conditions (Chan-Palay et al. 1982a, b, c; De Luca et al. 2015). Recently, a number of studies have shown that taurine may have a beneficial effect against metabolic syndrome, preventing obesity regulating glucose metabolism and lowering cholesterol plasma concentration (Bai et al. 2016; Zhang et al. 2016a, b; Chen et al. 2016).

Moreover, many sulfurous amino compounds and taurine metabolic derivatives such as cysteic acid, homocysteic acid, hypotaurine and homotaurine (Fig. 1) have important biological roles (Jacobsen and Smith 1968) and researchers are in need of analytical methods for their accurate determination. Accumulation of sulfite, taurine, S-sulfocysteine and thiosulfate contributes to the severe neurological impairment in molybdenum cofactor deficiency, a severe autosomal recessive inborn error of metabolism (Atwal and Scaglia 2016).

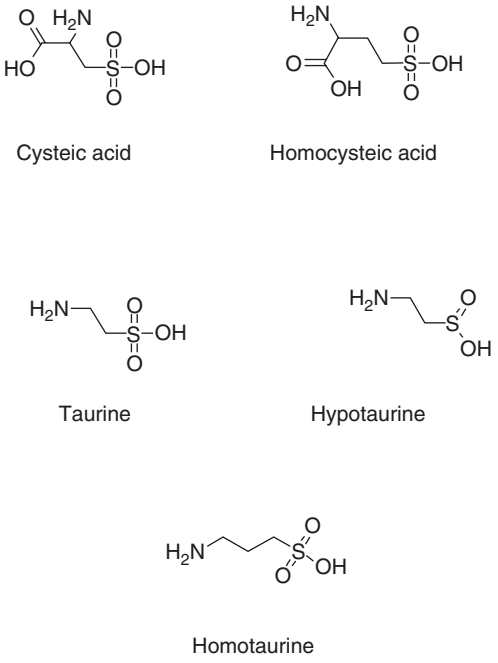


Fig. 1 Chemical structures of Taurine and related sulfur-containing bioactive compounds

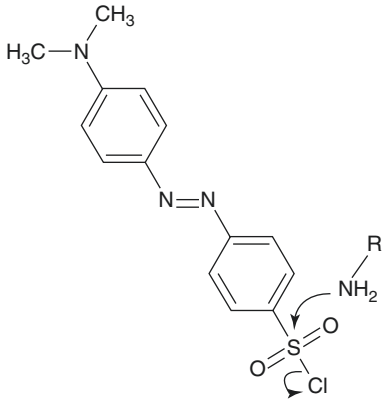


Fig. 2 Reaction of dabsyl chloride (DABS) with amines

For this purpose we developed a High Performance Liquid Chromatography (HPLC) analytical method for the determination of taurine, sulfur-containing molecules and other biogenic amines from different biological matrices. We improved a dabsyl chloride (DABS) pre-column derivatization method (Krause et al. 1995). DABS (4-N,N-dimethylaminoazobenzene-4′ sulfonyl chloride), is an amine derivatizing agent (Fig. 2) able to give rise to stable products that can be easily monitored spectrophotometrically at 460 nm (Krause et al. 1995; Lin and Wang 1980).

2 Materials and Methods

2.1 Chemicals

Amino acid standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). Gradient grade solvents used for chromatographic analyses were purchased from Carlo Erba Reagents (Milan, Italy). DABS was purchased from Supelco (595 North Harrison Road, Bellefonte, PA). All other reagents were analytical grade products from Sigma-Aldrich. S-Acetylglutathione (SAG) was purchased from GNOSIS S.p.A. (Desio, MB, Italy).

2.2 Derivatization Procedure

The amino acid standards were dissolved in 0.1 M HCl containing 0.2% thiodiglycolic acid (TDGA) to prevent—SH oxidation. One microliter of amino acid standards at different concentrations were added to 14 μL of reaction buffer (1 M $NaHCO_3$, pH 8.6) and then to 25 μL of 15 mM DABS in acetone. After vigorous vortexing, the standards were incubated at 40 °C for 30 min, vortexing at the first minute, twelfth minute and twenty-eighth minute. The resultant Dabsyl derivatives were put in an ice bath for 5 min and then centrifuged at 14000 *g* for 20 min. Supernatants were diluted 1:10 in mobile phase, filtered onto 0.2 μm filters, and then 50 μL were injected onto the column.

2.2.1 Treatment of Plasma and Urine Samples

Hundred microliter of fresh human plasma or urines were deproteinized by treatment with 100 μL of 10% TCA for 30 min at 4 °C and centrifuged at 14000 *g* for 30 min at room temperature. The supernatant was then lyophilized. Dry material was resuspended in 15 μL of reaction buffer and 25 μL of 15 mM DABS and derivatized as described above.

2.2.2 Treatment of Brain Tissues and Cultured Cell Samples

Selected mouse brain samples from either cortical or striatal regions (100 mg wet weight) and neuroblastoma cells (SH-SY5Y) pellet derived from 25 cm^2 flask were treated with 500 μL of 0.1 M HCl containing 0.2% TDGA, sonicated for 10 min (only for brain tissue), and then centrifuged at 14000 *g* for 30 min. The supernatant was freeze-dried. 50 μL of reaction buffer and 100 μL of 15 mM DABS were added to the tube and derivatized as described above.

2.3 High-Performance Liquid Chromatography (HPLC)

The apparatus consisted of a Waters HPLC 600 pump equipped with a controller, a Waters autosampler mod. Seven hundred and seventeen and a UV-Vis photodiode array detector mod. 2996. The chromatographic column was a reverse phase X-Bridge C18 column, 4.6 mm × 150 mm, 5 µm particle size, with a 10 mm guard column of the same material. Data analysis was performed using a dedicated application (Millennium[32]). Elution was performed with a binary gradient system with sodium acetate (30 mM, pH 6.5) and acetonitrile in a ratio of 80:20 (v/v) (solvent A) and propan-2-ol with acetonitrile 50:50 (v/v) (solvent B). The gradient was: 0–4 min, 5% B; 4–8 min, 20% B; 8–15 min, 25% B; 15–27 min, 60% B; 27–28 min, 100% B; 28–32 min, 100% B; 32–33 min, 5% B; 33–60 min, 5% B. The column was equilibrated for 20 min with 5% B at 1 mL/min and was maintained at 40 °C. Dabsylated products were monitored spectrophotometrically at 460 nm.

3 Results and Discussion

A variety of pre-column amine derivatizing methods are reported in the literature, among them *o*-phthalaldehyde (OPA) and dansyl chloride (DANS) are the main derivatizing agents (Fig. 3) (Kang et al. 2006; Bertollini et al. 2012; Mou 1997).

OPA reacts in the presence of thiols specifically with primary amines above their isoelectric point. One of the limits of this procedure is that not all of the isoindolic fluorescent OPA derivatives are stable, moreover the analyses with this method could be hampered by interferences of unknown thiol moieties potentially present in biological samples. Thiols can displace the organic thiolic additive for the reaction generating different isoindolic adducts for the same investigated molecule (Kand'ar et al. 2007; Mopper and Delmas 1984).

DANS derivatization is a good versatile tool in terms of chromatographic separation and determination but the instability of the products and the need of a fluorimetric detector, as for OPA derivatization, makes this method inaccessible to a large

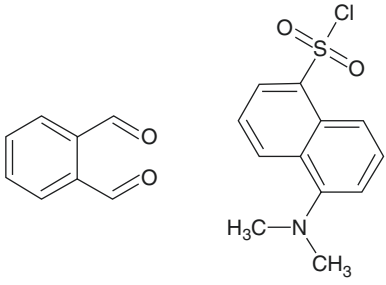


Fig. 3 Structures of OPA (*left*) and DANS (*right*)

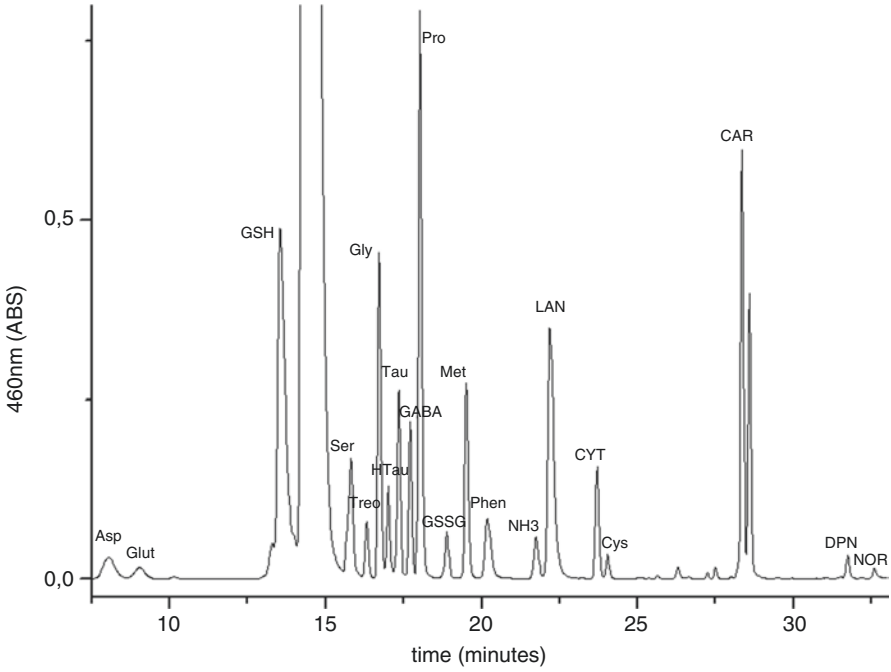


Fig. 4 HPLC chromatogram of selected dabsylated amines, amino acids and sulfurous amino compounds

number of research, commercial and clinical laboratories (Loukou and Zotou 2003). By contrast dabsyl chloride, 4-N,N-dimethylaminoazobenzene-4′ sulfonyl chloride (DABS), is an amine derivatizating agent that provides a simple derivatization, good stability, good reproducibility and good analytical detection limit (Lin and Wang 1980; Krause et al. 1995). HPLC analyses with DABS show a good separation of a large number of amines and amino acids, which are detected in the visible region at 460 nm (Krause et al. 1995). DABS, like the other amine derivatization agents, can give rise to mono-dabsyl and bis-dabsyl derivatives in the presence of multiple amino groups. Furthermore with respect to OPA derivatization, DABS can react with primary and also with secondary amines.

In our experimental conditions the chromatographic method allows the separation and the analysis of a wide range of dabsylated amino-derivatives from different complex biological matrices such as plasma, brain tissue, urines and cell lysate samples. As shown in Fig. 4 the following molecules were analyzed in this elution order: Aspartate (Asp), Glutamate (Glut), reduced Glutahione (GSH), Serine (Ser), Threonine (Threo), Glycine (Gly), Hypotaurine (HTau), Taurine (Tau), ɣ-aminobutirric acid (GABA), Proline (Pro), Oxidized glutathione (GSSG), Methionine (Met), Phenylalanine (Phe), Lanthionine (LAN), Cystathionine (CYT), Cysteine (Cys), Carnosine (CAR), Dopamine (DPN) and Norepinefrine (NOR).

Dabsylated amino acids and amphoteric molecules are eluted and separated according to their isoelectric point, from lower to higher value. DABS derivatization displays a good sensitivity (pmole level), linearity and efficiency of derivatization and has the important advantage of giving rise to stable derivatized products (24 h at 4 °C and at least 14 days at −20 °C) (Jansen et al. 1991; Kang et al. 2006; Krause et al. 1995).

With respect to other similar methods using DABS as a derivatizing agent, we introduced some important modifications of the procedure (Krause et al. 1995; Vendrell and Aviles 1986; Drnevich and Vary 1993).

Firstly, the derivatizing procedure was modified and the volumes of reaction were reduced to allow a better recovery of all the analytes from biological samples. The mobile phase was modified and no organic additives such as triethylamine or tetrahydrofuran were added to the hydrophilic phase (Solvent A, 30 mM sodium acetate pH 6.5 containing 20% CH_3CN). Many published methods in literature report the use of organic modifiers in the hydrophilic phase to avoid the interaction of the analytes with siloxan and silanol residues in non completely endcapped C18 stationary phases (Krause et al. 1995; Romero et al. 2000). In our setting the use of modifiers was not necessary because of the chemistry of the column. X-bridge columns have replaced a certain number of siloxan groups with ethylene bridges reducing the hydrophilic interaction of the molecules with the stationary phase and improving the stability of the column. The ethylene bridge also reduces the number of free silanols, minimizing adverse interactions with the injected sample and improving both chromatographic resolution and peaks shape.

The second point is the separation of some strictly related compounds and acidic molecules that is strongly dependent on the pH of the mobile phase. In our setting it is important to maintain mobile phase at pH 6.5. Higher pH values lead to loss of chromatographic resolution for taurine and GABA, with concomitant improvement in aspartate and glutamate peak separation. Conversely, with lower pH values a more suitable separation of taurine and GABA is obtained, at the expenses of aspartate and glutamate peaks resolution. This could be explained by the increase of the total protonated forms of acidic compounds at lower pH values.

The third important factor that we want to underline is the condition of derivatization reaction with DABS. We set up our method on the basis of some considerations with respect to the basicity of the reaction environment and the temperature. We chose values of pH 8.6 and 40 °C for 30 min after a series of tests. Many papers report a reaction temperature of 70 °C with different reaction times. We tested the efficiency of different derivatizing reaction conditions by modifying reagents concentration, pH, temperature and reaction times, based on the chemical stability of the molecules of interests, and we found that some conditions are more appropriate than others for the detection of specific compounds. For example, our results indicated that for plasma samples it is necessary to increase the ionic strength of reaction buffer to guarantee the same pH value for all the samples. For the analysis of such biological samples (Figs. 5 and 6) after deproteinization with acid precipitants such as TCA, the use of a concentrated reaction buffer (0.5–1 M) is strongly recommended to ensure the buffering of the acidity.

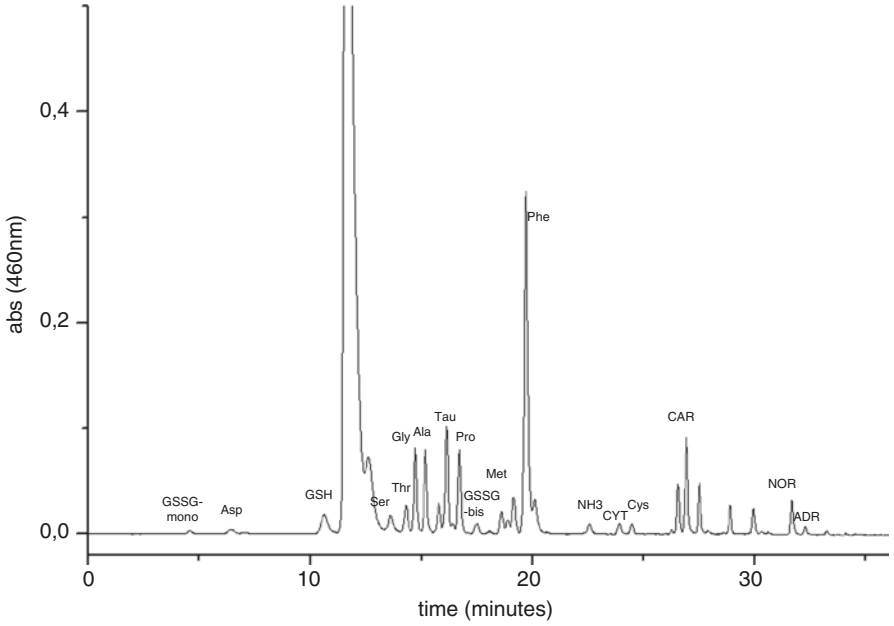


Fig. 5 HPLC chromatogram of dabsylated derivatives from human plasma

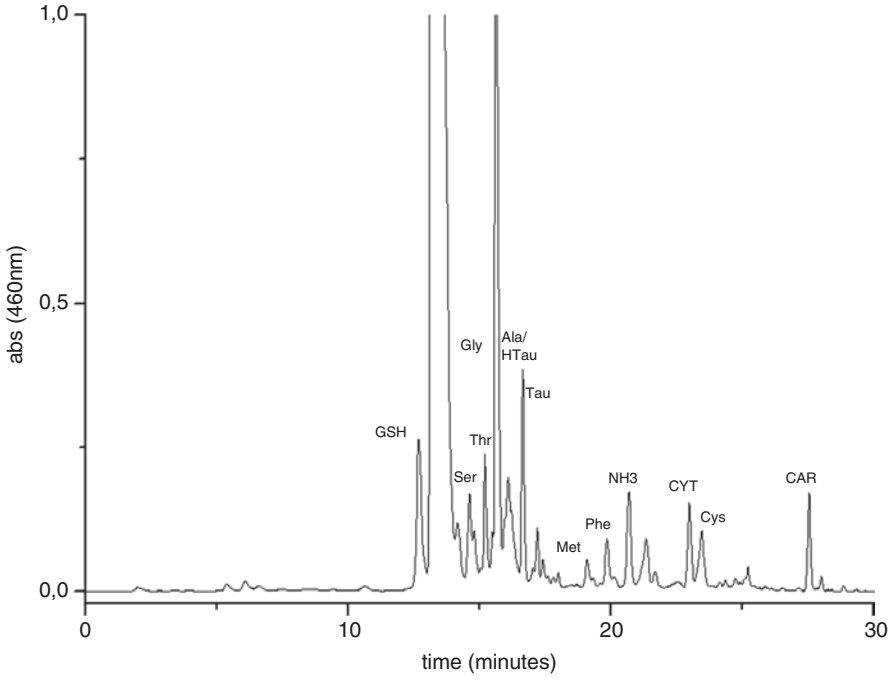


Fig. 6 HPLC chromatogram of dabsylated derivatives from human urine

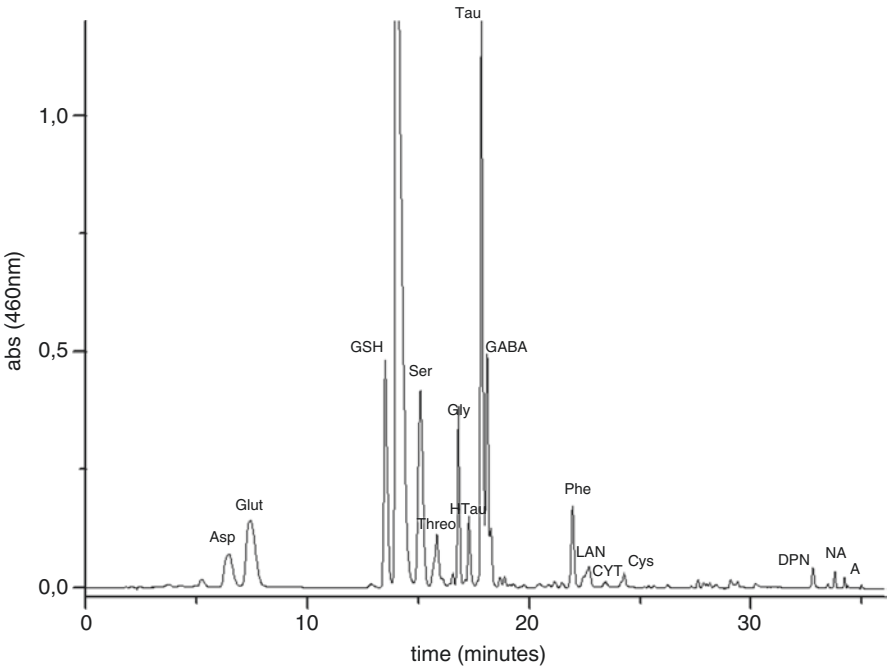


Fig. 7 HPLC chromatogram of dabsylated derivatives from brain tissue

As regards the pH of derivatization, we tested different reaction buffers (pH 8.0, 8.6 or 9.0). In our conditions the best derivatization efficiency of brain tissue samples is obtained with a reaction buffer at pH 9.0, with a reaction time of 15 min at 70 °C (Fig. 7).

Same results can be obtained by lowering the pH of the buffer and increasing the time of derivatization at lower temperatures. It should be underlined that for some molecules such as catecholamines, pH 8.0 is more convenient because of the lability of these molecules and their tendency to be oxidized to form quinones with subsequent degradation or loss of derivatization. At variance, some authors reported the derivatization of this molecules on the –OH moiety at pH 11.5 by forming phenolate which acts as nucleophile (Cai et al. 2010). In our conditions, the derivatization efficiency of dopamine is strictly dependent on the pH value of the reaction buffer; indeed, at pH values higher than 9.0 the derivatization is not efficient probably because of the oxidation of dopamine to the *o*-quinonic form. For this reason in our final protocol we adopted a pH 8.6 for the reaction buffer.

The last parameters that we adjusted in the procedure of derivatization were temperature and time. We tested the efficiency and the effect of different reaction conditions on the deacetylation of an S-acetylated sulfo-amino derivative, the S-acetylglutathione (SAG) (Fig. 8). SAG is the thioester of GSH and its detection in biological specimens could be very important as regards the study of thioesters (Fig. 9).

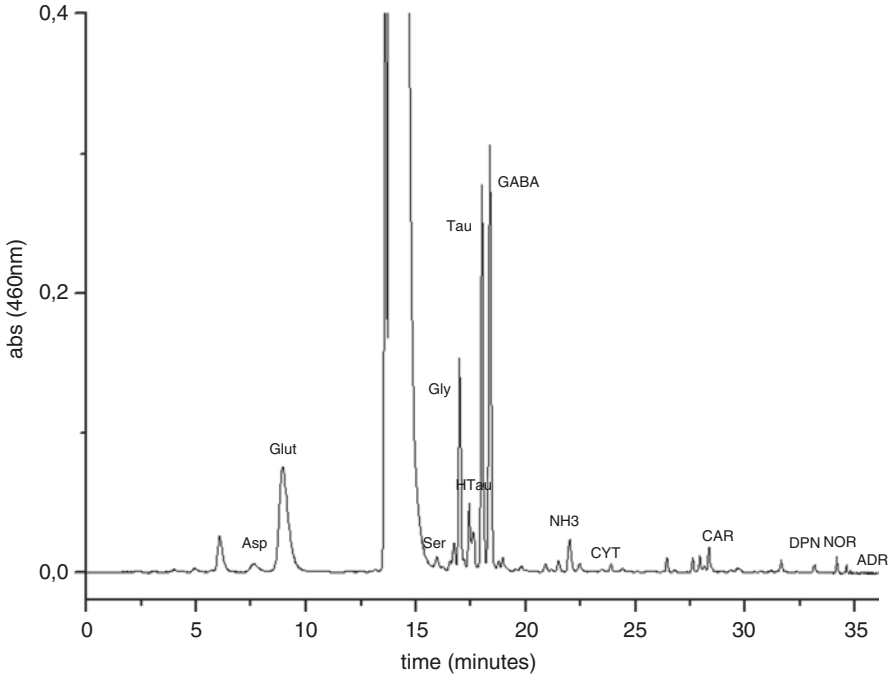
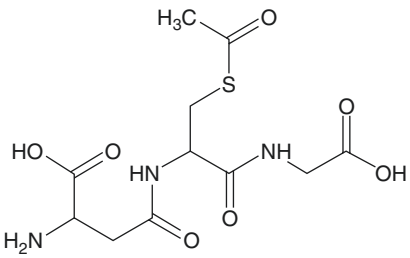


Fig. 8 S-acetylglutathione (SAG)

Fig. 9 HPLC chromatogram of dabsylated SAG



We tested different reaction conditions by analyzing the deacetylation of the molecule at different temperatures, pH and time of reaction. Dabsylation reaction carried out at pH 9 for 15 min at 70 °C gives a deacetylation of the thioester moiety of about 45%. Conversely, reaction conditions of 40 °C, 30 min and pH 8.6 reduces the extent of the deacetylation to about 8%. It is important to emphasize that reaction at pH values lower than 8.6 gives heterogeneous efficiencies of derivatization due to the fact that some amines have pKa values around 8.0. Hence, pH 8.6 is the best compromise that can ensure the homogeneous derivatization of all the studied amines and can minimize the degradation and the oxidation of labile molecules.

We also evaluated the derivatization of GSSG with DABS. As shown in Fig. 10, being GSSG a GSH dimer, its derivatization gives rise to the formation of two derivatives, the mono- and bis-dabsylated forms.

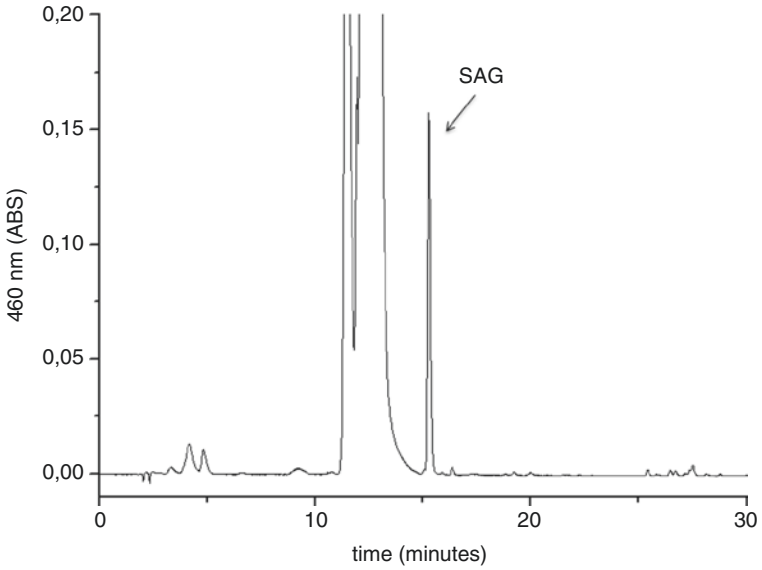


Fig. 10 HPLC chromatogram of Glutathione disulfide (GSSG)

Our data indicate that the dabsylation occurs in the same manner on the two amino groups and the rate of mono- and bis-dabsylated derivatives formation is constant in our reaction conditions.

As regards GSH and thiols in general, we used TDGA to protect thiol moieties from oxidation. The use of TDGA, together with the low temperature and the mild reaction conditions, allows the derivatization of GSH peptide to form a stable dabsyl derivative that can be monitored concomitantly with its bis-dabsylated oxidized form (GSSG) within the same chromatographic analysis. The simultaneous analysis of reduced/oxidized GSH forms, whose ratio is a useful index of oxidative stress (Zitka et al. 2012; Lakritz et al. 1997) enable the study of the redox state of cells or tissues.

GSH analysis shows the same linearity of derivatization and quantification as all the other studied amines, but it is important to underline that, when other thiols and sulfur-containing molecules are present in the derivatization process, some unknown byproducts are formed with a retention time that is strictly similar to GSH and in some cases not completely resolved from DABS peak.

We tested our chromatographic method by analyzing some important sulfur containing organic bioactive compounds. We analyzed simultaneously cysteic acid, homocysteic acid, hypotaurine, taurine and homotaurine (Fig. 11). As for GABA, taurine and homotaurine, resolution is improved at lower mobile phase pH, i.e. pH 6.45. This is due to the strictly related chemical structures and properties that homotaurine and GABA have.

As regards detectable sulfur-containing amines in the biological specimens analyzed, our data indicate that taurine relative content is higher in brain tissue samples with respect to all other biological samples analyzed. As described in the literature, the brain is one of the major body district in which taurine is present. Also in neuroblastoma cells

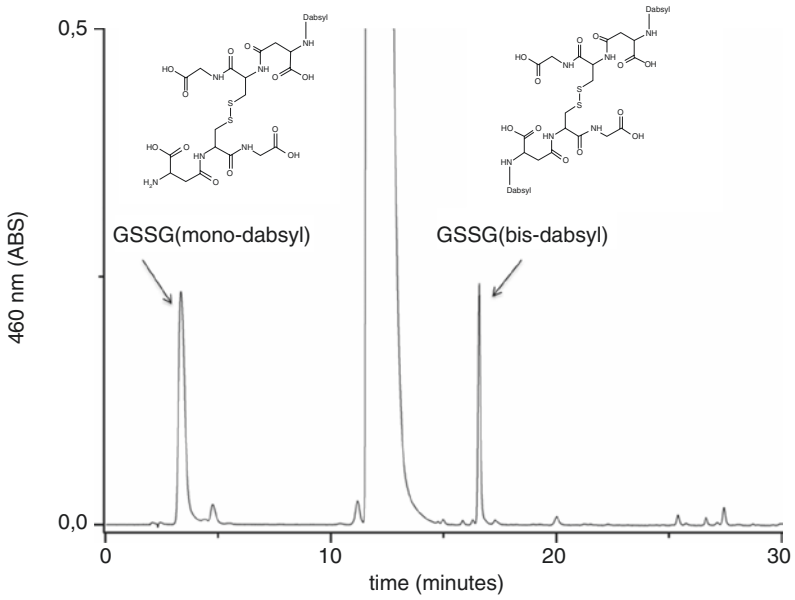


Fig. 11 HPLC chromatogram of Taurine and sulfur derivatives

the pattern of amino compounds (Fig. 12) reveals a relative content of taurine significantly higher with respect to other amines, whereas plasma and urine samples present lower relative amounts of taurine (Figs. 5 and 6).

It is well known that taurine is one of the end-products of cysteine metabolism. It is noteworthy, in brain tissues and neuroblastoma cells (Figs. 7 and 12) samples, we observed a lower amount of cysteine relative content along with higher amounts of taurine and hypotaurine, in agreement with cysteine metabolic cycle.

As regards the bioactive peptides carnosine and GSH, it can be noticed that urinary content of these two molecules is higher with respect to the other samples. GSH content in brain tissue samples is also relatively higher than in cells and in plasma, emphasizing the important role that this thiol tripeptide has at the cerebral level as a bioactive molecule.

4 Conclusion

The revised version of a relatively old analytical procedure, has enabled the improvement of a low-cost chromatographic method with high resolution and high diagnostic potential in order to investigate qualitatively and quantitatively many substances which can be implicated in the pathogenesis of several diseases.

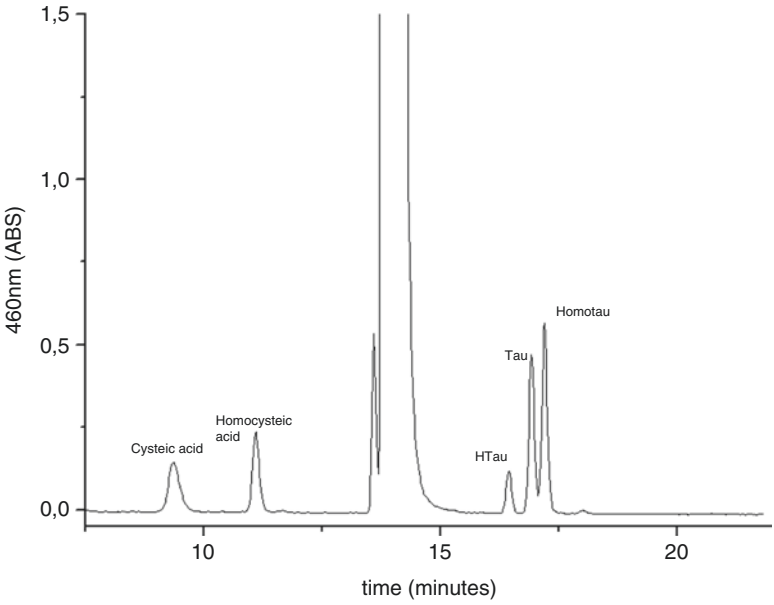


Fig. 12 HPLC chromatogram of dabsylated derivatives from neuroblastoma cell lysate

This method represents a good tool to study sulfur biochemical cycle from a metabolic point of view in relation to the pattern of biologic amines and provides a complete scenario of organic sulfur and amino metabolism. It allows also the study of amine level variations in mice and human samples derived from different treatments or environmental conditions (pollutants, drugs, etc.).

The use of spectrophotometric detection makes this method accessible to a large number of research, commercial and clinical laboratories that do not have access to fluorimetric or mass spectrometric detectors. Apart from the obvious advantages of lower costs and larger number of samples that can be analyzed within a working day, more importantly, an undoubted advantage from a qualitative and methodological point of view is the possibility to simultaneously analyze in each single sample a large number of amines, aminoacids and sulfur-amino compounds including GSH, GSSG, cysteic acid, cysteine, taurine, hypotaurine, lanthionine and cystathionine.

In conclusion, the use of this chromatographic method on different complex biological matrices, could pave the way for a new diagnostic tool.

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Carbonate Anion Radical Generated by the Peroxidase Activity of Copper-Zinc Superoxide Dismutase: Scavenging of Radical and Protection of Enzyme by Hypotaurine and Cysteine Sulfinic Acid

Alessia Baseggio Conrado, Simonetta Maina, Harry Moseley, Antonio Francioso, Luciana Mosca, Elisabetta Capuozzo, and Mario Fontana

Abstract Copper-zinc superoxide dismutase (SOD) is considered one of the most important mammalian antioxidant defenses and plays a relevant role due to its main function in catalyzing the dismutation of superoxide anion to oxygen and hydrogen peroxide. However, interaction between SOD and H_2O_2 produced a strong copper-bound oxidant (Cu(II)$^{\bullet}$OH) that seems able to contrast the self-inactivation of the enzyme or oxidize other molecules through its peroxidase activity. The bicarbonate presence enhances the peroxidase activity and produces the carbonate anion radical ($CO_3^{\bullet-}$). $CO_3^{\bullet-}$ is a freely diffusible reactive species capable of oxidizing several molecules that are unwieldy to access into the reactive site of the enzyme. Cu(II)$^{\bullet}$OH oxidizes bicarbonate to the $CO_3^{\bullet-}$, which spreads out of the binding site and oxidizes hypotaurine and cysteine sulfinic acid to the respective sulfonates through an efficient reaction. These findings suggest a defense role for sulfinates against the damage caused by $CO_3^{\bullet-}$. The effect of hypotaurine and cysteine sulfinic acid on the $CO_3^{\bullet-}$-mediated oxidation of the peroxidase probe ABTS to ABTS cation radical (ABTS$^{\bullet+}$) has been studied. Both sulfinates are able to inhibit the oxidation of ABTS mediated by $CO_3^{\bullet-}$. The effect of hypotaurine and

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cysteine sulfinic acid against SOD inactivation by H_2O_2 (~42% protection of enzyme activity) has also been investigated. Interestingly, hypotaurine and cysteine sulfinic acid partially avoid the H_2O_2-mediated SOD inactivation, suggesting that the two sulfinates may have access to the SOD reactive site and preserve it by reacting with the copper-bound oxidant. In this way hypotaurine and cysteine sulfinic acid not only intercept $CO_3^{\bullet-}$ which could move out from the reactive site and cause oxidative damage, but also prevents the inactivation of SOD.

Keywords Hypotaurine • Sulfonyl radicals • Sulfinates • Taurine • Superoxide dismutase • Antioxidants

Abbreviations

ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic)
CA	Cysteic acid
$CO_3^{\bullet-}$	Carbonate anion radical
CSA	Cysteine sulfinic acid
H_2O_2	Hydrogen peroxide
HCO_3^-	Bicarbonate
HTAU	Hypotaurine
RSO_2^-	Sulfinates
$RSO_2^{\bullet}$	Sulfonyl radicals
RSO_3^-	Sulfonates
SOD	Cu–Zn superoxide dismutase
TAU	Taurine

1 Introduction

Copper-zinc superoxide dismutase (SOD) is considered one of the most important mammalian antioxidant defenses and plays a relevant role in the knowledge of oxidative damage. The main function of SOD is to catalyze the disproportionation of superoxide anion ($O_2^{\bullet-}$) to oxygen and hydrogen peroxide thanks to its catalytic copper ion (Fridovich 1989). However, SOD also interacts with hydrogen peroxide, producing a powerful oxidant species. This can either undergoes through self-inactivation or oxidize exogenous substrates acting as a relatively nonspecific peroxidase (Hodgson and Fridovich 1975a, b; Gunther et al. 2002; Yim et al. 1993). It has been reported a potential correlation between a mutated form of superoxide dismutase and the neurodegenerative disease, familial amyotropic lateral sclerosis (Yim et al. 1996; Widedau-Pazos et al. 1996; Valentine and Hart 2003; Liochev et al. 1998).

The SOD peroxidative mechanism is due to the production of a copper(II) hydroxyl radical (Cu(II)$^{\bullet}$OH), a one-electron oxidant, at the enzyme active site (reactions 1–2).

$$SOD - Cu(II) + H_2O_2 \rightarrow SOD - Cu(I) + O_2^{\bullet -} + 2H^+ \quad (1)$$

$$SOD - Cu(I) + H_2O_2 \rightarrow SOD - Cu(II)^{\bullet}OH + OH^- \quad (2)$$

In this mechanism the inactivation of SOD (self-inactivation) is due to either the oxidative activity of the copper(II)$^{\bullet}$OH towards histidine residues which are close to the oxidant or towards other molecules that reach the catalytic site, such as small anions (Hodgson and Fridovich 1975a, b). In this latter circumstance, the inactivation is prevented as the active site seems to be preserved. For example imidazole, urate, and formate, seems to compete with histidine residues in the active site, reacting directly with Cu(II)$^{\bullet}$OH and preventing the SOD-inactivation due to hydrogen peroxide presence (Liochev and Fridovich 2002; Goldstone et al. 2006).

It has been suggested that in vivo, where the plasma concentration of bicarbonate (HCO_3^-) is 25 mM, the peroxidase action of the SOD could be relevant (Sankarapandi and Zweier 1999; Zhang et al. 2000). When bicarbonate (or CO_2) and H_2O_2 are present, strong evidences indicate that SOD is able to produce the carbonate anion radical ($CO_3^{\bullet -}$) (Liochev and Fridovich 2002, 2004; Goss et al. 1999). A relatively small anion like bicarbonate can access to the SOD active site and be oxidized through one-electron mechanism by Cu(II)$^{\bullet}$OH. $CO_3^{\bullet -}$ is a freely diffusible reactive species capable of oxidizing several molecules that are unwieldy to access the enzyme reactive site (Zhang et al. 2000, 2002; Goss et al. 1999). The copper-bound oxidant (Cu(II)$^{\bullet}$OH) oxidizes HCO_3^- to the carbonate radical anion, which diffuses out and consequently oxidizes substrates (reaction 3).

$$SOD - Cu(II)^{\bullet}OH + HCO_3^- \rightarrow SOD - Cu(II) + CO_3^{\bullet -} + H_2O \quad (3)$$

Augusto et al. (2002) reported that "$CO_3^{\bullet -}$ is a strong one-electron oxidant that oxidizes suitable electron donors via electron transfer mechanisms". Moreover, $CO_3^{\bullet -}$, formed by SOD-mediated peroxidase activity, has the property to diffuse rapidly away from the reactive site of the SOD, and promptly abstracts electrons from cellular target, such as tyrosine and/or tryptophan, and enhances the DNA damage through oxidation (Huie et al. 1991; Bonini and Augusto 2001; Yermilov et al. 1996; Shafirovich and Dourandin 2001).

Recently, it has been shown that hypotaurine and cysteine sulfinic acid are efficiently oxidized to the respective sulfonates by $CO_3^{\bullet -}$ originated by SOD through its peroxidase activity (Baseggio Conrado et al. 2014). Due to pulse radiolysis studies, the rate constants between $CO_3^{\bullet -}$ and sulfinates have been reported, with a value of 1.1×10^9 $M^{-1}s^{-1}$ for hypotaurine and 5.5×10^7 $M^{-1}s^{-1}$ for cysteine sulfinic acid. Moreover, this study has supported the evidence of the production of sulfonyl

radical ($RSO_2^{\bullet}$). In particular, $CO_3^{\bullet-}$ oxidizes, via a one-electron transfer mechanism, the sulfinic group (RSO_2^-) of hypotaurine and cysteine sulfinic acid to form the $RSO_2^{\bullet}$ radical that reacts with O_2 to lead to sulfonate formation (RSO_3^-). These results suggest a defense action for sulfinates against the damage caused by $CO_3^{\bullet-}$ (Fontana et al. 2005, 2006; Baseggio Conrado et al. 2014).

In order to explore the ability of sulfinates to prevent the oxidation mediated by carbonate radical anions generated by the SOD/H_2O_2/HCO_3^- system, the effect of both sulfinates on the oxidation of the peroxidase probe 2,2-azino-bis[3-ethylbenzothiazoline]-6-sulfonic acid (ABTS) to ABTS radical cation ($ABTS^{\bullet+}$) has been studied. As reported (Zhang et al. 2000), the addition of bicarbonate to SOD/H_2O_2 is required to perform the ABTS peroxidation. Due to the fact that ABTS is a large molecule and rarely reaches the SOD reactive site, it could undergo a process of oxidation with carbonate radical anion. As reported above, several small anionic molecules, such as azide, nitrite and formate, can enter into the SOD reactive site preventing the enzyme inactivation by H_2O_2, anagously hypotaurine is known to be capable of avoiding this inactivation (Pecci et al. 2000a; Liochev and Fridovich 2002; Goldstone et al. 2006). However, many of these experiments including ours were performed in bicarbonate buffer, not considering the oxidation and consequently the production of $CO_3^{\bullet-}$ from the same buffer. Consequently, we have investigated the effect sulfinates on the inactivation of SOD by H_2O_2 in more detail.

2 Methods

2.1 Oxidation of ABTS by SOD/H_2O_2/HCO_3^-

ABTS (20 μM) with SOD (1 mg/mL, from bovine erythrocytes EC 1.15.1.1) and sodium bicarbonate (0.025 M) was incubated in buffer (0.1 M K-phosphate) at pH 7.4 plus 100 μM DTPA added to prevent metal-catalyzed reactions. Addition of 1 mM H_2O_2 started the reaction where H_2O_2 concentration was previously analyzed at 240 nm with $\varepsilon = 43.6\ M^{-1}cm^{-1}$ (Hildebraunt and Roots 1975). The oxidation rates of ABTS were measured at 37 °C using a Cary 50 Scan spectrophotometer using ε $ABTS^{\bullet+} = 3.6 \times 10^4\ M^{-1}cm^{-1}$ at 415 nm (Childs and Bardsley 1975).

2.2 Assay of SOD Activity

1 mM hypotaurine (HTAU) or cysteine sulfinic acid (CSA) in the presence of 1 mg/mL of SOD plus 1 mM of H_2O_2 were incubated at 37 °C for 60 min in buffer (0.1 M K-phosphate) at pH 7.4, with 100 μM DTPA. Addition of 1 mM H_2O_2 started the reaction. To stop the reaction, catalase (220 units/mL) was added. The

ferri-cytochrome *c* (cyt *c*) reduction assay was used to measure the SOD activity at 550 nm. For the assay, Goss et al. (1999) method was followed with the difference in the K-phosphate buffer (0.1 M) and in the presence of DTPA (0.1 mM).

2.3 Oxidation of Sulfinates by SOD Activity and HPLC Analyses

HPLC analyses were performed following the methods discussed in our previous study based on the work of Hirschberger and collaborators (Baseggio Conrado et al. 2014; Hirschberger et al. 1985).

2.4 Statistical Analysis

The experiments performed were carried out for a minimum three separate time and each time in duplicate (mean ± SEM). GraphPad Prism 4 software was used to perform data analysis and graphics. Differences with a $P < 0.05$ are considered significant.

3 Results

3.1 Effect of Sulfinates on ABTS Oxidation by SOD/H_2O_2/HCO_3^-

$CO_3^{\bullet -}$ is known to oxidize ABTS with a mechanism of electron transfer to the radical cation $ABTS^{\bullet +}$ (Zhang et al. 2000). To investigate sulfinate ability to prevent carbonate radical anion-mediated oxidation, their effect on ABTS oxidation were studied.

To evaluate the rate of ABTS oxidation, the increase of the absorbance was analyzed at 415 nm owing to the radical cation formation ($\varepsilon = 3.6 \times 10^4\ M^{-1}cm^{-1}$) (Childs and Bardsley 1975). Sulfinates are able to inhibit dose-dependently the formation of $ABTS^{\bullet +}$ (Fig. 1). The ability of sulfinates, hypotaurine and cysteine sulfinic acid, to inhibit the ABTS oxidation rate has been investigated at pH 7.4. Cysteine sulfinic acid showed an inhibitory effect greater than hypotaurine.

3.2 Effect of Sulfinates on H_2O_2-Mediated SOD Inactivation

An enzyme copper-bound hydroxyl radical (SOD-Cu(II)$^{\bullet}$OH) is generated after the interaction of H_2O_2 with the active site of SOD. This powerful oxidant can oxidize an accessible substrate or attack amino acid residues at the active site, leading to

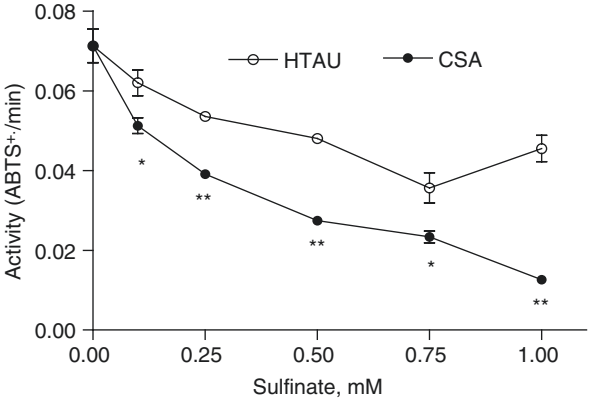


Fig. 1 Rate of ABTS formation in function of sulfinate concentration through SOD/H_2O_2/HCO_3^-. ABTS (20 μM) with SOD (1 mg/mL), H_2O_2 (1 mM), and $NaHCO_3$ (25 mM) in the absence (control) or in the presence of 1 mM HTAU or CSA, was incubated in buffer (0.1 M K-phosphate) at pH 7.4, plus DTPA (0.1 mM). The rate of ABTS formation was measured spectrophotometrically at 415 nm. Values are given as the mean ± SEM (n = 3). *$P < 0.05$ and **$P < 0.01$ CSA values compared to HTAU values

enzyme inactivation (self-inactivation) (Hodgson and Fridovich 1975a, b). To determine whether HTAU and CSA can affect the H_2O_2-mediated SOD inactivation, their effect on SOD activity was investigated.

Figure 2 shows that HTAU and CSA partially protected SOD from self-inactivation, suggesting that the two sulfinates may enter the reactive site of SOD scavenging the copper bound-•OH. This effect is similar to that exerted by bicarbonate, which is known to decrease H_2O_2-mediated SOD inactivation by reacting with the enzyme-bound oxidant (Goss et al. 1999).

The extent of protection is slightly affected when sulfinates are added simultaneously to bicarbonate, suggesting that the compounds compete for binding to the active site of SOD (Fig. 2).

The sulfinate protection on SOD self-inactivation were examined as a function of time (Fig. 3).

3.3 SOD Peroxidase-Mediated Oxidation of Sulfinates

We next evaluated the effect of the SOD/H_2O_2 system on the oxidation of both sulfinates, hypotaurine (HTAU) and cysteine sulfinic acid (CSA). The amount of the HTAU and CSA depletion and the formation of the corresponding sulfonates, taurine (TAU) and cysteic acid (CA) was monitored to determine the extent of sulfinate oxidation (Table 1). HPLC analysis showed that HTAU oxidation by SOD/H_2O_2 system produces mainly TAU. After 60 min incubation, 90% of depleted HTAU is recovered as TAU. On the contrary, when 1 mM CSA is reacted for 60 min in the SOD/H_2O_2

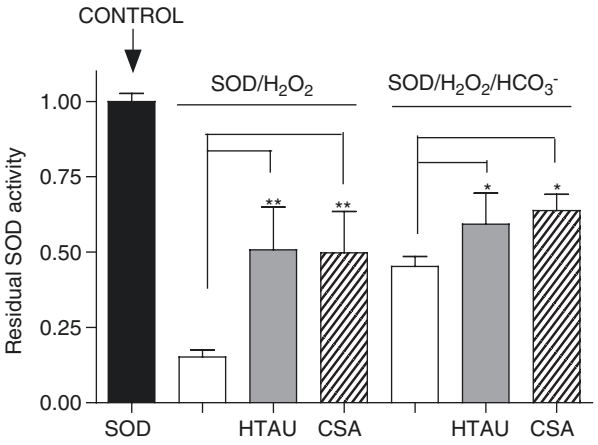


Fig. 2 Sulfinate effect on H_2O_2-mediated SOD inactivation. The cyt *c* reduction assay was used to measure the SOD activity. Enzyme activity is reported as residual SOD activity observed in the different mixtures. With the exception of the control, performed without H_2O_2, all mixtures contained SOD (1 mg/mL) with H_2O_2 (2 mM) with or without 1 mM HTAU or CSA. The "H_2O_2 + HCO_3^-" mixture also contained $NaHCO_3$ (25 mM). All mixtures were incubated for 2 h at 37 °C in buffer (0.1 M K-phosphate) at pH 7.4, plus DTPA (0.1 mM). Values are mean + SEM (n = 4). $^*P < 0.05$ and $^{**}P < 0.01$

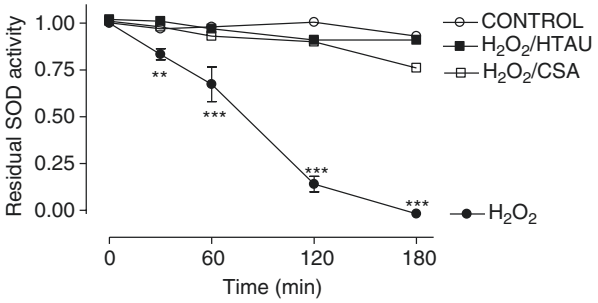


Fig. 3 Sulfinate effect on H_2O_2-mediated SOD inactivation as a function of time. The cyt *c* reduction assay was used to measure the SOD activity. Enzyme activity is reported as residual SOD activity observed. Mixtures contained SOD (1 mg/mL) with H_2O_2 (2 mM) in the absence or in the presence of 2 mM HTAU or CSA. At 0′, 30′, 1 h, 2 h and 3 h, aliquots (30 μL) of mixtures were analyzes in the cyt *c* reduction assay. Values are given as mean + SEM (n = 4). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to control and H_2O_2/sulfinate samples

Table 1 SOD/H_2O_2-mediated oxidation of sulfinates

Conditions[a]	Substrate (1 mM)	Sulfonate production (μM)[b]	Sulfinate depletion (μM)[b]
H_2O_2	HTAU	48 ± 2	n.d.
	CSA	34 ± 2	n.d.
SOD/H_2O_2	HTAU	140 ± 8	155 ± 6
	CSA	53 ± 2	146 ± 6

[a]1 mM H_2O_2; 1 mg/mL SOD; 60 min at 37 °C

[b]Sulfinates and sulfonates concentrations were determined by HPLC

system, under the same oxidative conditions used for HTAU, 53 ± 2 μM CA is produced. By comparing the values of the CA yield with those of depleted CSA, CA formation was roughly 36% of the depleted corresponding sulfinate (CSA).

4 Discussion

Several studies have proposed sulfinates, hypotaurine (HTAU) and cysteine sulfinic acid (CSA) as antioxidant biomolecules and free radical scavengers (Fontana et al. 2004, 2008; Baseggio Conrado et al. 2014, 2015). The present study demonstrates that HTAU and CSA can prevent carbonate radical anion-mediated oxidation of ABTS, indicating that sulfinates can act as protective agents against the $CO_3^{\bullet-}$-induced oxidative damage. Furthermore, HTAU and CSA partially prevent the H_2O_2-mediated SOD inactivation, suggesting that the two sulfinates may reach the SOD active site, thus protecting the enzyme by reacting with the copper-bound oxidant (Cu(II)$^{\bullet}$OH).

The ABTS oxidation to ABTS radical cation ($ABTS^{\bullet+}$) requires bicarbonate as the SOD/H_2O_2 system does not oxidize ABTS to $ABTS^{\bullet+}$ when bicarbonate is not present. The addition of bicarbonate induces the oxidation of ABTS. The ABTS formation is not affected in any way by hydroxyl radical. Therefore, in the SOD/H_2O_2/bicarbonate system free hydroxyl radicals are not produced and are not responsible for the oxidation of ABTS (Zhang et al. 2000). Instead, $ABTS^{\bullet+}$ is formed from the oxidation of ABTS by $CO_3^{\bullet-}$. $CO_3^{\bullet-}$ is a selective and strong oxidant that is able to spread out from the SOD reactive site and oxidizes ABTS to $ABTS^{\bullet+}$ by an electron transfer mechanism (Liochev and Fridovich 1999). In this way, a large molecule like ABTS, which is unlikely to reach the active site of SOD, could still be oxidized by the peroxidase activity of SOD in the presence of added HCO_3^-. The results shown in this study indicate that both HTAU and CSA can inhibit the oxidation of ABTS mediated by carbonate radical anions. Furthermore, cysteine sulfinic acid exhibits a protective effect higher than hypotaurine. The different fate of sulfonyl radicals ($RSO_2^{\bullet}$) resulting from the sulfinate reaction with carbonate radical anions can explain this finding. Differently, CSA-derived sulfonyl radical can degrade with production of sulfite (Pecci et al. 2000b; Harman et al. 1984; Fontana et al. 2005). Sulfite ions undergo one-electron oxidation by several radicals, including carbonate radical anions (Neta and Huie 1985). Thus, the observed higher inhibitory effect of CSA on carbonate radical anion-mediated ABTS oxidation could be attributed to the concomitant sulfite formation. Similar results were shown in our previous work, where sulfinates, hypotaurine and cysteine sulfinic acid, exert a protective effect on the tyrosine dimerization mediated by the carbonate radical anion (Baseggio Conrado et al. 2014; Fontana et al. 2008). Due to the sulfinate

ability to react with $CO_3^{\bullet-}$, HTAU and CSA can be included in the scavengers exerting protective effect on reactions mediated by carbonate radical anion such as ABTS oxidation and tyrosine dimerization.

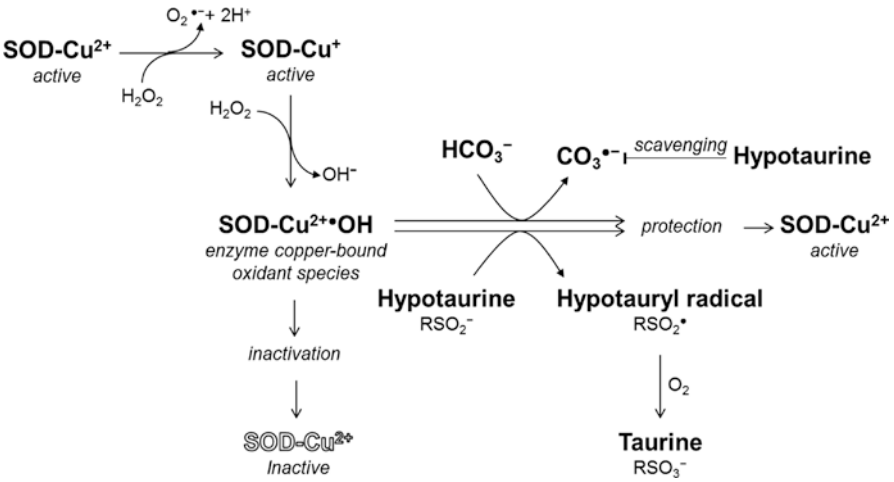
As reported, H_2O_2 can react with the SOD active site with formation of a copper-bound hydroxyl radical (Cu(II)$^{\bullet}$OH), which can either attack amino acid residues at the active site, leading to enzyme inactivation, or oxidize an accessible substrate, preventing enzyme inactivation in this case. According to this, bicarbonate, which is oxidized by the copper-bound oxidant to $CO_3^{\bullet-}$ (reaction 3), inhibits SOD self-inactivation (Goss et al. 1999 and this work). The results reported here show that HTAU and CSA partially prevent SOD inactivation by H_2O_2. This finding suggests that the two sulfinates may enter into the SOD active site and protect the enzyme by reacting with copper-bound $^{\bullet}$OH which causes the inactivation. In agreement, HTAU and CSA are oxidized, although at low level, by SOD/H_2O_2 system with formation of the sulfonates, taurine (TAU) and cysteic acid (CA), respectively. The production of taurine and cysteic acid by the SOD/H_2O_2 system reveals that the copper-bound oxidant is involved in the oxidative mechanism of sulfinates suggesting that the sulfinic group of sulfinates (RSO_2^-) rescues the enzyme in an active form (SOD-Cu(II)) and is concurrently oxidized to the sulfonyl radical ($RSO_2^{\bullet}$), as in reaction 4.

$$SOD - Cu(II)^{\bullet}OH + RSO_2^- \rightarrow SOD - Cu(II) + RSO_2^{\bullet} + OH^- \quad (4)$$

Subsequently, sulfonyl radicals ($RSO_2^{\bullet}$) trigger an oxygen-dependent radical chain reaction with sulfonates (RSO_3^-), TAU and CA, as final products. SOD copper-bound $^{\bullet}$OH radical oxidizes HTAU and CSA to the same extent as shown by sulfinate depletion measurements, in agreement with the reaction rate constants reported in our previous work, between hydroxyl radical and hypotaurine ($k = 5.2 \times 10^9$ $M^{-1}s^{-1}$) and cysteine sulfinic acid ($k = 4.5 \times 10^9$ $M^{-1}s^{-1}$) (Baseggio Conrado et al. 2014). However, only 36% of depleted CSA is recovered as CA. This result can be explained, as reported above, as CSA-derived sulfonyl radical presents a higher propensity to decay (Harman et al. 1984).

5 Conclusion

Sulfinates, such as hypotaurine, not only intercept the carbonate anion radical which could move out from the active site and consequently damage oxidatively relevant biomolecules but also prevent the H_2O_2-mediated inactivation of SOD (as summarized graphically in Scheme 1), which is a crucial antioxidant enzyme catalyzing the superoxide dismutation in vivo.



Scheme 1 Hypotaurine scavenger effect and its protection of SOD activity

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A Proteomic Approach to Study the Effect of Thiotaurine on Human Neutrophil Activation

Elisabetta Capuozzo, Alessandra Giorgi, Sonia Canterini, Alessia Baseggio Conrado, Pina Giarrusso, Maria Eugenia Schininà, and Mario Fontana

Abstract Thiotaurine, a thiosulfonate related to taurine and hypotaurine, is formed by a metabolic process from cystine and generated by a transulfuration reaction between hypotaurine and thiocysteine. Thiotaurine can produce hydrogen sulfide (H_2S) from its sulfane sulfur moiety. H_2S is a gaseous signaling molecule which can have regulatory roles in inflammatory process. In addition, sulfane sulfur displays the capacity to reversibly bind to other sulfur atoms. Thiotaurine inhibits PMA-induced activation of human neutrophils, and hinders neutrophil spontaneous apoptosis. Here, we present the results of a proteomic approach to study the possible effects of thiotaurine at protein expression level. Proteome analysis of human neutrophils has been performed comparing protein extracts of resting or PMA-activated neutrophils in presence or in absence of thiotaurine. In particular, PMA-stimulated neutrophils showed high level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression compared to the level of the same glycolytic enzyme in the resting neutrophils. Conversely, decreased expression of GAPDH has been observed when human neutrophils were incubated with 1 mM thiotaurine before activation with PMA. This result, confirmed by Western blot analysis, suggests again that

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thiotaurine shows a bioactive role in the mechanisms underlying the inflammatory process, influencing the energy metabolism of activated leukocytes and raises the possibility that thiotaurine, acting as a sulfur donor, could modulate neutrophil activation via persulfidation of target proteins, such as GAPDH.

Keywords Human neutrophils • Thiotaurine • Sulfhydration • Hydrogen sulfide • Inflammation

Abbreviations

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
TTAU	Thiotaurine
PMA	Phorbol 12-myristate 13-acetate PMNs, human neutrophils

1 Introduction

Thiotaurine (2-aminoethane thiosulfonate) is produced *in vivo* from cystine (Cavallini et al. 1959, 1960) and is generated spontaneously by transulfuration between the persulfide analogue of cysteine (RSSH) and hypotaurine (RSO_2H) (De Marco et al. 1961). In several animal tissue thiol oxidation to sulfinates and thiosulfonates can enzymatically occur when inorganic sulfur is present (De Marco and Tentori 1961; Cavallini et al. 1961). Furthermore, thiotaurine is formed by a sulfurtransferase catalyzing sulfur transfer from mercaptopyruvate to hypotaurine (Sörbo 1957; Chauncey and Westley 1983). Structurally, thiotaurine is a thiosulfonate (RSO_2SH) related to hypotaurine (RSO_2H) and taurine (RSO_3H). However, it exhibits peculiar biological properties distinct from those exerted by the structurally related sulfur compounds (Westley and Heyse 1971; Luo and Horowitz 1994; Capuozzo et al. 2015). It has been previously reported that thiotaurine can also produce hydrogen sulfide (H_2S) by its sulfane sulfur moiety (Capuozzo et al. 2013). Sulfane sulfur exhibits the singular capacity to reversibly bind to other atoms of sulfur (Toohey 1989). Related to this chemical behaviour, sulfane sulfur moiety has been reported to have regulatory effects in various biological processes (Beinert 2000; Mueller 2006). In addition, the gaseous signaling molecule H_2S promotes several physiological effects from cardioprotection to being an anti-inflammatory mediator and a neuromodulator (Zanardo et al. 2006; Whiteman and Winyard 2011; Whiteman et al. 2011). A dominant way for transmission of sulfide-based signals includes activation or inactivation of enzymes via post-translational modification of reactive cysteine thiols (RSH) to persulfide (RSSH) (Toohey 2011; Yadav et al. 2016). However, which are the chemical intermediates associated to H_2S signaling remain difficult to identify in spite of the various pathophysiological effects displayed by this gasotransmitter (Mishanina et al. 2015).

Previously, it has been shown that thiotaurine inhibits neutrophil activation in response to PMA, a diacylglycerol substitute that activates protein kinase C, or to fMLP, a ligand which binds to specific leukocyte receptors. Thiotaurine can attenuate leukocyte functions by the inhibition of PMA-induced ROS generation, and of superoxide anion production in human neutrophils activated by PMA or fMLP (Capuozzo et al. 2015). Moreover, thiotaurine reduced apoptosis of human neutrophils (Capuozzo et al. 2013). The protection of mouse cerebellar granule neurons from potassium deprivation-induced apoptosis by interfering with the activation of caspase-3 has been also observed (Dragotto et al. 2015). Here, we suggest that key enzymes of neutrophil activation cascade can be modulated by sulfane sulfur of thiotaurine. Relatively to this hypothesis, proteomic profiling of human neutrophils can be applied to understand possible effects of thiotaurine treatments at protein expression level. The aim of this approach is to identify and to analyze proteins that change their expression level or undergo post-translational modifications, such as phosphorylation, nitrosylation/nitration, persulfidation etc. In particular, we performed proteome analysis of human neutrophils comparing protein extracts of resting neutrophils, PMA-activated neutrophils, and PMA-activated neutrophils treated with thiotaurine.

2 Materials and Methods

2.1 Chemicals

Thiotaurine (2-aminoethane thiosulfonate) was synthetized reacting elemental sulfur and hypotaurine according to Cavallini et al. (1959). All chemicals were analytical grade.

2.2 Isolation of Neutrophils

Human leukocytes were purified from freshly drawn heparinized blood of healthy donors and isolated by one-step procedure with the Ficoll-Hypaque medium purchased from Axis-Shield, Oslo, Norway (Ferrante and Thong 1980). Ice-cold isotonic phosphate-saline buffer, pH 7.4, containing 5 mM glucose was used to suspend the cells. To check cell viability trypan blue exclusion test was used obtaining a survival value higher than 90% up to 6 h after purification.

2.3 Activation of Human Neutrophils

Human neutrophils were activated by 1 μg/mL PMA. The incubation mixture contained 15×10^6 cells/mL in phosphate-saline buffer with 5 mM glucose, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$. When present, thiotaurine was 1 mM. After 5 min at 37 °C,

the reaction was started by adding PMA to cell suspension to trigger the oxidative burst. Neutrophils were collected by centrifugation at 2750 × *g* after 15 min incubation at 37 °C and lysed in 0.5 mL ice-cold lysis buffer, pH 7.4, containing 10 mM Tris, 0.5% NP40, 60 mM KCl, and 1 mM EDTA. Protease inhibitors were added to a final concentration of 10 μM APMSF (4-amidinophenylmethanesulfonyl fluoride), 10 μg/mL aprotinin, and 10 μg/mL pepstatin.

2.4 Proteomic Analysis of Human Neutrophils

Cell lysates were centrifuged at 32,000 × *g* for 20 min and protein concentration in the supernatant was determined by Bradford assay. A volume corresponding to 300 μg protein of control and treated cell samples were precipitated with cold ethanol (overnight at −20 °C). The protein separation by pI and molecular weight was performed according to Cattaneo et al. (2015).

The interesting spots were processed via tryptic proteolysis, the peptide mixtures were analysed by MALDI-ToF mass spectrometry (AutoFlex II, Bruker Daltonics, Bremen, Germany) and the resulting peptide mass fingerprints used to identify proteins by Mascot search engine (Di Domenico et al. 2016).

2.5 Western Blot Analysis

Equal amount (15 μg) of total protein/lane were separated by electrophoresis on a 4–12% gradient SDS-polyacrylamide gel (Bolt® Bis-Tris Plus gels, Life Technologies, Carlsbad, CA, USA), as previously described (Canterini et al. 2013; Canterini et al. 2009). Membranes were incubated overnight at 4 °C with anti-GAPDH (Sigma-Aldrich, 1:8000 dilution) and anti-β actin (AbCam, 1:1000 dilution) primary antibodies, then washed and incubated with the appropriate secondary antibody. Blots were evaluated by using a Gel Doc 2000 videodensitometer (Biorad, Hercules, CA, USA). After normalization of the band densities against the β-actin, the percentage was averaged for three replicates of three different biological samples and compared between groups to find out statistically significant (two-way ANOVAs, $p \leq 0.05$) differences.

3 Results

3.1 Human Neutrophil Proteome Profiles

Two-dimensional gel electrophoresis (2-DE) method was applied to evaluate protein expression profiles in human neutrophils. Colloidal Coomassie staining of a 2-DE gel showed at least 300 protein spots in the cell lysates. A representative proteome profile of resting human neutrophils is shown in Fig. 1. Gels obtained from lysates of resting neutrophils, resting neutrophils treated with 1 mM thiotaurine,

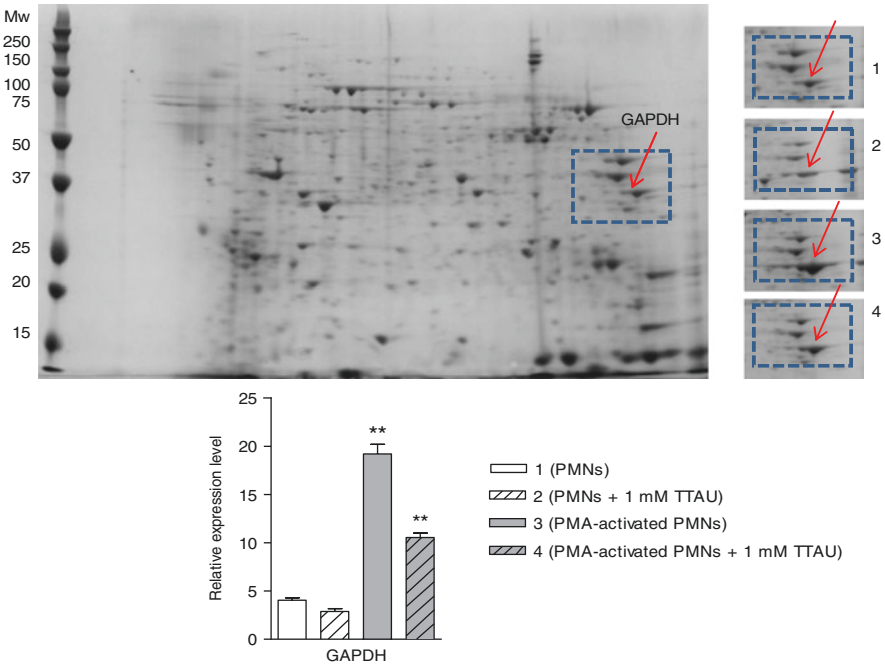

Fig. 1 2-DE human neutrophil proteome map: effect of thiotaurine on expression level of GAPDH. Proteins were separated in the pH 3–10 NL range and the 200–15 kDa molecular mass range and visualized by colloidal Coomassie staining. In the boxes, protein spot corresponding to GAPDH is indicated by a red arrow. The relative intensities of GAPDH expression in resting and PMA-activated neutrophils with/without 1 mM thiotaurine are shown in the bar graph. **$p \leq 0.05$ was evaluated by Student's t-test

PMA-activated neutrophils, and PMA-activated neutrophils treated with 1 mM thiotaurine were compared to identify differences in protein patterns. The protein pattern analysis focused on the protein spots with different expression levels. Colloidal Coomassie-stained gels were comparatively evaluated by dedicated software for image analysis in order to define quantitative changes of protein density spots from treated and untreated cells. Only spots with a statistically significant variation in abundance were excised from the gel, undergone proteolysis and MS analysis. The identification of protein spots was allowed by the database search considering experimental results from Peptide Mass Fingerprinting MALDI-ToF. Among the recognized proteins, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) showed a significantly different expression level in neutrophils activated by PMA and neutrophils treated with 1 mM thiotaurine before PMA activation as compared to resting neutrophils. As highlighted in the boxes of Fig. 1, the PMA-stimulated neutrophils showed high level of expression of glycolytic GAPDH enzyme compared to the level of the same enzyme in the resting neutrophils. Conversely, a decreased amount of GAPDH was observed when human neutrophils were incubated with 1 mM thiotaurine before activation with PMA. Tryptic peptide mass fingerprint of GAPDH spot is shown in Fig. 2.

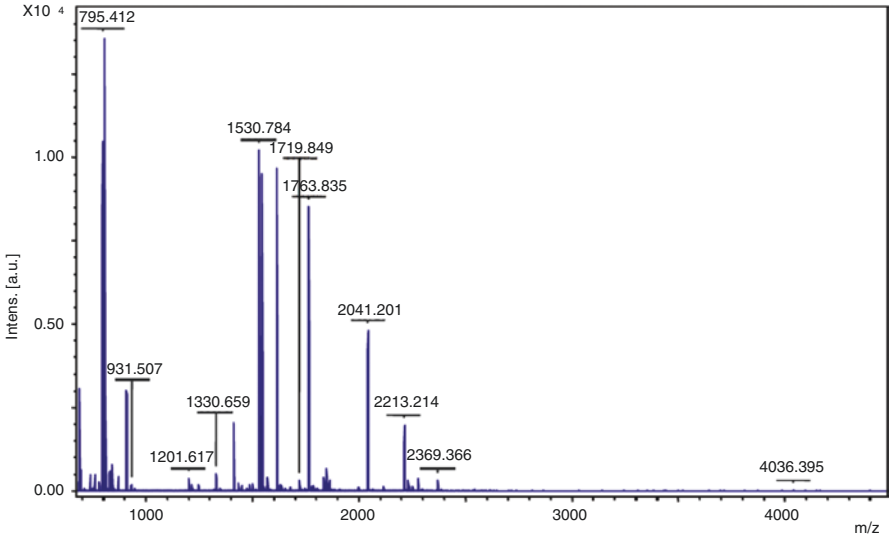

Fig. 2 MALDI-ToF mass spectrum of tryptic peptide fingerprint of GAPDH spot

3.2 Western Blot Analysis of GAPDH

To verify proteome data, Western blot analysis with antibodies directed against the GAPDH protein was performed. Results, reported in Fig. 3, show that 1 mM thiotaurine decreases the expression level of the glycolytic enzyme GAPDH in PMA-activated neutrophils (lane 4), thereby confirming the data obtained by proteomic analysis. Thiotaurine does not affect the expression level of GAPDH in resting human neutrophils.

4 Discussion

Proteome analysis of human neutrophils comparing protein extracts of resting neutrophils, PMA-activated neutrophils, and PMA-activated neutrophils treated with thiotaurine highlighted a different expression level of some proteins. The expression level of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as identified by mass spectrometry and Western blot analysis caught our attention. The PMA-stimulated neutrophils showed high level of expression of GAPDH compared to the level of the same enzyme in the resting neutrophils. Conversely, decreased amount of GAPDH was observed when human neutrophils were incubated with 1 mM thiotaurine before activation with PMA. This result suggests that thiotaurine exhibits a bioactive role in the mechanisms underlying the inflammatory process, influencing the energy metabolism of activated leukocytes,

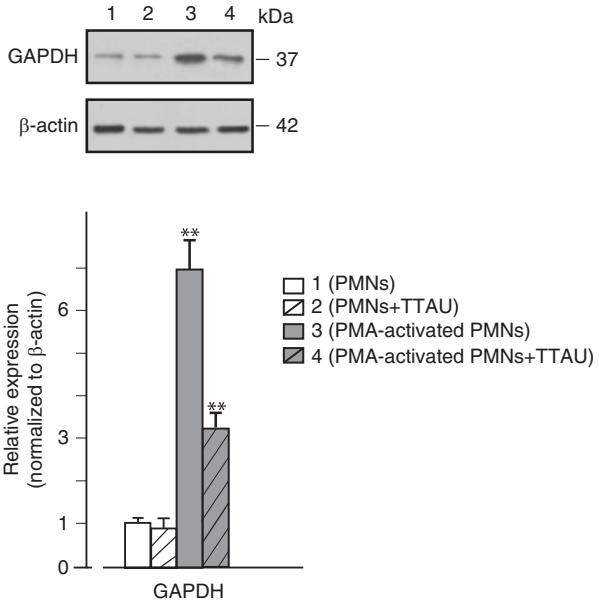

Fig. 3 Western blot analysis of human neutrophil proteins: effect of thiotaurine on expression level of GAPDH. Western blot analysis of protein extracts from human neutrophils and probed with primary antibodies direct against GAPDH. Histograms represent the GAPDH abundance (mean ± SEM) determined by protein band densitometry of three separate experiments, β-actin was used as internal standard. $^{**}p \leq 0.01$

but especially it gives insights in the cell mechanism involved in the modulation of human leukocyte activation by thiotaurine. Interestingly, one of the first proteins shown to undergo persulfidation, alternatively called sulfhydration, was GAPDH. It has been described a sevenfold increase of GAPDH activity after sulfhydration of cysteine 150 (Mustafa et al. 2009). Conversely, cysteine residue nitrosylation abolishes GAPDH activity (Hara et al. 2005). Protein persulfidation is considered a major pathway in sulfide signaling (Mustafa et al. 2009; Paul and Snyder 2012). Despite the various examples reported in literature, it is still unclear whether protein sulfhydration by H_2S is important in cell signalling and which are the mechanisms underlying this modification (Mishanina et al. 2015). The low intrinsinc reactivity of H_2S towards oxidized thiols like disulfides has also raised questions regarding the direct involvement of H_2S in signaling and led to the consideration of alternative sulfur donors in protein persulfidation reactions (Kabil and Banerjee 2014; Cuevasanta et al. 2015; Yadav et al. 2016). Moreover, biological H_2S donors and how this gasotransmitter is mobilized from cell stores remain to be identified (Paul and Snyder 2015). Up to now, the main source of H_2S *in vivo* is the desulfuration of cysteine by enzymes of the transulfuration pathway and 3-mercaptopyruvate sulfurtransferase (Singh et al. 2009; Kabil and Banerjee 2014). It has been suggested that sulfane sulfur pool can represent physiological stores able to release H_2S (Kimura 2011). At this regard, thiotaurine with its sulfane sulfur moiety can be part of the sulfur store pool and represent a biologically relevant sulfur donor in protein persulfidation reactions. It has been demonstrated that under reducing condition, such as in the presence of GSH, H_2S is released from thiotaurine (Chauncey and Westley 1983). Accordingly, in human neutrophils GSH induced the release of H_2S from

thiotaurine (Capuozzo et al. 2013). In conclusion, our result raises the possibility that thiotaurine, acting as a sulfur donor, could modulate neutrophil activation via persulfidation of target proteins, such as GAPDH. Furthermore, this result would confirm that thiotaurine takes part to mammalian biochemical pathways involved in sulfide transport, release and storage. This role is further supported by the ability of hypotaurine to readily incorporate H_2S with thiotaurine generation (De Marco and Tentori 1961). This reaction between H_2S and hypotaurine, the latter present at millimolar concentration in leukocytes, very likely occurs during inflammation (Learn et al. 1990).

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The Interaction of Hypotaurine and Other Sulfinates with Reactive Oxygen and Nitrogen Species: A Survey of Reaction Mechanisms

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Abstract Considerable strides have been made in understanding the oxidative mechanisms involved in the final steps of the cysteine pathway leading to taurine. The oxidation of sulfinates, hypotaurine and cysteine sulfinic acid, to the respective sulfonates, taurine and cysteic acid, has never been associated with any specific enzyme. Conversely, there is strong evidence that in vivo formation of taurine and cysteic acid is the result of sulfinate interaction with a variety of biologically relevant oxidants. In the last decade, many experiments have been performed to understand whether peroxynitrite, nitrogen dioxide and carbonate radical anion could be included in the biologically relevant reactive species capable of oxidizing sulfinates. Thanks to this work, it has been possible to highlight two possible reaction mechanisms (direct and indirect reaction) of sulfinates with reactive oxygen and nitrogen species.

The sulfinates oxidation, mediated by peroxynitrite, is an example of both reaction mechanisms: through a two-electron—direct—reaction with peroxynitrite or through a one-electron—indirect—transfer reaction. In the indirect mechanism, the peroxynitrite homolysis releases hydroxyl and nitrogen dioxide radical and in addition the degradation of short-lived adduct formed by peroxynitrite and CO_2 can generate carbonate radical anion. The reaction of hypotaurine and cysteine sulfinic acid with peroxynitrite-derived radicals is accompanied by extensive oxygen uptake with the generation of transient intermediates, which can begin a reaction by an

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oxygen-dependent mechanism with the sulfonates, taurine, and cysteic acid as final products. Due to pulse radiolysis studies, it has been shown that transient sulfonyl radicals ($RSO_2^{\bullet}$) have been produced during the oxidation of both sulfinates by one-electron transfer reaction.

The purpose is to analyze all the aspects of the reactive mechanism in the sulfinic group oxidation of hypotaurine and cysteine sulfinic acid through the results obtained from our laboratory in recent years.

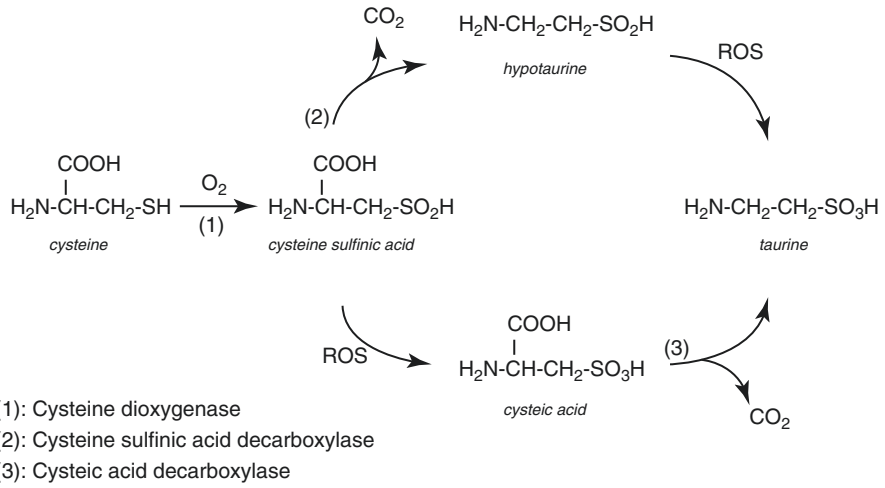
Keywords Hypotaurine • Sulfonyl radicals • Reactive sulfur species • Carbonate anion radical • Sulfinates • Cysteine sulfinic acid

Abbreviations

NO	Nitric oxide
$^{\bullet}NO_2$	Nitrogen dioxide
CA	Cysteic acid
$CO_3^{\bullet-}$	Carbonate anion radical
HTAU	Hypotaurine
CSA	Cysteine sulfinic acid
$ONOO^-$	Peroxynitrite
RSO_2^-	Sulfinates
$RSO_2^{\bullet}$	Sulfonyl radicals
$RSO_2OO^{\bullet}$	Sulfonyl-peroxyl radicals
RSO_3^-	Sulfonates
TAU	Taurine

1 Introduction

Hypotaurine (HTAU) and cysteine sulfinic acid (CSA) are the last two metabolic intermediates in the production of taurine in the mammalian cysteine pathway. This pathway is dependent upon three specific enzymes as well as a variety of biologically relevant oxidizing agents (Scheme 1) (Huxtable 1992; Stipanuk and Ueki 2011; Ricci et al. 1978; Fontana et al. 2005; Fellman et al. 1987; Aruoma et al. 1988; Pecci et al. 1999; Baseggio Conrado et al. 2014). It is well known that cysteine dioxygenase is the first enzyme involved in this pathway and its activity leads to CSA. With the addition of molecular oxygen to the cysteine, to its sulfur atom, there is a conversion to the sulfinic acid from the thiol (Stipanuk and Ueki 2011). At this point, the pathway can go in one of two directions both of which lead to taurine (TAU). In the first, a decarboxylation occurs and HTAU is produced due to the activity of CSA decarboxylase, and the sulfinic group of HTAU is subsequently oxidized



Scheme 1 Hypotaurine and cysteine sulfinic acid as intermediates in the mammalian pathway leading from cysteine to taurine

to the sulfonic group of TAU. CSA can also undergo oxidation to produce cysteic acid (CA) and, through subsequent decarboxylation, forms TAU (Scheme 1) (Huxtable 1992; Stipanuk and Ueki 2011). This crucial point, the sulfinic group oxidation to the respective sulfonic group, has never been associated with any specific enzyme (Wright et al. 1986; Huxtable 1992). Conversely, evidences that in vivo formation of TAU and CA is the result of sulfinate (RSO_2^-) interaction with a variety of biologically relevant oxidizing agents, are strong (Ricci et al. 1978; Fellman et al. 1987; Aruoma et al. 1988; Pecci et al. 1999; Fontana et al. 2005; Baseggio Conrado et al. 2014).

Even though reactive oxygen species (ROS) are known to be capable of oxidizing the sulfinic group of HTAU and CSA, only hydroxyl radicals ($^{\bullet}OH$) and singlet oxygen have shown high direct reactivity leading to TAU formation (Pecci et al. 1999, 2000; Aruoma et al. 1988). This is in spite of low direct reactivity between hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$) and RSO_2^- (Aruoma et al. 1988).

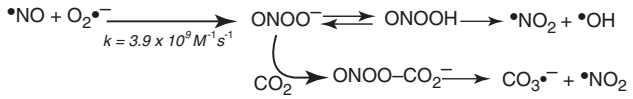
The oxidation of both HTAU and CSA to TAU and CA, respectively, can be also mediated by peroxynitrite ($ONOO^-$) (Fontana et al. 2005, 2006). The sulfinate oxidation mediated by $ONOO^-$ is an example of both direct and indirect reactions: via a two-electron mechanism (direct reaction) with $ONOO^-$ or by a one-electron transfer mechanism (indirect reaction). In the indirect mechanism, the $ONOO^-$ homolysis releases hydroxyl ($^{\bullet}OH$) and nitrogen dioxide ($^{\bullet}NO_2$) radical and in addition the degradation of short-lived adduct formed by $ONOO^-$ and CO_2 can generate carbonate radical anion ($CO_3^{\bullet-}$) (Fontana et al. 2005, 2008). Two previous studies, using the peroxidase activity of Cu-Zn superoxide dismutase and horseradish peroxidase, carried out from our group have demonstrated the importance of both carbonate anion and nitrogen dioxide radicals in the oxidation of HTAU (Baseggio Conrado et al. 2014, 2015). The reaction of HTAU and CSA with peroxynitrite-derived

radicals is accompanied by extensive oxygen uptake suggesting the generation of transient sulfonyl radicals ($RSO_2^{\bullet}$), which can begin a reaction by an oxygen-dependent mechanism with the sulfonates (RSO_3^-), TAU and CA, as final products (Fontana et al. 2005, 2006). Pulse radiolysis studies showed that transient sulfonyl radicals ($RSO_2^{\bullet}$) have been produced during the oxidation of both sulfinates by one-electron transfer reaction (Baseggio Conrado et al. 2014). The findings that HTAU and CSA efficiently react with hydroxyl radical, singlet oxygen as well as $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ provided further support for the proposed role of these compounds as antioxidants and free radical-trapping agents in vivo (Green et al. 1991; Tadolini et al. 1995; Fontana et al. 2004). Of note, in all studies performed on the antioxidant activity of both RSO_2^-, the scavenger effect of CSA on different targets, such as tyrosine dimerization, is higher than that exerted by HTAU, which can be explained considering the CSA-derived sulfonyl radicals. These transient radicals can undergo degradation to sulfite, a secondary product with an efficient protective effect against oxidative reactions. Instead, the $RSO_2^{\bullet}$ derived from the HTAU oxidation seems to have a reduced tendency to decay.

The mechanisms involved in the interaction of the reactive oxygen (ROS) and nitrogen species (RNS) with RSO_2^-, HTAU and CSA, have only recently been investigated in depth (Fontana et al. 2005; Baseggio Conrado et al. 2014). In particular, starting from the findings of Cavallini and coworkers (Ricci et al. 1978; Pecci et al. 1999, 2000) and subsequently, through experiments performed to understand whether peroxynitrite could be included in the biologically relevant reactive species capable of oxidizing RSO_2^-, it has been possible to highlight the reaction pathway mechanism of RSO_2^- leading to RSO_3^- (Fontana et al. 2005, 2006).

2 Sulfinic Group Oxidation by One-Electron Pathway (Indirect Reaction)

In recent years, through three different oxidants—$ONOO^-$, $^{\bullet}NO_2$ and $CO_3^{\bullet-}$—that were tested for inclusion as oxidant agents capable of oxidizing the sulfinic group of RSO_2^-, it has been possible to define the indirect one-electron pathway in the reaction from RSO_2^-, HTAU or CSA, to RSO_3^-, TAU or CA (Baseggio Conrado et al. 2014; Fontana et al. 2005). The extensive $ONOO^-$ studies were due to both its strong oxidizing and nitrating effect on substrates. It has been suggested that the damage in cellular and tissue in neurodegenerative disorders as well as in inflammatory and autoimmune disease can be related to the oxidizing and nitrating effect of $ONOO^-$ that acts as a reactive toxic species (Stewart and Heales 2003; Koppenol et al. 1992; Pryor and Squadrito 1995; Huie and Padmaja 1993; Eiserich et al. 1998;). In vivo, nitric oxide ($^{\bullet}NO$) and $O_2^{\bullet-}$ can react through a diffusion-controlled reaction and produce $ONOO^-$ (Scheme 2) (Beckman et al. 1990; Pryor and Squadrito 1995). It has been described that $ONOO^-$ could be present with its conjugate acid ($ONOOH$, $pK_a = 6.8$) which decays rapidly ($t_{1/2} < 1$ s). ONOOH homolysis can generate two radicals, $^{\bullet}OH$ and $^{\bullet}NO_2$, capable of oxidizing through an indirect



Scheme 2 Formation and fate of peroxynitrite at physiological pH

one-electron reaction other target substrates (Radi et al. 2001). Moreover, the decomposition of peroxynitrite-CO_2 adducts, produced under physiological conditions by peroxynitrite predominant reaction with CO_2, can generate $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ (Scheme 2) (Lymar and Hurst 1995; Meli et al. 2002; Augusto et al. 2002).

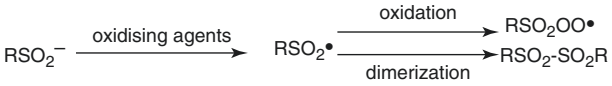
It has been shown that $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ stimulate the oxidation, peroxidation, and nitration of several biological targets (Beckman et al. 1990). Furthermore, Bonini and collaborators have suggested that the $ONOO^-$ production and peroxidase activity of superoxide dismutase (SOD) can play a key role as mediator in the oxidative damage (Bonini et al. 2004a, b).

$CO_3^{\bullet-}$ is a strong acid ($pK_a < 0$) that through a one-electron mechanism ($E° = 1.5$ V) can oxidize many biological targets that consequently produces radicals (Augusto et al. 2002; Bonini and Augusto 2001). $^{\bullet}NO_2$ is a neutral radical and shows a greater selectivity towards biomolecules, compared to $CO_3^{\bullet-}$. $^{\bullet}NO_2$ can mediate oxidation and nitration of various substrates and it can be generated by many physiological processes, such as $^{\bullet}NO$ autoxidation, homolysis of $ONOO^-$ or its addition to CO_2 (Farrel et al. 1992; Torre et al. 1996; Brennan et al. 2002). The relevance of these two radicals in the oxidation of RSO_2^- has been analysed through the peroxidase activity of two different enzymes, the Cu–Zn SOD and horseradish peroxidase (Baseggio Conrado et al. 2014, 2015). In particular, we have shown that diffusible $CO_3^{\bullet-}$, produced by the SOD/H_2O_2 system with bicarbonate, is involved in sulfinate oxidation suggesting the generation of transient intermediates in the oxidative step of RSO_2^- to RSO_3^- (Baseggio Conrado et al. 2014).

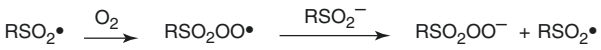
3 Sulfonyl Radical ($RSO_2^{\bullet}$)/Sulfonyl-Peroxyl Radical ($RSO_2OO^{\bullet}$) As Transient Intermediates in the One-Electron Pathway of Sulfinic Group Oxidation

In the indirect one-electron pathway of the RSO_2^- oxidation, the first step is the reaction between RSO_2^- and biological oxidizing agents, such as carbonate radical anion, hydroxyl radical and nitrogen dioxide, and is accompanied by the formation of $RSO_2^{\bullet}$ (Aruoma et al. 1988; Sehested and Holcman 1996; Fontana et al. 2005, 2008; Baseggio Conrado et al. 2014, 2015). Acting as a strong oxidizing species, $RSO_2^{\bullet}$ can produce the sulfonyl-peroxyl radical ($RSO_2OO^{\bullet}$) when oxygen is present (Scheme 3) (Sevilla et al. 1990).

The above fate for the $RSO_2^{\bullet}$ is not the only one, actually, $RSO_2^{\bullet}$ can dimerize and produce the disulfone derivative ($RSO_2–SO_2R$) (Fellman et al. 1987; Ricci et al. 1978; Green and Fellman 1994).



Scheme 3 One-electron pathway of sulfinate oxidation



Scheme 4 Formation and fate of sulfonyl-peroxyl radical

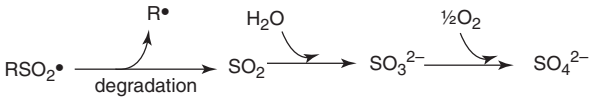
Pulse radiolysis studies carried out by our group, used to evaluate the one-electron oxidation rate between RSO_2^-, HTAU and CSA, with $^{\bullet}OH$, $CO_3^{\bullet-}$, and $^{\bullet}NO_2$, has supported the evidence of the production of $RSO_2^{\bullet}$ (Baseggio Conrado et al. 2014). We have observed that during the reaction between HTAU or CSA with $CO_3^{\bullet-}$ at pH 7.4 maximum absorption spectra around 320 nm (within a region of 300–350 nm), and at 600 nm was also observed a decay of $CO_3^{\bullet-}$ absorption spectra (Baseggio Conrado et al. 2014). Taking into account that Sehested and Holcman (1996) observed during the oxidation of methansulfinic acid by $^{\bullet}OH$ a sulfonyl radical (maximum optical absorption spectra around 325 nm), the spectra obtained in the reaction between RSO_2^- and oxidizing agents, such as $^{\bullet}OH$, $CO_3^{\bullet-}$ and $^{\bullet}NO_2$, can be attributed to $RSO_2^{\bullet}$ as a transient intermediate.

In the $ONOO^-$ experiments, the oxidation of RSO_2^-, HTAU and CSA has been shown to be associated with an extensive oxygen uptake (Fontana et al. 2005, 2006). The high O_2 consumption observed is related to the strong oxidizing activity of $RSO_2^{\bullet}$ which reacts with oxygen producing the sulfonyl-peroxyl radical ($RSO_2OO^{\bullet}$). $RSO_2OO^{\bullet}$ is an intermediate in this mechanism and also a highly reactive species that can react with excess RSO_2^- and produce peroxysulfonate (RSO_2OO^-) (Scheme 4) (Sevilla et al. 1990).

4 Different Fates of HTAU- and CSA-Derived Sulfonyl Radicals Generated by One-Electron Sulfinate Oxidation

In all experiments performed to detect the yield in the production of RSO_3^-, TAU and CA, from RSO_2^-, HTAU and CSA, a different behaviour has always been reported between the HTAU- and CSA-derived sulfonyl radical (Pecci et al. 2000; Fontana et al. 2005, 2006; Baseggio Conrado et al. 2014, 2015). It has been shown that only a fraction of the depleted CSA was oxidized to CA with a yield of around 17%, compared with the almost total oxidation of HTAU in TAU (Baseggio Conrado et al. 2014). Sulfonyl radical derived by CSA has a high tendency to degrade to acetaldehyde and concurrently producing ammonia, CO_2, and sulfite (Harman et al. 1984; Pecci et al. 2000). In detail, CSA-derived sulfonyl radicals partly undergo decomposition, producing sulfur dioxide (SO_2) and a highly

Scheme 5 Decomposition pathway of sulfonyl radical to sulfite/sulfate



Scheme 6 Two-electron mechanism of sulfinate oxidation

carbon-centred radical ($R^{\bullet}$) which can react with O_2 and further degrade to end products, such as ammonia or CO_2 (Scheme 5) (Pecci et al. 2000). In contrast, HTAU-derived sulfonyl and sulfonyl-peroxyl radicals are more stable. It is likely that these highly reactive radicals could also promote the oxidation of suitable target molecules (Fontana et al. 2008).

5 Two-Electron Mechanism of Sulfinic Group Oxidation (Direct Reaction)

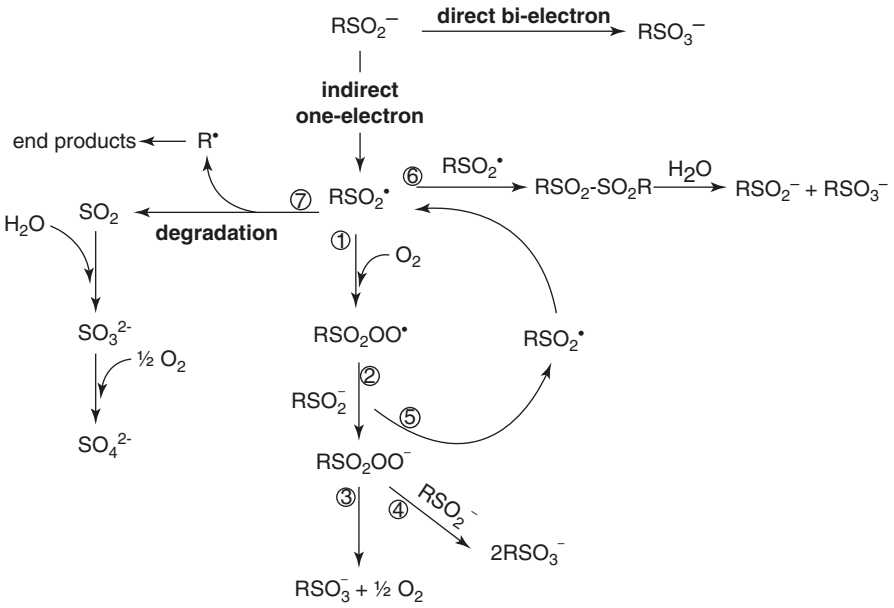
In the pathway leading from RSO_2^- to RSO_3^-, a route in addition to the one-electron mechanism can occur without any oxygen consumption. This route is a two-electron mechanism that can directly produce the RSO_3^- (Scheme 6) (Fontana et al. 2005, 2006).

In this reaction, oxygen is transferred directly from $ONOO^-$ to RSO_2^- with the production of RSO_3^- and nitrite in stoichiometric amounts. According to kinetic experiments, the direct reaction between $ONOO^-$ and HTAU or CSA appears to be a second-order rate with, respectively, 77.4 and 76.4 $M^{-1}s^{-1}$ constant. Moreover, ONOOH can be considered the reactant in the direct oxidative pathway as a correlation between k_{app} build-up and pH decrease has been observed (Fontana et al. 2005).

6 Conclusion

The oxidation of sulfinates, HTAU and CSA, by reactive oxygen and nitrogen species to form sulfonates, TAU and CA, may occur either through one- or two-electron pathways as shown in Scheme 7.

To briefly overview the one-electron mechanism: the reaction between sulfinates (RSO_2^-) and one-electron oxidants, such as $ONOO^-$, $CO_3^{\bullet-}$ and $^{\bullet}NO_2$, is accompanied by the generation of intermediate sulfonyl radicals ($RSO_2^{\bullet}$). Acting as a strong oxidizing species, $RSO_2^{\bullet}$ can produce the sulfonyl-peroxyl radical ($RSO_2OO^{\bullet}$) when oxygen is present (① in Scheme 7) (Sevilla et al. 1990; Flyunt et al. 2001). $RSO_2OO^{\bullet}$ is an intermediate in this mechanism and also a highly reactive species that can react with excess RSO_2^- and produce peroxysulfonate (RSO_2OO^-) (② in Scheme 7).



Scheme 7 One and two-electron pathway for the oxidative reactions of sulfinates

Subsequently, the RSO_2OO^- formed decomposes to yield sulfonate (RSO_3^-) and molecular oxygen or oxidizes the excess RSO_2^- to RSO_3^- (③ and ④ in Scheme 7).

Moreover, $RSO_2^\bullet$ can either proceed through other three routes. It may initiate an oxygen-dependent radical chain propagation with a possible amplification of the oxidative mechanism (⑤ in Ischeme 7) (Sevilla et al. 1990; Flyunt et al. 2001) or it can dimerize to form disulfone derivative (RSO_2–SO_2R), that subsequently hydrolyzes to yield RSO_3^- (⑥ in Scheme 7) (Ricci et al. 1978; Fellman et al. 1987; Green and Fellman 1994). The dimerization route does not require oxygen and can be operative in systems when low oxygen uptake is observed or in anaerobiosis (Ricci et al. 1978; Pecci et al. 1999; Baseggio Conrado et al. 2015).

The last possible route in the indirect pathway is the decomposition of $RSO_2^\bullet$. As reported above, especially CSA-derived sulfonyl radicals can partly undergo degradation with production of sulfite (⑦ in Scheme 7) (Harman et al. 1984; Pecci et al. 2000).

In the two-electron process, the direct transfer of oxygen from the oxidant, such as $ONOO^-$, to RSO_2^- generates RSO_3^- without any oxygen consumption. Which pathway occurs, between the one or two-electron mechanisms, depends mainly on RSO_2^- concentration. In fact, in the $ONOO^-$-mediated oxidation, it has been shown that at low RSO_2^- concentration, $RSO_2^\bullet$ formation is associated with high oxygen uptake via the one-electron mechanism. On the contrary, at high RSO_2^- concentration, the second-order reaction (between RSO_2^- and $ONOO^-$) becomes more significant, producing a decrease in the oxygen consumption and consequently the direct oxidation of RSO_2^- without the formation of $RSO_2^\bullet$ predominates (Fontana et al. 2005, 2006).

It is noteworthy that the sulfonyl and sulfonyl-peroxyl radicals produced in this mechanism are highly reactive oxidizing agents and could promote oxidative reactions (Pecci et al. 2003; Fontana et al. 2008; Baseggio Conrado et al. 2014). Looking forwards, it would be interesting to investigate the pathophysiological role of sulfonyl or sulfonyl-peroxyl radicals, particularly considering the increased significance of reactive sulfur species (RSS) in cellular oxidative stress (Schöneich et al. 1992; Giles and Jacob 2002; Jacob 2012).

As outlined in this review, many questions on the oxidative mechanism of sulfinates to sulfonates have been addressed, however, the actual biological relevance and the pathophysiological role of generated reactive intermediates deserve to be further investigated.

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Protective Effects of An Water Extracts Prepared from *Loliolus beka* Gray Meat Against H_2O_2-Induced Oxidative Stress in Chang Liver Cells and Zebrafish Embryo Model

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Abstract In this study, we first evaluated protective effects of *Loliolus beka* in a human liver cell line and zebrafish embryo model with its anti-oxidant activity. First, we prepared the water extract from *L. beka* meat (LBMW) at room temperature for 24 h and revealed it consisted of a rich taurine. LBMW exhibited the scavenging effects against 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and hydrogen peroxide (H_2O_2) as well as the high value of oxygen radical absorbance capacity (ORAC). Also, the hydroxyl radical-induced DNA damage was dose-dependently reduced by the treatment of LBMW. In addition, LBMW showed no cytotoxicity and reduced the production of reactive oxygen species (ROS) in H_2O_2-treated hepatocytes. Moreover, LBMW regulated the expression of an anti-apoptotic molecule, Bcl-2 and the expression of pro-apoptotic molecules, Bax and PARP in H_2O_2-treated hepatocytes as well as the increment of antioxidant mediated-HO-1 and Nrf2 protein expression. In further study, LBMW improved the survival rate and decreased the production of ROS in H_2O_2-treated zebrafish embryo model. Therefore, our results suggest that *Loliolus beka* has protective effects against H_2O_2-induced oxidative stress and may be used as a potential source for functional foods.

Keywords Loliolus beka Gray meat • Protective effects • Oxidative stress • Zebrafish embryo

1 Introduction

A free radical contains the unpaired electrons in its molecular orbitals (Thannickal and Fanburg 2000). The surplus generation of instable free radicals and reactive oxygen species (ROS) thought to be one of role fact in the oxidative damage of cellular DNA, lipids and protein, and their normal function (Park et al. 2014). Radical-related damage to DNA, lipids and proteins has been considered as an important factor in the development of aging-related disease and has been associated with a number of human diseases such as cancer, atherosclerosis, arthritis, neurodegenerative, disorders and other conditions (Halliwell and Gutteridge 1999).

ROS such as superoxide anion (O_2^-), hydroxyl radical (HO-) and hydrogen peroxide (H_2O_2) are physiological metabolites formed during aerobic circumstance (Heo et al. 2005). Such ROS can be generated by various enzymatic and nonenzymaic processes in mammalian cells. Also, this ROS is influenced food-mediated potentially toxic reaction products (Je et al. 2009). Especially, most oxidative stress conditions may inhibit cell growth and induce cell arrest and transient adaptive response (Davies 2000).

Antioxidants regulate various oxidative reactions frequently happened in cell or organization, Hence antioxidants can protect and regulate the oxidative process by scavenging free radicals and acting as electron donors (Senevirathne et al. 2006). Therefore, the antioxidants are useful needed in body system and

storage of food (Heo et al. 2005). Currently, synthetic antioxidants are commonly used to maintain foodstuff. However, those synthetic antioxidants have side effects such as toxicity, cancer and inflammation in body systems (Ito et al. 1986). With these points, many researchers have been performed for naturally derived antioxidants that induce no deleterious side effects. Cephalopoda is the third largest molluscan class which comprises more than 800 identified species (Lindgren et al. 2004). Among Cephalopoda species, about 125 species have been recorded in China (Xu 2008). Cephalopoda is potential protein sources containing abundant bioactive substances with potential biological activity (Dai et al. 2012). *Lolious beka* is one of the cephalopoda species, only few biological studies have studied this *L. beka* compared to the other cephalopoda species (Dai et al. 2012).

In this study, we evaluated potential protective effects of the protein sources from *L. beka* on H_2O_2-induced oxidative stress in human liver cell line and zebrafish embryo model.

2 Methods

2.1 Materials

Loliolus beka was purchased from Yeosu market of South Korea, and stored at −60 °C until required for analysis. All testing reagent including 1,1-diphenyl-2-picrylhydrazy (DPPH), bovineserum albumin (BSA), gallic acid, folin-Ciocalteau reagent, Hydrogen peroxide, 5,5-dimethyl-1-pyrroline N-oxide (DMPO), 2,2-azobis (2-methylpropion-amidine) dihydrochloride (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), fluorescein sodium salt, 2′,7′-dichlorodihydroflurescin diacetate (DCFH-DA), 3-(4-5-dimethyl-2yl)-2-5-diphynyltetrasolium bromide (MTT), monobromobimane (mBBr) and diphenyl-1-pyrenylphosphine (DPPP) and α-(4-pyridyl *N*-Oxide)-*N*-*tert*-butylnitrone (POBN) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The human Chang liver cells were obtained from the American Type of Culture Collection (Manassas, VA, USA). Dulbeco's modified Eagle's medium (DMEM) including antibiotics were purchased from Gibco BRL (Paisley, UK). Other chemical and reagent of the highest grade available commercially were used.

2.2 Proximate Composition of Loliolus beka *Meat (LBM)*

The contents of moisture, protein, lipid and ash from *Loliolus beka* meat (LBM) were determined according to the Official Analytical Chemists (Association of Official Agricultural Chemists (AOAC) 1980).

2.3 Preparation of the Water Extract from LBM (LBMW)

To prepare the water extract from LBM, LBM (100 g) was added into 5 L of distilled water and reacted for 24 h at 38 °C. Then, it was centrifuged and filtered through a Whatman No. 6 filter paper. The filtrate (LBMW) was freeze-dried and used for this study.

2.4 Determination of Antioxidant Activities

2.4.1 $ABTS^+$ Radical Scavenging Activity

The $ABTS^+$ radical scavenging activity of LBMW was measured according to the method presented by Park and Kim (2009). The working solution was prepared by distilled water to an absorbance of 1.50 ± 0.05 at 414 nm. The extracts and ABTS working solution (7 mM ABTS in 2.4 mM potassium persulfate) were mixed as the ratio 1:3 and then kept for 10 min at room temperature, and the $ABTS^+$ radical scavenging activity was identified by measuring the absorbance of the mixture at 414 nm using microplate reader (SpectraMax® M2/M2^e, CA, USA).

$ABTS^+$ radical scavenging activity (%)
$= [(Abs._0 - Abs._s)/Abs._0] \times 100$
$Abs._0$: Control absorbance at 414 nm
$Abs._s$: Sample absorbance at 414 nm

2.4.2 DPPH Radical Scavenging Activity

The scavenging effect of LBMW on DPPH radicals was evaluated by the slightly modified method indicated by Blois (1958). In brief, a 100 μL of LBMW was mixed with 100 μL of 150 μM DPPH reagent, and the mixture was incubated at room temperature for 30 min in the darkness. The DPPH scavenging activity of LBMW was calculated by the following equation.

DPPH scavenging activity (%)
$= [(Abs._0 - Abs._s)/Abs._0] \times 100$
$Abs._0$: Control absorbance at 517 nm
$Abs._s$: Sample absorbance at 517 nm

2.4.3 Hydrogen Peroxide Scavenging Activity

The hydrogen peroxide scavenging effect of LBMW was measured by Müller methods (Muller 1985). A 100 μL of phosphate buffer (pH 5.0, 0.1 M) was mixed with 100 μL of LBMW (0.25, 0.5 and 1 mg/mL) followed by the addition 20 μL of H_2O_2 (20 mM). After the incubation at 37 °C for 5 min, 30 μL of ABTS (1.25 mM) and 30 μL of peroxidase

(1 unit/mL) were added to the mixture and further incubated for 10 min at 37 °C. The absorbance values of LBMW were measured at 405 nm by using microplate reader.

2.4.4 ORAC Assay

The ORAC assay was performed by a slightly modified method of Zulueta et al. (2009). All solutions were made in 75 mM phosphate buffer (pH 7.0). In brief, 50 μL of LBMW (25 and 50 μg/mL) was mixed with 50 μL of fluorescein (78 nM) and incubated for 15 min at 37 °C. Then, 25 μL of AAPH (221 mM) was added rapidly, and the fluorescences were respectively recorded every 5 min for 1 h (excitation wavelength: 485 nm, emission wavelength: 582 nm). Different concentrations of trolox (0–20 μM) were used for a standard curve, and ORAC values of the sample were calculated by using the net area under the decay curves (AUC) and expressed as μM trolox equivalent (TE)/mg sample.

2.4.5 Reducing Power

The reducing power was measured using the method described by Oyaizu (1986). LBMW (0.25, 0.5, 1 mg/mL) was mixed with 300 μL of 0.1 M sodium phosphate buffer (pH 6.6) and 500 μL of potassium ferricyanide (1%). The mixture was kept at 50 °C for 20 min, and then 500 μL of 10% TCA was added, the mixture was centrifuged at 1036 × *g* for 10 min. Finally, 100 μL of the each supernatant solutions were mixed with 100 μL of distilled water and 20 μL of 0.1% $FeCl_3$, and the absorbance were checked at 700 nm.

2.4.6 DNA Damage Protective Activity

The protective effect of the LBMW against hydroxyl radical-induced DNA damage was performed. Twelve microliter of the solution (0.5 μg of pBR322 plasmid DNA, 2 mM $FeSO_4$, and 0.1 M sodium phosphate buffer, pH 6.0) was reacted with various concentrations of LBMW (200 μL) for 20 min at 37 °C. The each reactant was electrophoresed in 0.8% agarose gel including ethidium bromide, and the band was quantified using UV illuminator.

2.5 Antioxidant Effects of LBMW Against H_2O_2-Induced Oxidative Stress in Chang Liver Cells

2.5.1 Chang Liver Cell Culture

Chang liver cells were maintained in an incubator at 37 °C with 5% CO_2, and cultured in DMEM adding 10% heat-inactivated fetal bovine serum (FBS), antibiotic including penicillin (100 unit/mL) and streptomycin (100 μg/mL).

2.5.2 Cell Viability

To check the cytotoxic effect of LBMW, MTT assay was performed in Chang liver cells. MTT assay was based on the discoloration of MTT into formazan crystals by living cells, which fined mitochondrial activity (Johan et al. 2011). The Chang liver cells (1×10^4 cells/wells) were incubated with or without LBMW at various concentrations (from 0.125 to 1 mg/mL) for 2 h. Control cells were treated with only DMEM medium. Additionally, the cells were incubated with 1 mM H_2O_2 for 24 h. Then, MTT stock solution (10 μL; 5 mg/mL) was applied to the each cells for 4 h. The formazan crystals were dissolved in 100 μL of solublization buffer (pH 4.7) including 50% dimethylformamide (DMSO) and 10% sodium dodecyl sulfate (SDS), and the absorbance of the formazan was measured at 540 nm using micro plate reader.

2.5.3 Determination of Intracellular ROS

Intracellular ROS formation was measured with 2′,7′-dichlorofluorescin diacetate (DCF-DA). DCF-DA is oxidized to the fluorescent DCF by hydrogen peroxide (Park et al. 2015). Hepatocytes (1.0×10^4 cells/well) were seeded in 96-well black plates and incubated for 18 h. The cells were pretreated with LBMW (0.125, 0.25, 0.5 and 1 mg/mL) for 1 h, and then treated with 1 mM H_2O_2 at 37 °C. Twenty-four hours after the treatment of LBMW, 500 μM DCF-DA (in 100% ethanol) was treated to the all cells, the formation of DCF in the presence of ROS was read for 5 min at excitation 485 nm/emission 528 nm. The fluorescence intensity of LBMW treated cell was expressed as the percentage by comparing with that of the control cells, which were represented as 100%.

2.5.4 Western Blot

Chang liver cells (5.0×10^5 cells/well) were treated with H_2O_2 (1 mM) and LBMW (0.25, 0.5 and 1 mg/mL) for 24 h. After the incubation, the cells were lysed in a RIPA buffer (Thermo, Rockford, IL) and the obtained lysates were used as the cellular proteins. The 20 μg of cellular proteins were electrophoresed in SDS-polyacrylamide gels (12%) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). The membrane was incubated with 5% skim milk in Tris buffered saline (TBS) containing Tween-20 for 2 h, and then the membrane was incubated overnight at 4 °C with specific primary antibodies as Bax (1:1000 dilution, Cell Signaling Technology Inc.), Bcl-2 (1:1000 dilution, Cell Signaling Technology Inc.), PARP (1:1000 dilution, Cell Signaling Technology Inc.), and β-actin (1:3000 dilution, Sigma) in 5% skim milk. After the incubation, the HRP-conjugated secondary antibodies (anti-mouse IgG and anti-rabbit IgG, 1:5000, Cell Signaling Technology Inc.) were added into the membrane at room temperature for 90 min. The bands were detected using an enhanced chemiluminescence (ECL) and analyzed using NIH Image J software (US National Institutes of Health, Bethesda, MD).

2.6 Antioxidant Effect of LBMW in Zebrafish Embryo Model

Maintenance of zebrafish were kept in 3 L acrylic tank at 28.5 °C with a 14:10 h light:dark cycle. The fed was supplied three times a day. The embryo were provided in natural circumstances that turning one the light in the morning. The embryos were move to 12 well plates containing 900 μL embryo media at 7–9 hpf (hour post fertilization). LBMW at various concentrations (31.3, 62.5, 125, 250 μg/mL) were treated to each wells. After the incubation 1 h, a 0.5 mM H_2O_2 solution was added to the embryo exposed with or without LBMW for up to 1 dpf (days post-fertilization). Then, embryos were changed using fresh embryo media. The survival rate was observed every day until 7 dpf.

2.6.1 Estimation of Intracellular ROS Generation in Zebrafish Embryo

The generation of ROS in H_2O_2-induced oxidative stress zebrafish model was analyzed using 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA). The zebrafish embryos were divided to 96 well plates as experimental groups. The zebrafish embryos treated with samples and/or H_2O_2 was treated with 20 μg/mL of DCF-DA solution and incubated for 1 h in dark space at 28.5 °C. Then, the embryos were rinsed with embryo medium and anesthetized in anesthetic solution (tricaine methanesulfonate). The fluorescence intensity of the individual embryos was measured using a spectrofluorometer (Perkin-Elmer LS-5B, Norwalk, CT, USA). The images of embryos were observed using a fluorescent microscope equipped with a Moticam color digital camera (Motix, Xiamen, China).

2.7 Statistic Analysis

All results are expressed as the mean ± standard error of three determinations. Differences between means of each group were assessed by one-way analysis of variance followed by Duncan's test using PASW statistics 21.0 software (SPSS, Chicago, IL). A P-value <0.05 was considered statistically significant.

3 Results

3.1 Proximate Compositions of LBM and LBMW

As shown in Table 1, LBM had the higher protein and carbohydrate compositions at 46.67 ± 0.55% and 36.55% than those of moisture (2.20 ± 0.40%) and ash (3.13 ± 0.10%). Table 2 showed that the extraction yield of LBMW was

Table 1 Proximate composition (%) of LBM

Protein	Carbohydrate	Lipid	Moisture	Ash
46.67 ± 0.55	36.55	11.45 ± 1.38	2.20 ± 0.40	3.13 ± 0.10

Table 2 Protein, carbohydrate, and polyphenol contents (%) and yield (%) of the water extract of LBM (LBMW)

Yield	Protein	Carbohydrate	Polyphenol
55.47 ± 1.70	63.29 ± 0.32	30.86 ± 0.21	7.13 ± 0.22

55.47 ± 1.70%. Also, we identified that LBMW contained the plentiful protein and carbohydrate contents whereas contained the small quantity of polyphenol content. Particularly, LMBW exhibited the highest protein content (63.29 ± 0.32%), compared to those of carbohydrate (30.86 ± 0.21%) and phenol (7.13 ± 0.22%). This result indicates the plentiful protein content of LBMW might be affected to the biological effects of LBMW.

3.2 Free Amino Acid Contents of LBMW

As indicated in Table 3, LBMW consisted of the free amino acid contents such as plentiful leucine, lysine, phenylalanine, methionine and taurine.

3.3 Determination Antioxidant Activity

Antioxidant activity of LBMW was determined by measuring the $ABTS^+$, DPPH radicals, hydrogen peroxide scavenging activities, ORAC value and reducing power.

Normally, the assessment of $ABTS^+$ radical scavenging activity has been widely used as an antioxidant capacity assay (You et al. 2009). As shown in Fig. 1A, LBMW led to the highly $ABTS^+$ radical scavenging activity in a dose-dependent manner. Especially, this activity ranged from 43.97 ± 0.57% to 78.19 ± 0.12% at concentrations of 0.25 and 1 mg/mL, respectively. In Fig. 2B, LBMW dose-dependently scavenged the DPPH radicals. The effect of LMBW against hydrogen peroxide was shown in Fig. 1C. LBMW induced the hydrogen peroxide scavenging activity in a dose-dependent manner. In particular, the 1 mg/mL of LBMW exhibited the highly hydrogen peroxide activity (approximately 53.21 ± 0.89%). Additionally, we evaluated the ORAC values of LBMW. As shown in Fig. 1D, LBWM (50 μg/mL) had 1214.16 μM TE/mg sample. Furthermore, the result of LBMW on reducing power was indicated in Fig. 1E. The result showed that LBMW had the potent reducing power activity in the used all concentrations, particularly

Table 3 Free amino acid contents (%) of LBMW

Free amino acids	Contents (%)
Phosphoserine	0.17
Taurine	6.75
Phosphoethanolamine	1.82
Urea	–
L-Aspartic acid	0.70
L-Threonine	0.17
L-Serine	0.02
Asparagine	–
L-Glutamicacid	1.54
L-α-Aminoadipicacid	–
L-Proline	4.91
L-Glycine	1.75
L-Alanine	2.05
L-Citulline	0.61
L-α-Aminobutyricacid	0.10
L-Valine	3.62
L-Cystine	0.05
L-Methionine	7.58
L-Isoleucine	4.72
L-Leucine	14.34
L-Tyrosine	4.70
L-Phenylalanine	11.34
β-alanine	0.45
DL-β-Aminoisobutyric acid	1.06
γ-Aminobutyricacid	0.31
L-Histidine	1.63
3-Methyl-L-Histidine	0.10
L-Methyl-L-Histidine	–
L-Tryptophan	0.50
L-Carnosine	3.04
L-Orinithine	2.95
L-Lysine	23.53
L-Arginine	0.09
Total	100

1 mg/mL led to the highest activity (0.13 ± 0.00%). In further experiment, we measured the protective activity of LBMW on hydroxyl radical-induced DNA damage. Figure 1F exhibited LBMW protected DNA against damages caused by hydroxyl radical mediated DNA. Thus these activites of LBMW lead to the inhibition of oxidative induced-fenton reaction, and protected the SC DNA.

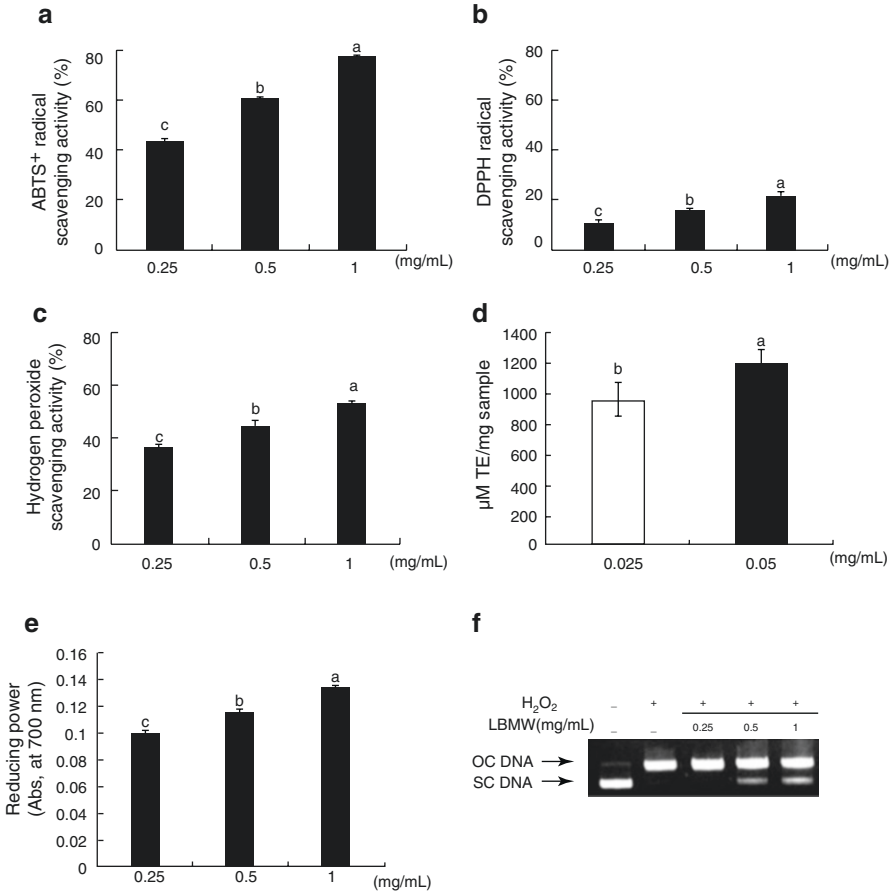

Fig. 1 Antioxidant effects of LBMW in vitro. (**a**) The ABTS$^+$ radical scavenging effect of LMBW, (**b**) the DPPH radical scavenging effect of LMBW, (**c**) the H_2O_2 scavenging effect of LMBW, (**d**) the ORAC value of LBMW, (**e**) the reducing power of LBMW, (**f**) protective effects of LBMW on the DNA damage. Data represented as means ± S.E. $^{a-c}$The values with different alphabet subscripts indicate significant difference between the each sample ($p < 0.05$)

3.4 Antioxidant Effects of LBMW Against H_2O_2-Induced Oxidative Stress in Chang Liver Cells

As shown in Fig. 2A, LBMW did not show the cytotoxic effects at the used all concentration. The non-cytotoxic concentrations of LBMW were applied to the next experiments. The cell viability of LBMW in H_2O_2-treated Chang liver cells was evaluated by MTT assay. Figure 2B exhibited that the exposure of H_2O_2 markedly decreased the cell viability (56.60 ± 1.81%), compared to the non-treated control cells (control). Interestingly, the cell viabilities in LBMW (0.25, 0.5, and 1 mg/mL)

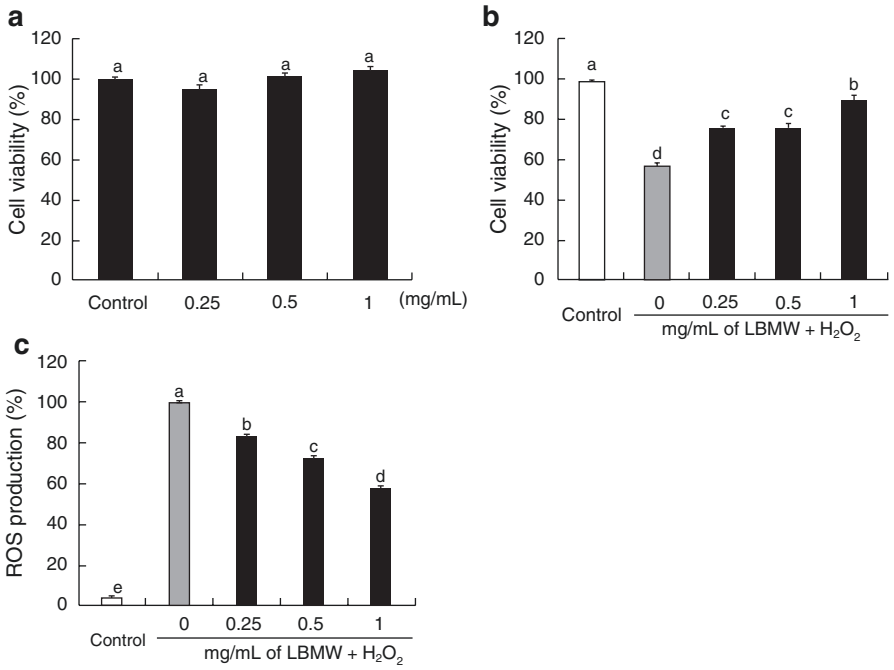

Fig. 2 Effects of LBMW on the cell viabilities (**a** and **b**) and the intracellular ROS production (**c**) in Chang liver cells. (**a**) The cell viability was checked in only LBMW-treated Chang cells by MTT assay. (**b**) The cell viability was checked in both H_2O_2- and LBMW-treated Chang cells by MTT assay. (**c**) The intracellular ROS production was checked in both H_2O_2- and LBMW-treated Chang cells by DCF-DA assay. Data represented as means ± S.E. [a–e]The values with different alphabet subscripts indicate significant difference between the each sample ($p < 0.05$)

treated cells were considerably ($p < 0.05$) increased in a concentration-dependent manner. Especially, the cell viability in 1 mg/mL of LBMW treated cells was significantly increased nearly 90.10 ± 2.2% at 1 mg/mL compared to non-treatment group (Fig. 2B). Hydrogen peroxide induced had been widely used to induce cell injury. And hydrogen peroxide was important role generate reactive oxygen species (ROS). In addition, it its homeostasis could have diverse physiological and pathological consequences (Halliwell and Gutteridge 1999). We also measured cellular antioxidant effect on hydrogen peroxide-stimulated cells. ROS-detecting in chang liver cells using DCF-DA assay. DCFH-DA was hydrolyzed to DCFH by intracellular esterase, followed by oxidation of DCFH to form fluorescent DCF in the presence of ROS (Je et al. 2015). This results was showed Fig. 2C, LBMW significantly inhibited ROS formation in dose-dependent manner, and ROS formation was decreased nearly 60% compared to hydrogen peroxide-stimulated cell at 1 mg/mL. This result suggests that LBMW had potent protective effect against hydrogen peroxide- induced oxidative stress.

3.4.1 LBMW Regulated the Expression Levels of Antioxidant and Apoptosis-Related Molecules in H_2O_2-Treated Chang Liver Cells

To confirm the effect of LBMW on the expression levels of apoptosis-related molecules, PARP, Bcl-2 and Bax, we performed western blot analysis. As shown in Fig. 3A, the treatment of H_2O_2 markedly decreased the expression levels of PARP and Bcl-2, whereas increased that of Bax, compared to non-treated control group. However, LBMW up-regulated the protein expression levels of anti-apoptosis molecules, PARP and Bcl-2 whereas down-regulated that of pro-apoptotic molecule, Bax, in comparison with the only H_2O_2-treated cells. They were in dose-dependent manners (Fig. 3B). This result suggest that LBMW protected the cells against H_2O_2-induced oxidative stress by regulating the protein expression levels of apoptotic molecules.

3.5 *Measurement of LBMW Against H_2O_2–Treated Oxidative Stress in* In Vivo *Zebrafish Model*

To identify the protective effect of LBMW in *in vivo* zebrafish model. First of all, we determined the non-toxic concentrations of LBMW in zebrafish embryos. Therefore, we evaluated survival rate at multiple LBMW concentrations (31.3, 62.5, 125, and 250 μg/mL). Therefore, LBMW did not show the toxic effects up to the concentrat of 250 μg/mL. As shown in Fig. 4A, the exposure of H_2O_2 markedly decreased the survival rate, compared with the non-treated zebrafish embryo group. Interestingly, they were significantly improved by the application of LBMW in a concentration-dependent manner. Especially, the 250 μg/mL of LBMW considerably increased the survival rate up to 98.00 ± 0.00%, compared with the H_2O_2-treated zebrafish embryo group. Additionally, we measured the effect of LBMW against the ROS production in H_2O_2-treated zebrafish embryos using by DCFH-DA reagent. The result showed that the treatment of H_2O_2 increased the ROS levels in zebrafish embryos, comparing to the non-treated control group (Fig. 4B, C). However, it was significantly inhibited by the application of LBMW at the used all concentrations. This results indicate that LBMW protects the zebrafish embryo against oxidative stress caused by the exposure of H_2O_2 via regulating the generation of ROS.

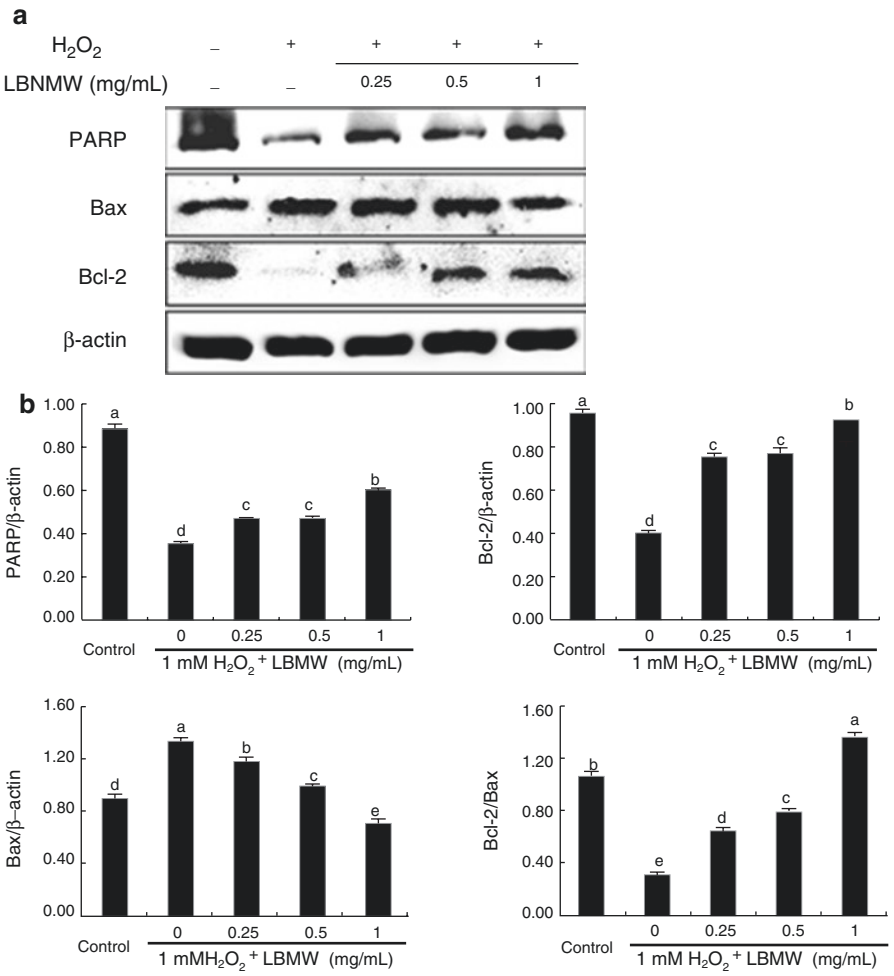

Fig. 3 Effect of LBMW on the expression of apoptosis-related proteins in H_2O_2-treated Chang liver cells (**a**) and the densitometry analysis (**b**). Data represented as means ± S.E. [a–d]The values with different alphabet subscripts indicate significant difference between the each sample ($p < 0.05$)

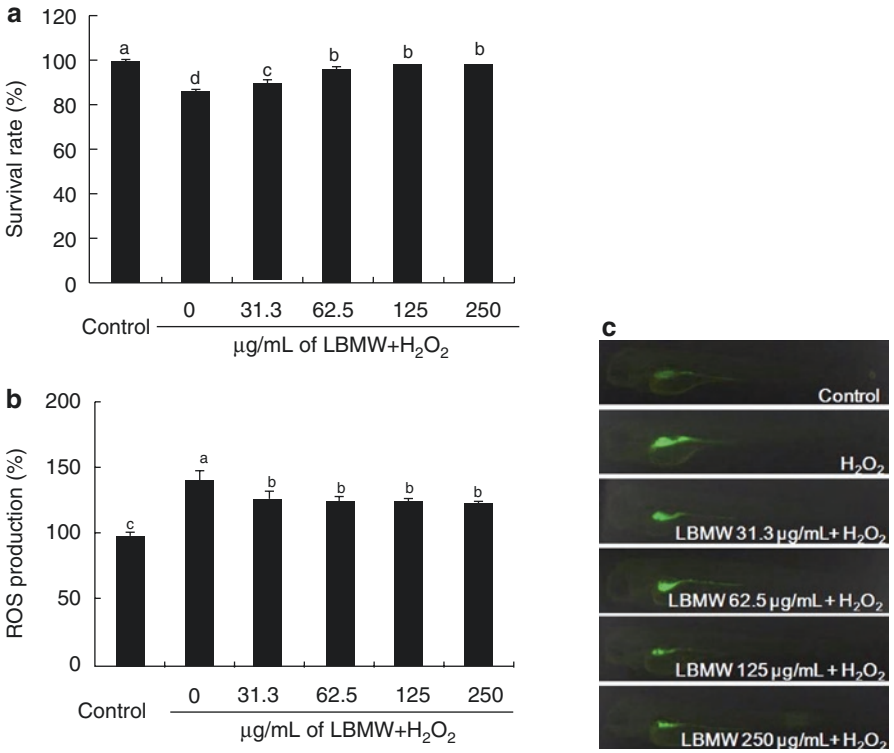

Fig. 4 Effect of LBMW on the survival rate (**a**) and the ROS production in H_2O_2-treated zebrafish embryo (**b** and **c**). Data represented as means ± S.E. [a–d]The values with different alphabet subscripts indicate significant difference between the each sample ($p < 0.05$)

4 Discussion

Taurine is one of the most abundant amino acids in the human body (Wright et al. 1986). Taurine is present in our daily foods and dietary supplements. A broad range of pharmacological and physiological effects are attributed to this amino acid (Huxtable 1992; Zhu et al. 2016; Rashid et al. 2013; Islambulchilr et al. 2015). *Loliolus beka* is widely consumed seafood in Asia, which contains high levels of taurine. Recently, many studies concerning the effect of taurine-rich food on oxidative stress have been reported. However, protective effect of *L. beka* on oxidative stress has not yet to be determined. Therefore, the purpose of the study was to investigate the protective effects of *L. beka* gray meat water extract (LBMW) containing high concentration of taurine against H_2O_2-induced oxidative stress in Chang liver cells and zebrafish embryo model.

In present study, we have confirmed that LBMW was rich in taurine, lysine and leucine. Kim et al. (1999) reported that species of squid was rich in taurine compared to other seafood. Recently, taurine has become an attractive candidate for attenuating various pathophysiological conditions through its antioxidant action

(Patrick 2006). Our data showed that LBMW containing high amounts of taurine has potent scavenging activities against hydrogen peroxide, $ABTS^{+}$ and DPPH radical. These results indicate that LBMW may be an effective antioxidant for prevention of various chronic diseases that are related with oxidative stress. Cellular DNA damage is susceptible to oxidation by high ROS levels and free radicals. In the present study, LBMW exhibited a clear inhibition of DNA oxidation. These results confirm that LBMW has an ability to protect DNA oxidation-related cellular damage.

In general, H_2O_2-induced oxidative stress leads to cell damage and eventually cell death (Trinh et al. 2014). Also, high level of ROS induces oxidative stress, which can cause various biochemical and physiological disorder. Based on the results, H_2O_2 treatment decreased Chang liver cells viability. However, treatment with LBMW together with H_2O_2 inhibited cell death, suggesting that LBMW protected Chang liver cells from H_2O_2-induced cytotoxicity. In addition, our results demonstrate that LBMW dose-dependently and significantly inhibited H_2O_2-induced intracellular ROS generation in Chang liver cells. According to these results, it was suggested that LBMW possessing high amounts of taurine scavenged intracellular ROS and thereby protected the cellular macromolecules from ROS-mediated damage. Some previous studies have indicated that taurine protects hepatocytes against the oxidative stress and cell damage (Roy and Sil 2012; Miyazaki and Matsuzaki 2014).

ROS are generated in the mitochondria and other sources through normal cellular oxidative stress processes. It causes serious damage to molecules including nucleic acids, DNA, protein, and membrane lipids, and results in regulation of the processes related in the initiation of apoptotic signaling. Several previous studies have revealed that ROS production is a crucial function in the pro-apoptosis. Molecules in the Bcl-2 family are critical regulators of the apoptotic pathway (Zanke et al. 1996). Bcl-2 is an upstream molecule in the apoptotic pathway and well known as a potent suppressor of apoptosis (Szatrowski and Nathan 1991). Previous reports have suggested that the activation of caspase-3 related Bcl-2 family induces the ROS-induced apoptosis (Chen and Chang 2009). In this study, we demonstrated that LBMW protects Chang liver cells under H_2O_2 treatment conditions. These protective effects of LBMW were induced by inhibition of apoptosis and the increase of Bcl-2 expression, one of anti-apoptotic molecule. Bcl-2 reduce in pro-apoptotic PARP and Bax expression levels.

Zebrafish have been valuably used in the study fields of molecular genetics and development biology, as a special animal model for drug discovery, bioactivity and toxicological studies because of various advantages including small size, easy observation, large clutches, as well as their physiological similarity to mammals (Pichler et al. 2003; Den Hertog 2005). In this study, we investigated that the *in vivo* protective effects of LBMW against the oxidative stress induced by the treatment of H_2O_2 in zebrafish model. Our data showed that survival rate was significantly increased by LBMW treatment to H_2O_2-treated zebrafish. It is proved that LBMW can protect the zebrafish from damage induced by H_2O_2. Also, H_2O_2-treated zebrafish significantly produces ROS, however, LBMW markedly inhibits H_2O_2-induced

ROS production. All of the results confirm that LBMW containing large amounts of taurine possesses a protective effect against oxidative damage by inhibiting ROS production. Cheong et al. (2015) reported that mussel extract possessing taurine has protective effect against AAPH-induced oxidative stress in the zebrafish model.

5 Conclusion

Taken together, these results suggest that LBMW containing large amounts of taurine possesses a protective effect in *in vitro* hepatocytes as well as *in vivo* zebrafish model by reducing the oxidative stress and apoptosis induced by ROS.

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Protective Effects of An Enzymatic Hydrolysate from *Octopus ocellatus* Meat against Hydrogen Peroxide-Induced Oxidative Stress in Chang Liver Cells and Zebrafish Embryo

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Abstract *Octopus ocellatus*, a marine cephalopod distributed in the coast of South Korea, China, Japan and tropical sea, contains high amounts of taurine. In this study, an enzymatic hydrolysate obtained from *O. ocellatus* meat was evaluated for its antioxidant effects using a human liver cell line and zebrafish embryo model. Enzymatic hydrolysates of the *O. ocellatus* meat (OOM) were prepared using six different enzymes. Among the enzymatic hydrolysates, Alcalase hydrolysate of OOM (OOMAH) showed the highest scavenging effects against 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) radicals and hydrogen peroxide (H_2O_2). Moreover, it showed a high oxygen radical absorbance capacity (ORAC). OOMAH treatment effectively reduced the hydroxyl radical-induced DNA damage. OOMAH reduced the production of

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reactive oxygen species (ROS) in H_2O_2-treated hepatocytes without cytotoxicity. Furthermore, OOMAH improved the survival rate and reduced the intracellular ROS levels in H_2O_2-treated zebrafish embryos. Compositional analysis of amino acids indicated a high content of taurine in OOMAH. Current results suggest that OOMAH possesses antioxidant bioactivities and could provide protective effects against H_2O_2-induced oxidative stress. Therefore, OOMAH might be used as a potential resource of functional foods.

Keywords Octopus ocellatus meat • An enzymatic hydrolysate • Protective effects • Oxidative stress • Zebrafish embryo

Abbreviations

ORAC	oxygen radical absorbance capacity
OOM	*Octopus ocellatus meat*
OOMAH	Alcalase hydrolysate of *O. ocellatus* meat
ROS	Reactive oxygen species

1 Introduction

A typical oxidative stress is an imbalance between the ROS production and the cellular antioxidant defense system (Kim et al. 2015). Oxidative stress is depicted by an increased accumulation of ROS (Olatunji et al. 2016). Among the major ROS, superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO^-) are produced during the normal cellular metabolism (Chen et al. 2016). Especially, hydrogen peroxide (H_2O_2) can easily penetrate the cell membranes and react with water molecules, leading to the production of hydroxyl radicals (HO^-) and thereby damaging cellular molecules such as lipids, proteins, nucleic acid and cellular DNA (Kim et al. 2016). Marine organisms compose of structurally diverse bioactive compounds with a range of valuable nutraceutical, pharmaceutical and cosmeceutical potentials (Ngo et al. 2011). Recent studies have reported that marine protein hydrolysates are an abundant natural source of antioxidants with other related biofunctional properties (Saidi et al. 2014). Zebrafish, a tropical freshwater fish, is used as a new vertebrate model organism because of its benefits such as small size, large clutches, transparency, low cost, and physiological similarity to mammals (Kang et al. 2015). Literature indicates that taurine have shown several biofunctional properties including anti-tumor (Sadzuka et al. 2009), anti-aging (Ito et al. 2015), and anti-oxidative (Jong et al. 2012). The mollusc *Octopus ocellatus* (*O. ocellatus*),

which belongs to Octopodidae under the phylum Mollusca, provides an important resource of natural proteins for human beings (Wei et al. 2015). There are no scientific reports about the composition, biological activity and mechanism of *O. ocellatus* until now; although it became known that it contains plentiful taurine contents for recovery of fatigue. Therefore, the current study was designed to analyze the composition of free amino acids obtained from *O. ocellatus* meat (OOM) enzymatic extracts and to investigate the protective effects against oxidative stress using human liver cells and *in vivo* zebrafish embryo model.

2 Materials and Methods

2.1 Materials

DPPH, ABTS, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein sodium salt, 2,2-azobis(2-amidino-propane) dihydrochloride (AAPH), 2′,7′-dichlorodihydroflurescin diacetate (DCFH-DA), 3-(4-5-dimethyl-2yl)-2-5-diphynyltetrasolium bromide (MTT), monobromobimane (mBBr) and diphenyl-1-pyrenylphosphine (DPPP) were purchased from Sigma Chemical Co. (St, Louis, MO, USA). The Chang liver cell line was obtained from the American Type of Culture Collection (Manassas, VA, USA). Dulbeco's modified Eagle's medium (DMEM), penicillin/streptomycin, and other materials required for culturing the cells were purchased from Gibco BRL (Paisley, UK). A commercial enzymatic assay kit was supplied by Biovision (CA, USA).

2.2 Analysis of Proximate Compositions

The ash, moisture, lipid and protein contents of *O. ocellatus* were determined by complying Association of Official Analytical Chemists standard methods (AOAC 1990).

2.3 Preparation of OOM Hydrolysate by Enzyme Assisted Extraction Technique

OOM were ground into powder and the each enzyme-assisted hydrolysis was performed for 24 h with optimum conditions as indicated in Table 1. After hydrolysis, the enzyme-assisted hydrolysis was boiled at 100 °C for 10 min to inactivate the proteases. The best enzymatic hydrolysate was selected based on the bioactivities.

Table 1 The enzyme extraction condition for the preparation of OOM hydrolysates

Enzyme	Temperature (°C)	pH
Alcalase	50	7
Flavourzyme	50	7
Neutrase	50	7
Pepsin	37	2
Protamex	50	7
Trypsin	37	8

OOM was again digested using the selected enzyme to determine the enzyme/substrate (E/S) ratio starting with a ratio of 1:100. The obtained OOM hydrolysates were freeze-dried at −80 °C and stored at −20 °C for further research.

2.4 Determination of the Total Carbohydrate, Protein, and Phenolic Contents

All the OOM hydrolysates were analyzed for their chemical compositions including carbohydrate, protein and phenolic contents. Total carbohydrate content was determined by the standard AOAC method (1990). Total phenolic content was measured using the Folin-Ciocalteu method. The protein concentration was estimated using a BSA assay kit (BioRad, CA, USA). Glucose, gallic acid and BSA were respectively used in each of the above experiments as the calibration standards (Athukorala et al. 2006; Heo et al. 2005; Lee et al. 2010; Siriwardhana et al. 2008).

2.5 Determination of the Amino Acid Composition

Amino acid composition of OOMAH was analyzed with an amino acid analyzer (S433-H, Sykam GmbH, Germany). A 50 mg of OOMAH was hydrolyzed using 2 mL of 6.0 M HCl in a sealed vacuum ampoule at 110 °C for 24 h. Hydrochloric acid was removed by rotary evaporator and a final volume was adjusted at 10 mL with 0.2 M sodium citrate buffer (pH 2.2). Amino acids were separated and detected using a cation separation column (LCA K06/Na, 4.6 × 150 mm) with a flow rate of 0.45 mL/min (buffer) and 0.25 mL/min (reagent) at wavelengths of 440 and 570 nm. For the determination of free amino acids, 2 g of OOMAH was homogenized at 12,000 rpm twice for 2 min with 75% ethanol, followed by centrifuging at 2000 × *g* for 30 min. The supernatant solvents were removed by using a rotary evaporator, and redissolved in 8.0 mL of distilled water containing

5′-sulfosalicylic acid (0.2 g) at 4 °C for 1 h. Then, the mixture was centrifuged at 2000 × *g* for 30 min, and the 2 mL of the supernatant was transferred to a new tube containing 1 mL of 0.2 M lithium citrate buffer (pH 2.2). Free amino acids were determined using the same amino acid analyzer.

2.6 Determination of Antioxidant Activities

2.6.1 DPPH Radical Scavenging Activity

The DPPH radical scavenging activity was determined according to the method of Blois (1958) with some slight modifications. Briefly, a 100 μL of OOM hydrolysates (0.5 mg/mL) were added to 100 μL of 150 μM DPPH solution. The mixture was kept at room temperature for 30 min in dark, and the absorbance was measured at 517 nm (Spectra Max Plus^{384}, USA).

The ability scavenging DPPH radical was calculated using the following equation:

DPPH radical scavenging activity (%) = ((A_{517} of control-A_{517} of sample)/A_{517} of control) × 100

The selected OOMAH was further evaluated for radical scavenging activity in a concentration-dependent manner (0.125, 0.25, 0.5 and 1 mg/mL) to obtain the DPPH radical scavenging activity.

2.6.2 $ABTS^+$ Radical Scavenging Activity

The $ABTS^+$ radical scavenging activity was measured according to the presented method by Park and Kim (2009). A volume of 150 μL of a stock solution containing 7 mM ABTS and 2.4 mM potassium persulfate was mixed with each 50 μL of the hydrolysates. The mixture was allowed to react for 10 min in the dark, and then the absorbance was measured at 414 nm.

2.6.3 Hydrogen Peroxide Scavenging Activity

The Hydrogen peroxide scavenging activity was determined in accordance with the method of Müller (1985). In Brief, 100 μL OOM hydrolysates (0.5 mg/mL) was mixed with 100 μL of 0.1 M sodium phosphate buffer (pH 5.0) in a 96 well plate. And then, 20 μL of 20 mM H_2O_2 solution was added to each well and the well plate was incubated at 37 °C for 5 min. After, 30 μL of 1.25 mM ABTS and 30 μL of 1 U/mL peroxidase were added to the mixture followed by incubation at 37 °C for 10 min. The absorbance of the reactant of OGM hydrolysates or buffer was measured at 405 nm.

2.6.4 ORAC Assay

The ORAC assay was performed based on a modified method described by Zulueta et al. (2009). All the solutions were made in 75 mM phosphate buffer (pH 7.0). Briefly, 50 μL of the OGM hydrolysates were mixed with 50 μL of 78 nM fluorescein and incubated for 15 min at 37 °C. Then, 25 μL of 221 mM AAPH was rapidly added to the mixture and the fluorescence was recorded at each 5 min for 60 min (excitation wavelength 485 nm, emission wavelength 582 nm). Finally, the ORAC values were calculated using the regression equation between Trolox concentration and the net area under the curve (AUC) and were expressed as μM trolox equivalent (TE)/mg sample.

2.6.5 DNA Damage

To study the protective effect of the OOMAH against hydroxyl radical-induced DNA damage, the reaction of the mixture containing 0.5 μg of pBR322 plasmid DNA (Life Technologies, Seoul, Korea), 2 mM $FeSO_4$ and the OOMAH solution (in D.W.) was performed using an Eppendorf tube. The mixture was incubated with 4.0 μL of H_2O_2 (10 mM) at 37 °C for 30 min. And then, the mixture was subjected to 0.8% agarose gel electrophoresis. The supercoiled and the open circular DNA bands were stained with ethidium bromide and quantified using Davinch-Chemi™ imaging system (Core Bio, Seoul, Korea).

2.7 Intracellular Antioxidant Activities

2.7.1 Cell Viability and Protective Effects of OOMAH Against H_2O_2-Induced Oxidative Stress

Chang liver cells were maintained in an incubator at 37 °C in a humidified atmosphere supplemented with 5% CO_2, and were cultured in a DMEM containing 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics (streptomycin (100 μg/mL) and penicillin (100 unit/mL)).

The cell viability of OOMAH was determined using the MTT assay by Johan et al. (2011). Chang liver cells were seeded in a 96 well plate at a density of 1.0×10^4 cells/well. After 24 h, the cells were pretreated with different concentrations of OOMAH for 1 h. The cells were then exposed to 1 mM H_2O_2 to induce oxidative stress, followed by incubation for 24 h at 37 °C. After 24 h incubation, the MTT assay was performed to determine the cell viability. The absorbance of the products through MTT assay was measured by using a microplate reader at 540 nm.

2.7.2 Intracellular ROS Scavenging Activities

Chang liver cells were seeded in a 96 black well plate at a density of 1.0×10^4 cells/well. After 24 h, the cells were treated with OOMAH. After 1 h, 1 mM H_2O_2 was added to each well and then the cells were incubated for an additional 1 h at 37 °C under a humidified atmosphere. Finally, the fluorescence emission of 2′7′-dichlorofluorescin due to oxidation was measured at Excitation 485 nm/Emission 528 nm. The percentage of fluorescence intensity of the OOMAH treated wells was compared with that of the non-treated wells, which were arbitrarily assigned a value of 100%.

2.8 Antioxidant Activities in Zebrafish Model

2.8.1 The Origin and Maintenance of Zebrafish

Adult zebrafish were acquired from a commercial dealer (World fish aquarium, Korea) and each group of ten fish was kept in a 3 L acrylic tank under 28.5 °C, in 14/10 h light/dark cycle. The zebrafish were fed with Tetramin flake supplemented with live brine shrimp (*Artemia salina*) three times a day. The zebrafish embryos used for the animal experiments were prepared through natural spawning method by turning on the light in the morning. The zebrafish embryos was collected within 30 min.

2.8.2 Application of OOMAH and Induction of Oxidative Stress Induced by H_2O_2 in Zebrafish Embryos

Nearly after 7–9 h post-fertilization (hpf), zebrafish embryos were transferred to a 24 well plate (25 per each group) and maintained in the embryo medium supplemented with OOMAH (62.5, 125 and 250 μg/mL) for 1 h. Then, the embryos were treated with 0.5 mM H_2O_2. After 24 hpf, the embryos were washed with embryo media and mounted in new embryo media.

2.8.3 Survival Rate, ROS Generation and Cell Death of Zebrafish Treated with OOMAH

Embryos (25 per each group) were treated with different concentrations of OOMAH to the plate. Embryos were incubated for 6 h, the embryo media were changed and the embryos were developed up to 2 dpf. Intracellular ROS generation and cell death in zebrafish embryos were estimated according to the method described by

(Kang et al. 2013) and (Kim et al. 2014). After incubation, the embryos were rinsed with fresh embryo media and anesthetized before the microscopic observation. Fluorescence intensities of each individual embryo were quantified using a spectrofluorometer (Perkin–Elmer LS-5B, Vienna, Austria) and the images of stained embryos were observed using a fluorescent microscope equipped with a CoolSNAP-Pro color digital camera (Olympus, Tokyo, Japan).

2.9 Statistical Analysis

Data were analyzed using the SPSS package (Version 21). Values were expressed as mean ± standard error (SE). The mean values were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test.

3 Results

3.1 Proximate Composition of OOM

The proximate composition was shown in Table 2. The proximate compositions of OOM indicated a higher amount of protein (56.48 ± 1.39%) and carbohydrate (30.1%) compared to that of the lipids (4.70 ± 0.42%). Especially, OOM had the highest protein content and it suggests that the plentiful protein content might be an important feature of OOM.

3.2 Yields, Total Carbohydrate, Phenolic and Protein Contents of OOM Hydrolysates

The extraction yield and the proximate composition of the enzymatic hydrolysates of OOM showed a considerably higher yield and protein content (Table 3).

OOMAH was obtained with a comparatively higher yield of 96.47 ± 1.19% and indicated a high protein content compared to carbohydrates (1.59 ± 0.34%) and polyphenols (4.98 ± 0.04%). Consequently, proteins in OOM which indicated a higher content could be the source of observed antioxidant activities.

Table 2 Proximate composition (%) of OOM

Protein	Carbohydrate	Lipid	Moisture	Ash
56.48 ± 1.39	30.1	4.70 ± 0.42	2.19 ± 0.12	6.62 ± 0.08

Value means ± SE of three determinations
Carbohydrate: 100-(moisture + protein + lipid + ash)

Table 3 Yields (%) and proximate composition (%) of enzymatic extracts prepared from OOM

Samples	Yields	Proteins	Carbohydrate	Polyphenol
OOMAH[1]	96.47 ± 1.19[a]	75.53 ± 0.10[a]	1.59 ± 0.34[c]	4.98 ± 0.04[b]
OOMFH[2]	76.00 ± 1.00[b]	46.92 ± 0.09[e]	2.21 ± 0.09[bc]	4.86 ± 0.15[b]
OOMNH[3]	77.67 ± 1.20[b]	66.48 ± 0.97[c]	1.88 ± 0.19[bc]	4.85 ± 0.02[b]
OOMPH[4]	79.00 ± 1.53[b]	64.45 ± 0.56[c]	2.81 ± 0.40[abc]	4.88 ± 0.11[b]
OOMPRH[5]	80.00 ± 2.08[b]	60.27 ± 0.05[d]	3.25 ± 0.40[ab]	4.86 ± 0.01[b]
OOMTE[6]	65.33 ± 0.88[c]	69.30 ± 0.10[b]	4.26 ± 0.58[a]	5.45 ± 0.02[a]

All data were statistically analyzed with three independent experiments and the data expressed as means ± S.E. [a–c]>The values with different subscripts indicated significant difference within the each samples ($p < 0.05$)
[1]OOMAH: The Alcalase hydrolysate of OOM
[2]OOMFH: The Flavourzyme hydrolysate of OOM
[3]OOMNH: The Neutrase hydrolysate of OOM
[4]OOMPH: The pepsin hydrolysate of OOM
[5]OOMPRH: The Protamex hydrolysate of OOM
[6]OOMTH: The trypsin hydrolysate of OOM

3.3 Antioxidant Activities of the Enzymatic Hydrolysates of OOM

3.3.1 DPPH Radical Scavenging Activity

DPPH radical scavenging activities of the enzymatic hydrolysates of OOM were ranged between 2 and 38% (Fig. 1a) at 0.5 mg/mL concentration. Moreover, OOMAH and OOMPH showed significantly high DPPH radical scavenging activity compared to the other hydrolysates. As illustrated in Fig. 2a, OOMAH showed effectively and dose-dependently on DPPH radical scavenging activity.

3.3.2 $ABTS^+$ Radical Scavenging Activity

$ABTS^+$ radical scavenging activities of OOM hydrolysates at 0.5 mg/mL concentration indicated considerably similar activities such as OOMPH (76%), OOMNH (72%) and OOMAH (71%) (Fig. 1b). In addition, the selected OOMAH showed $ABTS^+$ radical scavenging activity in a dose-dependent manner (Fig. 2b).

3.3.3 Hydrogen Peroxide Scavenging Activity

Hydrogen peroxide scavenging activities of OGM hydrolysates were ranged between 22 and 47% at 0.5 mg/mL concentration (Fig. 1c). Moreover, OOMAH, OOMTH and OOMPH showed significantly higher hydrogen peroxide scavenging activities compared to the OOMFH. OOMAH indicated the highest hydrogen peroxide scavenging activity with a concentration-dependent increase(Fig. 2c).

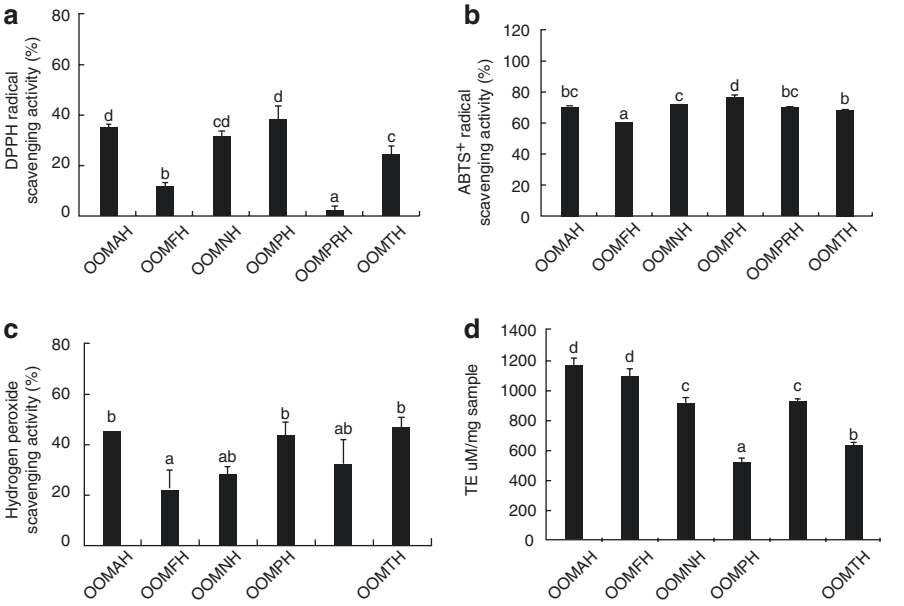

Fig. 1 Effects of OOM extracts on DPPH radical (**a**), ABTS$^+$ radical (**b**), hydrogen peroxide (**c**) and ORAC value (**d**). All data were statistically analyzed with three independent experiments and the data expressed as means ± S.E. $^{a-d}$>The values with different subscripts indicated significant difference within the each samples ($p < 0.05$)

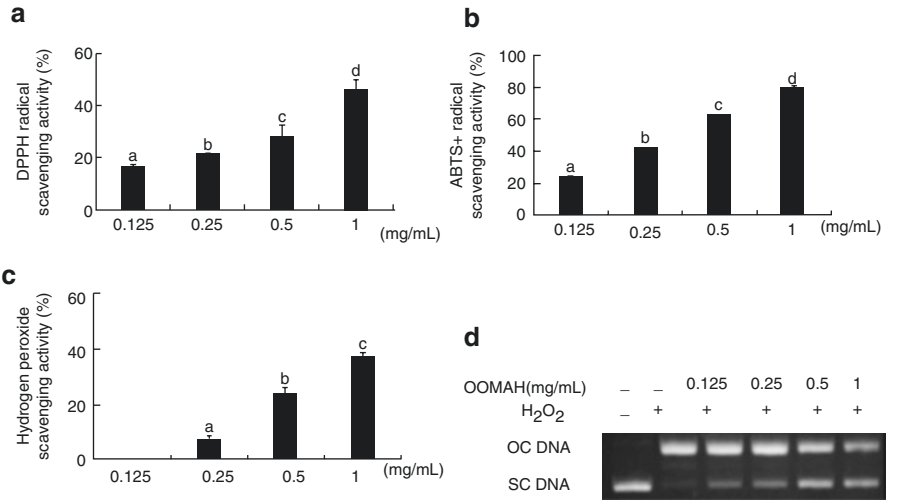

Fig. 2 Scavenging effects of OOMAH on DPPH radical (**a**), ABTS$^+$ radical (**b**), hydrogen peroxide (**c**) and protective effect of OOMAH on DNA damage (**d**). All data were statistically analyzed with three independent experiments and the data expressed as means ± S.E. $^{a-d}$>The values with different subscripts indicated significant difference within the same sample ($p < 0.05$)

3.3.4 ORAC Value

ORAC assay provides information regarding the sample's ability to scavenge peroxyl radicals through hydrogen atom transfer mechanism (Prior et al. 2003). Based on the results, ORAC values of the OOM hydrolysates were ranging between 523 and 1180 μM TE/mg (Fig. 1d). Moreover, OOMAH indicated an ORAC value of 1180 μM TE/mg. Our results indicate that ORAC value of OOMAH was the highest among all.

3.3.5 Protection Against DNA Damage

The genomic stability is an essential factor that affects the normal metabolic and regulatory processes of a cell. DNA damage could cause abnormalities in all kinds of cellular processes that associated with the cell cycle regulation and cell death through a variety of mechanisms (Liu et al. 2011). The presence and brightness of the supercoiled DNA bands increased with the increasing concentrations of OOMAH indicating the protective effect of OOMAH against Fenton's agent-induced DNA damage (Fig. 2d).

3.4 Amino Acid Composition of OOMAH

The amino acid compositions of the OOMAH show that OOMAH is rich in taurine, leucine, arginine and alanine which accounted for 23.16%, 12.12%, 9.95% and 6.93% respectively. Amino acid composition of OOMAH showed the presence of both essential and non-essential amino acids. Based on the results, OOMAH could possess a higher nutritional value (Table 4).

Table 4 Free amino acid composition (%) of OOMAH

Amino acids	Content (%)
Phosphoserine	0.34
Taurine	23.16
Phosphoethanolamine	0.00
Urea	0.00
Aspartic acid	0.00
Hydroxyproline	1.57
Threonine	0.00
Serine	2.08
Asparagine	0.00
Glutamic acid	0.81
Sarcocine	0.75
α-Aminoadipic acid	0.00

(continued)

Table 4 (continued)

Amino acids	Content (%)
Proline	0.07
Glycine	0.66
Alanine	6.93
Citrulline	0.00
α-Aminobutyric acid	0.11
Valine	1.94
Cystine	0.17
Methionine	5.05
Cysthathionine	0.00
Isoleucine	2.64
Leucine	12.12
Tyrosine	5.49
Phenylalanine	6.54
β-Alanine	0.70
β-Aminoisobutyric acid	5.86
γ-Amino-*n*-butyric acid	0.59
Histidine	0.55
3-Methylhistidine	0.00
1-Methylhistidine	0.00
Carnosine	0.59
Anserine	0.00
Tryptopan	4.28
Hydroxylysine	1.27
Ornitine	1.34
Lysine	4.41
Ethanolamine	0.04
Arginine	9.95
Total	100.00

3.5 Intracellular Antioxidant Activities

3.5.1 Cell Viability and Protective Effects of OOMAH Against H_2O_2-Induced Hepatotoxicity

As shown in Fig. 3, OOMAH did not indicate any cytotoxic effects upon the tested concentrations. Thus, non-toxic concentrations were used for further experiments. The treatment of 1 mM H_2O_2 to Chang liver cells resulted in the reduction of cell viability to 68%, however, pretreatment with OOMAH greatly increased the cell viability in a concentration-dependent manner. The cell viabilities were restored with the application of OOMAH (0.125, 0.25, 0.5, 1 mg/mL) up to 89.02%. The results show that the exposure of OOMAH to Chang liver cells could protect the cells against H_2O_2-induced hepatotoxicity.

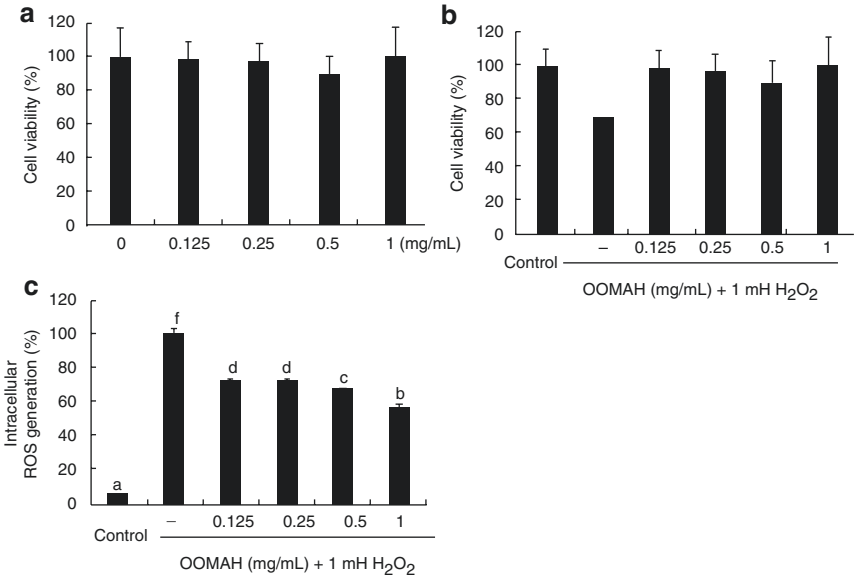

Fig. 3 Effects of OOMAH on the cell viabilities (**a** and **b**) and the ROS generation (**c**) in Chang liver cells. All data were statistically analyzed with three independent experiments and the data expressed as means ± S.E. $^{a–f}$>The values with different subscripts indicated significant difference within the each groups ($p < 0.05$)

3.5.2 Intracellular ROS Scavenging Activity

Oxidative stress, caused by the imbalance between ROS production and antioxidant system, has been considered to the cause of aging and various diseases in humans. Therefore, antioxidant therapy is vital in scavenging free radicals (Finkel and Holbrook 2000). The effects of the OOMAH on ROS scavenging were determined by DCF-DA assay using Chang liver cells. As shown in Fig. 3c, the treatment of H_2O_2 induced the production of ROS, however, the cells treated with OOMAH showed significant down-regulation of intracellular ROS generation compared with the 1 mM H_2O_2-induced cells.

3.6 Antioxidant Activity in Zebrafish Embryos

3.6.1 Effect of OOMAH on Survival Rate, Inhibition of ROS Generation and Cell Death in Zebrafish Embryos

According to the investigated survival rates, the non-treated group indicated a survival rate of 100% during the experimental period (Fig. 4a). The survival rate of H_2O_2 (0.5 mM) treated group reduced significantly less than 85%. Interestingly, the

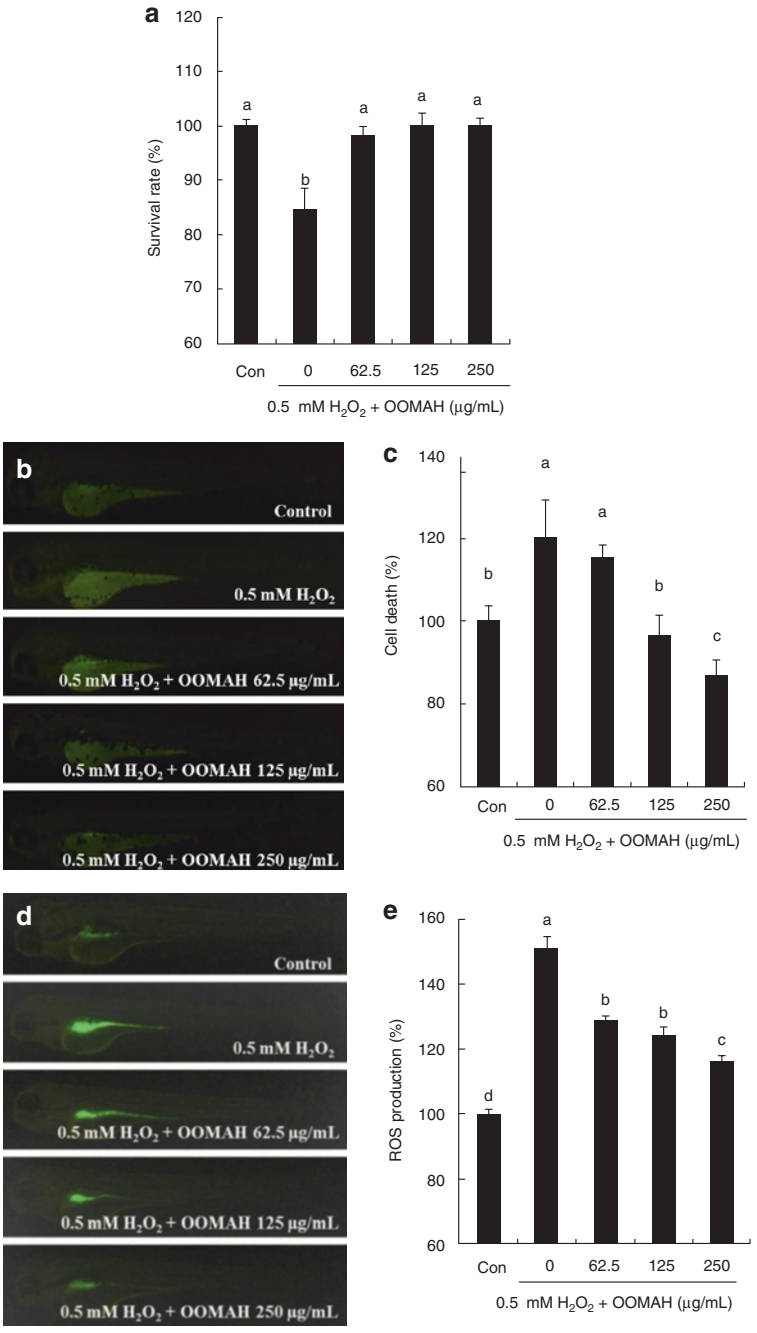

Fig. 4 Effects of OOMAH on the survival rate (**a**), the cell death (**b** and **c**) and ROS generation (**d** and **e**) in H_2O_2-treated zebrafish embryo models. All data were statistically analyzed with three independent experiments and the data expressed as means ± S.E. $^{a-d}$>The values with different alphabet subscripts indicated significant difference within the each groups ($p < 0.05$)

OOMAH-treatments (62.5, 125 and 250 μg/mL) dose-dependently increased the survival rates of H_2O_2 exposed zebrafish embryo and were reported as 98.30%, 100%, and 100%, respectively.

The antioxidant activities of OOMAH on the intracellular ROS levels and cell deaths were shown in Fig. 4b, c, d and e. Accordingly, a significant and a dose-dependent down-regulation of the elevated ROS and cell deaths levels in H_2O_2 treated zebrafish were observed with the treatment of OOMAH.

4 Discussion

Taurine is one of the major intracellular free β-amino acids (2-aminoethanesulfonic acid) in most mammalian tissues. Recently, many studies have demonstrated that taurine possesses pharmacological and physiological effects (Huxtable 1992; Zhou et al. 2011; Rashid et al. 2013; Islambulchilar et al. 2015). Generally, seafood and meat contain high levels of taurine, which are the major dietary supplements of this amino acid (Salze and Allen Davis 2015). *Octopus ocellatus*, a kind of cephalopod is plentifully produced in Korea, Japan and China. It is popular in Korea and Japan as taurine-rich seafood ingredients. Although various studies focusing on the anti-oxidant activities of taurine have been reported, the protective effect of taurine-rich seafood, *O. ocellatus*, on oxidative stress has not been validated. In the present study, we investigated whether the taurine-rich seafood, *O. ocellatus*, has protective effects against H_2O_2-induced oxidative stress in Chang liver cells and zebrafish embryo model.

Recently, enzyme-assisted hydrolysis has been successfully applied for the extraction of various biologically active compounds from a great variety of natural products. The extraction technique leads to high yields of bioactive compounds, which show enhanced biological activity (Lee et al. 2012; Puri et al. 2012; Ko et al. 2013). Therefore, in the present study, *O. ocellatus* gray meat was enzymatically hydrolyzed by using several commercial proteases (Alcalase, Flavourzyme, Neutrase, Pepsin, Protamex, and Trypsin) in order to evaluate their anti-oxidant activities. All enzymatic hydrolysate of *O. ocellatus* evidenced stronger anti-oxidant activities with higher extract yields than water extract. Among the *O. ocellatus* hydrolysates prepared with different enzymes, the Alcalase hydrolysate evidenced marked antioxidative activities on hydrogen peroxide, $ABTS^+$ and DPPH radical as well as ORAC value. Thus, the Alcalase hydrolysate was selected to investigate protective effect against oxidative stress.

In our present study, we have confirmed that taurine was the most abundant total amino acid of the *O. ocellatus* gray meat Alcalase hydrolysate (OOMAH). Kim et al. (1999) reported that species of octopus was rich in taurine compared to other seafood. Although biological functions of taurine are still unrevealed, valuable evidences demonstrate that taurine can work as a direct antioxidant by scavenging ROS (Niittynen et al. 1999) or as an indirect antioxidant by inhibiting the changes in membrane permeability induced by oxidative impairment. All of the results showed that OOMAH containing high amounts of taurine possesses the potent scavenging

activities against hydrogen peroxide, $ABTS^{+}$ and DPPH radical. These results indicate that OOMAH may be an effective agent for preventing various chronic diseases that are linked with oxidative stress. Cellular DNA damage is susceptible to oxidation by ROS and free radicals. In this study, OOMAH was analyzed for its protective effects against DNA oxidation using genomic DNA isolated from Chang liver cells. The isolated DNA was subjected to oxidation by •OH generated via the Fenton reaction. The effect of OOMAH on the •OH-induced DNA damage was observed after electrophoresing the DNA on agarose gel. The results demonstrated a clear inhibition of DNA oxidation with OOMAH treatment.

High ROS levels cause oxidative stress, which can result in a variety of physiological disorders. Such cellular damage often effects impairments in metabolic function, and leads to cell death (Finkel and Holbrook 2000). Based on the results, H_2O_2 treatment decreased Chang liver cells viability. However, treatment with OOMAH together with H_2O_2 inhibited cell death, suggesting that OOMAH protected Chang liver cells from H_2O_2-induced cytotoxicity. Next, we turn our attention to oxidative stress. H_2O_2 induced ROS generation. As expected, treatment with OOMAH coupled with H_2O_2 decreased ROS generation. Our data speculate the protective effect of OOMAH possessing high amounts of taurine on the cytotoxicity of Chang liver cells may in part be due to the scavenging ROS, thereby preventing H_2O_2-induced oxidative damage in Chang liver cells. Similar with our results, Roy and Sil (2012) reported that taurine protects hepatocytes from oxidative damage and apoptotic death against oxidative stress. Another previous study reported that taurine serves as a hepatoprotective agent to prevent liver injury (Miyazaki and Matsuzaki 2014).

The *in vivo* zebrafish model system is catching on new animal model because of many advantages in modern biotechnology such as a large clutch size, transparent embryos, low-cost, and easy handling. Recently, zebrafish as an *in vivo* model has been used to study human metabolism and diseases such as diabetes, oxidative stress, cancer, liver damage and inflammation (Kang et al. 2013). Therefore, we applied the H_2O_2-induced oxidative stressed zebrafish model to investigate the protective effects of OOMAH. Our data showed that survival rate was significantly increased by adding OOMAH to H_2O_2-treated zebrafish. For this result, it is proved that OOMAH can protect the zebrafish from damage induced by H_2O_2. Our results also demonstrate that H_2O_2-treated zebrafish significantly increase ROS levels. Therefore, OOMAH containing large amounts of taurine alleviates the oxidative damage by inhibiting ROS generation.

5 Conclusion

These results indicate that OOMAH containing large amounts of taurine possesses a beneficial effect on H_2O_2-induced oxidative stress *in vitro* as well as *in vivo* zebrafish model by inhibiting ROS formation, free radicals and DNA oxidation.

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Hepatoprotective Effects of Xylose-Taurine Reduced Against Hydrogen Peroxide-Induced Oxidative Stress in Cultured Hepatocytes

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Abstract In this study, Xylose-Taurine reduced (X-T-R) was synthesized to enhance biological activities. Hence, we investigated the hepatoprotective effects of X-T-R against H_2O_2-induced hepatocyte damage and apoptosis. The results showed that X-T-R led to the cytoprotective effect against H_2O_2-induced oxidative stress in cultured hepatocytes such as the improvement of cell viability and the reduction of reactive oxygen species (ROS) production. Additionally, pre-treatment with X-T-R increased the expression of nuclear factor erythroid 2-related factor 2 (Nrf2),

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NAD(P)H dehydrogenase:quinone 1 (NQO1) and heme oxygenase 1 (HO-1) in cultured hepatocytes. Furthermore, X-T-R protected the cells against apoptosis via regulating the expression level of Bcl-2/Bax as well as the activation of caspase-3. According to the results obtained, X-T-R may be a bio-material for the therapy of hepatic diseases.

Keywords Xylose-Taurine reduced • Hepatoprotective effects • Oxidative stress • Hepatocytes

Abbreviations

HO-1	Heme oxygenase 1
NQO1	NAD(P)H dehydrogenase:quinone
Nrf2	Nuclear factor erythroid 2-related factor 2
X-T-R	Xylose-Taurine reduced

1 Introduction

The liver is sensitive to damages caused by reactive oxidative species (ROS) and affects to the conservation of lipid homeostasis (Auroma 1994). Hepatocytes physiologically use ROS as secondary messengers, with plays a role in modulating normal cellular functions. However, the overproduction of ROS causes oxidative stress, resulting in lipid peroxidation, the mitochondrial membrane damage, the release of mitochondrial cytochrome C into the cytoplasm, followed by activation of caspases and apoptosis (Valko et al. 2007; Chiu et al. 2013; Ghosh et al. 2011). Therefore, intracellular antioxidant capability to eliminate the harmful effect of ROS, including endogenous and exogenous antioxidant systems, is crucial for maintaining normal cellular function (Chiu et al. 2013).

The nuclear factor erythroid 2-related factor 2 (Nrf2) known as a transcription factor regulates cellular protective effects by inducing antioxidant/phase II detoxifying enzymes including heme oxygenase 1 (HO-1) and NAD(P)H dehydrogenase:quinone 1 (NQO1) (Leiser and Miller 2010). Due to its cytoprotective capacity of Nrf2, it has been increased interests in various molecules that can initiate this pathway (Surh et al. 2009).

Taurine acts as a bioactive substance in a variety of physiological processes such as osmoregulation, brain development, immune response, inflammatory response, neuromodulation, anti-oxidation and detoxification. However, taurine

has the disadvantages with unfavorable pharmacokinetics, lower absorption, fast rate of extraction through urine, and high-dose requirement. Therefore, to resolve this disadvantage, various taurine derivatives have been developed (Nakagawa and Huxtable 1985; Yahn et al. 2013; Cho et al. 2014).

In this study, Xylose-Taurine reduced (X-T-R) was synthesized to enhance biological activities and we investigated hepatoprotective effects of X-T-R against H_2O_2-induced oxidative stress in cultured hepatocytes.

2 Materials and Methods

2.1 Chemicals and Materials

Xylose-Taurine is reduced to X-T-R and the chemical name of X-T-R is 2-(((2S,3R,4R)-2,3,4,5-tetrahydroxypentyl)amino)ethanesulfonic acid. 2′,7′-dichlorodihydroflurescin diacetate (DCFH-DA), 3-(4-5-dimethyl-2yl)-2-5-diphynyltetrasolium bromide (MTT) and ZnPP were purchased from Sigma Chemical Co. (St, Louis, MO, USA). Dulbeco's modified Eagle's medium (DMEM), antibiotics, and other materials required for culturing the cells were purchased from Gibco BRL (Paisley, UK).

2.2 Cell Culture

Chang liver cells, human hepatocytes were obtained from the American Type Culture Collection (Rockville, MD, USA). Hepatocytes were cultured in DMEM including 10% fetal bovine serum (FBS) and antibiotics at 37 °C under 5% CO_2.

2.3 Cell Viability and Hepatoprotective Effect of X-T-R

The cell viability was evaluated using the MTT assay. Hepatocytes (1.0×10^4 cells/well) in a 96-well plates were treated with various concentrations of X-T-R (25, 50, 100 and 200 μg/mL) at 37 °C for 24 h. A 100 μL of MTT solution (1 mg/mL) was added to each well after removal of medium, followed by incubation for 4 h at 37 °C. The formazan crystals in viable cells were dissolved in 100 μL DMSO after removal of medium. The optical density was measured at 540 nm using microplate reader (SpectraMax M2/M2e).

Non-cytotoxic concentrations of X-T-R were applied to examine hepatoprotective effect against H_2O_2-induced hepatocyte damage. The cells were pretreated with X-T-R (25, 50 and 100 μg/mL) for 2 h and then exposed to 1 mM H_2O_2 to generate oxidative stress. After 24 h incubation, MTT assay was performed as described in the above method.

2.4 Intracellular ROS Measurement

Intracellular ROS formation was measured according to the slightly modified method of Engelmann et al. (2005). Attached hepatocytes (7.5×10^4 cells/mL) were treated with X-T-R (25, 50 and 100 μg/mL) for 2 h and then exposed to 1 mM of H_2O_2. Cells were incubated for 1 h and labeled with DCFH-DA (5 μg/mL) for 30 min. Due to oxidation of DCFH in the presence of ROS, the formation of 2′7′-dichlorofluorescin (DCF) was measured after 30 min at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a spectrofluorometer.

2.5 Western Blot Analysis

Hepatocytes pretreated with X-T-R for 2 h were exposed to 1 mM of H_2O_2. After 24 h, Cytosolic and nuclear proteins were extracted from the cells using RIPA buffer (Sigma Chemical Co.). Their protein contents were examined with a BCA™ protein assay kit. The cell lysates (40 μg of total protein) were separated on 10% SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking with 5% skim milk, the membranes were incubated with the first antibodies and detected using the secondary antibody labeled with horseradish peroxidase and detected using chemiluminescence (ECL) Western blotting detection kit, and imaged on Davinch-Chemi™ imaging system (Core Bio, Seoul, Korea). The β-actin was performed as an internal control.

2.6 ZnPP Treatment

To confirm whether X-T-R protect hepatocytes against H_2O_2-induced hepatotoxicity through the up-regulation of HO-1 expression, hepatocytes were incubated with 5 μM ZnPP (HO-1 inhibitor) for 2 h with or without X-T-R. The cells were exposed by 1 mM H_2O_2 for 24 h at 37 °C. The cell viability was identified by MTT assay described in the above method.

2.7 Statistical Analysis

Data are expressed as means ± S.D. and the experiments were repeated three times. All statistical comparisons were compared using a one-way analysis of variance followed by Duncan's test using PASW Statistics 21.0 software (SPSS, Chicago, IL, USA) and a p-value was <0.05.

3 Results

3.1 Cytoprotective Effects of X-T-R Against H_2O_2-Treated Hepatocytes

Effect of X-T-R against H_2O_2-induced cytotoxicity were examined. First of all, X-T-R exhibited no cytotoxic effects in the used all concentrations (Fig. 1a). Figure 1b showed that H_2O_2 alone significantly ($p < 0.05$) reduced the cell viability (61.0%), compared to the untreated cells. But, -T-R significantly led to the increased cell viability with the increment of concentrations. The cell viabilities were restored up to 88.5% by X-T-R. This result indicates that X-T-R protects hepatocytes against cellular oxidative damages.

3.2 Effect of X-T-R on ROS Production in Cultured Hepatocytes

To evaluate the effects of X-T-R on intracellular ROS production, we performed the DCFH-DA assay. As summarized in Fig. 1c, treatments of H_2O_2 significantly increased the intracellular ROS production, comparing with the untreated cells. Pre-treatment of hepatocytes with X-T-R significantly ($p < 0.05$) suppressed the H_2O_2-induced production of intracellular ROS in a dose-dependent manner, compared to the only H_2O_2-treated hepatocytes.

3.3 X-T-R Regulated the Expressions of Apoptosis-Related Molecules in H_2O_2-Treated Cultured Hepatocytes

To analyze whether X-T-R can reduce apoptosis as regulating the mitochondrial signaling in H_2O_2-exposed hepatocytes, western blot assay were performed. As

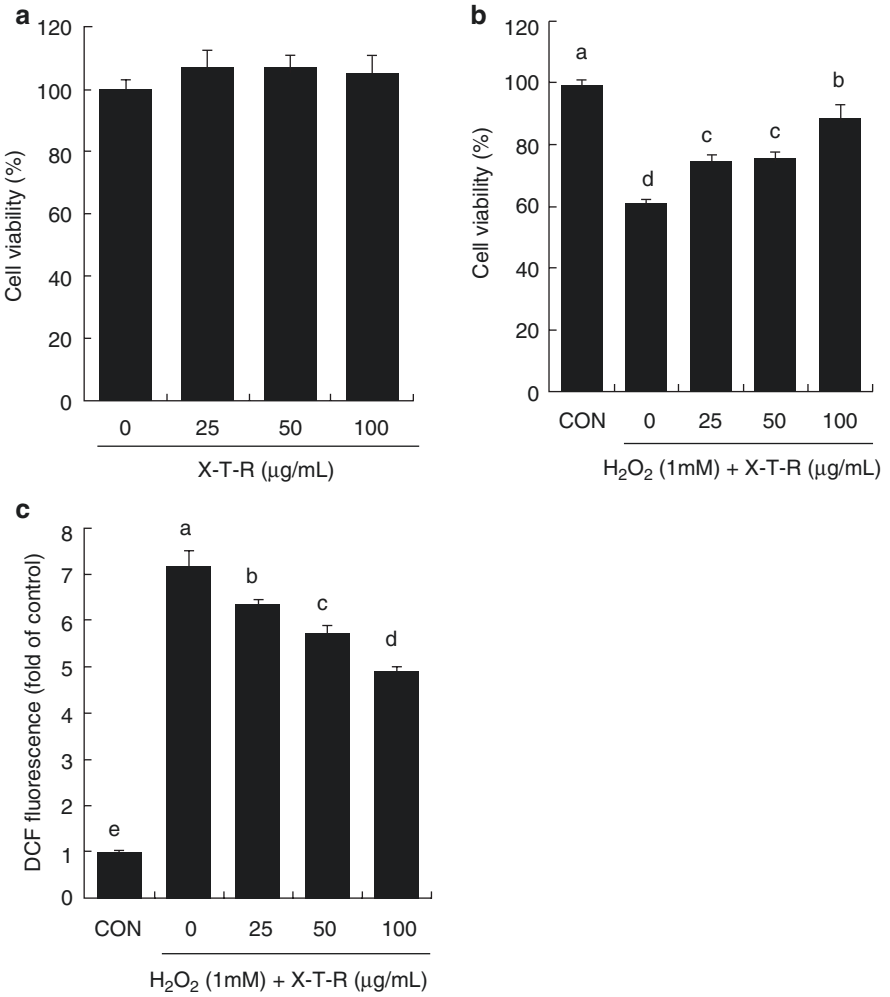

Fig. 1 Effect of X-T-R on the cell viabilities and the ROS production in H_2O_2-treated hepatocytes (**a**) Cell viability of X-T-R on hepatocytes. (**b**) Cell viability of X-T-R on H_2O_2-treated hepatocytes. (**c**) Inhibition of intracellular ROS generation by X-T-R in hepatocytes. [a–e]The error bars with different letters indicate significant differences ($p < 0.05$). Data are expressed as mean ± S.D. ($n = 3$)

summarized in Fig. 2, exposure of H_2O_2 induce considerably up regulation in the expression levels of Bax and cleaved caspase-3 known as pro-apoptotic molecules, whereas the expression of Bcl-2, an anti-apoptotic molecule was reduced in hepatocytes. However, they were modulated by X-T-R with the decreased expression of Bax and cleaved caspase-3 as well as the increased expression level of Bcl-2 in the H_2O_2-treated cells. This result suggests that X-T-R protects apoptosis caused by H_2O_2 treatment as modulating apoptosis-related proteins.

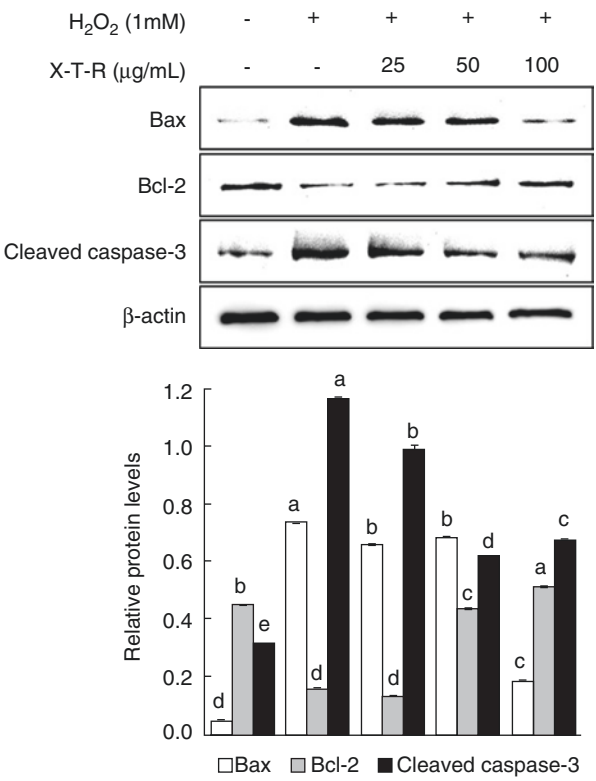

Fig. 2 Protective effect of X-T-R against H_2O_2-induced apoptosis in cultured hepatocytes. Total cell lysates were prepared and performed western blot analysis to expression levels of apoptotic proteins, namely Bax, Bcl-2 and cleaved caspase-3. Values are expressed as mean ± S.D. ($n = 3$). [a–d]The error bars with different letters indicate significant differences ($p < 0.05$)

3.4 *Effects of X-T-R on Nrf2-Mediated Defense System Proteins in H_2O_2-Induced Cultured Hepatocytes*

To elucidate cytoprotective mechanism underlying hepatoprotection against H_2O_2-induced oxidative stress in cultured hepatocyte, we checked protein expression levels of Nrf2, NQO1 and HO-1 under oxidative stress by Western bloting. Figure 3a showed pre-treatment with X-T-R dose-dependently increased Nrf2 expression. Activated Nrf2 causes the up-regulation of HO-1 and NQO1 known as major antioxidant enzymes that inhibit H_2O_2-caused oxidative stress in hepatocytes (Campbell et al. 2013; Xu et al. 2014). As expected, we observed that X-T-R significantly ($p < 0.05$) led to the increased expressions of HO-1 and NQO1 in H_2O_2-treated hepatocytes and it was dose-dependent. From these result, we indicate the cytoprotective effects of X-T-R are mediated through Nrf2 signaling.

To confirm that X-T-R exhibit cytoprotective effect through up-regulation of HO-1 expression, we employed HO-1 inhibitor ZnPP to cultured hepatocytes in the absence or the presence of X-T-R. As shown in Fig. 3b, pretreatment with X-T-R (100 μg/mL) exerted cytoprotective effects against H_2O_2-induced hepatotoxicity.

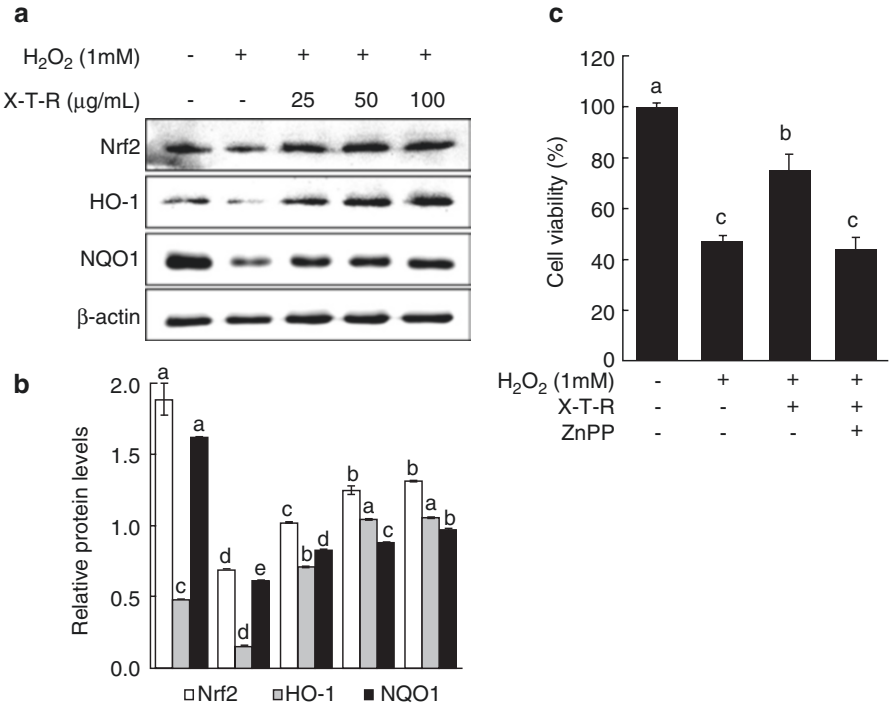

Fig. 3 Effects of X-T-R on Nrf2 signaling in cultured hepatocytes. Hepatocytes pretreated with X-T-R for 2 h were exposed to 1 mM H_2O_2 for an additional 24 h. (**a**, **b**) The protein expression levels of Nrf2, HO-1 and NQO1 and quantitative representations. (**c**) Protective ability of X-T-R in the presence of HO-1 inhibitor ZnPP against H_2O_2-induced hepatotoxicity in cultured hepatocytes. [a–d]The bars with different letters represent significant differences ($p < 0.05$). Values are expressed as mean ± S.D. ($n = 3$)

However, this protection ability of X-T-R was abolished by the presence of ZnPP, indicating that X-T-R exerted their cytoprotective effects through up-regulation of HO-1 expression.

4 Discussion

Oxidative stress is caused by an imbalance between reactive oxygen species (ROS) production and antioxidant defense system (Rigoulet et al. 2011). The over-production of ROS and oxidative damages leads to the increase of a number of human diseases such as hepatitis, cancer, rheumatoid arthritis, atherosclerosis inflammation and neurodegenerative diseases, and is also thought to accelerate the aging process (Okezie 1998). ROS are important both in terms of their elimination by various antioxidants (Kumar et al. 2012). Therefore, previous study has focused

on natural antioxidants showing chemo preventive effects and their biological mechanisms (Masella et al. 2005).

Here, we examined the hepatoprotective activities of X-T-R on H_2O_2-caused oxidative stress to cultured hepatocytes. Our data revealed that the pre-treatment of X-T-R increased the cell viability reduced by H_2O_2, implying that X-T-R could protect hepatocytes from H_2O_2-induced damage. Also, the oxidative stress-caused cellular damages are related to increase of ROS (Trachootham et al. 2008). In this study, we revealed treatment with X-T-R significantly removed H_2O_2-caused ROS production, possibly due to the potent antioxidant activity of X-T-R.

Apoptosis in hepatocytes has a considerable relation with the development of various liver diseases (Li et al. 2014). Apoptosis leads to cell death for cellular homeostasis, embryogenesis and metamorphosis (Renehan et al. 2001). During intracellular damage and apoptosis, Bax and caspase-3 are over-expressed in hepatocytes. Antiapoptotic protein, Bcl-2 is responsible for apoptosis and death of cells (Adachi et al. 2004; Kang et al. 2013). Here, we found out that X-T-R down-regulated Bax and cleaved caspase-3, and up-regulated Bcl-2. It indicates that X-T-R protected hepatocytes against H_2O_2-induced apoptotic responses by regulation of Bcl-2, Bax and cleaved caspase-3.

Nrf2, an activator of antioxidant molecules protects cells on cellular damages caused by oxidative stress and elevates cell viability (Niture et al. 2010). Upon stimulation, Nrf2 is detached from Keap-1 known as a its cytosolic inhibitor, translocated into the nucleus, and then binds to the promoter regions of many phase II enzymes (HO-1 and NQO1) (Kay et al. 2010). NQO1 leads to the metabolic detoxification of quinones (Nioi and Hayes 2004). HO-1 is a cytoprotective enzyme that could catalyze the rate-limiting step of heme catabolism, cause to the production of bilirubin, carbon monoxide, and ferrous iron which is sequestered by intracellular ferritin. Many researches have proved the strong cytoprotective and antioxidative effects of HO-1–produced heme derivates (Ryter et al. 2002; Kim et al. 2010). In the present study, we found that X-T-R clearly induces the activation of Nrf2, and this observation was consistent with increased NQO1 and HO-1 expressions (Fig. 3a). However, hepatoprotective abilities of X-T-R were abolished in the presence of ZnPP, HO-1 inhibitor, suggesting that X-T-R exerted their cytoprotective effects through upregulation of HO-1 expression.

5 Conclusion

These results of this study demonstrated that X-T-R can protect cell against H_2O_2-induced apoptosis by up-regulating Bcl-2 and down-regulating Bax and cleaved caspase-3 in cultured hepatocytes. Furthermore, we have demonstrated that X-T-R led to hepatoprotective effects against H_2O_2-induced oxidative damage through the activation of Nrf2 signaling including NQO1 and HO-1. Therefore, this study suggests that it may be a potential material for the treatment of liver diseases followed by oxidative stress.

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Xylose-Taurine Reduced Suppresses the Inflammatory Responses in Lipopolysaccharide-Stimulated Raw264.7 Macrophages

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Abstract Here, the anti-inflammatory effect of Xylose-Taurine reduced (X-T-R), a taurine derivate was investigated in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. X-T-R reduced the generations of nitric oxide (NO) and prostaglandin E_2 (PGE_2) induced by the stimulation of LPS in RAW 264.7 by suppressing the protein expression of iNOS and COX-2 known as inflammatory mediators. Also, X-R-T

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reduced the expression levels of the pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF-α). Moreover, X-T-R inhibited the activation of nuclear factor-κB (NF-κB) and the phosphorylation of inhibitor κB (IκB)-α. In conclusion, these results first indicate that X-T-R inhibits LPS-induced inflammation by regulating the NF-κB signal pathway in macrophages.

Keywords Xylose-taurine reduced • LPS • Anti-inflammatory effect • Macrophages

Abbreviations

COX-2	Cyclooxygenase-2
IL-1β	Interleukin-1β
iNOS	Inducible NO synthase
IκB-α	Inhibitor κB-α
LPS	Lipopolysaccharide
NF-κB	Nuclear factor-κB
NO	Nitric oxide
PGE_2	Prostaglandin E2
TNF-α	Tumor necrosis factor-α
X-T-R	Xylose-Taurine reduced

1 Introduction

Inflammatory response has an important role in immune system related to infection, toxin exposure and cell injury in human body (Heo et al. 2010; Wu et al. 2015). Despite its essential roles, the excessive or aberrant inflammation can induce various acute and chronic human diseases including atherosclerotic lesions, rheumatic disease, and type II diabetes (Kim et al. 2014).

Lipopolysaccharide (LPS), a bacterial endotoxin of gram-negative bacteria is the most potent initiator of the inflammatory response and can cause fever, septic shock and microbial invasion (Kim et al. 2010). Macrophages play a key role as innate immune system in resistance against pathogens. Stimuli of LPS can lead to the release of nitric oxide (NO) and prostaglandin E_2 (PGE_2) as well as pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α in macrophages. It can be regulated by the NF-κB signal pathway (Ham et al. 2015). In previous study focused on anti-inflammation, LPS-stimulated macrophage was used as *in vitro* model (Wen et al. 2016).

Taurine is a sulfonated β-amino acid in certain aspects of mammalian development (Redmond et al. 1998). The deficiency of taurine is related with various pathological diseases. Especially, deficiency of taurine during mammalian development causes such as cardiomyopathy, retinal degeneration, and growth retardation. Therefore, taurine has been used as successful therapeutic materials for various

diseases and disorder including cardiovascular diseases, hypercholesterolemia, hepatic disorders, alcoholism, cystic fibrosis, and macular degeneration (Birdsall 1998; Corte et al. 2002).

The purpose of this study is to evaluate anti-inflammatory capacity of Xylose-Taurine reduced (X-T-R), a taurine derivate, in lipopolysaccharide (LPS)-stimulated RAW264.7 cells.

2 Materials and Methods

2.1 Chemicals

Synthesized Xylose-Taurine reduced (X-T-R) was offered by Prof. *Sung Hoon Kim*, Kunkuk University. The chemical name of X-T-R is 2-(((2S,3R,4R)-2,3,4,5-tetrahydroxypentyl)amino)ethanesulfonic acid. RAW264.7 cells were purchased from Korean Cells Bank (KCLB; Seoul, Korea). Anti-bodies including iNOS, COX-2, NF-κB p65, phosphorylated IκBα, β-actin were offered from Cell signaling Technology (Beverly, MA, USA). NE-PER^R Nuclear and Cytoplasmic Extraction Reagents was purchased from Pierce (Rockford, IL, USA). Polyvinylidene fluoride (PVDF) membrane was purchased from GE healthcare (PA, USA).

2.2 Cell Culture

RAW264.7 cells were incubated in DMEM media (Invitrogen-Gibco, NewYork, USA) including 10% FBS (Invitrogen-Gibco) and antibiotics (100 U/mL of penicillin/streptomycin). The cells were incubated at 37 °C in an atmosphere of 5% CO_2.

2.3 Measurement of NO Production

RAW 264.7 cells (5×10^5 cells/mL) were treated with X-T-R for 2 h and then stimulated by LPS (1 μg/mL). After 24 h, the cultured medium was mixed with Griess reagent (1:1) at RT. After 10 min, the absorbances were checked at 540 mn (SpectraMax® M2/M2^e, CA, USA).

2.4 Measurement of PGE_2 Production

RAW 264.7 cells (5×10^5 cells/mL) were incubated with X-T-R for 2 h followed by the stimulation of LPS (1 μg/mL) for the additional 24 h. The secretion of PGE_2 was quantified in the culture media by the EIA kits, in accordance with the manufactural instructions.

2.5 Western Blotting

The cells were seeded in 60 pi dishes (1×10^6 cells/dish) for 16 h and stimulated by LPS (1 μg/mL) before 1 h of the treatment of X-T-R. After 20 min or 24 h, the cells were used for the preparation of cytosolic and nucleic proteins. Cytosolic and nucleic proteins were extracted from the cells with the NE-PER® Nuclear and Cytoplasmic extraction kit. The lysates (30 μg of protein) were used for western blotting by the method of Kim et al. (2010). The primary antibodies used in this study were rabbit polyclonal antibodies (iNOS and NFκB p65) and mouse monoclonal antibodies (COX-2 and β-actin) The targeted proteins were visualized by using enhanced chemiluminescence (ECL) reagents (Amersham, Arlington Heights, IL, USA).

2.6 RT-PCR

Total cellular RNA of the cell lysates was isolated using Trisol (Molecular Research Center, Inc., Cincinnati, Ohio, U.S.A) and then the cDNA synthesis was conducted with RNA (1 μg) by using a Promega A3500 kit, respectively. The primers prepared following Kim et al. (2010). PCR was performed with the method indicated Kim et al. (2010) using the TaKaRa PCR machine (Takara Bio Inc., Otsu, Japan). PCR products were electrophoresed in a 1.0% agarose gel including EtBr and analyzed by UV transillumination.

2.7 Statistical Analysis

All data are expressed as means ± S.E. (n = 3), and all statistical analysis was identified by means of one-way analysis of variance followed by Duncan's test using PASW Statistics 21.0 software (SPSS, Chicago, IL, USA). A p-value < 0.05 was considered as statistically significant.

3 Results

3.1 Protective Effect of X-T-R Against LPS-Induced NO and PGE_2 Productions

First of all, cytotoxicity of X-T-R on RAW264.7 cells was determined by MTT assays. As shown in Fig. 1a, X-T-R has no cytotoxic effect in RAW 264.7 cells at concentrations of up to 200 μg/mL. Thus, these concentrations were applied for next experiments. To assess the inhibition effect of X-T-R on NO production, we measured the NO levels with the Griess reagent. As depicted in Fig. 1b, LPS treatment stimulated

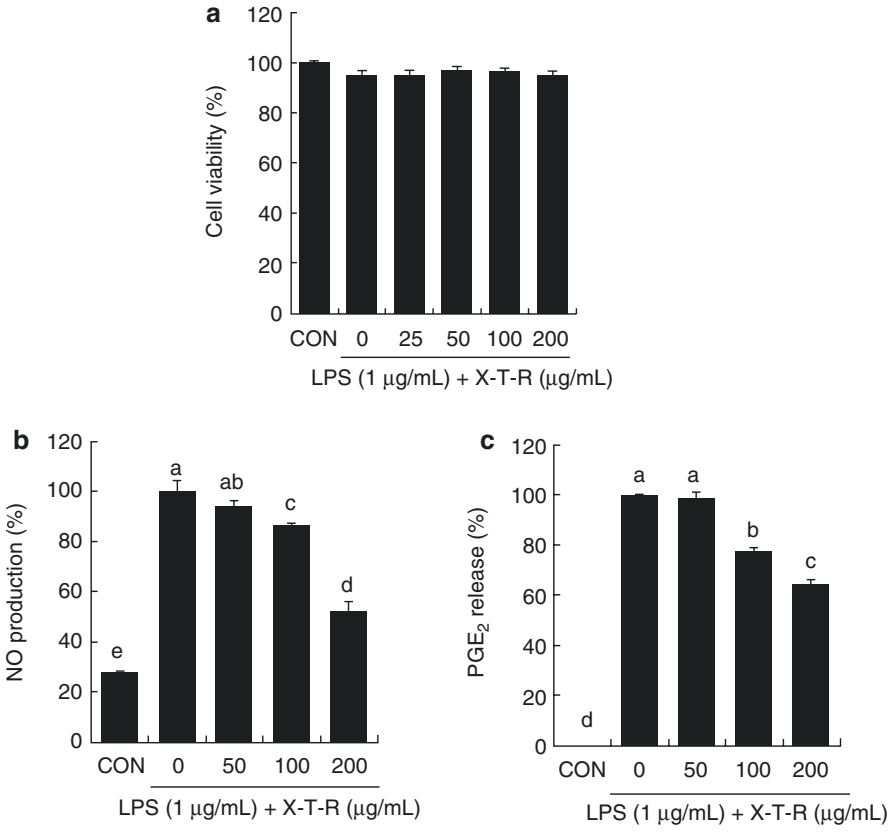

Fig. 1 The effect of xylose-taurine reduced (X-T-R) on cell viability (**a**), NO production (**b**), and PGE_2 release (**c**) in RAW 264.7 cells. The cells were stimulated with LPS and various concentra tions of X-T-R (25, 50, 100, and 200 μg/mL). After an incubation of 24 h, the viability of cells was determined by MTT and NO and PGE_2 levels were measured. The values are expressed mean ± SE by repeating thrice experiments. Different alphabet letter means that the values significantly differ with the others at $P < 0.05$ (Tukey-Kramer multiple comparison test)

the cells and markedly increased NO level as the approximate threefold higher compared to that of control cells. The pretreatment of X-T-R, however, dose-dependently reduced LPS-produced NO levels. Also, we identified that PGE_2 release increased by LPS stimulation was significantly decreased by the treatment of X-T-R.

3.2 Inhibitory Effect of X-T-R on the LPS-Upregulated iNOS and COX-2 Expression

To evaluate whether the reduced NO and PGE_2 levels are related to the inhibition of iNOS and COX-2 expression, their protein expression levels were analyzed by western blotting. The expression levels of iNOS and COX-2 were considerably

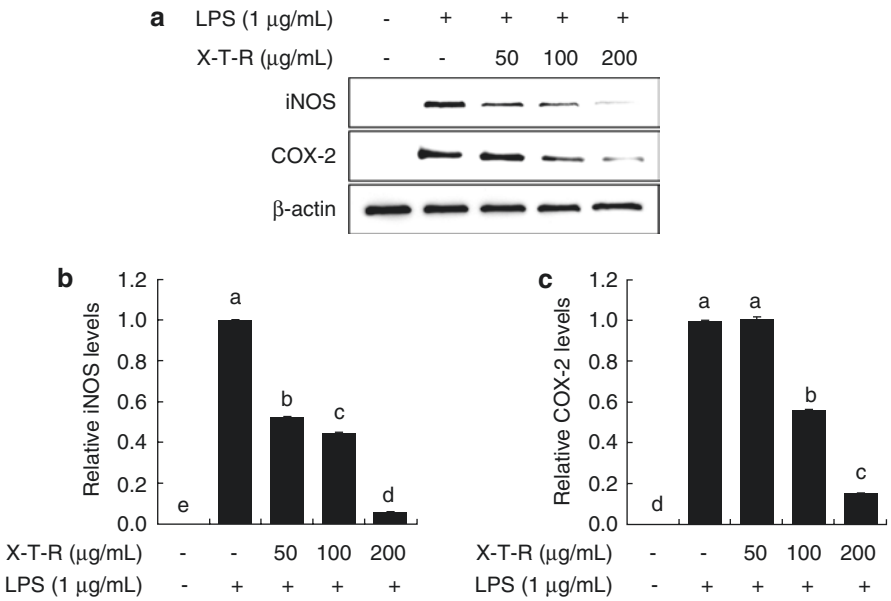

Fig. 2 Inhibition of X-T-R on the expression levels of iNOS and COX-2 in LPS-treated RAW 264.7 cells. (**a**) The expression levels of iNOS and COX-2. (**b** and **c**) Densitometry analysis. The values are expressed mean ± SE by repeating thrice experiments. Different alphabet letter means that the values significantly differ with the others at $P < 0.05$

increased in the presence of LPS (Fig. 2). X-T-R treatment, however, attenuated these expression levels in a dose-dependent manner.

3.3 Effects of X-T-R on LPS-Induced TNF-α, IL-1β, and IL-6

To investigative the effects of X-T-R on the mRNA expression levels of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α, RT-PCR was performed. Fig. 3 exhibited that the mRNA expression levels of cytokines increased by LPS were dose-dependently reduced by treatment of X-T-R at 200 μg/mL. Especially, the mRNA expression level of TNF-α was dramatically decreased. It was also found that X-T-R treatment was markedly inhibited the production of these pro-inflammatory cytokines in LPS-induced RAW 264.7 cells.

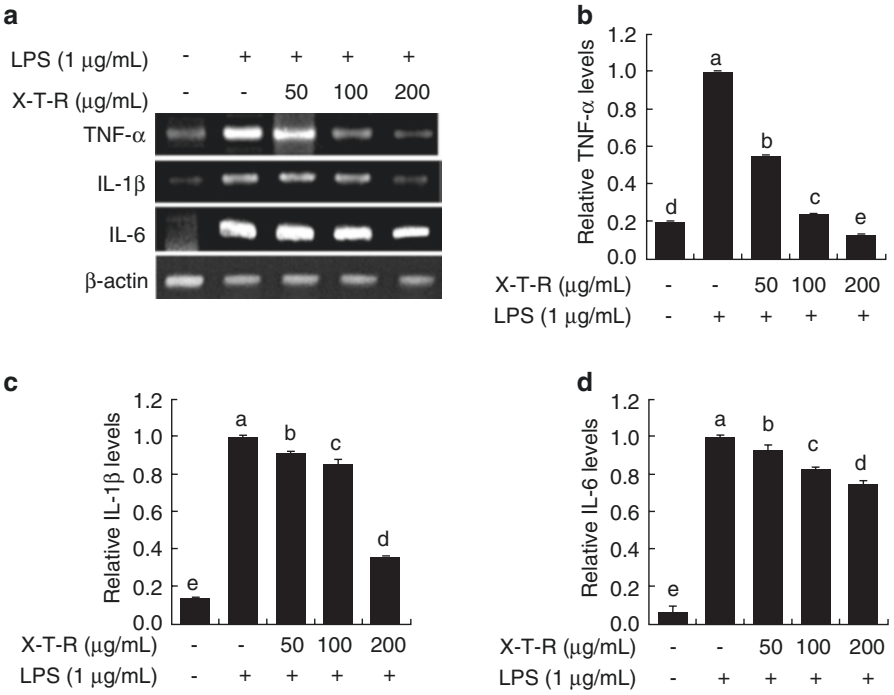

Fig. 3 Inhibition effect of X-T-R on the IL-1β, IL-6, and TNF-α expression levels in LPS-stimulated RAW 264.7 cells. (**a**) The mRNA expression levels of TNF-α, IL-1β, and IL-6 were determined by RT-PCR. (**b**, **c** and **d**) The density ratio represented the relative intensity of each band against that of the β-actin. The values are expressed mean ± S.E (n = 3). Different alphabet letter means that the values significantly differ with the others at $P < 0.05$

3.4 The Effect of X-T-R on NF-κB Signaling

LPS finally induces inflammation by translocating NF-κB p65 into the nucleus and phosphorylating IκB-α. To check the biological mechanism of X-T-R, nuclear NF-κB p65 and cytosolic phosphor-IκBα protein levels in RAW264.7 cells were measured with western blotting (Fig. 4). X-T-R significantly decreased LPS-stimulated nuclear NF-κB p65. In addition, cytosolic phosphor-IκBα protein level was reduced in a concentration-dependent manner in the presence of X-T-R. These results indicate that X-T-R inhibits LPS-induced activation of NF-κB by inducing IκB-α phosphorylation, as well as inhibiting translocation of p65 into the nucleus. As a result, X-T-R inhibits LPS-induced inflammation through NF-κB signaling.

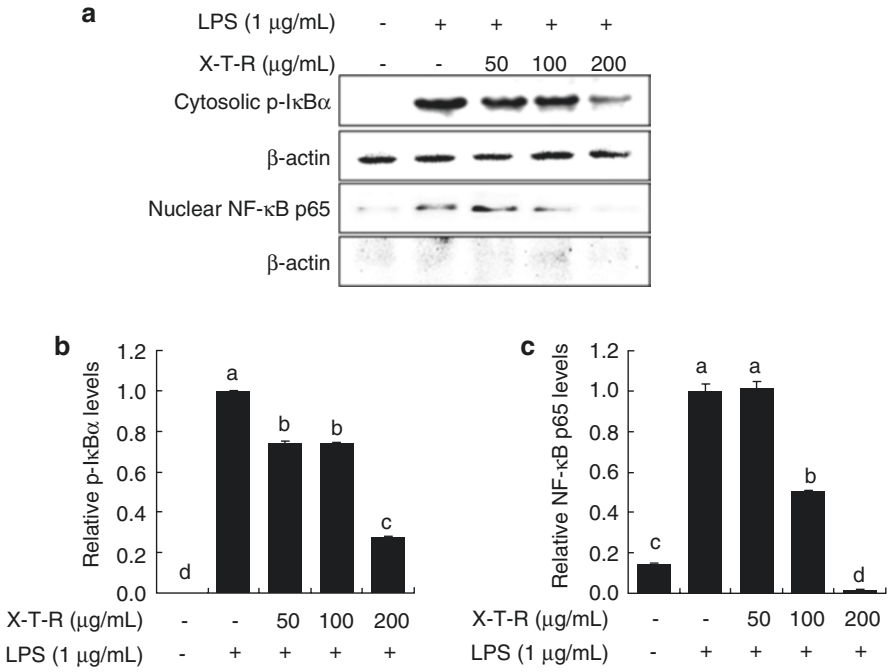

Fig. 4 The effect of X-T-R on the NF-κB activation in RAW 264.7 cells. The cells were stimulated with 1 μg/mL of LPS after 1 h of X-T-R treatment (50, 100, and 200 μg/mL). After an incubation of 20 min, p-IκB-α and p65 levels were determined by immunoblotting analysis. (**a**) Western blot images (**b**, **c**) Densitomitric anlysis. The values are expressed mean ± SE by repeating thrice experiments. Different alphabet letter means that the values significantly differ with the others at $P < 0.05$ (Tukey-Kramer multiple comparison test)

4 Discussion

In the present study, we demonstrated that X-T-R, a taurine derivate, has anti-inflammatory effect via suppressing the pro-inflammatory mediators and cytokines induced by LPS treatment in RAW 264.7 cell, a murine macrophage.

Generally, LPS can stimulate and activate various cells, especially, RAW 264. cells. Also, previous reports have indicated that LPS stimulation significantly induces iNOS production and expression which catalyze the oxidative deamination of L-arginine to generate a potent pro-inflammatory mediator such as NO (Jacobs and Ignarro 2001). Also, LPS-increased COX-2 expression affected to the over-expression of PGE_2 (Posadas et al. 2000). Our present study showed that X-T-R significantly attenuated the generation of NO and PGE_2 via the inhibition of iNOS and COX-2 protein expressions without cytotoxicity in LPS-stimulated RAW 264.7 cells. These results indicated that X-T-R led to the anti-inflammatory activity as reducing the release of NO and PGE_2 via the inhibition of iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells.

Normally, major pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α released from macrophages play considerable roles in the pathophysiology of the inflammatory response (Lebovic et al. 2000; Bergqvist et al. 2001; Zhang et al. 2005). Additionally, the transcription of target genes such as pro-inflammatory mediators and cytokines, including iNOS, and IL-1β, IL-6, and TNF-α, is related with the activation of NF-κB signal pathway (Surh et al. 2001; Ghosh and Baltimore 1990; Verma et al. 1995). Many researchers have showed that the inhibition of NFκB activation led to anti-inflammatory effects as reducing the generation of pro-inflammatory mediators and cytokines (Surh et al. 2001). Our data demonstrated that X-T-R significantly decreased the productions of IL-1β, IL-6, and TNF-α in LPS-stimulated RAW 264.7 cells. X-T-R also inhibited the phosphorylation of IκB as well as the translocation of NF-κB p65 into nuclei after the exposure of LPS to RAW 264.7 cells. With these results, this study indicated that X-T-R inhibited the LPS-induced inflammation response as decreasing the expression and production levels of inflammatory mediators and cytokines via inhibiting the NF-κB activation in RAW 264.7 cells.

5 Conclusion

In conclusion, we revealed that xylose-taurine reduced (X-T-R), a taurine derivate has the anti-inflammatory effect via the suppression of NF-κB signaling, which may result in the inhibition of NO and PGE_2 production caused by LPS, as well as decreased inflammatory mediators and cytokines.

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Protective Effects of Xylose-Taurine Reduced against Damages Caused by Oxidative Stress in Zebrafish Embryos *In Vivo* Model

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Abstract The zebrafish (*Danio rerio*) is useful and convenient vertebrate models in various studies in human disease and drug discovery. In this present study, we first evaluated whether Xylose-Taurine reduced (X-T-R), a taurine derivate protects zebrafish embryos against oxidative stress caused by AAPH (2,2′-Azobis(2-amidinopropane) dihydrochloride). First of all, we selected the concentration of X-T-R showing no toxicity in zebrafish embryos. We identified that X-T-R signifi-

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cantly increased the survival of zebrafish embryo reduced by treatment of AAPH. Also, X-T-R effectively inhibited the productions of reactive oxygen species (ROS) and nitric oxide (NO) as well as the formation of cell death in zebrafish embryos. Moreover, X-T-R down-regulated the expression levels of Bax, caspase-3, caspase-9 and p53 known as pro-apoptotic molecules, whereas up-regulated those of Bcl-2, an anti-apoptotic molecule in AAPH-treated zebrafish embryos. From these results, this study reveals that X-T-R, a taurine derivate might be a potential protector against various damages caused by oxidative stress.

Keywords Xylose-Taurine reduced • Protective effects • Oxidative stress • Zebrafish embryos

Abbreviations

AAPH	2,2′-azobis (2-amidinopropane) dihydrochloride
DAF-FM-DA	Diaminofluorophore 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate
iNOS	Inducible NO synthase
NO	Nitric oxide
ROS	Reactive oxygen species
X-T-R	Xylose-Taurine reduced
COX-2	Cyclooxygenase-2

1 Introduction

Zebrafish (*Danio reiro*) and its embryos have various advantages for *in vivo* experiments dues to their small size and optical transparency as well as very fast development as the basic body plan is laid out 24 hours post-fertilization (hpf), hatch approximately 48 ~ 72 hpf. Also, they attain maturity at about 3 months and develop organs including the brain, heart, liver, kidney, intestines, bone, and muscles except lung within 120 hpf (McGrath and Li 2008; Scholz et al. 2008). Additionally, their physiological features are so similar to mammals (Driever et al. 1996; Kimmel 1989; Den Hertog 2005; Pichler et al. 2003; Kim et al. 2015). With these points, zebrafish and zebrafish embryos have been used in various fields of drug discovery, toxicology, genetic engineering, physiology and diverse human disease including cardiovascular diseases, diabetes, and obesity (Driever et al. 1996; Kimmel 1989; Den Hertog 2005; Pichler et al. 2003; Kim et al. 2015).

Free radicals play an important role in living organisms and is comprise of superoxide (O^{2-}), peroxyl (ROO^{-}), alkyl (RO^{-}), hydroxyl (OH^{-}), and nitric oxide (NO^{-}) (Kang et al. 2014). A high level of free radicals or reactive oxygen species (ROS) causes oxidative stress leading to degradation of DNA, cell membranes, proteins and other constituents and caused cellular damages and apoptosis via both

mitochondria-dependent and mitochondria-independent pathways (Fang et al. 2002; Kim et al. 2014; Sunha et al. 2013). Accordingly, oxidative stress can lead to serious harmful effects in human such as cancer, aging, atherosclerosis, rheumatoid arthritis, neurological disorders, and muscular dystrophy (Kovatcheva et al. 2006; Giusooe et al. 2001; Sarma et al. 2010).

Taurine, a free amino acid plays an important role in several essential biological activities such as anti-apoptotic (Zhang et al. 2010), anti-oxidant (Stapleton and Bloomfield 1993), and immune function (Sturman 1993). However, several taurine derivatives have been developed and their beneficial functions have been reported to resolve disadvantages such as a rapid renal extraction rate and poor absorption (Cho et al. 2014).

Therefore, this study first evaluated the anti-oxidative activities of xylose-taurine reduced (X-T-R) against oxidative stress caused by AAPH (2,2′-azobis (2-amidinopropane) dihydrochloride) that generates alkyl and peroxyl radicals in *in vivo* zebrafish embryo model.

2 Methods

2.1 Origin and Maintenance of Parental Zebrafish

Adult zebrafish were obtained from a commercial dealer (Seoul aquarium, Seoul, Korea); following conditions; 28.5 ± 1 °C, and fed twice times a day (Tetra GmgH D-49304 Melle Made in Germany), 14/10 h light/dark cycle. The day before, Breeding 1 female and 2 males interbreed. In the morning (On set of light), embryos were obtained from natural spawning collection of embryos were completed within 30 min in petri dishes (containing media).

2.2 Waterborn Exposure of Embryos to X-T-R and AAPH

The embryos (n = 15) were moved to individual wells of 12-well plates containing 900 μL embryo media from approximately 7–9 hpf, X-T-R was added to the wells. After the incubation 1 h, a 10 mM AAPH solutions was treated to the embryo exposed with X-T-R for up to 24 hpf. Then, embryos were changed to fresh embryo media.

2.3 Measurement of Intracellular ROS Production and Image Analysis

The zebrafish larva were transferred to individual well of 24-well plate at 3 dpf (days post fertilization), treated with DCFH-DA solution (20 μg/mL) and incubated for 1 h in the dark at 28.5 ± 1 °C. After the incubation, the zebrafish larvae

were rinsed by fresh embryo media and anaesthetized by 0.002% MS222 (ethyl 3-aminobenzoate methanesulfonate) before observation and photographed under the microscope CoolSNAP-Pro color digital camera (Olympus, Japan). A fluorescence intensity of individual larva was quantified using the image J program.

2.4 Measurement of NO Production and Image Analysis

The zebrafish larva were transferred to individual well of 24-well plate at 3 dpf, treated with 5 μM of DAF-FM-DA (diaminofluorophore 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate) thereafter incubated for 2 h in the dark at 28.5 ± 1 °C. The rest of the procedure was as described in Sect. 2.3.

2.5 Measurement Cell Death and Image Analysis

The zebrafish larva were transferred to individual well of 24-well plate at 3 dpf, treated with acridine orange solution (7 μg/mL) and incubated for 30 min under the dark at 28.5 ± 1 °C. The rest of the procedure was as described in Sect. 2.3.

2.6 Real-Time PCR Analysis

The embryos (n = 50) were transferred to individual wells of 6-well plates at 7–9 hpf, X-T-R was added to the wells. After the incubation 1 h, a AAPH solution (10 mM) was treated to the embryo exposed with X-T-R for up to 24 hpf. Then, embryos were rinsed using fresh embryo media. At 3 dpf, embryos were transferred into e-tube, and then washed twice. The zebrafish were homogenized in trizol reagent using a homogenizer. Total RNA was isolated by TRIzol reagent (Ambion) according to the manufacture's instruction. The oligo (dT)-based first strand cDNA was synthesized from 1 μg of total RNA using Transcriptor first strand cDNA synthesis kit (Roche). RCR reactions were performed using LightCyler 480 SYBR green 1 master kit (Roche) according to the manufacture's instruction. The expression levels of genes were estimated using LightCycler 480 system (Roche). The level of expression was measured with the crossing point (Cp) method implemented by LightCycler 480 software. The sequences of primer sets used were: Bcl-2 sense, 5′-TCACTCGTTCAGACCCTCAT-3′ and antisense, 5′-ACGCTTTCCACGCACAT-3′; Bax sense, 5′-GGCTATTTCAACCAGGGTTCC-3′ and antisense, 5′-TGCGAATCAATGCTGT-3′; Caspase-3 sense, 5′-CCGCTFCCCATCACTA-3′ and antisense, 5′-ATCCTTTCACGACCATCT-3′; Caspase-9 sense, 5′-AAATACATAGCAAGGCAACC -3′ and antisense,

5′-CACAGGGAATCAAGAAAGG -3′; p53 sense, 5′-GGGCAATCAGCGAGCAA-3′ and antisense, 5′-ACTGACCTTCTTGAGTCTCCA-3′; β-actin sense, 5′-GCTGACA-GGATGCAGAAGGA-3′ and antisense, 5′-TAGAAGCATTTGCGGTGGAC-3′.

2.7 Statistical Analysis

All results are expressed as means ± SD, and all statistical comparisons were made by means of one-way analysis of variance followed by Duncan's test using PASW Statistics 21.0 software (SPSS, Chicago, IL, USA). A p-value < 0.05 was considered to be statistically significant.

3 Results

3.1 Toxicity of X-T-R Against AAPH-Treated Oxidative Stress in Zebrafish

The survival rates of zebrafish embryos exposed to AAPH with or without X-T-R (12.5, 25 μg/mL) are shown in Fig. 1. We determined that the survival rate was 90% in the AAPH-treated group compared with the control group (without X-T-R and AAPH). Interestingly, X-T-R and AAPH-treated groups showed a

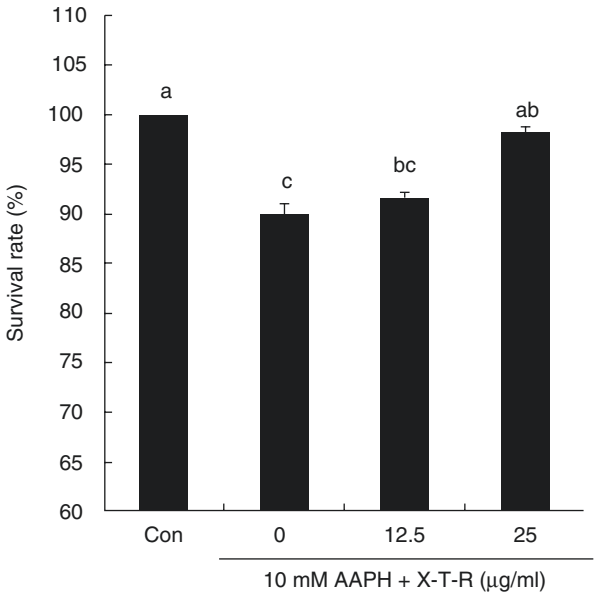

Fig. 1 Effect of X-T-R on survival rate in AAPH-treated zebrafish larvae. Experiments were performed in triplicate and the data are expressed as mean ± SE

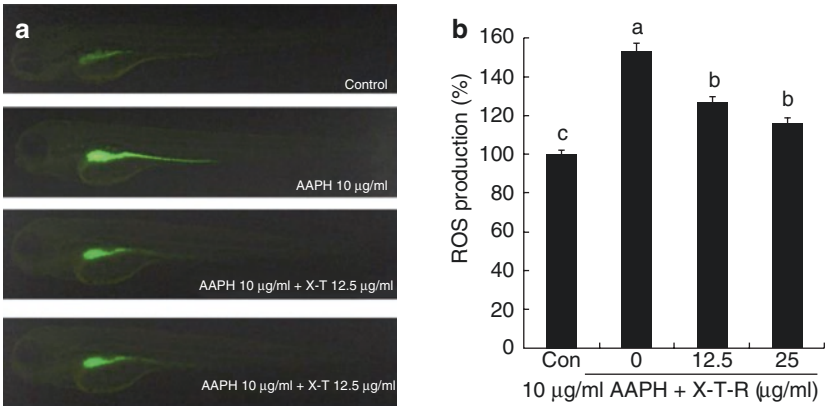

Fig. 2 Protective effect of X-T-R against AAPH-induced ROS production in zebrafish larvae. (**a**) the ROS production and (**b**) the image analysis. Experiments were performed in triplicate and the data are expressed as mean ± SE

dose-dependent increased survival rate to 91% and 98% at 12.5 and 25 μg/mL at 7 dpf, respectively. However, X-T-R exhibited reduced survival rate at concentration of up to 50 μg/mL (data not shown). Therefore, Lower concentrations were used in subsequent experiments.

3.2 Protective Effect of X-T-R Against AAPH-Treated ROS Production in Zebrafish

The inhibition effects of X-T-R on AAPH-treated ROS production was measured using DCF-DA dye in zebrafish (Fig. 2). ROS level was 153% in AAPH-treated group compared with the control group (without X-T-R and AAPH). In contrast, the treated concentration of X-T-R at 12.5 and 25 μg/mL in the AAPH-treated group decreased to 126% and 116% in ROS production compared with control group, respectively (Fig. 2b). As a result, X-T-R was shown protective effect of ROS production against AAPH.

3.3 Protective Effect of X-T-R Against AAPH-Treated NO Production in Zebrafish

As shown Fig. 3, the inhibition effect of X-T-R on AAPH-treated production of NO was measured using DAF-FM-DA dye in zebrafish. The AAPH-treated group leveled 126% expression of NO, compared to the non-treated control group. On the other hand, zebrafish groups treated with 12.5 and 25 μg/mL of X-T-R showed the significantly reduced NO production to 107% and 102%, respectively.

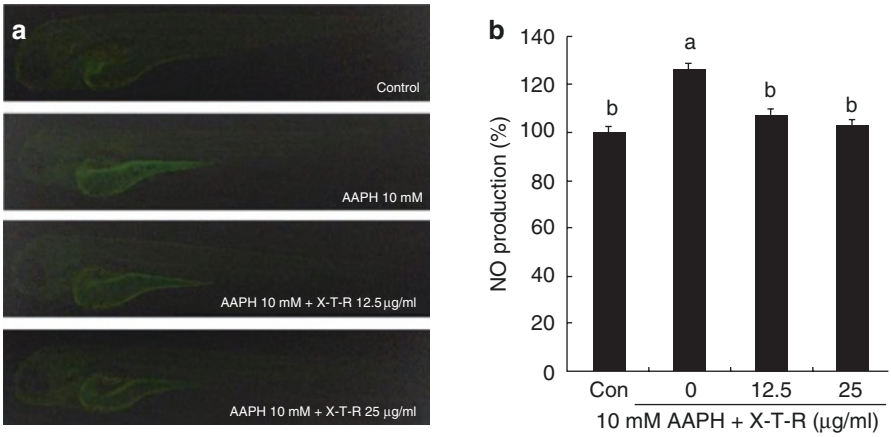

Fig. 3 Protective effect of X-T-R against AAPH-induced NO production in zebrafish larvae. Experiments were performed in triplicate and the data are expressed as mean ± SE.

3.4 Protective Effect of X-T-R Against AAPH-Treated Cell Death in Zebrafish

The inhibition effect of X-T-R on AAPH-treated cell death was confirmed using acridine orange dye in zebrafish (Fig. 4). The level of cell death was 1.5 fold inductions in the AAPH-treated group, compared with the control group (Fig. 4b). In contrast, the AAPH-caused cell death was dose-dependently reduced (1.3 and 1.1 fold) by the adding of X-T-R (12.5 and 25 µg/mL), comparing to the only AAPH-treated group (Fig. 4b). These results showed that X-T-R can protect zebrafish from cellular damage caused by AAPH.

3.5 The Effect of X-T-R Against AAPH-Treated on mRNA Expression Levels of Associated Genes

To confirm the effect of X-T-R on the expression levels of apoptosis-related molecules such as Bcl-2, Bax, caspase 3, caspase 9 and p53 in AAPH-treated zebrafish model, we performed real time-PCR (Fig. 5). As shown in Fig. 5, the expression levels of pro-apoptotic molecules such as Bax, caspase 3, caspase 9 and p53 were highly increased by treatment of AAPH. However, they were significantly reduced by treated with X-T-R (12.5 and 25 µg/mL) in a dose dependently manner compared with control group. Interestingly, the level of Bcl-2 decreased by treatment of AAPH was up-regulated by the treatment of X-T-R (12.5 and 25 µg/mL), compared with the only AAPH-treated group. The result indicates that X-T-R protects zebrafish embryos against oxidative stress via the regulation of apoptosis-related molecules in AAPH-treated zebrafish.

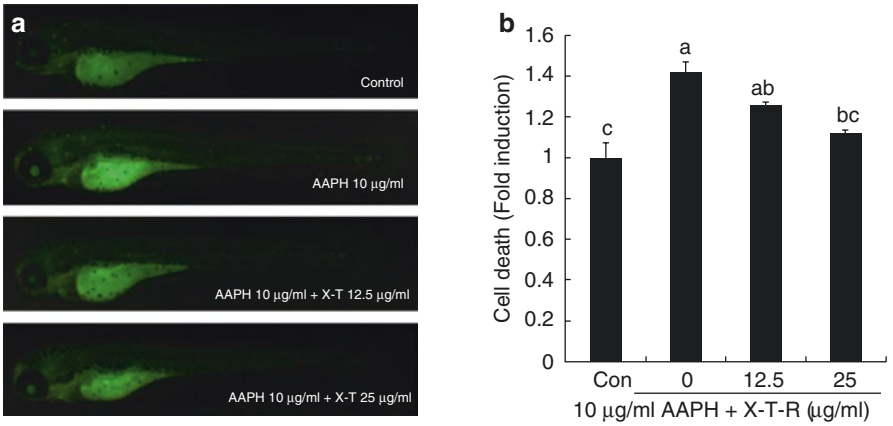

Fig. 4 Protective effect of X-T-R against AAPH-induced cell death in zebrafish larvae. (**a**) the ROS production and (**b**) the image analysis. Experiments were performed in triplicate and the data are expressed as mean ± SE

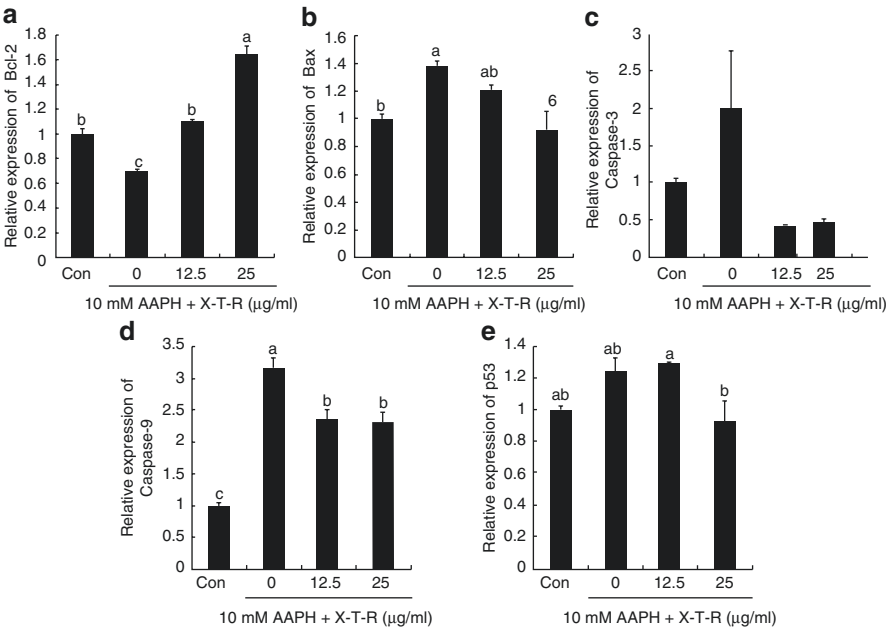

Fig. 5 The effects of X-T-R against AAPH-treated regulation of apoptosis on mRNA expression levels of Bcl-2 (**a**), Bax (**b**), caspase3 (**c**), caspase9 (**d**) and p53 (**e**). Experiments were performed in triplicate and the data are expressed as mean ± SE

4 Discussion

In the present study, we investigated the protective effect of Xylose-Taurine reduced (X-T-R) which is a taurine derivate against oxidative stress in AAPH-treated zebrafish embryo *in vivo* model. We first revealed that X-T-R improved the survival rate via the inhibition of ROS and NO generations and cell death in AAPH-treated zebrafish *in vivo* model. Moreover, we indicated that the regulation of apoptosis-related molecules plays important roles as a biological mechanism for its protective effects.

Normally, AAPH treatment can cause oxidative stress with the production of free radicals including ROS and NO and cells death and finally affects to the reduced survival rate in cells and zebrafish models (Kang et al. 2012). The over generations of ROS and NO lead to cellular damages or death in cells and tissue and indirectly affects to the development of various human diseases such as skin diseases, cancer, inflammation, rheumatoid arthritis and neurodegenerative diseases and so on (Kang et al. 2012; Choi et al. 2002; Shibata et al. 2008). So, many researchers have noticed the importance of ROS removal as an essential key in various antioxidants and protectors against oxidative stress (Kumar et al. 2007). Interestingly, our data showed that X-T-R effectively improved the mortalities reduced by the treatment of AAPH, an inducer of free radicals and ROS in zebrafish embryo models as well as the reduction of ROS generation. Recent our study also observed the cytoprotective capacity of X-T-R such as the improvement of cell viability and the reduction of ROS against oxidative stress in human liver cells (Park et al. in press). In addition, previous reports have indicated the considerable evidence that taurine contents can act as a direct or indirect antioxidant by scavenging ROS including free radicals (Niittynen et al. 1999). With these points, our study indicates that X-T-R, a taurine derivate has the important value as a ROS scavenger in cells and *in vivo* models.

As well as the over generation of ROS, when NO is abnormal over generated, it subsequently brings about cytotoxicity, cellular damages and tissue injury under pathological condition (Kim et al. 1999). Recently, the inhibitory capacities of taurine and its derivatives on the generation of NO in primary cultured mouse mammary epithelial cells as well as LPS-stimulated mouse macrophages have been reported (Marcinkiewicz et al. 2006; Miao et al. 2012). Our result also demonstrated the inhibitory effect of X-T-R on the NO generation and the induction of cell death as well as the inhibition of ROS generation in AAPH-treated zebrafish model. With the reports, we suggest that X-T-R protected zebrafish embryos against cell death via the inhibition of ROS and NO generations.

Oxidative stress is associated to apoptosis responses which are programmed cell death and the apoptotic phenomenon plays an important role in embryogenesis, metamorphosis and cellular homeostasis (Renehan et al. 2001). There are apoptotic molecules such as Bcl-2, an anti-apoptotic molecule and caspase-3 and -9, P53 and Bax, pro-apoptotic molecules (Kang et al. 2012; Park et al. in press). At the early

stage of apoptosis, the levels of pro-apoptotic molecules are increased in cells and zebrafish models (Kang et al. 2012; Park et al. in press). In contrast, an anti-apoptotic protein is responsible for the inhibition of cell apoptosis and cell death (Adachi et al. 2004; Kang et al. 2012). In the present study, we discovered that the pre-treatment with X-T-R down-regulated the expression levels of Bax, p53 and cleaved caspase-3 and -9, whereas up-regulated the expression level of Bcl-2. These results suggested that X-T-R protects zebrafish embryos against AAPH-induced apoptosis via modulating the expression levels of apoptosis molecules.

5 Conclusion

In conclusion, the findings of this study demonstrate that X-T-R improved the survival rate of zebrafish embryos as inhibiting the ROS and NO generations and the cell death via modulating the expressions of apoptotic molecules. These results also provide the therapeutic or preventive potential of X-T-R in the treatment of various diseases associated with oxidative stress.

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Anti-inflammatory Effects of Galactose-Taurine Sodium Salt: A Taurine Derivate in Zebrafish In Vivo Model

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Abstract Taurine, the plentiful amino acids in mammalian cells exerts various biological activities including antioxidant and anti-inflammatory effects. Inflammation can cause several diseases such as cancer, heart disease, rheumatoid arthritis and immune system reactions. Here, we investigated anti-inflammatory effects of Galactose-Taurine sodium salt (Gal-Tau), a newly synthesized taurine derivate in LPS-stimulated zebrafish embryos *in vivo* model. The result showed that G**al**-Tau improved the survival rate and the edema in LPS-treated zebrafish embryos. Also, Gal-Tau effectively reduced the productions of nitric oxide (NO), reactive oxygen

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species (ROS) and cell death induced by LPS in zebrafish embryos. In addition, Gal-Tau regulated the expression levels of inflammatory mediators such as inducible NOS (iNOS) and cycloxygenase 2 (COX-2) as well as IL-6 and TNF-α, inflammatory cytokines known as important key mediators of inflammation. Taken together, this study first indicates that Gal-Tau could be considered as an effective anti-inflammatory material with its anti-inflammatory activity.

Keywords Galactose-Taurine sodium salt • Anti-inflammatory effect • Macrophages • Zebrafish

Abbreviations

Gal-Tau	Galactose-Taurine sodium salt
LPS	Lipopolysaccharide
NO	Nitric oxide
PGE_2	Prostaglandin E2
iNOS	Inducible NO synthase
COX-2	Cyclooxygenase-2

1 Introduction

Zebrafish (*Danio rerio*) have well-developed innate and acquired immune systems that very similar to the mammalian immune system (Trede et al. 2001). Recently, adult zebrafish and embryos have been used to study on assessment of bioactivities against various human diseases. Recently, zebrafish animal model is used as rapid and simple methods to assess the anti-inflammatory effect on the LPS-related inflammation *in vivo*.

Inflammatory responses are related to the progression and of several diseases. In response to inflammatory stimuli, macrophages release various pro-inflammatory mediators and cytokines, although it is an important defense mechanism to remove the harmful stimuli. In initial state, macrophages increase nitric oxide (NO) and reactive oxygen species (ROS) production and it is widely accepted that the overproductions of NO and ROS are the important key of cellular events leading to the inflammatory process as well as prostaglandin E_2 (Park et al. 2009). Also, NO is an inorganic free radical that is implicated in pathological processes such as chronic and acute inflammation (Salerno et al. 2002). NO is occurred by the oxidation of L-arginine, catalyzed by NO synthase (NOS). Among the NOS family, iNOS (iNOS), in particular, is involved in the pathological overexpression of NO, and it can be expressed in response to pro-inflammatory agents such as TNF-α (tumor necrosis factor α) and lipopolysaccharide (LPS) in various cell types, including

macrophages. Therefore, effective inhibitions of both NO and ROS production as well as these inflammatory mediators have been interested in the prevention and/or treatment of diseases associated with chronic inflammatory conditions.

Taurine (2-aminoethane sulfonic acid) is a free amino acid and can be synthesized from other dietary sulfur-containing amino acids such as cysteine, endogenous, methionine and exists in most animal tissues (Sturman 1993). Also, previous studies have reported that taurine plays an important role in several essential biological activities such as anti-oxidant (Stapleton and Bloomfield 1993), anti-apoptotic (Zhang et al. 2010), calcium modulation (Takahashi et al. 1992), membrane stabilization on lymphoblastoid cells (Pasantes-Morales et al. 1985), and immune function (Sturman 1993). However, Taurine has disadvantages as the followings; a rapid renal extraction rate and poor absorption. With these disadvantages, many researchers have tried to develop several taurine derivatives with beneficial functions (Cho et al. 2014).

In the present study, we confirmed the anti-inflammatory effects of galactose-taurine sodium salt (Gal-Tau), a synthetic material derived from taurine, by measuring survival rate and edema in LPS-stimulated zebrafish embryo model. We also examined that ROS and NO productions, cell death and pro-inflammatory cytokines expression (IL-6, IL-1β, and TNF-α), as well as the expression of inflammatory mediators (iNOS and COX-2) in LPS-stimulated zebrafish embryo model.

2 Methods and Methods

2.1 Origin and Maintenance of Parental Zebrafish

Adult zebrafish were acquired from a commercial dealer (Seoul aquarium, Seoul, South Korea) and maintained with a 14/10 h light/dark cycle at 28.5 ± 1°C. The feeding was conducted twice times a day (Tetra GmbH D-49304 Melle Made in Germany). The embryos used for experiments were collected through natural spawning (containing media) in the morning (On set of light).

2.2 Preparation of LPS-Induced Inflammatory Zebrafish Model and Application of Gal-Tau

Gal-Tau was synthesized and offered by prof. Kim at Konkuk University. The embryos (n = 15) were transferred to individual wells of 12-well plates containing 900 μL of embryo media at 7–9 hpf (hour post-fertilization), Gal-Tau was added to the wells. After the incubation 1 h, a LPS solution (10 μg/mL) was treated to the embryo exposed with Gal-Tau for up to 24 hpf. Finally, the embryos were rinsed using fresh embryo media for the observation.

2.3 Measurement of the Survival Rate

To examine the effects of Gal-Tau on the survival rates, zebrafish embryos (n = 15) were treated with Gal-Tau (from 12.5 to 50 μg/mL) at 7–9 hpf and the number of surviving zebrafish embryos was monitored daily until 7 dpf (day post-fertilization). For the next experiments, we used Gal-Tau at the nontoxic concentrations.

To test the effects of Gal-Tau on the survival rates in LPS-treated zebrafish embryos, the embryos (n = 15) were applicated with Gal-Tau (12.5 and 25 μg/mL) at 7–9 hpf for 1 h and treated with LPS. The number of surviving zebrafish within each group was monitored daily until 7 dpf.

2.4 Measurement of Edema

To examine the effects of Gal-Tau on the edema, zebrafish embryos (n = 15) were treated with Gal-Tau (12.5 and 25 μg/mL) at 7–9 hpf and the edema of zebrafish embryos was monitored at 2 dpf. For the next experiments, we used Gal-Tau at the nontoxic concentrations.

2.5 Measurement of Intracellular ROS Production and Image Analysis

At 3 dpf, the zebrafish larvae were transferred to 24-well plate, treated with 20 μg/mL of DCFH-DA solution (2,7-dichlorodihydrofluorescin diacetate) and reacted for 1 h in the dark at 28.5 ± 1 °C. Before observation and photographed under the microscope CoolSNAP-Pro color digital camera (Olympus, Japan), The zebrafish larvae were rinsed using fresh embryo media and anaesthetized by 0.002% MS222 (ethyl 3-aminobenzoate methanesulfonate). A fluorescence intensity of individual larva was investigated using the image J program.

2.6 Measurement of NO Production and Image Analysis

At 3 dpf, the zebrafish larvae were transferred to each group in 24-well plates, treated with 5 μM of DAF-FM-DA (diaminofluorophore 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate) and incubated for 2 h in the dark at 28.5 ± 1 °C. The rest of the procedure was as described in Sect. 2.5.

2.7 Measurement of Cell Death and Image Analysis

At 3 dpf, the zebrafish larvae were transferred to each group in 24-well plates, treated with acridine orange solution (7 μg/mL) and incubated for 30 min under the dark at 28.5 ± 1 °C. The rest of the procedure was as described in Sect. 2.5.

2.8 Measurement of Inflammatory Mediator Expressions

The embryos (n = 50) were transferred to individual groups of 6-well plates containing 2700 μL of embryo media from approximately 7–9 hpf, Gal-Tau (12.5 and 25 μg/mL) was added to the wells. After the incubation 1 h, a LPS solution (10 μg/mL) was treated to the embryo exposed with Gal-Tau for up to 24 hpf. Then, embryos were rinsed using fresh embryo media. At 3 dpf, embryos were transferred into eppendorf tube, and then washed twice for fresh media. The zebrafish were homogenized in trizol reagent using a homogenizer. Total RNA was isolated by TRIzol reagent (Ambion) according to the manufacture's instruction. The oligo (dT)-based first strand cDNA was synthesized from 1 μg of total RNA concentration using Transcriptor first strand cDNA synthesis kit (Roche). RCR reactions were performed using LightCyler 480 SYBR green 1 master kit (Roche) according to the manufacture's instruction. The expression levels of genes were estimated using LightCycler 480 system (Roche). The level of expression was measured with the crossing point (Cp) method implemented by LightCycler 480 software. The sequences of primer sets used were: iNOS sense, 5′-GAGCAGGCCCAATGCATTT-3′ and antisense, 5′-TGCGCTGCTGCCAGAAAC-3′; COX-2 sense, 5′-TGCGGATCAAACTTGAGC-3′ and antisense, 5′-TCGGCAGGCTCATCCTTATTGGTGAGACTA-3′; TNF-α sense, 5′-ACAAGGCAATTTCACTTCCA-3′ and antisense, 5′-AGCTGATGTGCAAAGACACC-3′; IL-6 sense, 5′-TCAACTTCTCCAGCGTGATG-3′ and antisense, 5′-TCTTTCCCTCTTTTCCTCCTG-3′; IL-1β sense, 5′-TTGTGGGAGACAGACAGTGC-3′ and antisense, 5′-GATTGGGGTTTGATGTGCTT-3′; β-actin sense, 5′-GCTGACAGGATGCAGAAGGA-3′ and antisense, 5′-TAGAAGCATTTGCGGTGGAC-3′.

2.9 Statistical Analysis

All results are expressed as means ± SE, and all statistical comparisons were made by means of one-way analysis of variance followed by Duncan's test using PASW Statistics 21.0 software (SPSS, Chicago, IL, USA). A p-value <0.05 was considered to be statistically significant.

3 Results

3.1 Gal-Tau Improved the Survival Rate in LPS-Treated Zebrafish Embryos

First of all, to determine effect of Gal-Tau on the toxicity of zebrafish embryos, we examined the survival rate. We additionally checked the effect of Gal-Tau on survival rate in LPS-stimulated zebrafish embryos. In Fig. 1a, we identified that the survival rate was markedly reduced by the exposure of LPS stimulation in zebrafish embryos. Interestingly, it was improved by the pre-treatment of Gal-Tau (12.5 and 25 μg/mL) in a dose-dependent manner, comparing to the only LPS-stimulated zebrafish embryos. But, the higher concentration of Gal-Tau showed the toxic effect, we used the 12.5 and 25 μg/mL of Gal-Tau for the next experiments. This result indicates that Gal-Tau protects zebrafish embryos against death caused by LPS stimulation.

3.2 Gal-Tau Inhibited the Formation of Yolk Edema in LPS-Stimulated Zebrafish Embryos

We examined the effect of Gal-Tau on the formation of yolk edema caused in LPS-stimulated zebrafish embryos. When zebrafish embryos were exposed to LPS-stimulation, the formation of yolk edema was markedly increased in zebrafish embryos comparing with the non-treated zebrafish embryos (Fig. 1b). However, the increased yolk edema was recovered by the pre-treatment of Gal-Tau at all used concentrations, compared to the only LPS-stimulated zebrafish embryos. Form the result, we found out the protective effect of Gal-Tau against the yolk edema in zebrafish embryos.

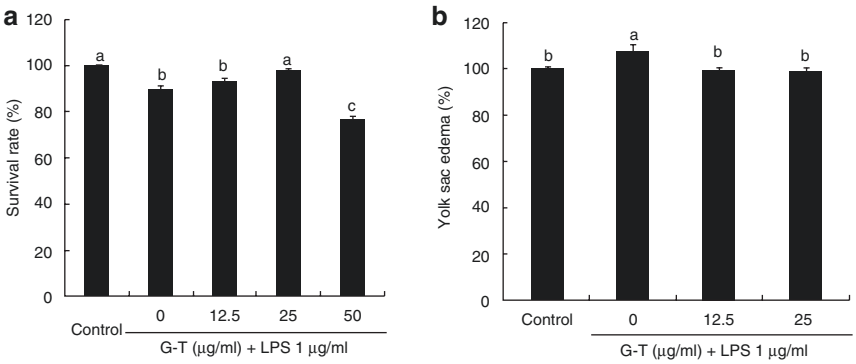

Fig. 1 Effect of Gal-Tau on survival rate (**a**) and yolk edema (**b**) in LPS-treated zebrafish larvae. These values are expressed as average value and SE through triplicate experiments

3.3 *Protective Effect of Gal-Tau Against LPS-Induced ROS Production in Zebrafish Embryos*

To measure the effects of Gal-Tau on ROS generation, we carried out the DCFH-DA fluorescence staining assay. As shown in Fig. 2a, b, the LPS stimulation markedly caused the increased ROS production in zebrafish embryos, compared to the non-treated zebrafish embryos. In contrast, Gal-Tau significantly decreased the ROS levels produced by the exposure to LPS, compared to the only LPS-treated zebrafish embryos. This result indicates that Gal-Tau can inhibit the ROS productions in zebrafish embryo model

3.4 *Gal-Tau Decreased the Production of NO in LPS-Stimulated Zebrafish Embryos*

To elucidate the effect of Gal-Tau on the production level of NO of LPS-stimulated zebrafish embryos, the staining assay using a fluorescent probe dye, DAF-FM DA were performed. In zebrafish embryos, the exposure to LPS stimulation highly increased the fluorescence intensity based on the NO production level up to approximately 136% (Fig. 3a, b). Interestingly, the 25 μg/mL of Gal-Tau considerably inhibited the NO production levels with the reduction of the detected fluorescence intensity, compared to the only-LPS-stimulated zebrafish embryos. But, the lower concentrations of Gal-Tau did not affect the NO production in zebrafish embryo

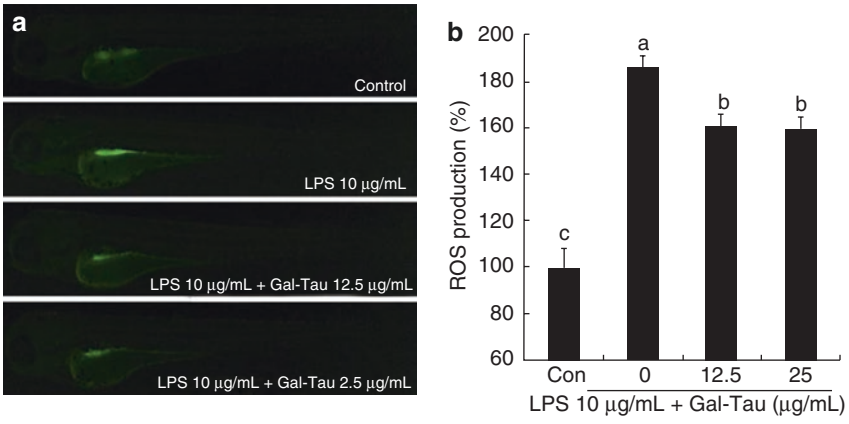

Fig. 2 Effect of Gal-Tau on the ROS production (**a**) and the image analysis (**b**) in LPS-treated zebrafish embryos. Zebrafish embryos were pretreated with Gal-Tau for 1 h and then exposed to LPS stimulation for up to 24 hpf. [a,b]The bars with different letters represent significant differences ($p < 0.05$). These values are expressed as average value and SE through triplicate experiments (n = 30)

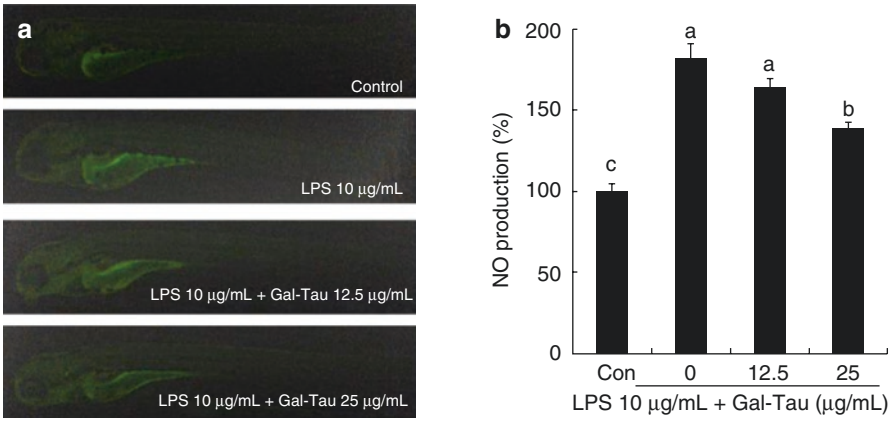

Fig. 3 Effect of Gal-Tau on the NO production (**a**) and the image analysis (**b**) in LPS-treated zebrafish embryos. Zebrafish embryos were pretreated with Gal-Tau for 1 h and then exposed to LPS stimulation for up to 24 hpf. [a,b]The bars with different letters represent significant differences ($p < 0.05$). These values are expressed as average value and SE through triplicate experiments (n = 30)

after the exposure of LPS stimulation. This result suggests that Gal-Tau can inhibit the inflammation response generating by abnormal NO production.

3.5 Gal-Tau Protected Zebrafish Embryos Against Cell Death Caused by LPS Stimulation

The protective effect of Gal-Tau on cell death was examined by the acridine orange staining assay in LPS-stimulated zebrafish embryos. After the LPS stimulation, the fluorescence intensity of cell death was increased up to approximately 118% at 3 dpf of zebrafish embryos, compared to the non-treated zebrafish embryos (Fig. 4a, b). In contrast, the increased cell death induction was decreased by the pre-treatment of Gal-Tau (25 μg/mL), compared to the only-LPS-stimulated zebrafish embryos. This result indicates that Gal-Tau protects zebrafish embryos against the induction of cell death as well as the productions of ROS and NO caused LPS stimulation.

3.6 Inhibitory Effect of LPS-Induced iNOS and COX-2 Protein Expression by Gal-Tau

In the present study, we also investigated the expression of iNOS and COX-2 protein to confirm the effects of Gal-Tau on inflammatory mediator expressions. As shown in Fig. 5a, b, the expressions of iNOS and COX-2 proteins were markedly

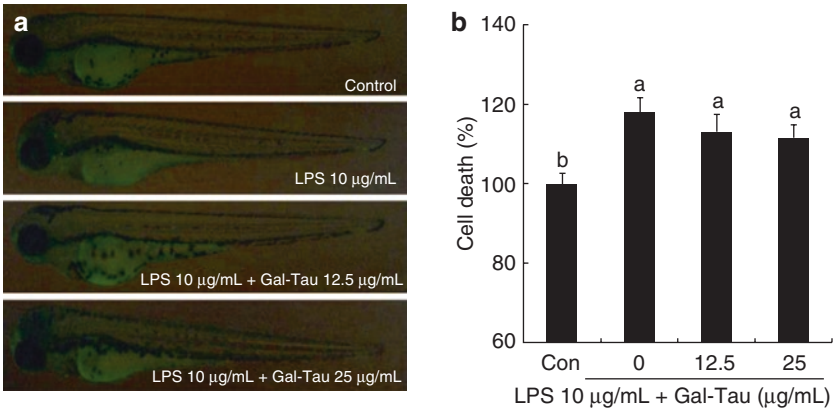

Fig. 4 Effect of Gal-Tau on the cell death (**a**) and the image analysis (**b**) in LPS-treated zebrafish embryos. Zebrafish embryos were pretreated with Gal-Tau for 1 h and then exposed to LPS stimulation for up to 24 hpf. [a,b]The bars with different letters represent significant differences ($p < 0.05$). These values are expressed as average value and SE through triplicate experiments (n = 30)

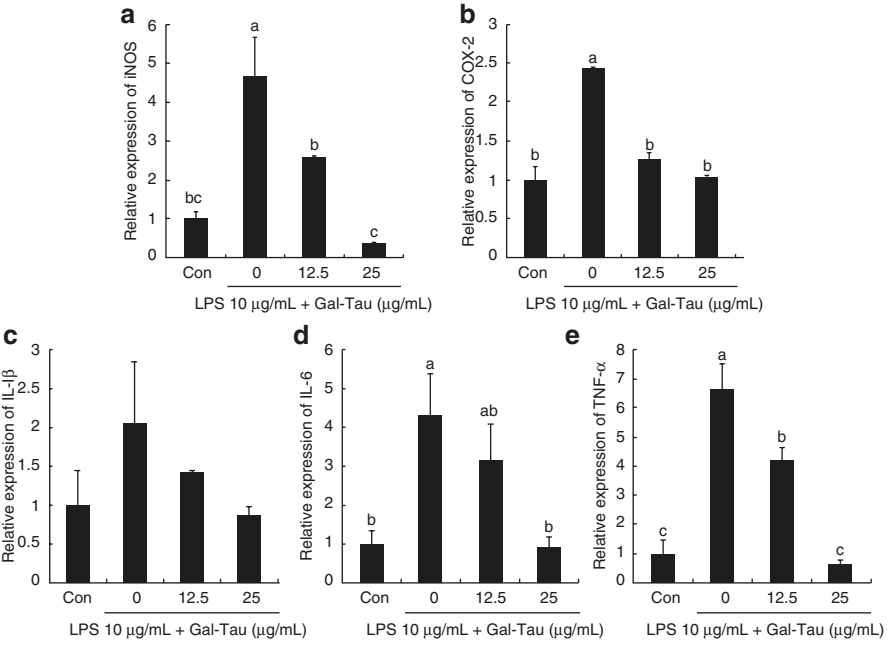

Fig. 5 Effect of Gal-Tau on the expression levels of iNOS (**a**), COX-2 (**b**), IL-1β (**c**), IL-6 (**d**) and TNF-α (**e**) in LPS-treated zebrafish embryos. Zebrafish embryos were pretreated with Gal-Tau for 1 h and then exposed to LPS stimulation for up to 24 hpf. [a,b]The bars with different letters represent significant differences ($p < 0.05$). These values are expressed as average value and SE through triplicate experiments (n = 50)

increased after LPS treatment. However, Gal-Tau treatment significantly suppressed the rise in the expression of iNOS and COX-2 protein in a dose-dependent manner.

3.7 Effects of Gal-Tau on LPS-Induced TNF-α, IL-1β, and IL-6 Expression Levels

To determine the effects of Gal-Tau on the expression levels of pro-inflammatory cytokines containing IL-1β, IL-6 and TNF-α, the zebrafish embryos were incubated with Gal-Tau in the presence or absence of LPS (10 μg/mL), and the pro-inflammatory cytokine levels were measured by real time PCR. All the mRNA levels of IL-6 and TNF-α were increased by treatment of LPS, however these increases were markedly decreased in a concentration dependent manner by treatment with Gal-Tau (Fig. 5).

4 Discussion

Zebrafish embryo can be applied as new *in vivo* animal model to rapidly and simply assess the anti-inflammatory materials (Park and Cho 2011). Recently, our study has reported that Gal-Tau inhibited the production of NO and the expression levels of the inflammatory mediators and cytokines in LPS-stimulated macrophages (Kang et al. in press). In the present study, we suggested the anti-inflammatory effect of Gal-Tau synthesized from taurine on the survival rate, yolk edema, production of ROS and NO, cell death, and expression of inflammatory mediators and cytokines in LPS-treated zebrafish embryos *in vivo* model.

Stimulation of LPS leads to the prolonged and profound production of NO in macrophages and zebrafish embryos that can cause cytotoxicity and tissue injury under pathological condition as well as amplification of inflammation (Kim et al. 1999; Tripathi et al. 2007). The abnormal NO production can attribute to overproduction of ROS production from activated macrophages and zebrafish model (Conforti et al. 2008; Kang et al. in press). The overproduction of ROS causes cell or tissue injury related to degenerative diseases including inflammation by attacking several biological molecules in cells and zebrafish models (Choi et al. 2002; Shibata et al. 2008). Therefore, the suppression of both NO and ROS productions and cell death provides a potential strategy for the development of beneficial anti-inflammatory materials. In this study, we have shown that the levels of NO and ROS increased by the stimulation of LPS were inhibited with the application of Gal-Tau and it finally affected to the reduced yolk edema as well as the improved survival rate in zebrafish embryo models. Interestingly, this was supported by our previous results that Gal-Tau controlled the production of NO in LPS-activated macrophages (Kang et al. in press). In addition, Gal-Tau inhibited the expression levels of iNOS and COX-2 under the LPS stimulation in zebrafish embryos and cells (Kang et al. in press). It has

been demonstrated that LPS-increased iNOS and COX-2 expressions affected to the over-productions of NO and PGE_2 (Posadas et al. 2000). These results indicate that the modulation of Gal-Tau on the overproduced NO and PGE_2 leads to the reduced expression levels of inflammatory mediators, iNOS and COX-2, after the stimulation of LPS.

Moreover, LPS, a strong immune activator is known to elicit produce of inflammatory cytokines including TNF-α, IL-1β, IL-6, and IL-8, which trigger the production of NO and ROS (Higashimoto et al. 2006; Yoon et al. 2010). In this study, we confirmed that the expression levels of TNF-α and IL-6 were inhibited by Gal-Tau in zebrafish embryos after LPS stimulation. In similar, recent our study demonstrated that the productions of TNF-α and IL-6 were does-dependently inhibited by Gal-Tau in LPS-stimulated macrophages. Furthermore, several researchers reported that taurine and its derivatives have the inhibitory capacities against the generation of inflammatory cytokines such as TNF-α, IL-1β and IL-6 as well as inflammatory mediators, NO, ROS and PGE_2 in LPS-stimulated mouse macrophages as well as zebrafish embryos (Marcinkiewicz et al. 2006; Miao et al. 2012). With these results, this suggest that taurine and its derivatives such as Gal-Tau can decrease NO and ROS secretion via down-regulating the expression of pro-inflammatory cytokines as well as inflammatory mediators, and be a potent molecule in regulation of the inflammation-related responses.

5 Conclusion

Taken together, we revealed that Gal-Tau has the anti-inflammatory effects via the inhibition of NO and ROS production, and cell death as well as the expression levels of inflammatory mediators and cytokines in LPS-stimulated zebrafish embryo *in vivo* model. Also, this study suggests that Gal-Tau can be a potential material for therapy and prevention of inflammation-related to diseases.

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Effect of N-(D-Ribopyranosyl) Taurine Sodium Salt on the Differentiation of Human Preadipocytes and Expression of Adipokines Through Inhibition of STAT-3 Signaling in Differentiated Human Adipocytes

Kyoung-Soo Kim and Sung Hoon Kim

Abstract We investigated whether a taurine-ribose derivative, N-(D-ribopyranosyl) taurine sodium salt, inhibits the differentiation process of preadipocytes or modulates the expression of cytokines from adipocytes as does taurine chloramine (TauCl) in vitro. To know the inhibitory effects of taurine-ribose (Tau-Ribose) on differentiation process and adipokine expression, preadipocytes were incubated with Tau-Ribose in differentiation medium for 14 days. Differentiated adipocytes were also stimulated at the concentration of IL-1β 1 ng/ml with addition of Tau-Ribose. After 7 days of incubation, the levels of adiponectin, leptin, IL-6, and IL-8 were measured from the culture supernatants. At concentrations of 10–40 mM, Tau-Ribose dose-dependently inhibited the process of adipogenesis. The treatment of Tau-Ribose decreased the expression of transcription factors, which are necessary for adipogenesis and are known as adipocyte marker. Treatment with Tau-Ribose significantly modulated the production of IL-8 and IL-6. However, it did not modulate the production of adiponectin and leptin in IL-1β-activated adipocytes. As with taurine chloramine, Tau-Ribose also inhibited STAT-3 signaling, independent of MAPK signaling. In conclusion, Tau-Ribose inhibits the signaling pathway of

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STAT-3 and can change adipokines production; thus, it may have a potential as an agent for treating obesity-related diseases.

Keywords Taurine derivatives • Adipokines • Adipogenesis • STAT-3 signalling

Abbreviations

TauCl	Taurine chloramine
Tau-Ribose	Taurine-ribose derivative
TauT	Taurine transporter

1 Introduction

Taurine, which is an abundant free intracellular amino acid in animal cells, seems to contribute to be involved in numerous physiological and biological functions for health benefits due to its unique chemical structure. Thus, taurine gives cytoprotective property to tissues and also control various cellular functions such as antioxidation, ion movement, osmoregulation, modulation of neurotransmitters, and conjugation of bile acids (Kim et al. 2013c). During inflammation, taurine is changed to taurine chloramine (TauCl) and taurine bromamine through halogenation in phagocytes. TauCl is formed by a combination with hypochlorite (HOCl) which is produced by the halide-dependent myeloperoxidase system in activated neutrophils. Activated neutrophils releases TauCl after apoptosis and then the released TauCl is known to suppresses the expression of inflammatory mediators, such as superoxide anion, tumor necrosis factor (TNF)-α, nitric oxide (NO), interleukins, and prostaglandins, in inflammatory cells of inflammatory tissues (Kim and Cha 2014). Furthermore, we previously reported that the adipogenesis was significantly inhibited by TauCl. It was suggested that TauCl may have the potential as an agent for treating obesity-associated diseases (Kim et al. 2013a). We also showed that it affects the production of cytokines in adipocytes under inflammation through inhibition of the signaling pathway of STAT-3 in adipocytes (Kim et al. 2013b).

Even though TauCl has various beneficial effects, it has not been easily developed into a therapeutic drug because of its unstable properties. Thus, we tried to develop taurine derivatives that have effects similar to TauCl but are more stable. To develop nontoxic and stable taurine derivatives, taurine was conjugated to carbohydrates such as D-glucose, D-xylose, D-galactose, D-arabinose, D-lyxose, and D-ribose. The taurine-carbohydrates showed anti-adipogenic effects similar to those of TauCl (Cho et al. 2014). In this study, we study whether a taurine-ribose derivative (Tau-Ribose), N-(D-Ribopyranosyl)taurine sodium salt, shows the same effect and utilizes the same molecular mechanism as TauCl.

2 Methods

2.1 Cell Culture and Adipogenesis

Human preadipocytes, which were commercially available, were seeded into six-well plates (1.5×10^5 cell/2 ml of medium) or 60-mM dishes (2.5×10^5cell/2 ml of medium/dish) and incubated until confluent. The confluent cells were incubated in medium for adipogenesis with or without Tau-Ribose for 14 days, with media changes every 2 days, as indicated previously (Kim et al. 2013a).

2.2 Oil Red O Staining

Oil Red O staining was used to quantitate lipid accumulation in cells as indicated previously (Kim et al. 2013a). In brief, cells after culture were washed twice with PBS and fixed in 10% (v/v) formaldehyde for 60 min. The cells were washed three times with ddH_2O and stained with Oil Red O. The dye from the cells was dissolved with isopropanol.

2.3 Reverse Transcription Polymerase Chain Reaction

cDNA was made from 1 μg total RNA of cells in a 20 μl reaction mixture and were amplified with the specific primers for semiquantitative PCR using TaKaRa systems (TaKaRa Bio, Kyoto, Japan), as described previously (Kim et al. 2013a).

2.4 Western Blotting

The cells for the analysis of cell signaling pathway were starved in basal medium for 12 h and incubated with IL-1β. After 120 min, the activated cells were then lysed at time points for the analysis of cell signaling proteins. Cell lysates were processed as previously indicated (Kim et al. 2013b).

2.5 ELISA

The level of IL-8, IL-6, adiponectin, and leptin were analyzed by an ELISA (R&D Systems, Inc., USA) as indicated previously (Kim et al. 2013b).

2.6 Statistical Analysis

The mean ± standard error of the mean (SEM) of quadruplicate samples was used for data. GraphPad Prism 4 software was used for statistical analysis and graphing. The Mann–Whitney test was used for comparing the differences between groups. Differences were considered significant at $P < 0.05$.

3 Results

3.1 Effect of Tau-Ribose on Adipogenesis

To study the inhibitory effects of Tau-Ribose on adipogenesis of human adipocytes, preadipocytes were incubated in differentiation medium for 14 days with or without Tau-Ribose. The preadipocytes differentiated into adipocytes in the absence of Tau-Ribose (Fig. 1a), and this adipogenesis was dose-dependently inhibited by

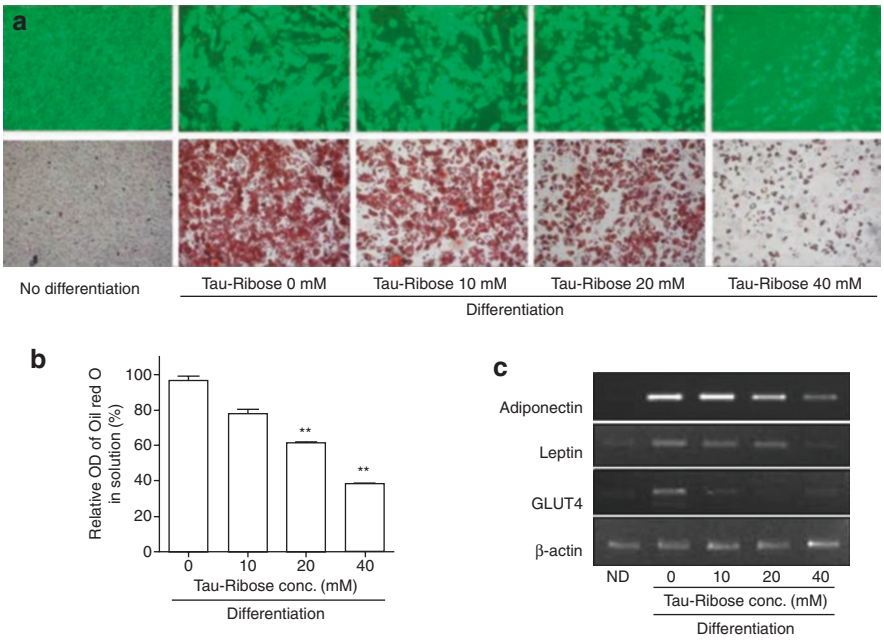

Fig. 1 Effect of Tau-Ribose on adipogenesis of human preadipocytes. Human preadipocytes were cultured in growth medium of six-well plates. For cell differentiation, the cells were incubated in differentiation medium with or without Tau-Ribose at different concentrations for 2 weeks, with media changes every 2 days. (**a**) Photos of adipocytes with oil droplet before (*top row*) and after (*bottom row*) Oil Red O staining. (**b**) Quantification of the dye retained in adipocytes. (**c**) RT-PCR of adipogenic gene expression. Independent experiments in triplicate were performed three times. The data are obtained from three independent experiments. Data are expressed as the mean ± standard error of the mean (SEM). $^{**}P < 0.01$ versus no treatment

Tau-Ribose. Also, the differentiated adipocytes accumulated intracellular lipid. The oil droplets of the cells were stained by Oil Red O. According to the degree of adipogenesis, the staining degree of Oil Red O was dose-dependently decreased by Tau-Ribose (Fig. 1b). Tau-Ribose at 40 mM completely blocked fat droplet formation. MTT assays were conducted for the toxic effects of Tau-Ribose. Cell viability was not deteriorated by 100 mM Tau-Ribose for 14 days. It suggests that cytotoxicity of Tau-Ribose inhibited the adipogenesis and lipid accumulation (data not shown).

3.2 Tau-Ribose Affects the Cytokine Expression from Inflammatory Adipocytes

To know whether Tau-Ribose affects the expression of adipokines from inflammatory adipocytes, the adipocytes after adipogenesis were activated with IL-1β (1 ng/ml) and Tau-Ribose, and cultured for 7 days to test if it affects the expression level of adiponectin, leptin IL-6, and IL-8. Tau-Ribose treatment at concentrations from 0 to 80 mM significantly and dose-dependently inhibited the increase in IL-6 expression in adipocytes stimulated with IL-1β (Fig. 2). However, it didn't dose-dependently inhibit the

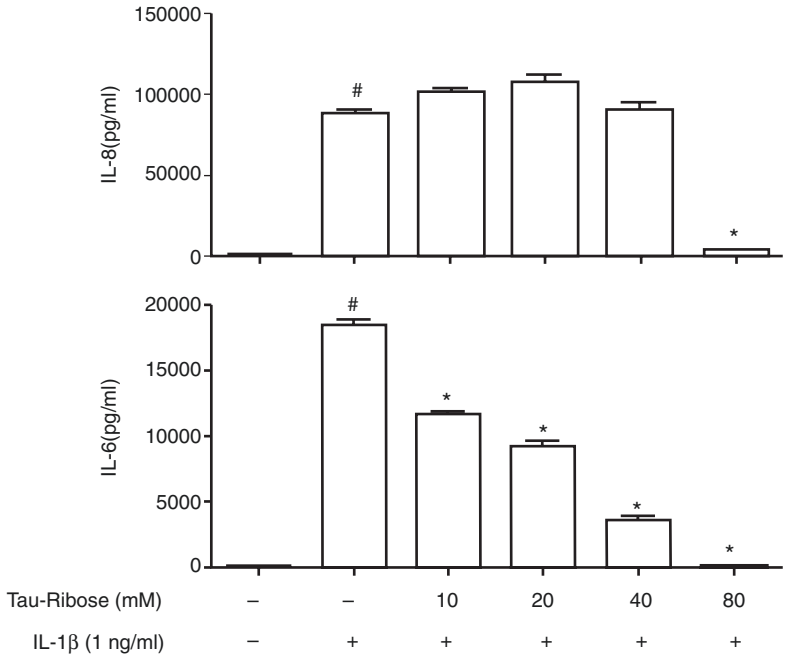

Fig. 2 Comparative effect of Tau-Ribose on the production of IL-6 and IL-8 in IL-1β-activated differentiated adipocytes. The adipocytes were incubated in basal media for 7 days at various combined conditions of Tau-Ribose and inflammation. Expression levels of two cytokines were analyzed by ELISA. After independent experiments in quadruplicate were performed three times, values are expressed as the mean ± SEM. $^{\#}P < 0.05$ versus no stimulation. $^{*}P < 0.05$ no treatment with Tau-Ribose in stimulated adipocytes

elevated production of IL-8. At 80 mM Tau-Ribose, the increase in IL-8 expression was completely inhibited. In contrast, Tau-Ribose treatment at concentrations of 0–80 mM didn't significantly reverse the production of leptin and adiponectin in IL-1β-activated adipocytes (data not shown). These results indirectly indicate that Tau-Ribose is specifically involved in modulating the production of cytokines, IL-6 and IL-8 through inhibition of signaling pathways in IL-1β-stimulated adipocytes.

3.3 Tau-Ribose Specifically Suppresses the Signaling Pathway of STAT-3 in IL-1β-Stimulated Adipocytes

To show if Tau-Ribose affects the signaling pathways in IL-1β-activated adipocytes, IL-1β-stimulated adipocytes were lysed to analyze the activation level of the signaling pathways of ERK, P-38, JNK, IκB, and Akt, as previously described (Kim et al. 2013b). Tau-Ribose did not significantly inhibit the signaling pathways of MAPK in IL-1β-activated adipocytes as TauCl affected MAPK signaling, suggesting that Tau-Ribose doesn't affect adipokine expression levels through the signaling pathways of MAPK and PI3K/Akt in adipocytes (Fig. 3a). We next

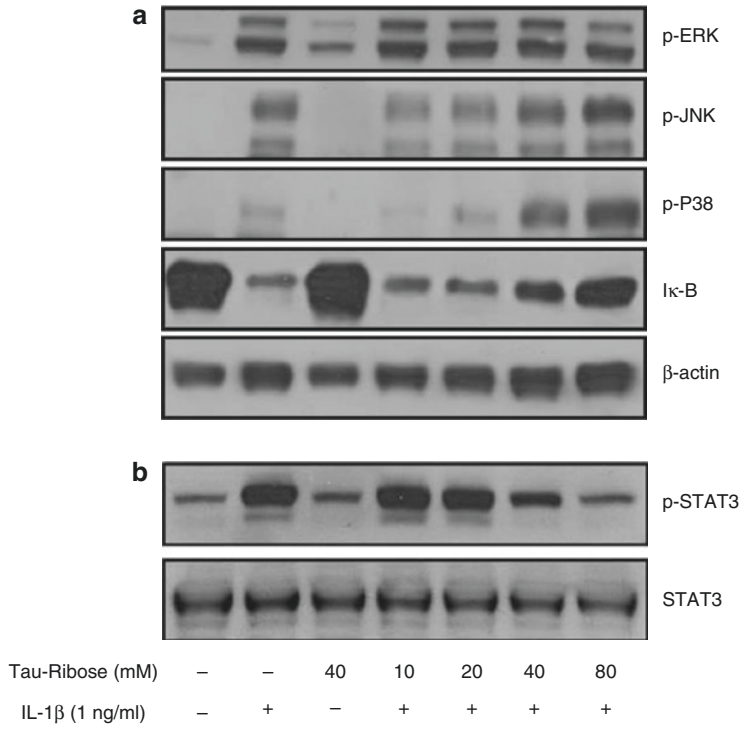

Fig. 3 Effect of Tau-Ribose on the signal pathways in IL-1β-activated adipocytes. (**a**) Adipocytes in the presence or absence of Tau-Ribose were activated with IL-1β for 45 min. (**b**) Adipocytes with or without Tau-Ribose were stimulated with IL-1β for 90 min. The data were obtained from three independent experiments

evaluated if Tau-Ribose inhibits the signaling pathway of STAT-3 (Fig. 3b). As shown in Fig. 3b, while the signaling pathways of MAPK were stimulated at 45 min after stimulation, phosphorylated STAT-3 was upregulated after 90 min of stimulation. In contrast, Tau-Ribose greatly inhibited the level of phosphorylated STAT-3 in a dose-dependent manner. It suggests that Tau-Ribose modulates the production level of IL-6 and IL-8 in IL-1β-activated adipocytes by inhibiting the STAT-3 signaling pathway.

4 Discussion

We investigated if Tau-Ribose affects the production of adipokines in differentiated adipocytes. As the adipocytes were activated with IL-1β, the production levels of leptin and adiponectin decreased, while the levels of IL-8 and IL-6 increased. TauCl dose-dependently inhibited this increase in IL-8 and IL-6 in a previous study (Kim et al. 2013b). The decreased expression levels of adiponectin and leptin were reversed by TauCl in IL-1β-stimulated differentiated adipocytes. However, in the current study, Tau-Ribose significantly inhibited IL-6 expression in a dose-dependent way, while significant inhibition of IL-8 expression required 80 mM Tau-Ribose. In addition, decreased expression of adiponectin and leptin was not reversed by Tau-Ribose, suggesting that the mode of action of Tau-Ribose is different from that of TauCl. In particular, a physiological level of TauCl (200–600 μM) is effective in inhibiting inflammation in tissue. In contrast, Tau-Ribose is effective at concentrations of 20–80 mM. This effective concentration is about 100-fold higher than that of TauCl. This finding also indirectly indicates that Tau-Ribose has a different mode of action. Taurine passes through its transporter, and TauCl also seems to pass through the taurine transporter because chloride is a small molecule compared to taurine. However, Tau-Ribose is not thought to pass through the taurine transporter (TauT). The molecular weights of taurine and ribose are 125.1 and 150, respectively, indicating that Tau-Ribose is capable of being transported through the ribose transporter. Furthermore, it may be transported through other monosaccharide transporters such as the glucose transporter (Naula et al. 2010). However, the transporters require high specificity; consequently, Tau-Ribose may not be easily transported through these transporters. Thus, higher concentrations of Tau-Ribose relative to TauCl may be needed in order for Tau-Ribose to be effective. To partly answer the question of whether Tau-Ribose passes through the taurine transporter or ribose transporter, our future studies will test Tau-Ribose in TauT knockout cells (Ito et al. 2010). If TauCl has no an anti-inflammatory effect in the TauT knockout cells, it may indirectly indicate that TauCl may pass through only the TauT. If Tau-Ribose shows any physiological effect in TauT knockout cells, Tau-Ribose may pass through not TauT but a monosaccharide transporter.

5 Conclusion

Tau-Ribose, which shares the ability to depress the STAT-3 signal pathway with TauCl, can affect the production level of adipokines and inhibit the differentiation of preadipocytes into adipocytes during adipogenesis. Thus, it may have the potential to be developed as a therapeutic agent for obesity-related diseases.

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Synthesis of N-Chloroamino Acids and Their Biological Activities in LPS Stimulated RAW 264.7 Cells

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Abstract Amino acids (AAs) are essential for protein synthesis, neurotransmission and macro molecule biosynthesis. Ala, Gln, Gly, Lys, Val and taurine (Tau) are the most abundant free AAs in mammals, and some of these react with hypochlorite ($HOCl/OCl^-$) produced by myeloperoxidase in activated phagocytes to form N-chloroamino acids (NCAA). In this study, we reacted 20 AAs and Tau with sodium hypochlorite (NaOCl), then classified the products into five types (I–V) based on the change in their absorbance. Type I AAs (Ala, Arg, Gln, Gly, Ile, Lys, Phe, Ser, Tau, Thr and Val) generated a typical monochloramine peak at 252 nm, while Type II AAs (Asn and Tyr) and Type III AAs (Glu and Leu) produced peaks at 275 nm and 225 nm, respectively. The Type IV AAs (His, Met and Trp) did not show any distinct absorption peak, and Type V AAs (Asp, Cys and Pro) did not appear to react with NaOCl. The ArgCl and TauCl were stable, while GlnCl, GlyCl, IleCl, LysCl, PheCl and ValCl were less stable and AlaCl, SerCl and ThrCl were the least stable. Tau is the most abundant non-proteinogenic free AA in cellular fluid and has many physiological functions in the nervous, cardiovascular, renal and immune systems. Tau reacts with HOCl to form TauCl, which inhibits the production of proinflammatory mediators such as superoxide, nitric oxide (NO) and interleukins, while increasing the antioxidant proteins in macrophages. We determined the effects of Type I NCAA on cell viability, NO and TNF-α production in LPS-activated RAW 264.7 cells. All Type I NCAA showed dose-dependent cytotoxicity and inhibited LPS-induced NO production. However, only GlnCl, GlyCl, IleCl, LysCl, SerCl and TauCl inhibited LPS-induced TNF-α production. In summary, Type I NCAA showed dose-dependent cytotoxicity and inhibited NO production,

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while their effects on TNF-α varied. Our results suggest that Type I NCAA may serve as biological regulators similar to TauCl during inflammation.

Keywords Amino acids • N-chloroamino acids • Taurine • Taurine chloramine • Inflammation

Abbreviations

AAs	Amino acids
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MPO	Myeloperoxidase
MTT	3-(4,5 Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCAA	N-chloroamino acids
NO	Nitric oxide
PBS	Phosphate buffered saline
PGs	Prostaglandins
RT	Room temperature
Tau	Taurine
TauCl	Taurine chloramine
TNF	Tumor necrosis factor
UV	Ultraviolet

1 Introduction

All animal cells require amino acids (AAs) for growth, reproduction, and maintenance of their physiological functions. AAs are either ingested (essential AAs) or synthesized endogenously (non-essential AAs) (Miyagi et al. 2011). Among the 500 AAs (Wagner and Musso 1983), only 22 are responsible for the protein structure and function (Kawashima and Kanehisa 2000), and these serve as substrates for protein synthesis (Kraemer et al. 2009). Plasma free AAs circulate to link organ systems and play important roles in metabolism, and serve as targets for biochemical modification of metabolic reactions (Miyagi et al. 2011). Although abundance varies depending on separation methods, Ala, Gly, Glu/Gln and Val are most abundant in normal human blood (Lewis et al. 1980), while Ala, Gln, Lys, Pro and Val were high in plasma (Bergström et al. 1990; Canepa et al. 2002). Inter-organ transport of free AAs by the blood involves different distributions between the blood

cells and plasma (Lobley et al. 1996), and the intracellular AAs patterns in the three cellular (plasma, RBC and PMN) compartments are qualitatively similar (Canepa et al. 2002).

Hypochlorous acid (HOCl/OCl^-) produced by myeloperoxidase (MPO) in activated phagocytes is a major bactericidal factor that serves as a chemical defense against infectious pathogens (Robaszkiewicz et al. 2008). Indeed, bacteria exposed to HOCl are killed within milliseconds (Schraufstätter et al. 1990). HOCl directly oxidizes a variety of biological molecules, including carbohydrates, nucleic acids, peptide linkages, AAs, and lipids (Marquez and Dunford 1994). Moreover, HOCl reacts with cellular free AAs to generate their oxidation or chlorination products. It has been reported that most AAs react rapidly with HOCl (Alexander and Gough 1951). Thus, AAs present in cells are potential targets of oxidation or chlorination by HOCl (Robaszkiewicz et al. 2008). N-monochloroamino acids are formed when the ratio of AA to HOCl is 1:1 or higher. When hypochlorous acid is present in excess, N,N′-dichloroamino acids are generated (Robaszkiewicz et al. 2008). HOCl can also react with the carbon–carbon double bonds of fatty acyl groups in phospholipids to produce chlorohydrins (Carr et al. 1996).

Taurine (2-aminoethane sulfonic acid, Tau) is one of the most abundant non-proteinogenic free AAs present in mammalian tissues (Marquez and Dunford 1994). Tau produced from cysteine by decarboxylation and oxidation is considered to be an essential AA for felines and a conditionally indispensable AA for humans and non-human primates (Schuller-Levis and Park 2003). Tau readily reacts with HOCl to form TauCl, which is more stable and less toxic than HOCl. TauCl modulates the production of many pro-inflammatory mediators, including nitric oxide (NO), superoxide anions, tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8 and prostaglandins (PGs) (Kim and Cha 2014).

In this study, we reacted 20 proteinogenic AAs and Tau with HOCl, classified the products into five types (I–V) based on changes in their absorbance characteristics, and then determined the cytotoxicity and biological effects of Type I products on LPS-induced production of the inflammatory mediators, NO and TNF-α.

2 Methods

2.1 Antibodies and Reagents

Dulbecco's modified eagle medium (DMEM), characterized fetal bovine serum (FBS), penicillin, streptomycin and phosphate buffered saline (PBS) were purchased from Hyclone (Logan, UT, USA). Cell culture dishes and 96-well plates were purchased from SPL (Pocheon, South Korea). Duoset mouse TNF-α ELISA kit was obtained from R&D systems (Minneapolis, MN, USA). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.2 Synthesis of N-Chloroamino Acids

AAs were mixed with equimolar amounts of sodium hypochlorite (NaOCl) solution as previously described (Marcinkiewicz et al. 1995; Kim and Kim 2005). The production of N-chloroamino acids (NCAA) and presence of unreacted HOCl/OCl^- were monitored by measuring the ultraviolet (UV) absorption spectra (200–400 nm) before and after the reaction. The concentrations of synthesized NCAA were determined after reaction by the molar extinction coefficient 415 M^{-1} cm^{-1} at 252 nm. NCAA were kept at 4°C for 2–3 days, during which time their stability was determined by measuring the absorbance at 252 nm.

2.3 RAW 264.7 Cell Culture

The murine macrophage cell line, RAW 264.7 cells (ATCC, Manassas, VA, USA), were grown in DMEM containing 10% FBS, 100 units/ml of penicillin and 100 μg/ml of streptomycin at 37°C in 5% CO_2.

2.4 Measurement of Cell Viability by MTT Assay

To determine the cytotoxicity of Type I NCAA on RAW 264.7 cells, we determined their effects on viability using the conventional MTT reduction assay. Briefly, cells (2×10^4) were incubated with Type I NCAA (0.2, 0.5 and 0.7 mM) for 24 h at 37°C in 5% CO_2. Thereafter, 10 μl of 2 mg/ml 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well. After 3 h of incubation with MTT at 37°C, media were removed and the cells containing MTT formazan were dissolved by adding dimethyl sulfoxide. The absorbance of the reduced MTT at 570 nm was then measured using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA) and the associated Softmax software.

2.5 Measurement of Nitric Oxide Production

The amount of NO present in the phenol red free conditioned culture media was determined using Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylethyenediamine and 2.5% phosphoric acid) as previously described (Park et al. 1993, 1995). Briefly, 100 μl of the conditioned media from RAW 264.7 cells (1×10^5/well) stimulated with 1 μg/ml LPS and NCAA for 24 h were reacted with an equal volume of Griess reagent for 10 min at room temperature (RT), after which the absorbance was read at 550 nm. NO concentration was calculated from a sodium nitrite standard curve.

2.6 Measurement of TNF-α Production

The TNF-α concentration in the culture media of LPS-treated RAW 264.7 cells was measured using Duoset mouse TNF-α ELISA kit as previously described (Kang et al. 2014). Briefly, 96-well immune-plates (Capitol Scientific, Austin, TX, USA) were coated with rat monoclonal antibody against murine TNF-α overnight at RT, then blocked for 1 h with 1% bovine serum albumin. Plates were subsequently washed three times with PBS containing 0.05% Tween 20. TNF-α standards (0–2000 pg/ml) and samples were added to each well and incubated for 2 h at RT. Biotinylated antibodies against TNF-α were subsequently added and samples were incubated for 2 h at RT. The plate was then developed with streptavidin-HRP for 20 min, after which the substrate, 3,3′,5,5′-tetramethylbenzidine (TMB) solution was added. The reaction was stopped with 2 M H_2SO_4 and the absorbance was measured at 450 nm.

3 Results and Discussion

3.1 Synthesis of N-Chloroamino Acids

The reaction was performed by mixing equimolar amounts of each AA with NaOCl, and the formation of reaction product was monitored by scanning their UV absorption. The products formed were classified into five types (I–V) based on their UV absorption spectra (Fig. 1). The Type I AAs (Ala, Arg, Gln, Gly, Ile, Lys, Phe, Ser, Tau, Thr and Val) have a typical monochloramine peak at 252 nm as was observed with TauCl (Thomas et al. 1986), while the type II AAs (Asn and Tyr) and Type III AAs (Glu and

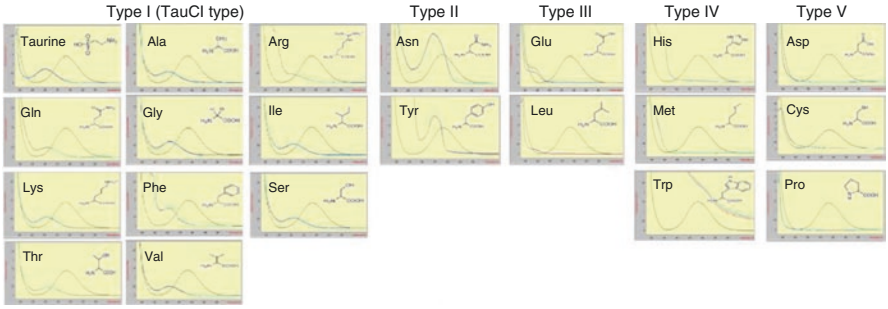

Fig. 1 Synthesis of NCAA and their classification. NCAA were synthesized by mixing equimolar amounts of AAs with NaOCl and classified into five types (Type I–V) based on the absorption spectrum. Type I AAs produced a typical monochloramine peak at 252 nm, while Type II and III generated peaks at 275 nm and 225 nm, respectively. Type IV did not show any new distinct absorption peaks, and Type V did not appear to react with NaOCl. Type I NCAA were referred to as TauCl type. The results shown are representative of three independent experiments

Leu) produced peaks at 275 nm and 225 nm, respectively. The Type IV AAs (His, Met and Trp) reacted with NaOCl resulting in disappearance of the NaOCl peak, but did not show any new distinct absorption peaks, and Type V AAs (Asp, Cys and Pro) did not appear to react with NaOCl. The ArgCl and TauCl were stable, while GlnCl, GlyCl, IleCl, LysCl, PheCl and ValCl were less stable, and AlaCl, SerCl and ThrCl were the least stable. The concentrations of Type I NCAA were calculated using the molar absorption coefficient of N-monochloroamine, 415 M^{-1} cm^{-1} at 252 nm.

The formation of several chlorination products of AAs has been reported by several groups. TauCl and GlyCl were prepared by mixing taurine or glycine with HOCl at a molar ratio of 5:1 in Hank's balanced salt solution, and the presence of a fivefold excess of AA ensured that only monochloramine and no dichloramine was formed (Midwinter et al. 2006). Robaszkiewicz et al. (2008) reported the synthesis of AlaCl, AspCl, LysCl, PheCl and SerCl by reacting AAs with HOCl at a ratio of 5:1. Although dependence of the reaction rates on pH has been suggested (Armesto et al. 1993; Na and Olson 2007), we did not consider it in the current study. We reacted AAs with NaOCl at equimolar ratios based on our previous studies (Kim and Kim 2005; Piao et al. 2011).

3.2 Cytotoxicity of the Type I NCAA

We examined the cytotoxic effects of Type I NCAA on RAW 264.7 cells by the MTT reduction assay. Type I NCAA inhibited cell survival in a dose-dependent manner (Fig. 2). TauCl (0.2 mM) inhibited 59% of cell survival, while other Type I

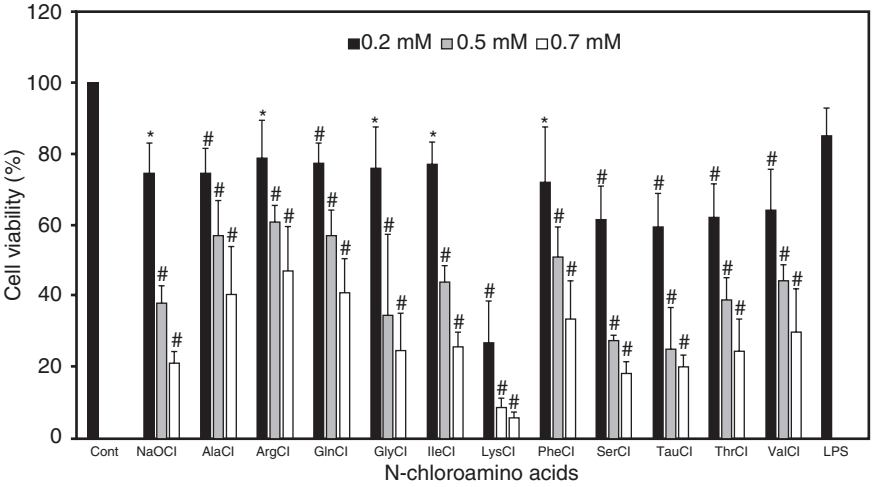

Fig. 2 Effects of Type I NCAA on cell viability. RAW 264.7 cells were incubated with Type I NCAA for 24 h and the viability was determined by MTT reduction. The results shown are the means ± SD of three independent experiments. #$p < 0.01$ and *$p < 0.05$ compared to control

NCAA showed the cytotoxicity (Fig. 2). These results are inconsistent with those of our previous study in that 1.0 mM TauCl showed a decrease, but non-significant MTT reduction and no apoptosis in RAW 264.7 cells (Kim and Kim 2005). We have no clear explanation for this discrepancy; accordingly, further studies to clarify this are warranted. Treatment of A549 cells with AlaCl, LysCl, SerCl and AspCl showed concentration-dependent cytotoxicity, with AspCl having the highest cytotoxicity (Robaszkiewicz et al. 2010). However, further investigation is necessary to see if the cytotoxic effects of Type I NCAA including TauCl are associated with changes in mitochondrial membrane potential and induction of apoptosis.

3.3 Type I NCAA Inhibited LPS-Induced NO Production in RAW 264.7 Cells

TauCl at non-cytotoxic doses was previously shown to inhibit the LPS and interferon-γ induced production of NO in murine macrophages (Kim and Kim 2005). In this study, LPS (1 μg/ml) stimulation enhanced NO production, and all of the Type I NCAA showed inhibition of LPS-induced NO production at cytotoxic dose in RAW 264.7 cells (Fig. 3). These findings suggest that Type I NCAA can play a role in inflammatory processes by inhibiting NO production. In our previous studies, TauCl inhibited LPS-induced inducible nitric oxide synthase (iNOS) expression and NO production in RAW 264.7 cells and murine peritoneal macrophages (Kim et al. 1996, 2005). However, 0.7 mM TauCl was not cytotoxic in those studies. These findings suggest that NO inhibition was greatly influenced by the cytotoxicity

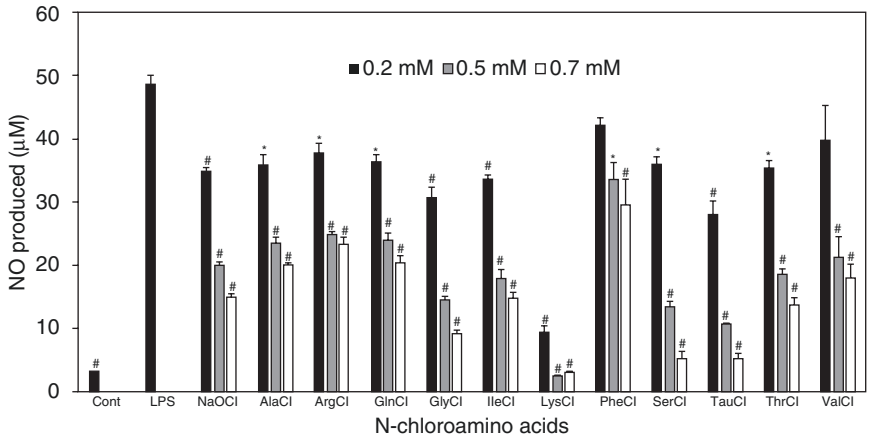

Fig. 3 Effects of Type I NCAA on NO production in RAW 264.7 cells. Cells were incubated for 24 h and NO production was measured by Griess reaction. The results shown are the means ± SD of three independent experiments. #$p < 0.01$ and *$p < 0.05$ compared to LPS

of Type 1 NCAA, and further study is necessary to determine the reason for the discrepancy between the results of the current study and previous studies.

3.4 Type I NCAA Inhibited LPS-Induced TNF-α Production in RAW 264.7 Cells

TNF-α is a prominent pro-inflammatory mediator produced by activated immune cells that induces further production of several other pro-inflammatory mediators, including NO, IL-6, IL-8, PGs and TNF-α itself (Marcinkiewicz and Kontny 2014; Kim and Cha 2014). Release of TNF-α was inhibited by TauCl in a dose-dependent manner, similar to the inhibition on NO production (Kim et al. 1996). We determined the effects of Type I NCAA on TNF-α production in RAW 264.7 cells. Cells activated with LPS-produced TNF-α, while treatment with GlnCl, GlyCl, IleCl, LysCl, SerCl and TauCl inhibited TNF-α production and other Type 1 NCAA had little or no effect on TNF-α production (Fig. 4).

4 Conclusion

We reacted 20 proteinogenic AAs and taurine with NaOCl. The AAs were classified into five types based on their reactivity and the absorbance characteristics of products. The Type I NCAA showed a typical monochloroamine spectrum similar to TauCl. In addition, these NCAA inhibited cell viability of RAW 264.7 cells and NO

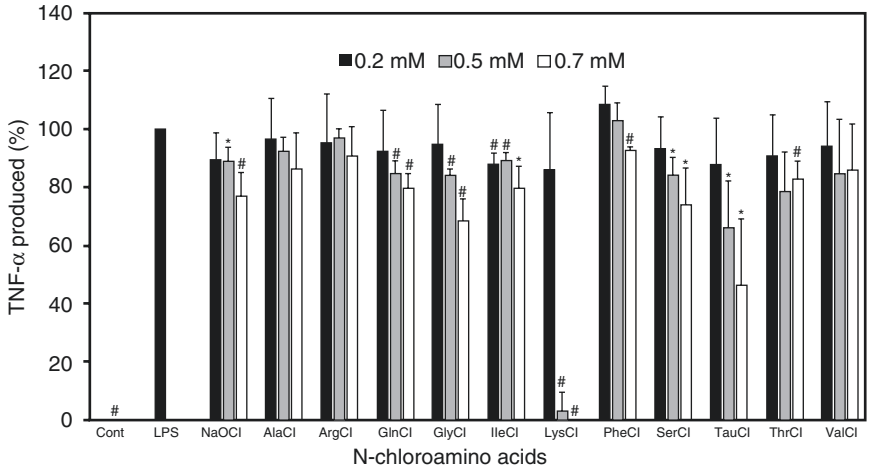

Fig. 4 Effects of Type I NCAA on TNF-α production in RAW 264.7 cells. Cells were incubated for 24 h and TNF-α production was determined. The results shown are the means ± SD of three independent experiments. #$p < 0.01$ and *$p < 0.05$ compared to LPS

production in LPS-activated RAW 264.7 cells in a dose-dependent manner. Moreover, GlnCl, GlyCl, IleCl, LysCl, SerCl and TauCl inhibited TNF-α production. These results suggest that NCAA could be produced by the MPO in phagocytic cells upon inflammation and modulate the production of inflammatory mediators such as NO and TNF-α, thereby regulating the inflammatory process.

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Part VI
Effects of Taurine on Organ Disorders

Taurine Promotes Retinal Ganglion Cell Survival Through $GABA_B$ Receptor Activation

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Abstract Retinal ganglion cell (RGC) degeneration occurs in numerous retinal diseases, either as a primary process like in glaucoma, or secondary to photoreceptor loss and no efficient compound targeting directly RGC neuroprotection is yet available. We previously described that taurine exerts a direct protective effect on RGCs cultured under serum-deprived conditions. Because taurine was known to have an agonist-like activity for GABA/glycine receptors, we investigated here if the taurine-elicited neuroprotective effect may be mediated through the activation of these receptors using selective antagonist ligands. RGCs were purified, seeded in 96-well plate and maintained in culture during 6 days *in vitro*. Viable cells were labelled with calcein and densities in full-well area were then automatically counted. Here we show that the protective effect of taurine against RGC loss observed under

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serum deprivation can be mediated through the $GABA_B$ receptor stimulation. Hence, two selective agonists, including baclofen, at this metabotropic $GABA_B$ receptor were found to reproduce taurine action by enhancing RGC survival in culture. This study suggests that $GABA_B$ receptor stimulation provides direct neuroprotection for RGCs. Accordingly, drugs targeting $GABA_B$ receptor may represent a new way for the prevention of RGC degeneration.

Keywords Retinal ganglion cells • Neuroprotection • Taurine • $GABA_B$ receptors • Baclofen

1 Introduction

Taurine, the common name of 2-amino-ethanesulfonic acid, is a free amino-sulfonic acid which is present in large amounts in the central nervous system (Brosnan and Brosnan 2006). It is even the most abundant amino-acid in the retina, showing higher content than glutamate, and reaching up to 50 mmol/g tissue in rat (Froger et al. 2014). Taurine is mainly provided by nutrient intake, although endogenous synthesis can occur in most of species (Huxtable 1989). The exogenous supplying of taurine is dependent of the taurine transporter (Tau-T) function (Liu et al. 1992), which specifically uptakes taurine from the extracellular to intracellular compartments. When taurine is uptaken into the intracellular compartment, it exerts powerful antioxidant properties, through the neutralization of reactive oxygen species production (Froger et al. 2014). This intracellular function at the mitochondrial level may support the neuroprotective role of taurine. However, cellular action of taurine also involve its capacity to modulate both the glycinergic the GABAergic neurotransmissions. Taurine is indeed described as an enhancer of $GABA_A$ and glycine channels, and as an agonist at $GABA_B$ metabotropic receptors (Albrecht and Schousboe 2005).

Retinal ganglion cells (RGCs) are spiking neurons which send visual information to the brain through the optic nerve (Roska and Werblin 2001). Degeneration of these neurons occurs in different retinal diseases, either as primary process like in glaucoma (Quigley 1999), or secondary to the photoreceptor loss, like in *Retinitis pigmentosa* (Humayun et al. 1999). The taurine depletion was found dramatically harmful for both photoreceptor and RGC survival (Jammoul et al. 2009, 2010). On other hand, taurine supplementation can prevent RGC degeneration in animal models of glaucoma (Froger et al. 2012). Such neuroprotective efficacy of taurine was observed *in vitro* in purified RGCs maintained in culture under deprived conditions (Froger et al. 2012, 2013).

The cellular mechanisms underlying the taurine dependence of RGC survival still remains unclear. However, the requirement of Tau-T in the maintain of photoreceptor survival, as evidenced by administering an inhibitor substrate of Tau-T in rats (Pasantes-Morales et al. 1983) or knocking-out Tau-T in mice (Rascher et al. 2004), has suggested that Tau-T may also support the neuroprotective role of taurine for RGCs. Hence, the pharmacological blockade of Tau-T induced a significant

degeneration of RGC layer in mice (Gaucher et al. 2012; Hadj-Saïd et al. 2016) indicating that intracellular antioxidant actions of taurine are crucial for RGC survival. However, the involvement of GABA and glycine signaling in the neuroprotective role of taurine was never explored. As previously mentioned, taurine is a non-selective agonist at $GABA_A$ and glycine ionotropic receptors, while it can also activate the $GABA_B$ metabotropic receptors (Albrecht and Schousboe 2005; Balse et al. 2006). Here, we investigate the role of the GABA and glycine receptors in the taurine-enhanced RGCs survival in culture (Froger et al. 2012). For this purpose, we used specific ligands to block either $GABA_A$/Glycine ionotropic receptors or $GABA_B$ metabotropic receptors, in presence of taurine. The RGC survival was evaluated with an automatic counting of viable calcein-positive RGCs.

2 Materials and Methods

2.1 Animals

Eight-week old male Long-Evans rats purchased from Janvier (Le Genest Saint-Isle, France), were used to performed primary cultures of retinal ganglion cells. Animals were housed with a 12 h dark/light cycle with food (standard diet) and water available ad libitum. All Experiments have been carried out in accordance with the European Community Council Directives of November 24th 1986 (86/609/EEC) and with the ARVO (Association for Research in Vision and Ophthalmology) statement for the Use of animals in ophthalmic and visual Research. All efforts have been made to minimize the number of animals used and their suffering.

2.2 Drugs

Taurine, Strychnine (blocker of glycine receptor), Picrotoxin (blocker of $GABA_A$ receptor), CCP35348 (antagonist at $GABA_B$ receptor), and baclofen (selective agonist at $GABA_B$ receptors) were purchased from Sigma-Aldrich (Saint-Louis, USA). SKF-97,541 (potent selective agonist at $GABA_B$ receptor) was supplied by Tocris Bioscience (Bristol, UK). Drugs were incubated directly into the neurobasal medium (see below) during the whole period of the RGC culture (6 DIV).

2.3 Pure Retinal Ganglion Cell Cultures

RGCs were isolated from retinas of adult Long-Evans rat with an immunopanning technique, according to the protocol previously described in young rats by Barres et al. (1988) and subsequently adapted on adult rats (Fuchs et al. 2005; Froger et al.

2012). Six rats were anesthetized and killed by cerebral dislocation. Their eyes were removed and placed in a solution of phosphate-buffered saline (PBS) containing 1 g/L of glucose (PBS-glucose; Invitrogen, Carlsbad, USA). The 12 retinas were isolated and rinsed in PBS-glucose and incubated in this same buffer containing 33 UI/ml of papaïn (Worthington, Lakewood, USA) and 200 UI/ml of DNase (Sigma-Aldrich) for 30 min at 37 °C. They were then rinsed in PBS-glucose, containing 0.15% ovomucoid (Roche Diagnosis, Basel, Switzerland) and 0.15% bovine serum albumin (BSA; Sigma-Aldrich) to stop the enzymatic reaction. Retinas were dissociated in this same latter buffer, supplemented with 333 UI/ml of DNase and a rabbit anti-rat macrophage (5 mg/ml; Accurate Chemical & Scientific Corporation, Westbury, USA) in three steps, using pipettes with decreasing tip diameters. The cell suspension was centrifuged at 115 × *g* during 13 min at room temperature. The supernatant was removed and cells were suspended in PBS-glucose, containing 1% ovomucoid and 1% BSA. After a second centrifugation (115 × *g*, 13 min), cells were suspended in the PBS-glucose, containing 0.02% BSA. Cell suspension was filtrated using a Sefar Nitrex mesh (48 µm, Dutscher, Brumath, France) and then incubated in a dish (Ø 150 mm), previously coated with a goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA), during 36 min at room temperature. After a vigorous shaking of the dish, the cell suspension was moved into a second dish (Ø 150 mm), previously coated with the same antibody, and incubated during 33 min at room temperature. After another vigorous shaking, the remaining cell suspension was transferred into a dish (Ø 100 mm), previously coated successively with (i) a goat anti-mouse IgM (Jackson Immunoresearch, West Grove, USA) and (ii) a medium containing a mouse anti-Thy-1 antibody, previously prepared in our laboratory from a T11D7 hybridoma cell line (ATCC, Manassas, USA). After 45 min incubation, the dish was rinsed ten times with PSB-glucose. Adherent cells remaining into the dish were RGC specifically selected by the mouse anti Thy-1 antibody. Cells were incubated with Earle's Balanced Salts Solution (EBSS; Sigma-Aldrich) containing 0.125% of trypsin (Sigma-Aldrich) for 10 min at 37 °C, in humidified atmosphere (5% CO_2). Trypsin action was blocked by adding PBS-glucose containing 30% inactive fetal bovine serum (FBS; Invitrogen) in the Earle's solution. Cells were detached by ten successive pipette flows with the mixed Earle-PBS solution, and the resulting cell suspension was centrifuged at 115 × *g* for 15 min. Pure RGCs were then suspended in Neurobasal-A (Invitrogen) supplemented with 2 mM L-glutamine (Invitrogen). As previously described, RGC purity was estimated between 92 and 98% (Froger et al. 2012). Pure RGCs were seeded in 96-well plates at an initial density of 8000 cells/well in serum-deprived conditions (Neurobasal-A + glutamine, without B27 supplement). Cells were kept in a humidified chamber (37 °C containing 5% CO_2) for 6 DIV.

2.4 Calcein Labeling

After 6 days in culture, alive cells were labeled with calceinAM (producing a green fluorescence; Invitrogen). For this purpose, cells were incubated in a calceinAM solution (1.3 µg/ml, diluted in Neurobasal-A medium) for 1 h in the incubator (humidified chamber, 37 °C, 5% CO_2).

2.5 Automated Counting of Live Cells

Calcein-positive RCG were then automatically counted after a frame per frame scanning of the full well surface (objective ×10), by using an inverted epifluorescence microscope (TiE, Nikon, Champigny-sur-Marne, France) equipped with a motorized platine and a CCD camera (Roper Scientific, Evry, France).

2.6 ATP Measurement

ATP amounts were measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, USA) which allows evaluating the cell viability. Indeed, the amounts of ATP produced by the cell directly indicate the presence of metabolically active cells. The assay is based on the mono-oxygenation of luciferin, which is catalyzed by luciferase in the presence of Mg^{2+}, ATP and molecular oxygen. The luminescent assay was performed following the instruction of the kit.

2.7 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA was extracted from freshly purified RGCs using the RNeasy plus mini kit (Qiagen, Courtaboeuf, France). Reverse transcription were made in three successive incubations (room temperature, 10 min; 42 °C, 60 min; 70 °C, 15 min) in presence of the SuperScript II (Invitrogen), the Random Hexamers (25 ng/ml; Promega, Madison, WI, USA), dNTP (500 mM; Invitrogen) and RNasin (2 U/ml; Promega). The resulting cDNA was purified with phenol/chloroforme/isoamyl mixture, precipitated with ethanol and suspended in Tris/EDTA solution (10 mM/1 mM; pH = 8.0). PCR experiments were conducted from purified cDNAs, in the presence of 200 nM of each primer (forward and reverse) for each gene amplified, 200 mM dNTP (Invitrogen), 1.5 mM $MgCl_2$ and 1.0 U Taq DNA polymerase (Invitrogen). Amplification was performed for 40 cycles (30 s, 95 °C; 45 s, 60 °C and 60 s, 72 °C) with the thermocycler, iCycler IQ system (Biorad, Marnes-la-coquette, France) and PCR products were separated by electrophoresis in 2% agarose gel, stained by ethidium bromide.

2.8 Statistical Analysis

All data are expressed as the mean ± SEM. All statistical analyses were performed using GraphPad Prism (GraphPad Prism Software, Inc. San Diego, USA). A non-parametric analysis of variance (Kruskal-Wallis) was performed and followed, in case of significance by a Dunn's post-hoc test to compared means between each groups. Differences were considered significant at $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$.

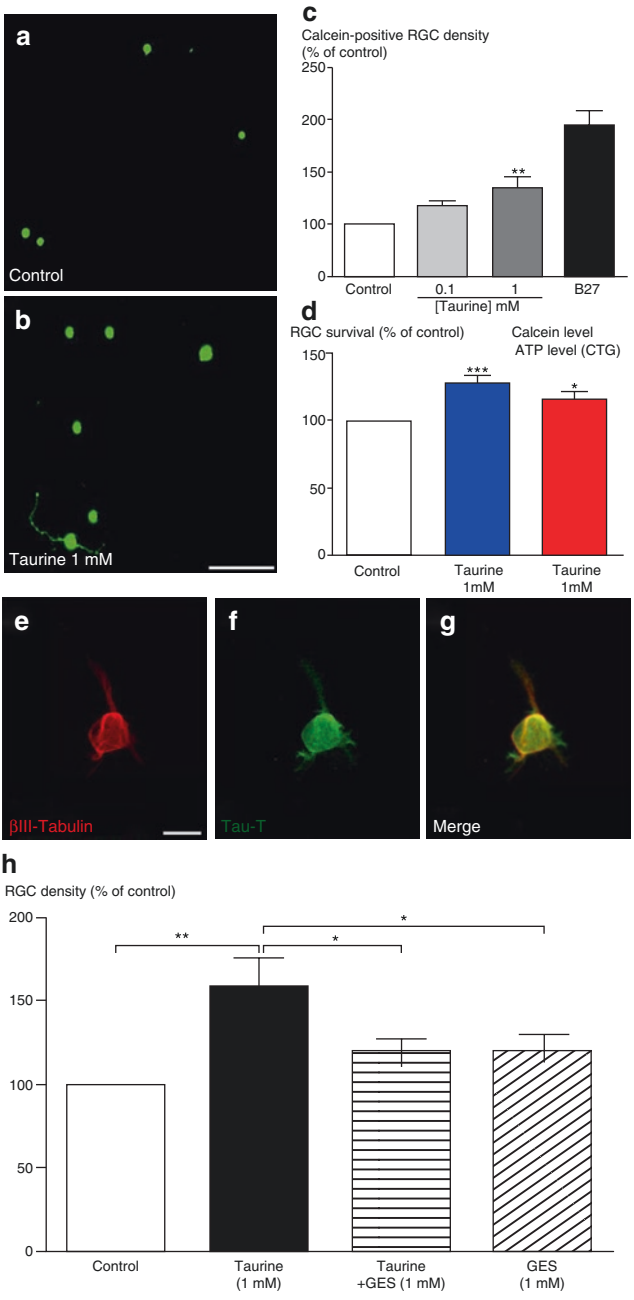
3 Results

3.1 Taurine Exerts a Neuroprotective Effect on RGCs Subjected to Serum Deprivation

The effect of taurine on RGC survival was assessed on purified RGCs (from adult rats) cultured under serum-deprivation condition. After 6 DIV, a calceinAM dye revealed a significant increase of density of viable RGCs in 1 mM Taurine added culture medium compared to the control untreated conditions by 68% ($p < 0.001$; Fig. 1a–c). Serum addition to cultured cell is considered as a positive control that increased RGC survival by 190%. A second test of viability consisting in measuring the intracellular ATP level showed that application of taurine during 6 DIV significantly increased the ATP level as compared to untreated cells (Fig. 1d). These experiments support the neuroprotective role of taurine on RGC.

Because taurine is provided to cells through the taurine transporter (Tau-T) function, we investigated the role of Tau-T in the neuroprotective action of taurine on RGC. Firstly, we controlled whether purified RGCs in culture express the taurine transporter. For this purpose, Tau-T immunostaining were performed on cultured RGCs (Fig. 1f, in green), counterstained with ßIII-tubulin (Fig. 1e, in red). Pictures show that ßIII-tubulin-positive RGGs expressed also Tau-T protein ($n = 3$ independent cultures; Fig. 1g). These data suggest that RGCs could generate a taurine uptake *in vitro*. The involvement of taurine uptake in taurine-induced RGC survival increase was investigated using a specific blocker of Tau-T: Guanidoethane sulfonate (GES). Co-incubation of GES with taurine (1 mM) for 6 DIV in RGC cultures significantly suppressed the protective effect exerted by taurine on pure RGC survival ($p < 0.05$, Fig. 1h), while the application of 1 mM GES alone did not significantly modify RGC survival as compared to untreated control conditions (Fig. 1h). These findings indicated that the protective effect of taurine on RGCs is critically dependent on the Tau-T activity.

Fig. 1 Effect of taurine on RGC survival *in vitro.* (**a**, **b**) Representative images showing calcein-positive viable RGCs, cultured for 6 days *in vitro* (DIV) under serum deprivation, in control untreated condition (**a**) or under 1 mM taurine application (**b**). (**c**) Automatic quantification of calcein-positive RGC densities after 6 DIV in the control condition, with 0.1 or 1 mM taurine application or with the B27 supplement (providing a positive control condition). In each experiment, the respective RGC densities were expressed as a percentage of the control condition (Data are means ± SEM from n = 21 independent experiments). (**d**) Comparative quantification of calcein-positive RGC densities after 6 DIV in the control condition, with 1 mM taurine application and of ATP level. In each experiment, the respective RGC densities were expressed as a percentage of the control condition (Data are means ± SEM from n = 7 independent experiments). (**e**–**g**) Representative confocal images of Tau-T immunolabelling (*green*, **f**) in ßIII-tubulin-positive RGCs (*red*, **e**) showing that Tau-T is localized with ßIII-tubulin-positive RGCs (*merge*, **g**) after 6 DIV. (**h**) The selective blockade of Tau-T function by GES (1 mM) abolished the taurine-enhanced RGC survival. Quantification of calcein-positive RGCs at 6 DIV, after incubation with 1 mM taurine alone (*black bar*), 1 mM taurine plus 1 mM GES (*horizontal hatched bar*) or GES alone (*oblique hatched bar*). In each experiment, viable RGCs were normalized with respect to control values (Data are means ± SEM from n = 9–11 independent experiments). From Froger et al. 2014


a
Control
b
Taurine 1 mM
c
Calcein-positive RGC density
(% of control)
250
200
150
100
0
**
Control
0.1
1
B27
[Taurine] mM
d
RGC survival (% of control)
Calcein level
ATP level (CTG)
150
100
50
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***
*
Control
Taurine
1mM
Taurine
1mM
e
βIII-Tabulin
f
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h
RGC density (% of control)
200
150
100
50
0
**
*
*
Control
Taurine
(1 mM)
Taurine
+GES (1 mM)
GES
(1 mM)

3.2 *$GABA_B$ Receptor Mediates the Taurine-Induced RGC Neuroprotection*

Taurine is considered as an inhibitory amino-acid, through its activity of partial agonist at glycine (Fig. 2a) and $GABA_A$ (Fig. 2b) ionotropic receptors, while taurine can also activate the $GABA_B$ metabotropic receptor (Fig. 2c).

To investigate whether the GABA and glycine receptors may be involved in the neuroprotective effects of taurine, we added into medium of cultured cells, together with taurine (i) pharmacological blockers of $GABA_A$ and glycine receptors (picrotoxin and strychnine respectively) and (ii) antagonists at $GABA_B$ receptors. A first step of experiments consisted in co-incubating taurine with both picrotoxin (10 μM) and strychnine (10 μM) to inactivate ionotropic receptors. As previously observed, application of 1 mM of taurine significantly increases the RGC survival (+58%, $p < 0.01$; Fig. 3a) as compared to control untreated cells. Addition with taurine of the mix of blockers (picrotoxin + strychnine) did not modify the neuroprotective effect of taurine (+41% of RGC survival, $p < 0.05$ as compared to untreated condition; Fig. 3a), while the incubation of the mix of blockers alone has no effect the density of viable RGC (Fig. 2a).

In a second step of experiment, taurine was co-incubated with a $GABA_B$ receptor antagonist: CGP35348 (30 μM). We found again a large increase in RGC survival in presence of 1 mM of taurine (+52%, $p < 0.01$; Fig. 3a). Addition of CGP35348 with taurine on RGC culture significantly reduces the taurine-enhanced RGC survival which reached only +20% of the basal survival ($p < 0.05$, Fig. 3b), while incubation of CGP35348 alone had not significant effect on the basal RGC survival.

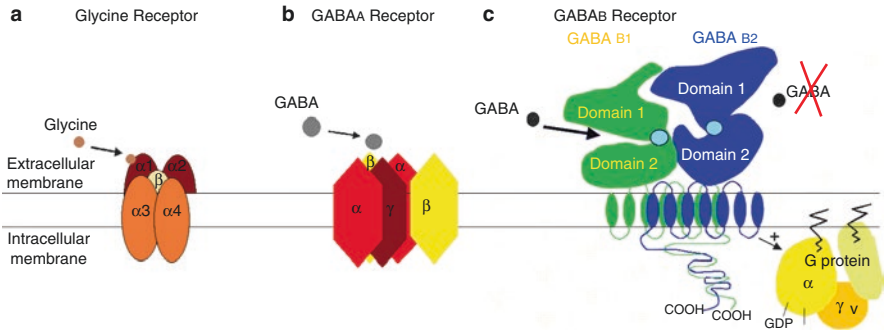

Fig. 2 Schematic structures of glycine and GABA receptors. (**a**) Scheme depicting the pentameric structure of the ionotropic glycine receptor-channel composed by four α-helical transmembrane segments (four isoforms: α1, α2, α3 and α4) and a single β-sheet subunit, surrounding a central pore. (**b**) Scheme illustrating the most common pentameric structure of the ionotropic $GABA_A$ receptor, composed by two α-subunits, two β-subunits and one γ-subunit. (**c**) Scheme representing the structure of the $GABA_B$ metabotropic receptor. It is composed by two transmembrane subunits $GABA_{B1}$ and $GABA_{B2}$ that assemble to form the heterodimeric receptor. It is believed that GABA binding at $GABA_{B1}$ subunit can activate the heterodimerization with $GABA_{B2}$, triggering of the G-protein coupling

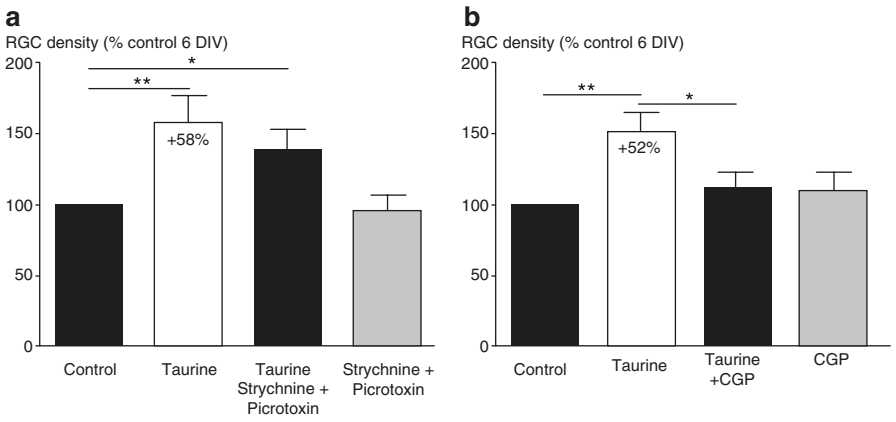

Fig. 3 Involvement of GABA/Glycine receptors in the taurine-elicited RGC survival. (**a**) Quantification of calcein-positive RGC densities after 6 DIV, in the control condition, with 1 mM Taurine alone; with Taurine + Strychnine + Picrotoxin or with Strychnine + Picrotoxin (Data are means ± SEM from n = 18 independent experiments). (**b**) Quantification of calcein-positive RGC densities after 6 DIV, in the control condition, with 1 mM Taurine alone, with Taurine + CGP or with CGP alone (Data are means ± SEM from n = 29 independent experiments). In each experiment, the respective RGC densities were expressed as a percentage of the control condition at 6 DIV

These data suggests that $GABA_B$ metabotropic receptors may be involved in the taurine-induced RGC neuroprotection.

3.3 $GABA_B$ Receptor Agonists Reproduce the Neuroprotective Effect of Taurine

In order to further investigate the neuroprotective role of the GABA-B receptor stimulation, we assessed whether incubation of $GABA_B$ receptor agonists directly into the medium of RGC cultures can affect the RGC survival *in vitro*. We used two compounds: (i) SKF-97,541, an extremely potent selective agonist at $GABA_B$ receptor and (ii) baclofen, a less potent $GABA_B$ receptor agonist, widely used as skeletal muscle relaxant.

Interestingly, addition of growing doses of SKF-97,541 (1–1000 μM) improved the RGC survival. A significant increase of alive RGC density was obtained with the dose of 10 μM ($p < 0.05$), and the efficacy of SKF-97,541 was growing with a dose-dependent manner (Fig. 4a). This neuroprotective effect of the $GABA_B$ receptor stimulation on RGC was reproduced with baclofen (100 μM) which also stimulated the RGC survival ($p < 0.01$ when compared survival of untreated cells).

In order to check the specificity of the $GABA_B$ receptor stimulation in the neuroprotective efficacy of SKF-97,541, it was co-incubated on RGC cultures with the specific antagonist CGP35348. The presence of CGP35348 (100 μM) together with

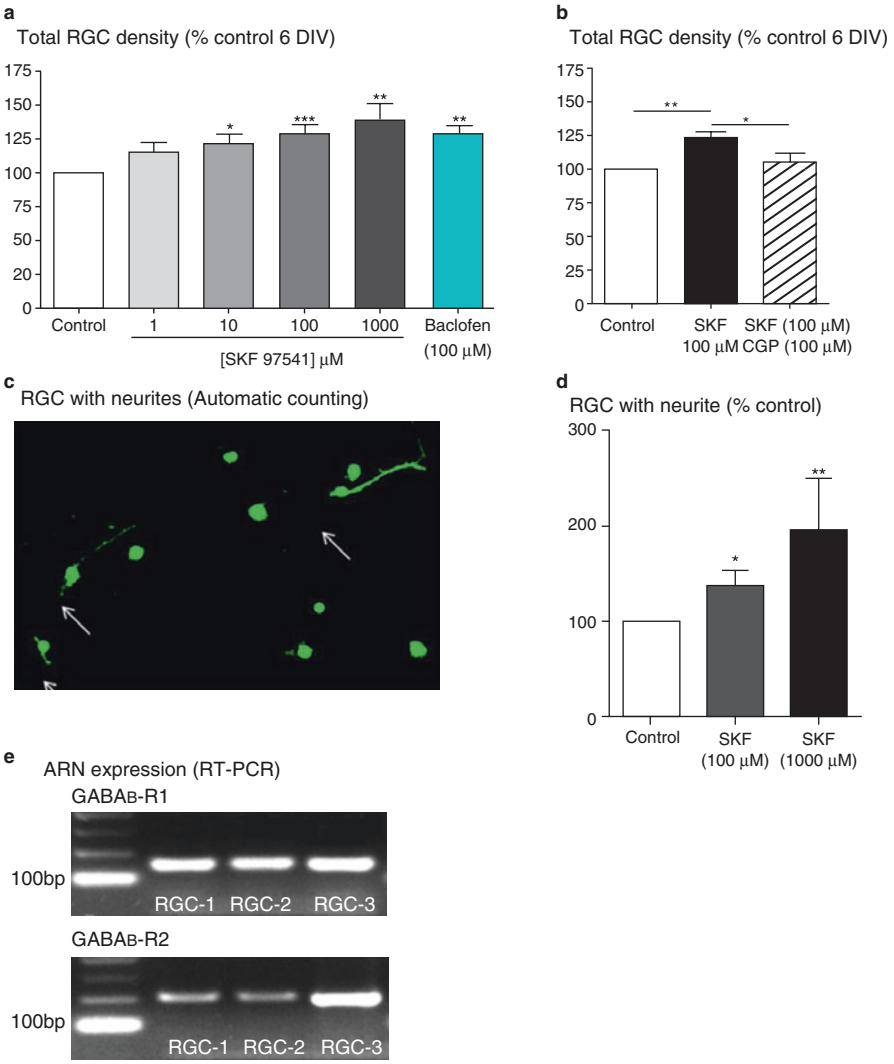

Fig. 4 $GABA_B$ receptor stimulation enhanced RGC survival. (**a**) Quantification of calcein-positive RGC densities after 6 DIV, in the control condition, with successive increased concentrations of SKF-97541: 1, 10, 100 and 1000 µM or with 100 µM of baclofen (Data are means ± SEM from n = 16 independent experiments). (**b**) Quantification of calcein-positive RGC densities after 6 DIV, in the control condition, with 100 µM of SFK or with both 100 µM SFK and 100 µM CGP (Data are means ± SEM from n = 7 independent experiments). (**c**) Representative images showing calcein-positive viable RGCs presenting detectable neurites, cultured for 6 days in vitro (DIV). (**d**) Quantification of viable RGC presenting neurite processing after 6 DIV, in the control condition, with 100 µM of SFK or with 1000 µM SFK (Data are means ± SEM from n = 4 independent experiments). (**e**) ARN expression of subtypes $GABA_B$ receptors 1 and 2. In each experiment, the respective RGC densities were expressed as a percentage of the control condition at 6 DIV

SKF-97,541 (100 μM) abolished the enhancement of RGC survival induced by SKF-97,541 (Fig. 4b).

To go even farther in our study, we focused on the effect of SKF-97,541 on RGC density with neurites to investigate the neurotrophic efficacy of the $GABA_B$ agonist. After 6 DIV, one third of cultured RGCs (30 ± 8.0%, n = 10; Fig. 4c) developed neurite outgrowth measurable by automatic counting. The survival of RGCs with neurites increased significantly with the concentrations of SKF-97,541 added in the culture medium (+37% with 100 μM, $p < 0.056$ and +95% with 1000 μM, $p < 0.01$), when compared to control untreated cells (Fig. 4d).

Finally, RT-PCR experiments were performed on purified RGCs in order to control the expression of both subunits of $GABA_B$ receptor, *i.e.* $GABA_B$-R1 and $GABA_B$-R2. The presence of the two subunit of $GABA_B$ receptor is needed to underlie the $GABA_B$ receptor function (see Fig. 2). Using three independent RNA samples from purified RGCs, we found after gel migration de presence of the amplified sequence of the $GABA_B$-R1 subunit in the three samples analyzed. Similarly, the amplified sequence of the $GABA_B$-R2 subunit was found in the three RNA samples analyzed. These results confirmed the expression of the two subunits of $GABA_B$ receptor in pure RGCs, suggesting that functional GABA metabotropic receptors could be present in primary cultures of RGCs.

Altogether, these data show that adding $GABA_B$ receptor agonists, baclofen or SKF-97,541 can reproduce the neuroprotective property of taurine on cultured RGCs. According, we demonstrated here that stimulation of $GABA_B$ receptors exerts a neuroprotective efficacy to promote RGC survival, and these receptors are believed to be involved in part of the neuroprotective role of taurine.

4 Discussion

Since 1970s, numerous studies have concluded that taurine plays a critical role to preserve retinal structure and function (Huxtable 1992). In particular, taurine depletion induced by either nutritional deprivation (Hayes et al. 1975) or pharmacological agents (Jammoul et al. 2009; Gaucher et al. 2012; Hadj-Saïd et al. 2016) leads to severe photoreceptor damages. More recently, taurine was also described as an efficient neuroprotective amino-acid against RGC degeneration in animal models of glaucoma (Froger et al. 2012) and oppositely, taurine depletion induced RGCs (Jammoul et al. 2010; Hadj-Saïd et al. 2016). Using primary culture of purified RGCs, we confirmed that application of taurine directly enhances the RGC survival (Froger et al. 2012).

Pharmacological action of taurine is mediated through two distinct pathways: (i) taurine is uptaken by the selective Tau-T (Uchida et al. 1993) to exert intracellular actions, mainly as an antioxidant agent, and (ii), on the other hand, taurine is a non-selective agonist at $GABA_{A/C}$ (Kletke et al. 2013) and glycine (Schmieden et al. 1992) ionotropic receptors, while it can also activate $GABA_B$ metabotropic receptors (Albrecht and Schousboe 2005) (For review see Froger et al. 2014). Here, we

addressed the involvement of these two cellular pathways in the neuroprotective action exerted by taurine against RGC degeneration. We firstly demonstrated that the selective blockade of Tau-T precluded the taurine-enhanced RGC survival, suggesting that the neuroprotective efficacy of taurine is mediated through its intracellular actions (Fig. 5), in particular at the mitochondrial level where taurine promotes the antioxidant processes. In addition, taurine can also modulate the intracellular calcium concentrations leading to a cytoprotective action (Froger et al. 2014).

Secondly, we investigated the role of the other cellular signaling triggered by taurine in the neuroprotection, i.e. the GABA and glycine receptors. GABA receptor family is composed by two subtypes: the $GABA_{A/C}$ ionotropic receptors and the $GABA_B$ metabotropic receptors (Fig. 2) (Barnard et al. 1998). The ionotropic $GABA_{A/C}$ receptor is an inhibitory anionic channel permeable to chloride and selectively blocked by picrotoxin, while the glycine receptor is selectively blocked by strychnine. The addition of both picrotoxin and strychnine with taurine, in order to block simultaneously the $GABA_{A/C}$ and glycine receptors did not affect the neuroprotective properties of taurine on serum-deprived RGCs. By contrast, when the

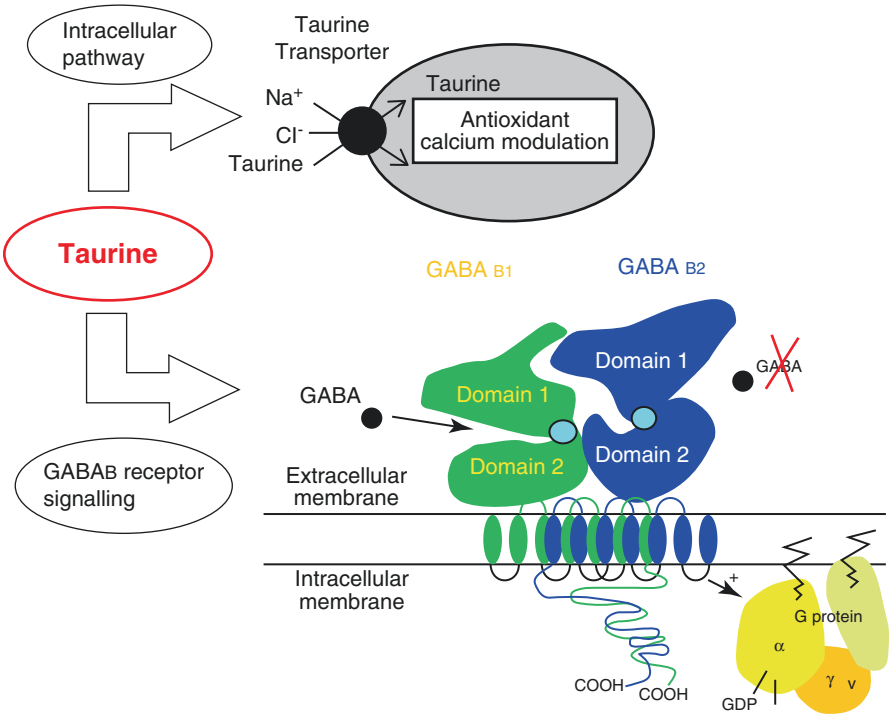

Fig. 5 Cellular pathways underlying neuroprotective action of taurine on RGCs. The mechanistic hypothesis involves either (i) the taurine transporter allowing taurine to penetrate into cytosol to exert its antioxidant properties at the mitochondrial level and modulate the intracellular concentrations of calcium or (ii) the $GABA_B$ metabotropic receptor which once stimulated activates intracellular signaling for neuroprotection

selective antagonist CGP35348 at $GABA_B$ receptors was added together with taurine, we found a significant alteration of the neuroprotective potency of taurine. These data suggested that the protective effect of taurine against RGC degeneration is mediated through the $GABA_B$ receptor stimulation. More interestingly, the selective agonists at this metabotropic GABA receptor, like baclofen, were found to reproduce the neuroprotective action of taurine by enhancing RGC survival under serum-deprived conditions. This study firstly demonstrates that $GABA_B$ receptor stimulation provides neuroprotection on RGCs. However, baclofen was recently described to mediate neuroprotection for hippocampic neurons under cerebral hypoperfusion (Liu et al. 2015). Other studies have revealed that baclofen displayed neuroprotective therapeutic possibilities in experimental traumatic brain injury or in stoke (Tuttolomondo et al. 2009; Jackson et al. 2012).

Taken together, our data indicate that taurine-mediated neuroprotection shares to cellular pathways (Fig. 5). The first involves the taurine uptake through the selective transporter (Tau-T, Fig. 5, upper part), necessary to allow to taurine to produce antioxidant actions at the mitochondrial level and calcium modulation. This pathway was also found dramatically crucial to preserve retinal neurons because the absence of taurine uptake leads to irreversible retinal damages (Pasantes-Morales et al. 1983; Lake and Malik 1987; Heller-Stilb et al. 2002). Invalidation of this cellular pathway in Tau-T knock-out mice, also produced damages of cardiac tissue and skeletal muscles (Ito et al. 2008), suggesting that taurine can preserve various cells through its intracellular actions. The second pathway, never described, involves the stimulation of $GABA_B$ metabotropic receptors (Fig. 5 lower part). The mechanisms leading to $GABA_B$-mediated RGC neuroprotection remain to be elucidate. $GABA_B$ receptor is a G-protein coupled receptor whose activation triggers an intracellular signaling leading to the phosphorylation of ERK (Im and Rhim 2012) and Akt (Lu et al. 2012) in hippocampal neurons. Activation of ERK and Akt plays an important role in regulating apoptosis, but it is also implicated in autophagy. It means that the neuroprotective effects of baclofen may be linked to the capacity of $GABA_B$ to reduce the cytodestructive autophagy (Liu et al. 2015). Further investigations are needed to clearly elucidate the mechanisms involved in the $GABA_B$ receptor-mediated RGC neuroprotection.

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Taurine Administration Mitigates Cisplatin Induced Acute Nephrotoxicity by Decreasing DNA Damage and Inflammation: An Immunocytochemical Study

Masahiro Tsunekawa, Shumin Wang, Toshihiro Kato, Takenori Yamashita, and Ning Ma

Abstract Cisplatin (CDDP) is one of the most effective chemotherapeutic agent used in the treatment of many kind of solid tumors. Its primary side effect is nephrotoxicity. The aim of this study to investigate the effects of taurine on cisplatin-induced acute nephrotoxicity. A single intraperitoneal injection of CDDP (15 mg/kg, or 25 mg/kg) deteriorated the kidney functions as reflected by histopathological changes. Histopathological changes were observed in all cisplatin groups. In the cisplatin group, oxidative stress was evident in the cisplatin group by observing an increase in 8-OHdG expression, an indicator of oxidative DNA damage. CDDP also resulted to an increase in CD68 expression in the renal tissues of CDDP groups. Taurine transporter (TauT) was down-regulated, and p53 was up-regulated in renal tissues as indicated by immunohistochemical analysis. Administration with taurine prior to a cisplatin injection was able to protect against deterioration of kidney function, to abrogate the decline in anti-oxidants and to suppress the increase in DNA damage. Moreover, taurine inhibited p53 activation and improved the pathological changes induced by cisplatin. This study demonstrates the protective effects of taurine in attenuating the expression of pro-inflammatory mediators and in improving antioxidant capacity in the kidney of cisplatin-injected rats. Thus, taurine could be a beneficial dietary supplement to attenuate cisplatin induced nephrotoxicity.

Keywords Cisplatin • Nephrotoxicity • Oxidation • Inflammation • Immunocytochemistry

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Abbreviations

8-OHdG	8-Hydroxy-2′-deoxyguanosine
CDDP	Cis-diammine dichloroplatinum II (cisplatin)
TauT	Taurine transporter

1 Introduction

Cisplatin is an important chemotherapeutic agent that has been widely used for its potent cytotoxic effects on a variety of tumor types including ovarian, testicular and cervical carcinoma (Fontanelli et al. 1992; Loehrer and Einhorn 1984; Ozols 1995; Panici et al. 1991). However, its full clinical utility is limited because of intrinsic toxicity to the kidney and liver (Arany and Safirstein 2003), with 25–35% of patients experiencing a significant decline in real function after its administration (Ries and Klastersky 1986). CDDP causes tubular injury, mainly at the proximal tubular level, through mechanisms that include hypoxia, oxidative stress, inflammation and apoptosis (Townsend et al. 2003; Francescato et al. 2007; Yao et al. 2007). Despite its adverse effects, CDDP remains a drug of choice in chemotherapy because of its efficacy and low cost. The kidney accumulates CDDP to a greater extent than other organs and is the major route for its excretion. Since the concentration of CDDP in proximal tubular cells is about five times the serum concentration, its disproportionate accumulation in renal tissues contributes to its acute nephrotoxicity (Arany and Safirstein 2003). The mechanism of CDDP nephrotoxicity is complex and involves processes such as inflammation, production of reactive oxygen and nitrogen species, as well as cell apoptosis (Faubel et al. 2007; Davis et al. 2001; Chirino and Pedraza-Chaverri 2009; Camano et al. 2010). Although the production of reactive oxygen species (ROS) by the CDDP has been implicated in the pathogenesis of renal tissues, the precise mechanism underlying the nephrotoxicity is, to this date, still a subject of debate.

Taurine, 2-aminoethylsulfonic acid, is the most abundant amino acid in mammalian tissues, including the kidney (Ma et al. 1994). The high intracellular level of taurine is maintained by active uptake and endogenous synthesis, mainly in the liver where taurine is an end product of sulfur amino acid catabolism (Hosokawa et al. 1990). One of the important actions of taurine is its antioxidant activity, which protects tissues from oxidative and nitrosative stress. During exposure to cytokines, such as TNF-α and IFN-γ, the halide-dependent myeloperoxidase of immune cells protects cells by promoting the conversion of taurine to taurine chloramines, in the process neutralizing the toxic metabolite, HOCl (Giri et al. 2000). Taurine chloramines prevent cellular damage arising from NO toxicity by suppressing NO synthase. Taurine can also protect cells from apoptosis and DNA fragmentation induced by NO generated outside the cell (Kim et al. 2006).

The aim of the present work was to investigate the effect of a taurine administration on the acute renal injury induced by CDDP in rats. The efficacy of taurine against

CDDP induced acute nephrotoxicity was evaluated in terms changes in oxidative/nitrosative stress markers, inflammatory markers and histopathological changes.

2 Methods

2.1 Reagent and Antibody

CDDP was obtained from Sigma-Aldrich Japan (Tokyo, Japan). Taurine was purchased from Wako Pure Chemicals Industries Inc. (Osaka, Japan). Rabbit taurine-specific antibodies was prepared essentially as described previously (Ma et al. 1994). Mouse 8-OHdG-specific antibody was purchased from Japan Institute for the Control of Aging (Fukuroi, Japan). CD68-specific antibodies (ab31630) purchased from abcam. (Chuo-ku, Tokyo, Japan). Taurine transporter (TauT) antibody (SC6A6) was obtained from Enogene. P53-specific antibody was purchased from Calbiochem (San Diego, CA, USA).

2.2 Animals and Treatments

Male Wistar rats, weighing 180–200 g, were used in this study. They were maintained in an animal room at a temperature of 22 ± 2 °C with a relative humidity of 55 ± 10% and a light/dark cycle of 14/10 h (light period 8:00–22:00). A commercial diet (NMF, Oriental Yeast, Tokyo, Japan) and tap water were supplied ad libitum.

The animals were divided into six groups: control rats (Control, n = 10) received i.p. injection of saline, Taurine-treated control rats (Tau + Control, n = 10), Low-dose CDDP rat (CDDP-Low, n = 10) received i.p. injection of CDDP (15 mg/kg body weight), High-dose CDDP rats (CDDP-High, n = 10) received i.p. injection of CDDP (25 mg/kg body weight), Taurine-treated Low-dose CDDP rats (Tau + CDDP-Low, n = 10), Taurine-treated High-dose CDDP rats (Tau + CDDP-High, n = 10). Taurine was administered at a concentration of 5% (w/v) in drinking water to which rats had unlimited access before four days from the injection of CDDP to killing (Fig. 1).

2.3 Fixation and Sectioning of Tissue

Rats were deeply anesthetized with an IP injection of sodium pentobarbital. Kidneys were dissected and allowed to stand in a fixative solution, containing 4.0% paraformaldehyde and 0.5% glutaraldehyde (GAL) in phosphate buffered saline (PBS), for 4 h. Then they were rinsed several times with PBS, dehydrated with a graded alcohol series and acetone, and embedded in paraffin. Sections 7 μm in thickness were mounted on albumin-coated slides.

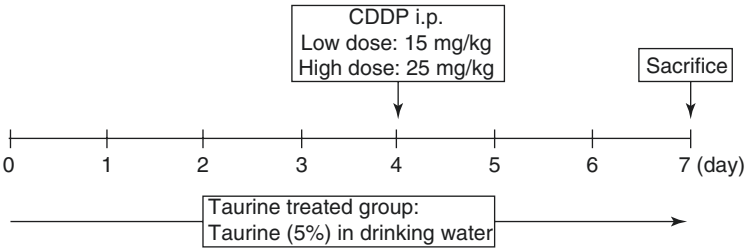

Fig. 1 Taurine administration and CDDP treatment schedule. Rats were divided into six groups. Taurine was administered at a concentration of 5% (w/v) in drinking water in all experiment period

2.4 Immunocytochemical Processing

Sections were deparaffinized in xylene, hydrated by passage through a graded alcohol series, and washed twice with PBS buffer for 5 min each. Next, they were incubated in 0.5% H_2O_2 in PBS for 15 min to block endogenous peroxidase activity, and incubated in 1% skim milk for 15 min to block nonspecific binding at room temperature (RT).

The sections for taurine and taurine transporter (TauT) immunocytochemical staining were incubated with primary antibodies, rabbit taurine-specific antibodies (150× diluted in PBS) overnight at RT. They were then incubated with goat anti-rabbit IgG antibodies (100× diluted in PBS) for 3 h at RT, and the staining was visualized by the peroxidase anti-peroxidase method. These sections were counter stained with hematoxylin.

The sections for p53, 8-OHdG and CD68 staining were incubated with mouse 8-OHdG antibodies (150 × diluted in PBS overnight at RT. Then they were incubated with goat anti-mouse IgG horseradish peroxidase (Medical & Biological Laboratories, Nagoya, Japan, 200× diluted in PBS), for 3 h in a moist chamber at RT. The sections were washed several times between each step with PBS. To identify the immune-negative structures of the kidney, some neighboring sections were stained with hematoxylin and eosin (H&E).

2.5 Immunohistochemical Grading and Statistics

Immunohistochemical (IHC) grading, which, is based on intensity and frequency of staining, was performed by two independent investigators. The staining intensity was scored as negative (−), minimal (+), mild (++), moderate (+++), or severe (++++). The χ^2-test was performed. Differences were considered statistically significant at $P < 0.05$.

3 Results

3.1 HE Staining

H&E staining was used to observe the pathological changes of the kidney caused by CDDP (Fig. 2). No marked changes were seen in sections from the control (no CDDP) treated group. Necrosis, degeneration, tubular dilatation, and hyaline casts were seen in the proximal tubular at 3 days after a CDDP treatment. CDDP-High group turned worse than CDDP-Low group. The acute kidney injury was more

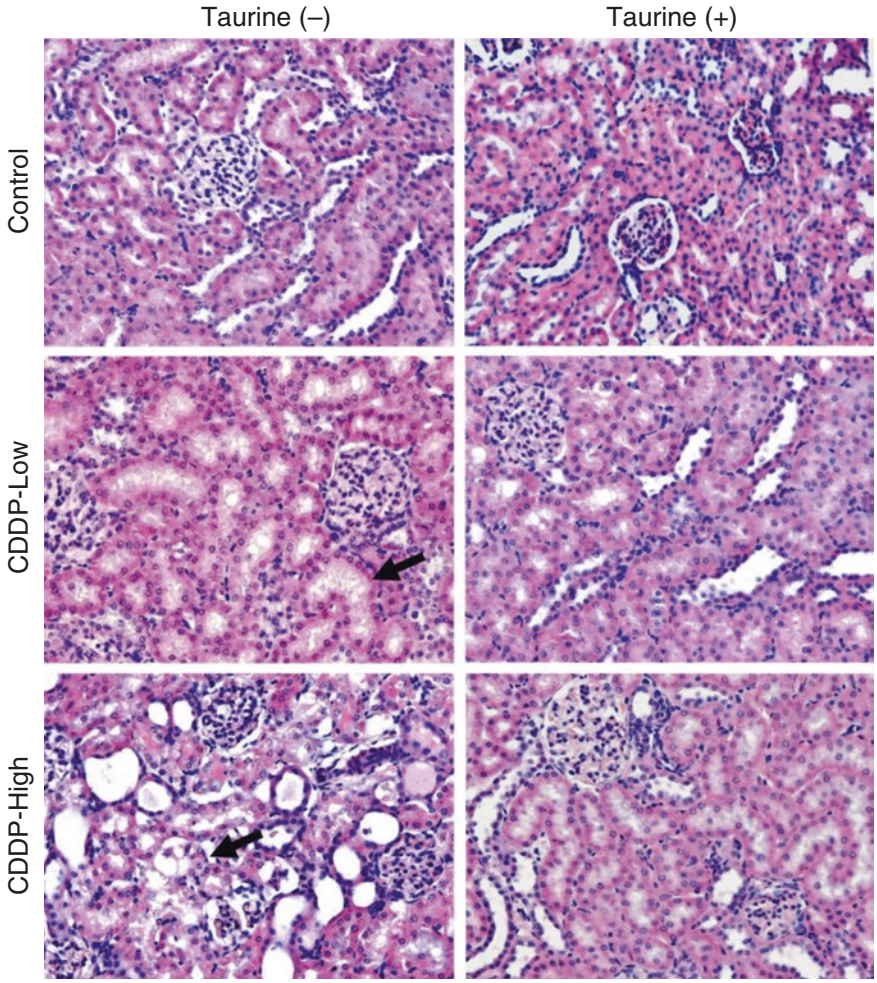

Fig. 2 Histopathological findings in the kidney of a CDDP-treated rat. Necrosis, degeneration, tubular dilatation, and hyaline casts were observed in the proximal tubules of the CDDP treated group (*arrow*). The Tau + CDDP group showed histological features that were comparable to those of the control group (×200)

severe and had spread into the distal tubules and Henle's loops. Sections from the Tau + CDDP group displayed marked improvement in the histological appearance, to the point that they were comparable to that of the control group.

3.2 Local Existence of Taurine and TauT

Taurine and TauT antibodies were used to confirm the local existence of taurine and TauT in the kidneys by the PAP method (Figs. 3 and 4). Intense taurine immunoreactivities of the collecting tubes and proximal tubules were observed in the Control

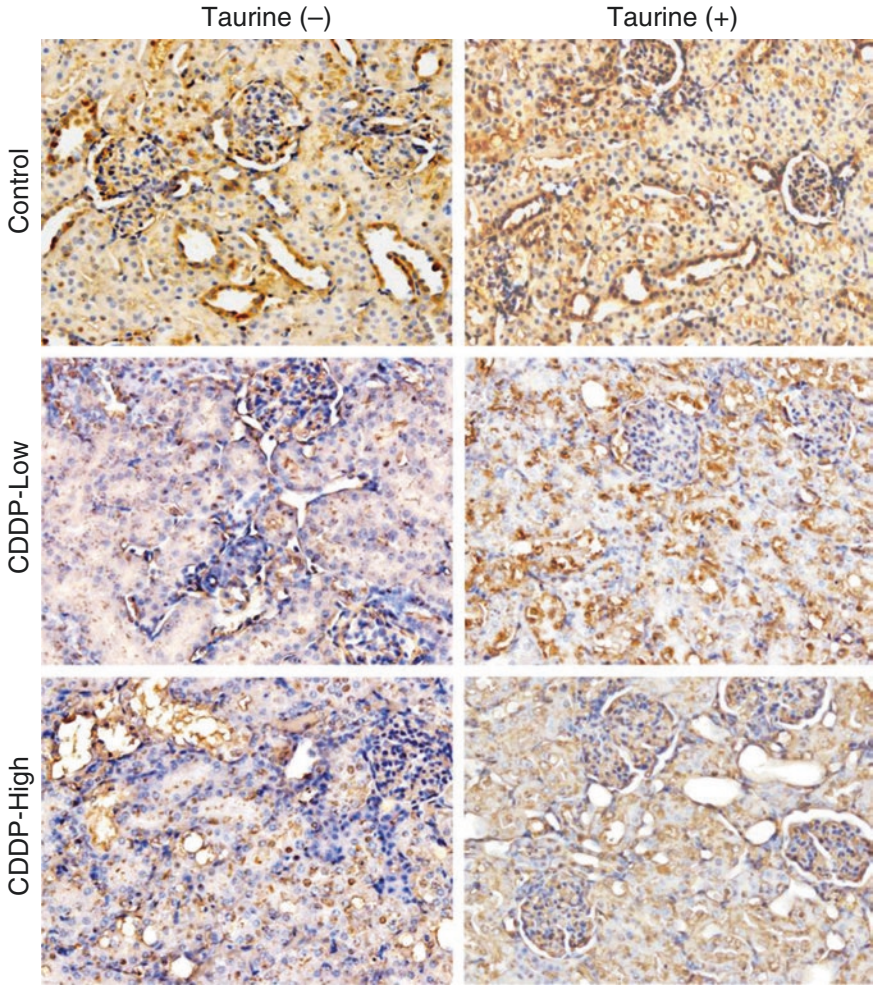

Fig. 3 Distribution of taurine-specific immunoreactivity in the kidney. Taurine immunoreactivities are observed in the collecting tubes and proximal tubules of Control group. Taurine immunoreactivities were reduced after CDDP treatment, but were increased in the proximal tubules of CDDP-taurine treated group (Nuclear staining: Hematoxylin, ×200)

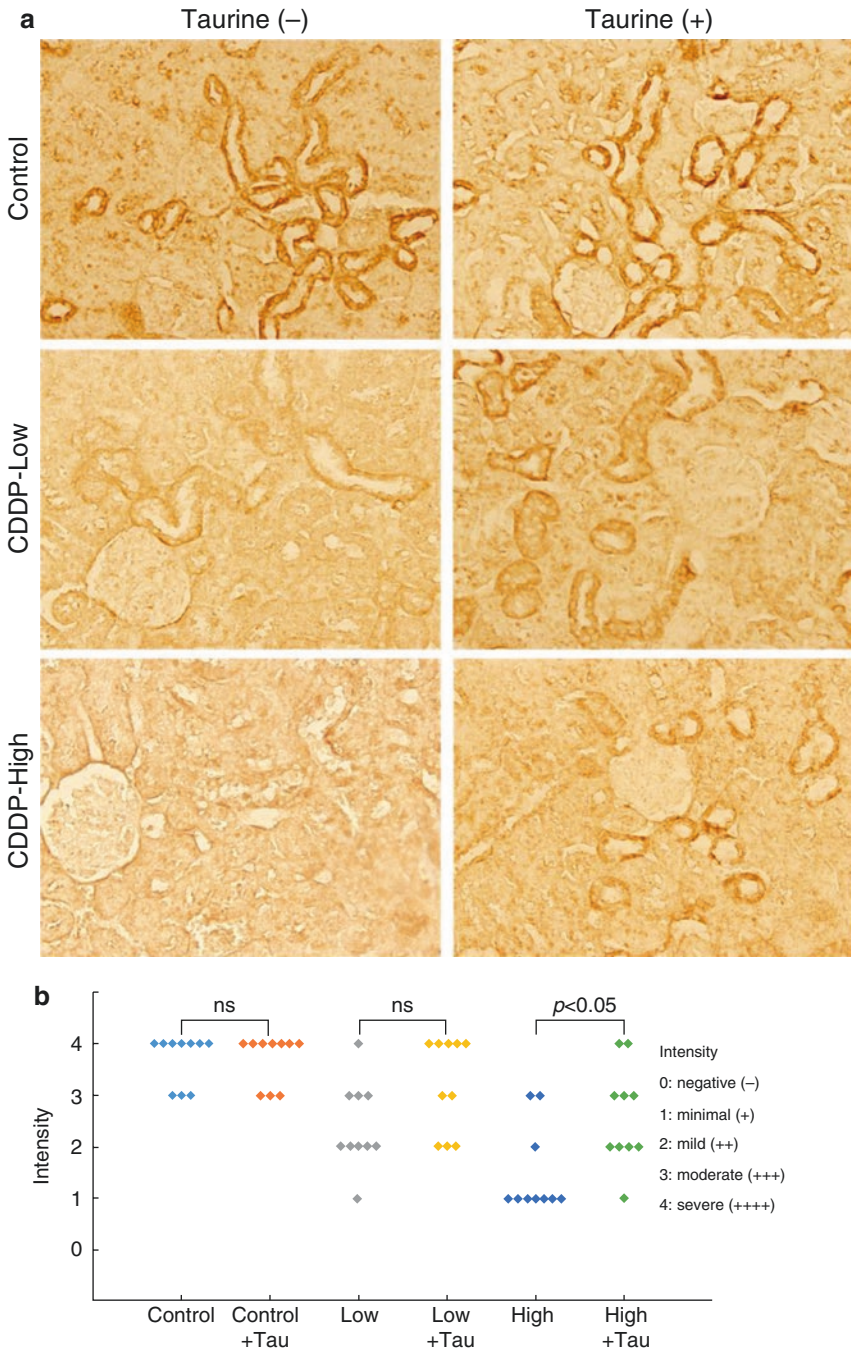

Fig. 4 Expression of TauT in the kidney. (**a**) TauT expression was reduced after CDDP treatment, and increases in the proximal tubules were observed in the CDDP-Tau group (×200). (**b**) Effect of taurine on expression levels of TauT (χ^2-test), $p < 0.05$ significantly different from CDDP-Low dose treated group; $p < 0.001$ significantly different from CDDP-High dose-treated group

group. Taurine immunoreactivities were reduced in the CDDP treated group because of the severe damage to the proximal tubules in the renal cortex. Taurine immunoreactivities were found to be increased in the proximal tubules of rats in the taurine-treated CDDP group compared to similar rats not receiving taurine.

TauT immunoreactivies were observed in proximal tubule of the Control group. There was no distribution alteration in comparison with the Tau + Control group. Expression of TauT was decreased in CDDP-Low group and CDDP-High group. TauT immunoreactivies were increased in Tau + CDDP-Low and Tau + CDDP-High group in comparison with CDDP-Low and CDDP-High group.

3.3 Expression of p53 in the Kidney of After CDDP Treatment

P53 was immunostained by the Avidin-Biotin Complex method using p53-specific antibodies to confirm expression of p53 (Fig. 5). Weak or no immunoreactivity of p53 was observed in the proximal tubule of Control group. Expression of p53 was increased in the CDDP-Low group. Expression was more intense in the CDDP-High group. In comparison with CDDP-Low and CDDP-High group, p53 immunoreactivity was decreased in Tau + CDDP-Low and Tau + CDDP-High groups, respectively.

3.4 Expression of CD68 · 8-OHdG in CDDP-Induced Nephrotoxicity

CD68 and 8-OHdG were immunostained by the HRP method using CD68-specific antibodies and mouse 8-OHdG-specific antibodies to confirm expression of CD68 and 8-OHdG (Figs. 6 and 7). The immunoreactivies of CD68 were increased in the CDDP treatment group. Renal stroma cells were strongly stained in the CDDP-High group. The expression of CD68 was suppressed in the taurine-administered group.

Weak immunoreactivity of 8-OHdG was observed in the collecting duct of the CDDP-Low group, but showed a significant increase in in the proximal tubule and distal tubule of the CDDP-High group. Taurine-treated groups demonstrated a significant reduction in 8-OHdG immunostaining.

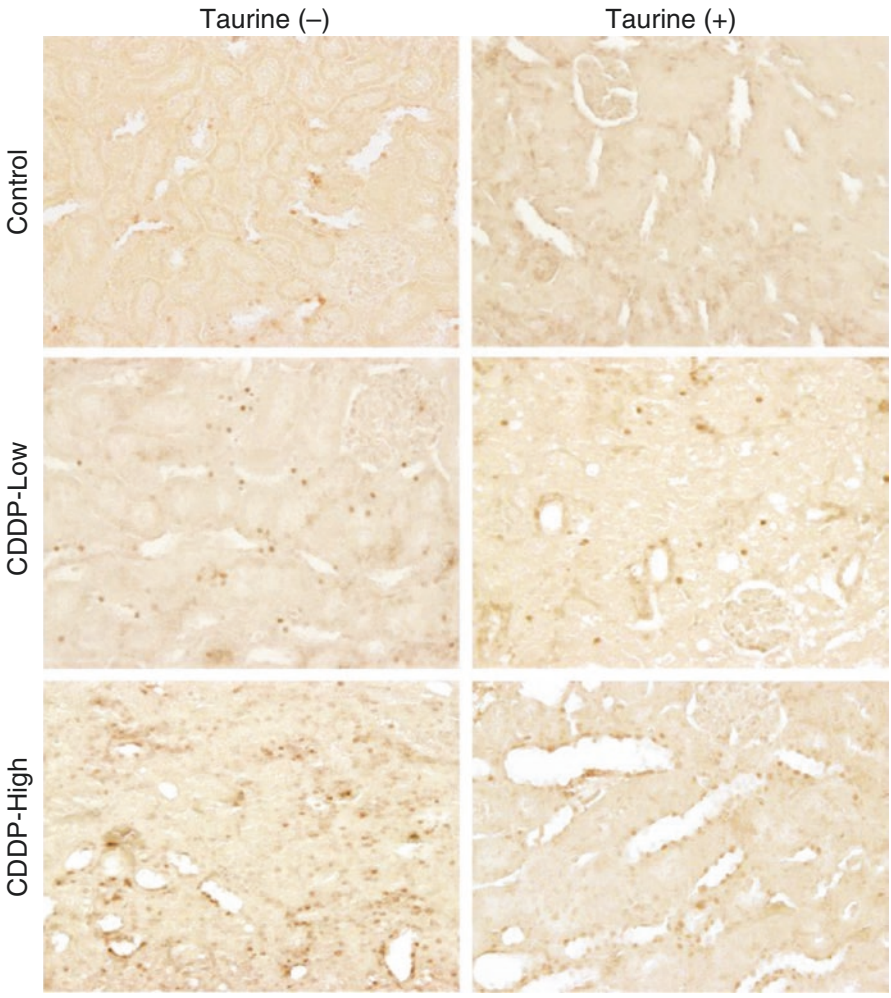

Fig. 5 Expression of p53 in the kidney. Weak or no immunoreactivity of p53 was observed in the proximal tubule of Control group. TauT was reduced after a CDDP treatment, and decreased p53 expression in the proximal tubules was observed in the CDDP-Taurine administered group (×200)

4 Discussion

Acute nephrotoxicity is one of the most important side effects of CDDP therapy for a wide range of solid tumors, by affecting primarily the proximal tubules. Previous studies have reported that fibrotic areas in the kidney of CDDP-taurine treated rats

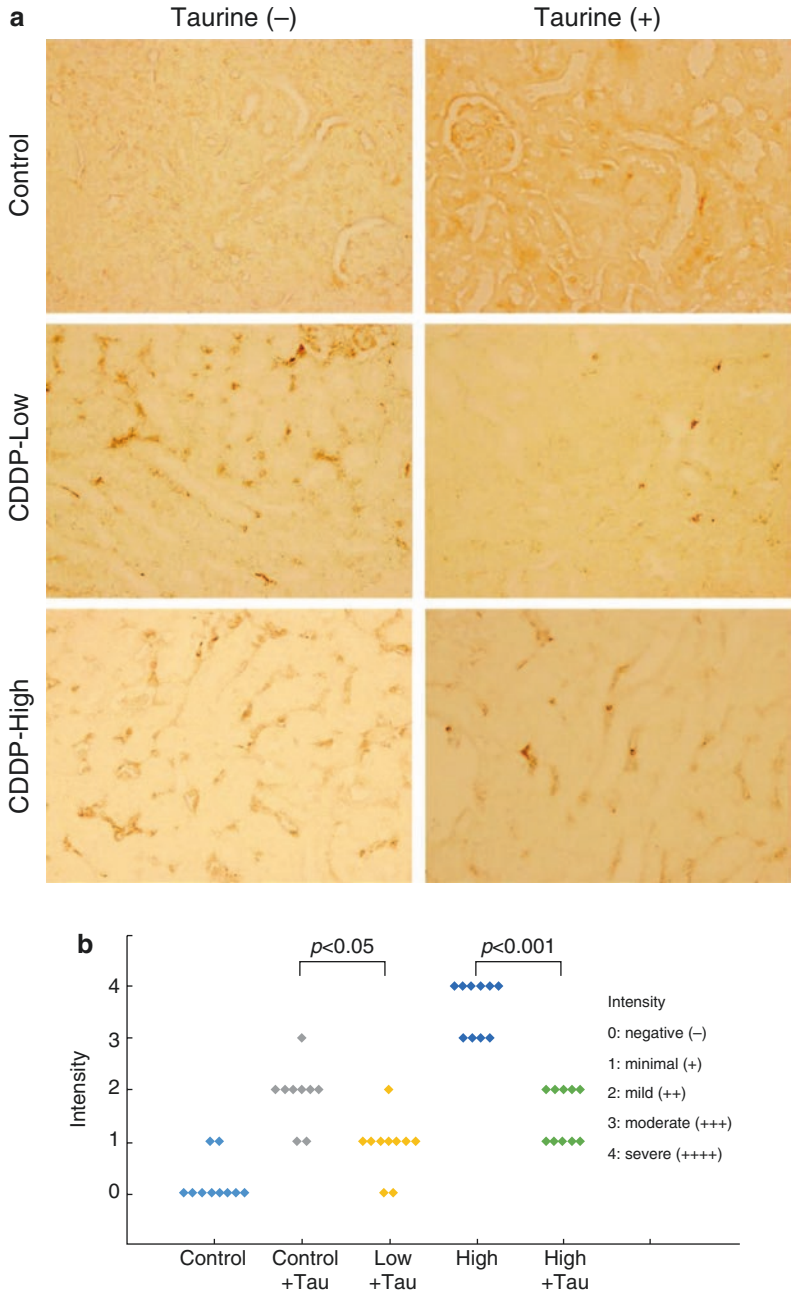

Fig. 6 Expression of CD68 in the kidney. (**a**) Expression of CD68 was increased in renal stroma cells of the CDDP-treated group. Taurine administration suppressed the expression of CD68 (×200). (**b**) Effect of taurine on the expression of CD68 (χ^2-test), $p < 0.05$ significantly different from CDDP-Low dose treated group; $p < 0.001$ significantly different from CDDP-High dose treated group

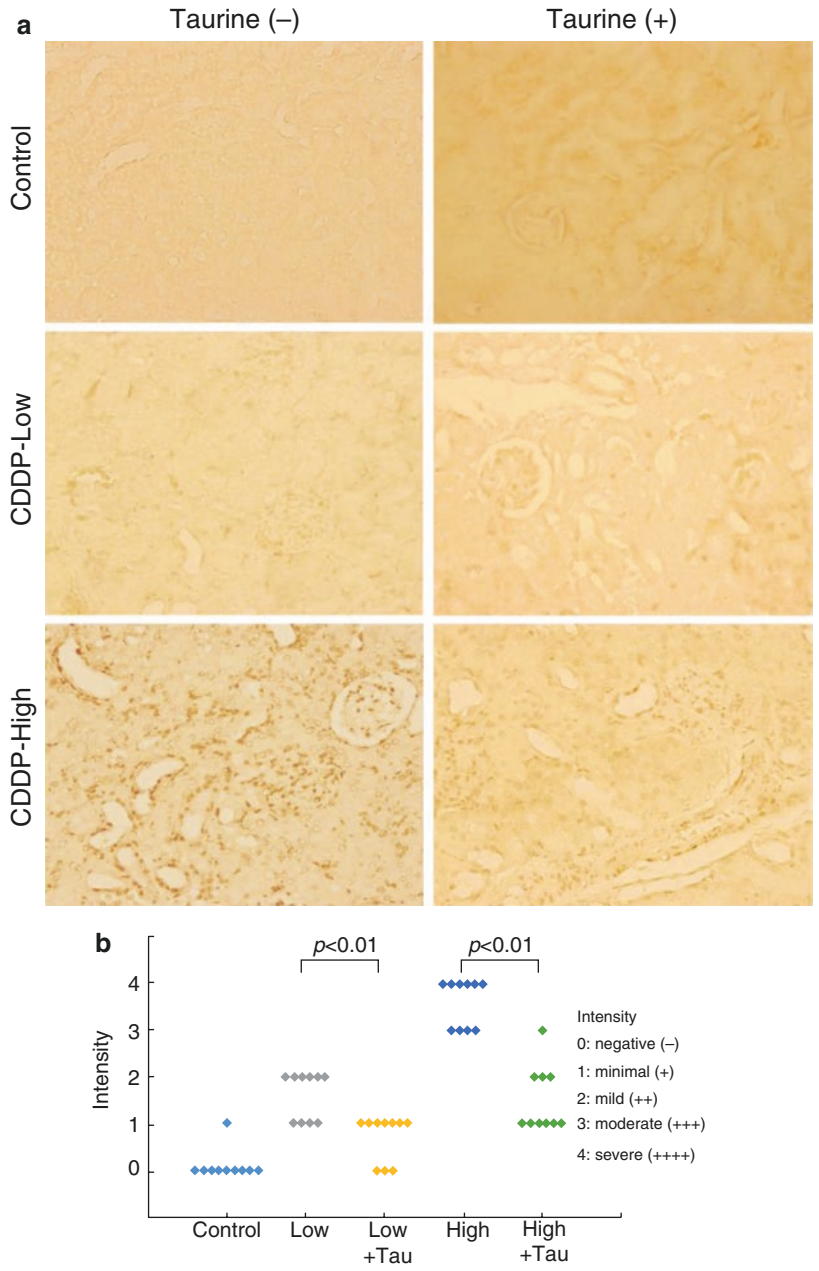

Fig. 7 Expression of 8-OHdG in the kidney. (**a**) Expression of 8-OHdG was observed in the collecting duct of the CDDP-Low group, and intense expression of 8-OHdG was observed in the proximal tubule and distal tubule of the CDDP-High group. Expression of 8-OHdG was suppressed in the taurine-treated group (×200). (**b**) Effect of taurine on the expression of 8-OHdG (χ^2-test), $p < 0.05$ significantly different from CDDP-Low dose treated group; $p < 0.001$ significantly different from CDDP-High dose treated group

were significantly less than in that of CDDP-treated rats. Compared with control rats, the macrophages in rats receiving only CDDP were significantly increased. In contrast, the number of macrophages in rats receiving CDDP-taurine was less than that in rats on CDDP alone (Sato et al. 2002). The mechanism of the anti-tumor activity of CDDP is not completely understood, and the oxidative stress associated with it is known to be one of the most important factors involved in the pathogenesis of renal injury (Pabla and Dong 2008; Perez-Rojas et al. 2011). Our study was aimed at investigating the effect of a taurine administration on CDDP-induced nephrotoxicity.

In our histological analyses, we found that no pathological changes were observed in the Control and Tau + Control groups. Necrosis, degeneration, tubular dilatation, and hyaline casts were observed in the proximal tubular after CDDP injection. While the pathological changes in the CDDP-High group were more than that in the CDDP-Low group, a marked improvement in the renal histological changes was achieved after a treatment with CDDP plus taurine in the Tau + CDDP-Low and Tau + CDDP-High. Taurine immunoreactivities were reduced in the collecting tubes and proximal tubules in the CDDP-treated group in comparison with the Control group. Localization of taurine in the proximal tubules was increased in the taurine treated group. Expression of TauT was evident in proximal tubule of Control group and Tau + Control group. Expression of TauT was increased in CDDP-Taurine treated groups in comparison with CDDP treated groups. This might be attributed to an alteration in the osmoregulatory activity of taurine, thus resulting in a reduction of taurine controlled natriuresis and diuresis (Ideishi et al. 1994), in activation of the renin-aldosterone axis, and in disturbance of the taurine modulation of atrial natriuretic factor secretion and its putative regulation of vasopressin release (Schaffer et al. 2000).

Previous studies have reported that the expression of TauT is decreased after activation of the p53 tumor suppressor gene (Han et al. 2006; Mochizuki et al. 2005; Han and Chesney 2010). In general, the activity of TauT is regulated by extracellular taurine concentration, osmotic cell swelling, p53 tumor suppressor gene and proto-oncogenes such as c-Jun (Lambert et al. 2014; Han et al. 2006; Mochizuki et al. 2005). In our study, the immunoreactivity expression of p53 was weak in the proximal tubule of the Control group but increased in both the CDDP-Low group and CDDP-High group. In comparison with the CDDP treated group, the immunoreactivity of p53 was found decreased in CDDP-taurine administration group.

Mozaffan et al. reported that taurine-deficient kidney specimens displayed periarterial mononuclear cell infiltrates with strong immunoreactivity to the histiocyte marker CD68 in the taurine-deficient rat (Mozaffari et al. 2006). The immunoreactivity of CD68 was increased in the CDDP treated group. Likewise, the renal stromal cells of the CDDP-High group also showed an intense immunoreactivity. In contrast, the expression of CD68 was decreased in the taurine administration group. Weak immunoreactivity of 8-OHdG was observed in the collecting duct of the CDDP-Low group; but it was increased in the proximal tubules and distal tubules of rats in the CDPP-High group. On the other hand, a significant reduction of

8-OHdG immunostaining was observed in the group receiving only taurine. Taurine appears to reduce the production of O^{2-} via redox reaction, particularly in places with high production of O^{2-} (Hansen et al. 2006). Our findings suggest that inflammation and ROS play a crucial role in the pathogenesis of CDDP renal damage, and that taurine can suppress both the generation of ROS and ensuing oxidative stress and the generation of pro-inflammatory cytokines in kidney tissues. In this connection, taurine administration prior to CDDP injection is found to protect against deterioration in kidney function, to abrogate the decline in antioxidants, and to suppress the increase in DNA damage. Moreover, taurine is found to inhibit p53 activation and to improve the histological changes induced by CDDP. These results indicated that since taurine exhibits protective effects on CDDP-induced renal injury it may find use as an adjunct in the cancer chemotherapy and in which CDDP is as first line treatment drug.

5 Conclusion

In brief, the present study demonstrates that taurine can protect against the nephrotoxic effects of CDDP by attenuating the generation of pro-inflammatory mediators and by improving the antioxidant competence of the kidney. Hence, taurine could be a dietary supplement of benefit in attenuating CDDP-related nephrotoxicity.

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Effect of Taurine on iNOS-Mediated DNA Damage in Drug-Induced Renal Injury

Toshihiro Kato, Masahiro Tsunekawa, Shumin Wang, Takenori Yamashita, and Ning Ma

Abstract Owing to an outstanding wide antitumor spectrum and excellent antitumor effect cisplatin has been used in chemotherapy for malignant tumor. However, cisplatin has strong side effects such as renal injury. Taurine has been found to protect against inflammatory tissue damage in a variety of experimental models. The aim of the present study was to investigate the effect of taurine against iNOS dependent DNA damage in cisplatin-induced renal injury in rats. With the help of a rat model of drug-induced kidney damage, we have assessed the nephrotoxic effects of different doses of cisplatin in the presence and absence of taurine. Immunohistochemical methods were used to examine the distribution of arginine, iNOS, citrulline and 8-nitroguanine in renal tissue. The expression levels of citrulline, iNOS, and 8-nitroguanine immunoreactivities were found to increase as a function of the dose of cisplatin used, and to decrease in the presence of taurine. The expression level of arginine immunoreactivity was reduced as a function of the dose of cisplatin used. On the other hand, iNOS, 8-nitroguanine and citrulline immunohistochemical staining showed an intense immunoreactivity in the renal tubule of cisplatin-treated animals; and arginine immunoreactivity was localized in the renal tubule of taurine-treated animals. We also confirmed the decrease of citrulline and iNOS expression in the renal tubule after taurine administration as well as the expression level of 8-nitroguanine, a nitrative stress marker in the same animals. The present results support the concept that taurine may have a protective role in the formation of cisplatin-related DNA lesions arising through iNOS-mediated nitrative stress.

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Keywords Cisplatin • *iNOS* • Inducible nitric oxide synthase • Renal injury

1 Introduction

Cisplatin (cis-diamminedichloroplatinum II: CDDP), one of the most effective and widely used chemotherapeutic agents, is a first-line therapy for the treatment of a variety of solid tumors including head and neck, ovary, testis, and lung malignancies (Perazella and Moeckel 2010). Cisplatin has been extensively utilized over the last decades and its therapeutic action is found to be dose dependent. However, its use is severely restricted as a result of severe side effects seen with high doses and including neurotoxicity, ototoxicity, emetogenicity, and nephrotoxicity (Hanigan and Devarajan 2003; McWhinney et al. 2009; Rybak 2007). Its nephrotoxicity is of great concern because of the high morbidity and mortality associated with its use (Domitrovic et al. 2013). Cisplatin-induced kidney injury has been reported to be the major factor limiting its use in cancer chemotherapy (Kelland 2007; Barabas et al. 2008). In renal tissue, cisplatin accumulates in the proximal tubule followed by the distal collecting tubule and the convoluted tubule (Kroning et al. 2000). Although the mechanism of cisplatin-induced kidney injury is not entirely known, it t may be multifactorial since it involves inflammation, oxidative stress and tubular apoptosis (Pabla and Dong 2008; Dasari and Tchounwou 2014). Reactive oxygen species (ROS) are of particular importance because they can cause DNA damage. Overall, these factors account for the proximal tubular damage and dysfunction associated with cisplatin chemotherapy. Therefore, protective strategies to alleviate cisplatin nephrotoxicity are crucial for ensuring the uneventful survival of cancer patients receiving this drug (Kintzel 2001).

Taurine (2-aminoethanesulfonic acid) is one of the most abundant amino acid in the body. In vertebrates is found in high concentrations in most tissues, including the skeletal muscle, heart, nerve, brain, and liver (Jacobsen and Smith 1968). Its cytoprotective activity is the result of several biological, physiological, and pharmacological mechanisms, including membrane stabilization (Pasantes-Morales and Cruz 1985), antioxidation (Gordon and Heller 1992; Kato et al. 2015) and osmoregulation (Thurston et al. 1980). Taurine is not incorporated into proteins, and by participating in renal biologic processes it can influence the kidney homeostasis (Han et al. 2000). Taurine and its derivatives have been as the subject of study as a renoprotective agent in several rat models based on its antioxidant properties (Mozaffari et al. 2003; Mozaffari and Schaffer 2002). The major mechanism accounting for this antioxidant action is the reaction with hypochlorous acid (HOCl) to form taurine chloramine. HOCl is an example of a reactive oxygen species (ROS) that can cause DNA oxidation, protein nitration, and lipid peroxidation of renal cells. Taurine has been found to protect the kidney against the renal interstitial fibrosis that develops as a result of

cisplatin use in rats (Sato et al. 2002). ROS produced by cisplatin-induced kidney injury produce reactive nitrogen species which, in turn, can cause nitrative DNA damage. Taurine has the effect of reducing nitrative DNA damage by inhibiting iNOS (Sugiura et al. 2013). The aim of the present study was to investigate whether taurine can protect against iNOS dependent DNA renal damage by cisplatin in a rat model.

2 Methods

2.1 Animals and Experimental Design

Male Wistar rats, 7-weeks old, were obtained from SLC (Hamamatsu, Japan) and weighed 200–250 g on arrival. They were housed in cages (2 per cage) and maintained on water and food ad libitum. All animals were housed in a room kept at 22.0 °C (45–55% of humidity) with a 12 h light and 12 h dark cycle. The rats were randomly divided into five group, each consisting of ten rats: cisplatin-low group taurine plus cisplatin-low group, cisplatin-high group, taurine plus cisplatin-high group, and control group. The experimental protocols were approved by the Animal Ethics Committee of Suzuka University of Medical Science, Suzuka, Mie, Japan.

2.2 Reagent

Cisplatin was obtained from Sigma-Aldrich Japan (Tokyo, Japan). Taurine was purchased from Wako Pure Chemical Industries (Osaka, Japan). Rabbit polyclonal anti-8-nitroguanine antibody and rabbit anti-arginine antibody and anti-citrulline antibody were prepared essentially as described previously (Ma et al. 1994). Mouse anti-iNOS antibody was obtained from abcam (ab15323). 3,3′-diaminobentidine tetrahydrochloride was obtained from Dojindo Chemical Institute (Kumamoto, Japan).

2.3 Cisplatin Treatment and Taurine Administration

In cisplatin-treated animals, 15 or 25 mg/kg cisplatin was injected intraperitoneally at once. In taurine-treated animals, the taurine was added to the drinking water at a concentration of 5% and made available on an unlimited basis from 4 days before a cisplatin treatment until the sacrifice time. In control animals, saline was injected intraperitoneally.

2.4 Surgical Method and Kidney Collection

The two kidneys were removed 3 days after a cisplatin treatment. The, renal tissue was fixed in 4.0% paraformaldehyde, dehydrated in a graded alcohol series, and embedded in paraffin. Six-micrometer thick sections were mounted on albumin-coated slides.

2.5 Immunohistochemical Study of 8-Nitroguanine, iNOS, Arginine and Citrulline

8-Nitroguanine, iNOS, arginine and citrulline immunoreactivities in the rat renal sections were observed by a peroxidase anti-peroxidase (PAP) method study. Briefly, paraffin sections (6 μm in thickness) were incubated with rabbit polyclonal anti-8-nitroguanine antibody (2 μg/ml), anti-iNOS antibody (2 μg/ml), anti-arginine antibody (2 μg/ml), and anti-citrulline antibody (2 μg/ml) overnight at room temperature, respectively. Then, the sections were incubated for 2 h with goat antibody against rabbit IgG (1:200), which was followed by a peroxidase anti-peroxidase complex (1:200) for another 2 h. The sections that had been treated with first and second antibodies were incubated for 10 min at room temperature with 3,3′-diaminobenzidine tetrahydrochloride serving as a chromogen, and which had been freshly prepared as a solution of 20 mg in 100 ml PBS that contained 0.01% H_2O_2.

3 Results

3.1 Immunoreactivitiy of 8-Nitroguanine

No immunoreactivity for 8-nitrogunanine was observed in the control group. Renal tissue of the cisplatin-treated groups expressed 8-nitroguanine in the nuclei of the proximal tubule and collecting duct, -in direct proportion to the dose of cisplatin used. On the other hand, in renal tissue of taurine treated animals, the immunoreactivity of 8-nitroguanine was considerably decreased in the nucleus of proximal tubule and collecting duct cells (Fig. 1).

3.2 Immunoreactivity of iNOS

A wake immunoreactivity of iNOS was observed in the control group. Renal tissue of cisplatin-treated animals expressed iNOS in epithelial cells of the proximal tubule and collecting duct, which was cisplatin's dose dependent. On the other hand,

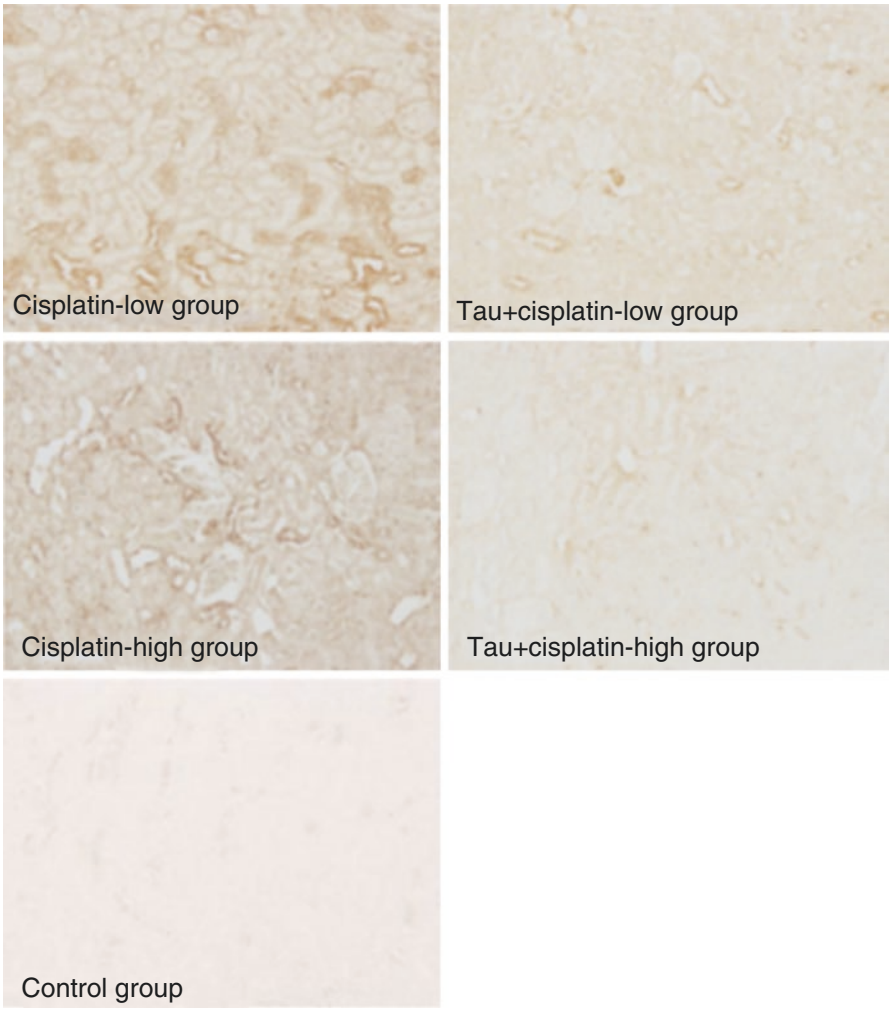

Fig. 1 No immunoreactivity of 8-nitrogunanine was observed in the control group. Immunoreactivities of 8-nitroguanine were increased in the proximal tubule and collecting duct of cisplatin-treated animals, and decreased in taurine-treated animals

in renal tissue of taurine-treated animals, the immunoreactivity of iNOS was decreased on epithelial cells of the proximal tubule and collecting duct. In animals receiving the lowest dose of cisplatin, the immunoreactivity was almost the same as that of the control groups (Fig. 2).

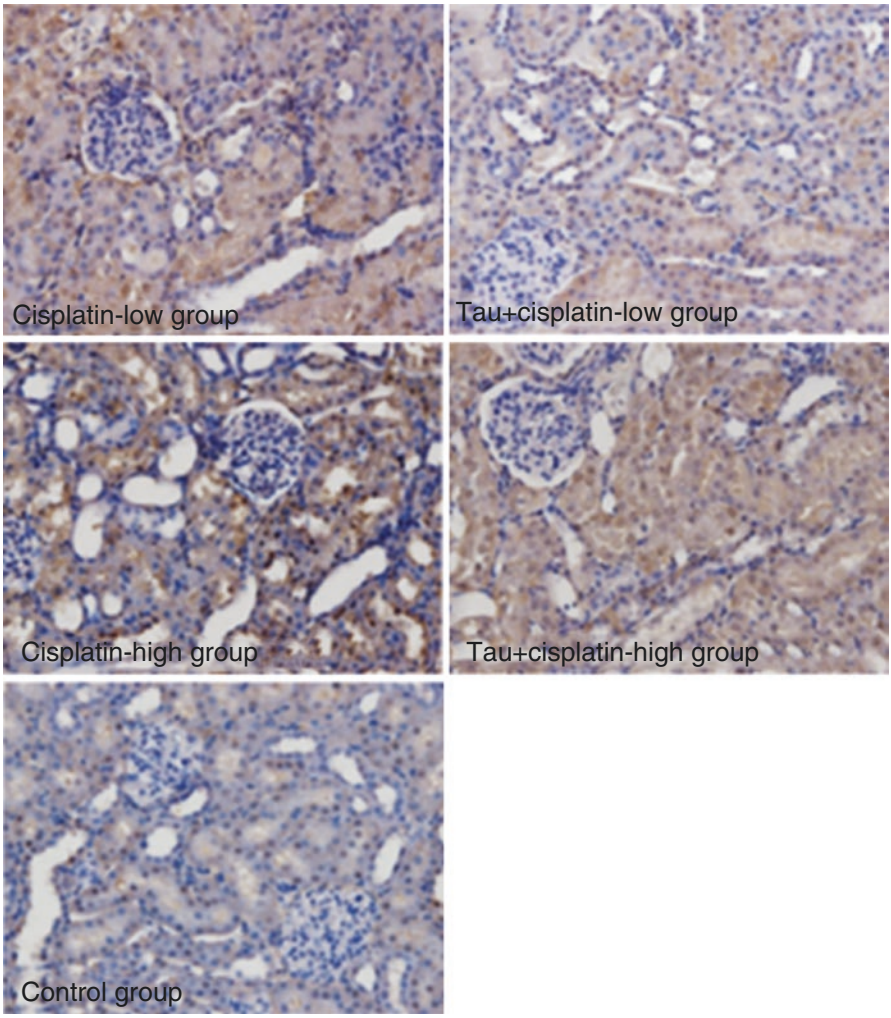

Fig. 2 Weak immunoreactivity for iNOS was observed in the control group. The immunoreactivity of iNOS was increased in the proximal tubule and collecting duct of animals receiving cisplatin. In contrast, the immunoreactivity of iNOS was decreased in the collecting duct and part of the proximal tubule of animals receiving taurine. The tissue sections were counter stained with hematoxylin

3.3 Immunoreactivities of Arginine and Citrulline in the Nitric Oxide Pathway

High arginine immunoreactivity was present in epithelial cells of the proximal tubule of control group. In cisplatin-treated animals the immunoreactivity of arginine was reduced in epithelial cells of the proximal tubule proportionally to the dose

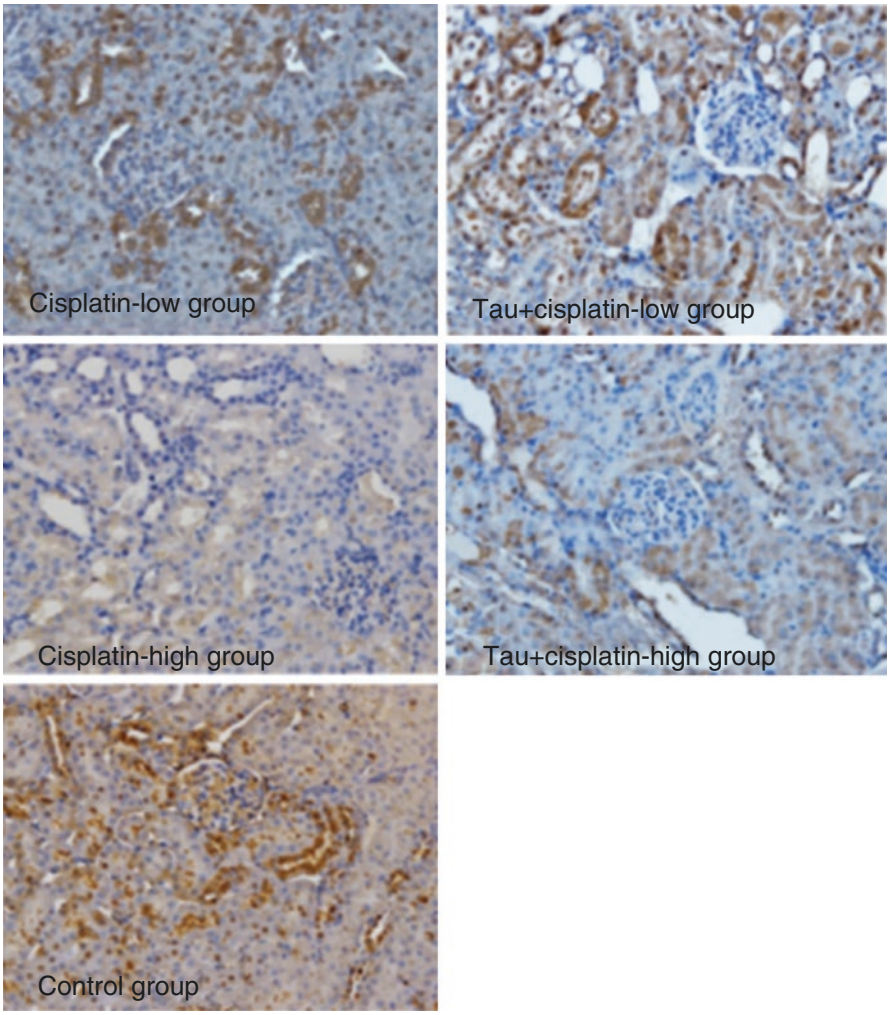

Fig. 3 High immunoreactivity of arginine was observed in the control group. The immunoreactivity of arginine was decreased in the proximal tubule of animals in the cisplatin-low group and cisplatin-high group. A treatment with taurine increased the immunoreactivity of arginine in the collecting duct and part of the proximal tubule. All sections were counterstained with hematoxylin

of cisplatin. A treatment with, reduced the immunoreactivity of arginine in epithelial cells of the proximal tubule (Fig. 3).

Weak citrulline immunoreactivity was present in epithelial cells of the proximal tubule of control animals. A treatment with cisplatin increased citrulline immunoreactivity in epithelial cells of the proximal tubule, which was proportional to the dose of cisplatin used. A treatment with taurine inhibited the immunoreactivity of citrulline in epithelial cells of the proximal tubule (Fig. 4).

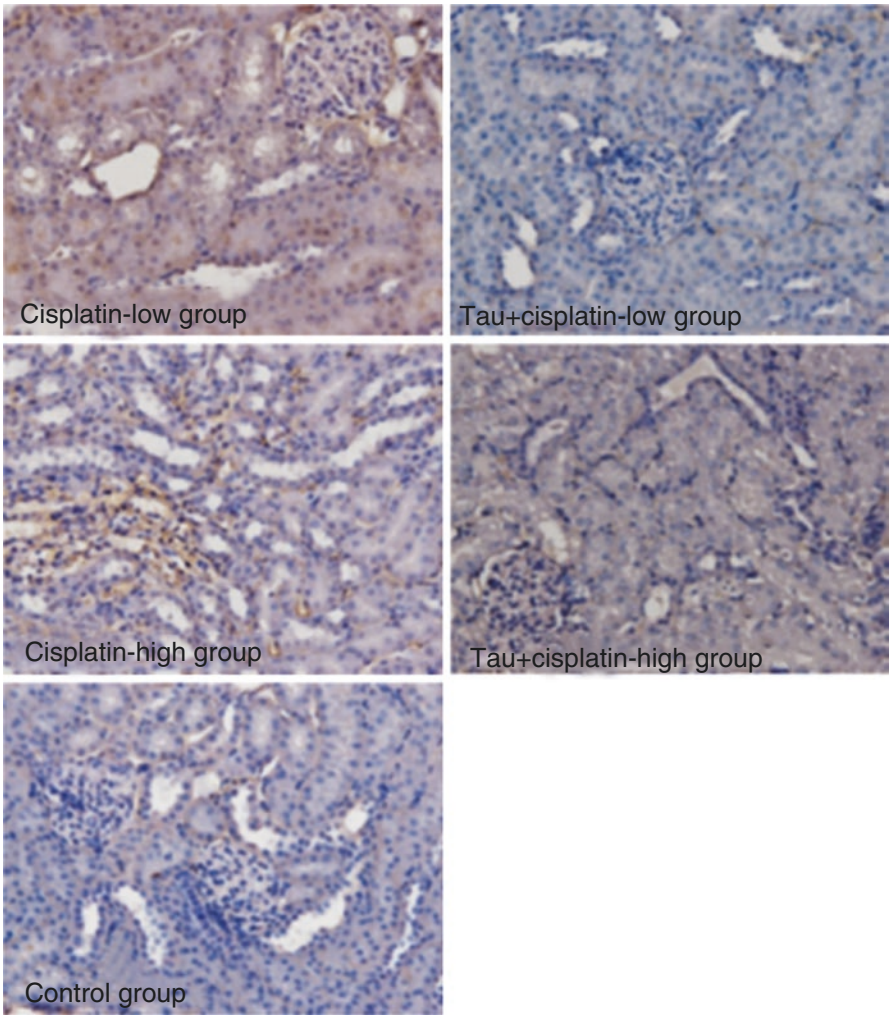

Fig. 4 A weak immunoreactivity for citrulline was observed in the control group. The immunoreactivity of citrulline increased in the proximal tubule of animals in the cisplatin-low group and cisplatin-high group. A treatment with taurine-lowered the immunoreactivity of citrulline in the collecting duct and part of the proximal tubules

4 Discussion

In the present study, we examined the effect of taurine on iNOS-dependent DNA damage as a result of cisplatin induced renal injury in a rat model. Between 25 and 35% of patients experience a significant decline in renal function after receiving a single dose of cisplatin (Ries and Klastersky 1986).

Therefore, understanding of mechanisms whereby cisplatin induces kidney injury is crucial to developing an effective and safe therapeutic strategy to cancer treatment with cisplatin. In renal tissues, cisplatin accumulates in the proximal tubule followed by the distal collecting tubule and the convoluted tubule (dos Santos et al. 2012). Reactive nitrogen species are regarded as the causes of DNA damage associated with cisplatin chemotherapy (Chirino and Pedraza-Chaverri 2009).

The extent of the nitrative DNA damage was found increased in the nucleus of proximal tubule and collecting duct cells of animals receiving cisplatin. The expression of iNOS was found increased in epithelial cells of the proximal tubule and collecting duct. Furthermore, iNOS decreased arginine and increased citrulline entering the pathway of nitric oxide synthesis. On the other hand, the occurrence of nitrative DNA damage was ameliorated by taurine to the point that the appearance of the renal structures approximated that of the control group, while the expression of iNOS was found decreased in epithelial cells of the proximal tubule and collecting duct; the level of arginine entering the nitric oxide pathway was decreased but that of the byproduct citrulline increased in the cisplatin-treated groups.

Two major conclusions about cisplatin emerge from the above results. First, cisplatin can induce nitrative DNA damage in the kidney that results in renal damage when used at doses as low as 15–25 mg/kg. The target areas of cisplatin are the proximal tubule followed by the distal collecting tubule and the convoluted tubule, and, as a result, renal function can become reduced. A decrease of arginine accompanied by an increase of citrulline is found to correlate with an increase in iNOS activity. In the kidney, the nitric oxide pathway may be important to maintain homeostasis, but it also may contribute to nitrative stress in the kidney. Second, taurine plays a protective role against renal inflammation and the formation of DNA lesions by iNOS-mediated nitrative stress in cisplatin-induced renal injury by suppressing the expression of iNOS. Reduction of 8-nitroguanine formation/increase of arginine in renal tissue, and arginine increase/citrulline decrease are all response to repression of iNOS activity in the kidney during cisplatin treatment. Taurine, by preventing iNOS-mediated activity, interrupts the inflammatory cycle and ablates renal damage.

5 Conclusion

Cisplatin is one of the most effective chemotherapeutic agents in current use. However, its use in cancer treatment is limited by its intrinsic nephrotoxicity. Therefore, protective strategies to alleviate cisplatin nephrotoxicity are crucial for the survival of cancer patients can serve as on this drug. Taurine is not incorporated into protein, and participates in biologic processes in the kidney that influence the kidney homeostasis. The present study has investigated the protective effect of taurine against cisplatin induced renal injury in a rat model.

Our study has indicated to two major findings. First, cisplatin can induce nitrative DNA damage in the renal proximal tubules. Second, taurine can play a protective role in the formation of nitrative DNA lesions though iNOS-mediated nitrative stress by cisplatin.

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Effect of Radiation on the Expression of Taurine Transporter in the Intestine of Mouse

Takenori Yamashita, Toshihiro Kato, Masahiro Tunekawa, Yeunhwa Gu, Shumin Wang, and Ning Ma

Abstract There has been a growing interest on the effects of radiation since the Fukushima nuclear power plant accident of 2011. Taurine has been reported to have a radioprotective effect in irradiated mice. However, the detailed mechanism of this radioprotective effect is still awaiting clarification. The aim of this study was to investigation how radiation affects the expression of taurine and to shed light on the mechanism accounting for radioprotective and radiation mitigating effect. Six-week-old male mice were randomly divided into two groups: IR group (7 Gy irradiation) and IR + Tau group (7 Gy irradiation + taurine 3000 mg/kg/day). We examined the survival rate, the expression of taurine and taurine transporter in the small intestine and the urinary taurine concentration. In this study, no statistically significant difference was found in the survival rate between IR Group and IR + Tau Group. Three days and 7 days after irradiation, the urinary taurine concentration of IR + Tau group increased more than that of IR group. Three days and 10 days after irradiation, the expression of taurine and taurine transporter in the small intestine of IR group and IR + Tau group decreased more than that of normal small intestine. It is reported that radiation exposure increases the urinary taurine concentration. We found that the radiation exposure decreases the expression of the taurine transporter in the small intestine of mouse. This finding suggests that a decrease in the expression of the taurine transporter promotes the release of taurine from the tissue into the urine.

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Keywords Radiation • Taurine transporter • Intestine • Oxidation • Inflammation

Abbreviations

IR	X-irradiation
ROS	Reactive oxygen species
Tau	Taurine

1 Introduction

The impact of damage by ionizing radiation exposure in both humans and mice is well known. Radiation generates reactive oxygen species (ROS), free radicals such the hydrogen radical (H^+) and hydroxyl radical (HO^-) in irradiated cells. The free radicals induce injury to living cells DNA (Rostami et al. 2016). Radioprotective agents can offer protection through an ability to remove free radicals. Radioprotectors have been studied for their effect to reduce the cell damage of free radicals in normal tissues for a long time (Weiss and Landauer 2009; Poggi et al. 2001). However, several radioprotectors do not protect irradiated cells when administered after a radiation exposure (Singh et al. 2012; Grebeniuk et al. 2012; Ciorba et al. 2012). This finding suggests that prevention of radiation-induced cellular damages requires the radioprotector to present at the time of irradiation (Grdina et al. 2002; Gu et al. 2000). Unexpected exposure to radiation due to a nuclear plant accident is a common cause of accidental exposure to radiation and one that requires the use of agents that will reduce injury to normal tissue as a result of exposure to radiation. In this case, it is expected that the agent used will accelerate either the recovery from or repair of the radiation injury (Singh et al. 2013). Research on the radioprotective effect of taurine has been carried out since the 1960s (Sugahara et al. 1969). Taurine (2-aminoethanesulfonic acid) is a major intracellular amino acid possessing several important effects, including antioxidant and anti-inflammatory ones (Oliveira et al. 2010; Ma et al. 2010; Kato et al. 2015). Taurine appears to be an attractive candidate for use as a radioprotectors and as a radiation mitigators but at the present time it is not known how it protects against radiation induced cell damage. It is reported that taurine is taken up by cells via taurine transporter (Kwon and Handler 1995). Since reduction of radiation induced cell damage by taurine might be associated with the expression of taurine transporter, it appeared of interest to investigate the expression of the taurine transporter after a radiation exposure. The purpose of this communication is to report that a radiation exposure affects the expression of the taurine transporter in the small intestine of the mouse.

2 Methods

2.1 Animals and Drug Administration

Male ICR mice 6-weeks-old, weighing 26–28 g, were obtained from Japan SLC (Shizuoka, Japan) and handled according to Guidelines for the Regulation of Animals, as provided by animal ethics committee of Suzuka University of Medical Science (Suzuka, Mie-ken, Japan). The animals were maintained in a controlled room at 22 ± 3 °C with a relative humidity of 65 ± 5% and a 12-h light/dark cycle (08:00–20:00). 3000 mg/kg b.w. per day of taurine was given orally by dissolving it in the drinking water to each mouse. Taurine was administered 30 min after irradiation.

2.2 Irradiation

All irradiation experiments were conducted at the X-radiation facility of Suzuka University of Medical Science (Suzuka, Mie-ken, Japan). Mice were irradiated in well ventilated boxes (five mice in each box) to 7 Gy whole body irradiation at a dose rate of 0.331 Gy/min at 200 kV and 9 mA (Phillips MG226, Tokyo, Japan). The beam was filtered through a 0.2 mm copper and 1 mm aluminum board. After irradiation, the mice were returned to their cages and maintained on food and water on an ad libitum basis.

2.3 Animal Groups

The mice were randomly divided into two groups of nine each: IR: 7 Gy whole body X-irradiation and IR + Tau: 3000 mg/kg b.w. per day of taurine after 7 Gy whole body X-irradiation.

2.4 Survival Studies

Two groups of five mice each were used in the experiments. Mice were exposed whole body X-irradiation (7 Gy/mouse). The mice were randomly divided into two groups: IR group (7 Gy irradiation) and IR + Tau group (7 Gy irradiation + taurine 3000 mg/kg b.w. per day). Survival and apparent behavioral deficit of these mice were monitored for a period of 13 days.

2.5 Measurement of Peripheral Blood Lymphocytes

Two groups of nine mice each were used in the experiments. Mice were exposed whole body X-irradiation (7 Gy/mouse). Mice were randomly divided into two groups: IR group and IR + Tau group. Peripheral blood was collected with a capillary tube from the tail vein and then counted lymphocytes with an automated blood cell counter (Celltac-α MEK-6318, Nippon Koden INC, Japan).

2.6 Measurement of Urinary Taurine Concentration

Three mice from each group were killed on days 3 and 10 and their urine collected in 1.5 ml Eppendorf tubes through a needle inserted into the bladder. Samples were frozen at −80 °C immediately after they were collected. The urine taurine concentration was measured by a photometric method based on the dinitrophenylation of the sample followed by chloroform extraction as described by Wilbraham et al. (1971).

2.7 Immunohistochemical Studies

Mice were exposed to 7 Gy of radiation. Three mice from each group were killed on days 3, 7 and 10 for the immunochemical analysis of their small intestines. Small intestines were isolated and fixed in 37% paraformaldehyde overnight and embedded in paraffin. Then, 7 μm-thick paraffin sections were stained with hematoxylin and eosin (H&E). Rabbit taurine-specific antibodies was prepared essentially as described previously (Ma et al. 1994). Taurine transporter (TauT) antibody (SC6A6) was obtained from EnoGene (Tokyo, Japan). Taurine and taurine transporter antibodies immunoreactivities in the intestine sections of mice were observed by a peroxidase anti-peroxidase (PAP) method study. Briefly, paraffin sections (6 μm in thickness) were incubated with rabbit polyclonal anti-taurine antibody (2 μg/ml), anti-taurine transporter antibody (2 μg/ml) overnight at room temperature, respectively. Then, the sections were incubated for 2 h with goat antibody against rabbit IgG (1:200), and were followed by peroxidase anti-peroxidase complex (1:200) for 2 h. The sections that had been treated with first and second antibodies were incubated for 10 min at RT with 3, 3′-diaminobenzidine tetrahydrochloride as chromogen, which had been freshly prepared as a solution of 20 mg in 100 ml PBS that contained 0.01% H_2O_2. Images of tissue sections were captured using an Olympus optical microscope (Olympus Corp., Tokyo, Japan).

2.8 Statistic Analysis

The comparison of means was performed by *t-test* for two-group comparisons. The survival curve analysis was assessed by log-rank test. Each value was expressed as the mean ± SEM. For all tests, significance was accepted when $P < 0.05$.

3 Results

3.1 Taurine Administration Effect of After X-Irradiation in Mouse

The effect of taurine on the survival rate of mice with whole body X-irradiation (7 Gy/mouse) are shown in Fig. 1. The survival rate was found not to be significantly different the between IR Group and IR + Tau Group. The effect of radiation on the peripheral blood lymphocytes of mice are shown in Fig. 2. Taurine was administered 30 min after irradiation. After 7 Gy irradiation, mice exhibited a considerable decrease in the number of lymphocytes. There was no effect on the recovery of lymphocytes on 3 and 7 days. However, we observed early recovery of lymphocytes from the IR + Tau group in 10 days. The effect of radiation on the urinary taurine concentration of mice is shown in Fig. 3. The urinary taurine concentration of the IR + Tau group increased more than that of IR group.

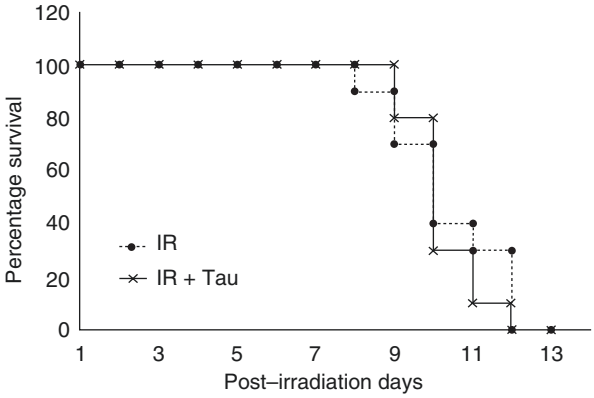

Fig. 1 The effects of the administration of taurine and of irradiation on survival using a 13-day survival curve. Male ICR mice (n = 10) underwent a whole body X-ray radiation (7 Gy/mouse). 3000 mg/kg b.w. per day of taurine was given orally to IR + Tau group. Taurine was administered 30 min after irradiation. Data are shown as the mean ± SE. No statistically significant difference was found in survival rate between IR group and IR + Tau group

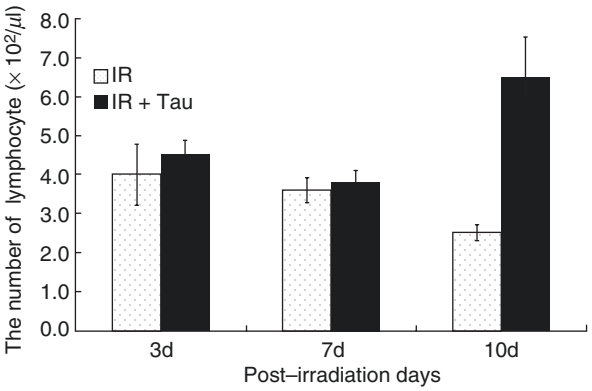

Fig. 2 The effects of the administration of taurine and irradiation on lymphocytes were analyzed using an automated blood cell counter. Male ICR mice (n = 9) were exposed whole body X-irradiation (7 Gy/mouse). 3000 mg/kg b.w. per day of taurine was given orally by dissolving it in the drinking water to IR + Tau group. Taurine was administered 30 min after irradiation. Data are shown as the mean ± SE. Ten days after irradiation, the number of lymphocytes of IR + Tau group increased more than that of IR group

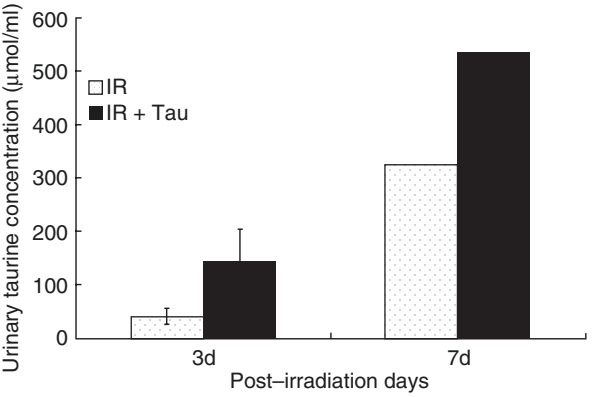

Fig. 3 The effects of the administration of taurine and irradiation on the urinary taurine concentration. Male ICR mice (n = 9) were exposed to whole body X-ray irradiation (7 Gy/mouse). Taurine (3000 mg/kg/day) taurine was given as part of the drinking water to rats in the starting at 30 min after irradiation. Data are shown as the mean ± SE. The urinary taurine concentration of IR + Tau group increased more than that of IR group

3.2 Localization of Taurine and Taurine Transporter After X-Irradiation in Mouse Small Intestine

The effect of radiation on the small intestine of mice is shown in Fig. 4. Ten days after irradiation, villus in the small intestine of IR group and IR + Tau group were shorter than that of a normal small intestine. The effect of radiation on the

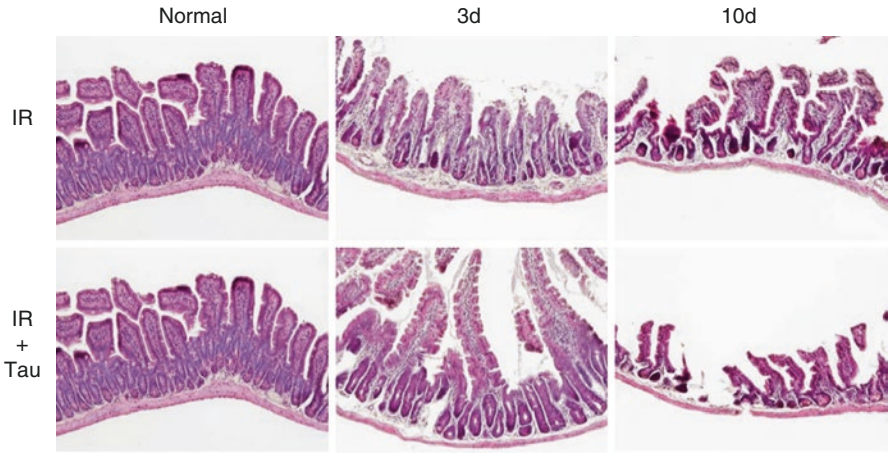

Fig. 4 Histological evidence of intestinal injuries—in mice after 7 Gy whole body X-irradiation. Representative intestinal sections stained with H&E are shown (200×). Taurine was given orally at 3000 mg/kg/day as part as part of the drinking water to rats in the IR + Tau group at 30 min after irradiation. Ten days after irradiation, the villi in the small intestine of IR group and IR + Tau group were found shorter than those in a normal small intestine

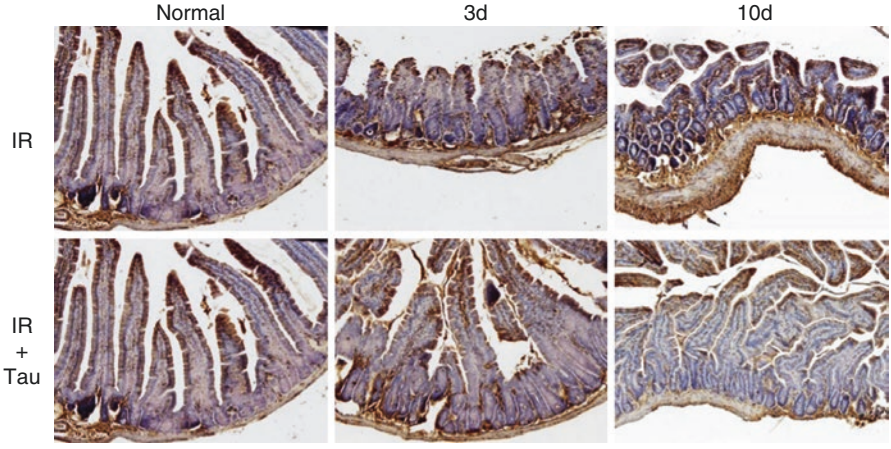

Fig. 5 Histological evidence of intestinal injuries in mice after 7 Gy whole body X-ray irradiation. Representative intestinal sections stained with H&E are shown (200×). Taurine, 3000 mg/kg/day was given orally as part of the drinking water to rats in the IR + Tau group. Taurine was administered at 30 min after irradiation. Three days and 10 days after X-ray irradiation, the expression of taurine in the small intestine of IR group and IR + Tau group decreased more than that of normal small intestine

expression of taurine/taurine transporter in the small intestine of mice is shown in Figs. 5 and 6. The expression of taurine/taurine transporter was reduced after radiation exposure.

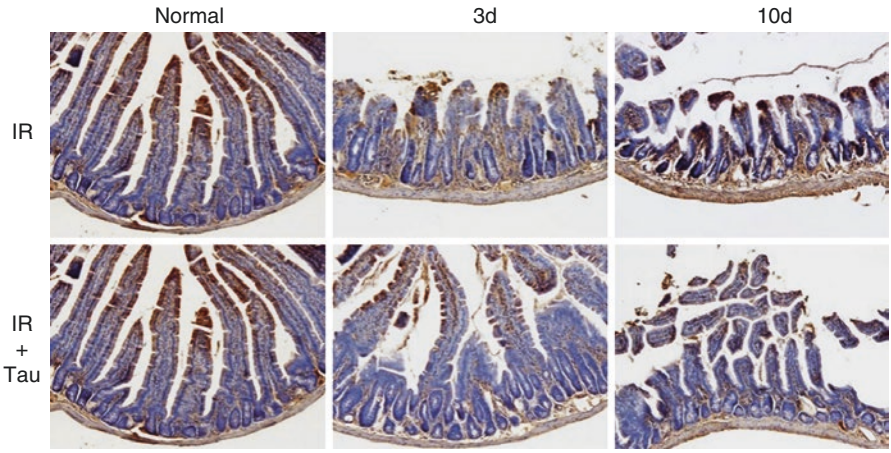

Fig. 6 Histological evidence of intestinal injuries in mice after 7 Gy whole body X-ray irradiation. Representative intestinal sections stained with H&E are shown (magnification 200×). Taurine, 3000 mg/kg/day was given as part of the drinking water to rays in the IR + Tau group. Taurine was administered 30 min after irradiation. Three days and 10 days after irradiation, the expression of taurine transporter in the small intestine of IR group and IR + Tau group decreased more than that of a normal small intestine

4 Discussion

Radiation exposure generates ROS and free radicals in irradiated cells. Excessive free radical generation is leading cause of oxidative stress. Physiological roles of taurine include an antioxidant action and protection of the body by inhibiting ROS and free radical formation (Johnson et al. 2012). The protective effect of taurine against organ damage caused by ischemia and reperfusion may be due to antioxidant action (Hanna et al. 2004). Ueno et al. (2007) has reported the functional recovery of rat administered of taurine after reperfusion Organ damage caused by ischemia/reperfusion and radiation exposure may be due to oxidative stress (Kingston et al. 2004; Asghari et al. 2016; Agrawal and Kale 2001). Therefore, the administration of taurine before or after irradiation might have a protective effect or aid in the recovery from organ damage.

Radiation can cause injury to hematopoietic and gastrointestinal systems depending on the dose of radiation received (Suman et al. 2012). Morphological changes of the intestinal mucosa after a high radiation exposure dose have been well documented (Driák et al. 2008; Labéjof et al. 2002), but molecular events that regulate intestinal epithelial cells radio-sensitivity and radiation-induced gastrointestinal injuries are not fully understood (Li et al. 2015). Abe et al. (1968) have reported an increase in the survival of mice to radiation exposure upon receiving taurine after the exposure although the effect was less than when administered before the exposure possibly because of a decrease in uptake after irradiation. Therefore, we examined the effect of taurine administration after a radiation exposure on the survival

rate, blood lymphocytes count and urinary taurine concentration in the whole body irradiation mouse model. This model is a well-established one that has been used to evaluate the radioprotective and radiation mitigation effect of chemical or of dietary compounds such as yeast-derived beta-glucan, melatonin and vitamin C against cellular damage (Gu et al. 2008; Rostami et al. 2016).

In the present study, taurine was not able to improve the survival rate after exposure to a high dose of radiation. This result is most likely due to incomplete recovery from an intestinal injury and to immune response deficiency. Therefore, to examine the role of taurine on the immune system and on the count of peripheral blood lymphocytes in mice after irradiation. In the present study, the administration of taurine led to an early recovery pf the lymphocytes count. Since taurine has been reported to function as a growth factor for lymphocytes or for lymphocyte progenitor cells (Fazzino et al. 2010), it is likely that this amino acid is promoting the growth factor for lymphocytes after exposure to a high dose of radiation.

Several studies have shown that the urinary excretion of taurine increases after irradiation (Goyer and Yin 1967; Johnson et al. 2012; Watson 1962). However, the precise cause for such an increase remains unknown. We also observed that a radiation exposure increased the urinary taurine concentration. Similarly, a high dose of radiation affects the gastrointestinal system. Radiation-induced gastrointestinal injury is primarily due to death of epithelial stem cells of the crypts (Ghosh et al. 2012). Finding that radiation increases the urinary elimination of taurine suggests that taurine might be release from the injury tissue. A taurine depletion will be harmful because it may inhibit the recovery of physiological functions depending on cellular growth, immune system and intestinal mucosa function.

Severe damage to the small intestine could the lead to malabsorption of taurine. It is reported that taurine uptake is associated with an increased expression of the taurine transporter (Warskulat et al. 2004). Therefore, to evaluate the mechanism of the radioprotective effect and radiation mitigation effect of taurine in the small intestine of mouse, we evaluated the expression of the taurine transporter. Histopathology is a well-established model that has been used to evaluate radioprotection and radiation mitigators against hematopoietic/gastrointestinal damage in mouse after irradiation (Zhang et al. 2003). Radiation exposure has been shown to induce apoptosis in cells (Singh et al. 2013). It is reported that taurine attenuates apoptosis (Maher et al. 2005). In the present study, radiation exposure inflicted severe damage to villi in the small intestine. This result suggests that taurine cannot attenuate apoptosis after a high dose of radiation. In fact, the expression of taurine/taurine transporter was reduced after radiation exposure. The intestinal injury after exposure to a high dose of radiation was associated with a decreased expression of the taurine transporter. Taurine uptake might be decreased in the small intestine after irradiation. These results indicate that exposure to a high dose of radiation induces cell damage and that taurine is unable to mediate recovery or repair against these changes. Thus, although taurine can mobilize hematopoietic cells to protect against the cellular damage by a high dose of radiation it, however, cannot aid in recover from intestinal injuries when given after a radiation exposure. It is also known that taurine losses have occurred after irradiation (Bezkrovnaia and Kostesha

1990). Therefore, taurine administration after irradiation might be beneficial to compensate for the taurine losses. In any event, a future study will be necessary to examine the effect of taurine after exposure to a low dose of radiation.

Taurine transporter, oxidative stress and inflammatory response-associated mechanisms are involved in radiation-induced gastrointestinal injury. We found that exposure to a high dose of radiation decreases the expression of the taurine transporter in the small intestine of the mouse. It is conceivable that the recovery/repair of radiation injury caused by taurine administration, as used in the present study, is not sufficiently pronounced to have a beneficial effect on intestinal injury after high dose of radiation exposure. Hence, the change of taurine transporter expression in response to different doses of radiation needs to be further investigated. These findings have significant implications to radiation exposure of civilians in nuclear plant accidents such as the one that took place in Fukushima, Japan.

5 Conclusion

In summary, a high dose of radiation exposure is found to decrease the expression of taurine transporter in the small intestine of mouse. Taurine may not necessarily be a good protectant when administered after high dose of radiation exposure but it might have a mitigating effect after low dose radiation exposure. The results of this study also indicate that by using a low dose of radiation it might be possible to explain the relationship between the mitigating mechanism of taurine on the radiation exposure and the expression of the intestinal taurine transporter.

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Taurine Supplementation Ameliorates the Adverse Effects of Perinatal Taurine Depletion and High Sugar Intake on Cardiac Ischemia/Reperfusion Injury of Adult Female Rats

Supaporn Kulthinee, Sasipa Rakmanee, J. Michael Wyss, and Sanya Roysommuti

Abstract Perinatal taurine depletion followed by high sugar intake after weaning adversely affects myocardial and arterial pressure function following a myocardial ischemia and reperfusion (IR) insult in adult female rats. This study tests the hypothesis that taurine supplementation ameliorates this adverse effect. Female Sprague-Dawley rats were fed normal rat chow and drank water containing β-alanine from conception until weaning (taurine depletion, TD). After weaning, female offspring were fed normal rat chow and drank either water containing 5% glucose (TDG) or water alone (TDW). At 6–7 weeks of age, half the rats in each group were supplemented with taurine and 1 week later subjected to cardiac IR. Body weight, heart weight, plasma electrolytes, plasma creatinine, blood urea nitrogen, and hematocrit were not significantly different among the four groups. The mean arterial pressures significantly increased in all groups after IR, but values were not significantly different among the four groups. Heart rates were significantly increased after IR only in TDW group. Compared to TDW, TDG displayed increased plasma cardiac injury markers (creatinine kinase-MB, troponin T, and N-terminal prohormone brain natriuretic peptide), increased sympathetic activity, decreased parasympathetic activity, and decreased baroreflex sensitivity after IR. Taurine supplementation completely restored the baroreflex and autonomic dysfunction of TDG to TDW levels and partially decreased myocardial injury after cardiac IR. The present study indicates that in adult female rats, perinatal taurine depletion followed by high sugar intake after weaning exacerbates cardiac IR injury and arterial pressure dysregulation and these adverse effects can be partially prevented by taurine supplementation.

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Abbreviations

AST	Aspartate aminotransferase
BSHR	Baroreflex sensitivity control of heart rate
BSRA	Baroreflex sensitivity control of renal nerve activity
BW	Body weight
CK-MB	Creatine kinase-MB
CPR	Cardiopulmonary resuscitation
Hct	Hematocrit
HF	High frequency
HW	Heart weight
IR	Ischemia/reperfusion
LF	Low frequency
NT-proBNP	N-terminal prohormone brain natriuretic peptide
TDG + T	Perinatal taurine depletion with high sugar intake plus taurine
TDG	Perinatal taurine depletion with high sugar intake
TDW + T	Perinatal taurine depletion with water intake plus taurine
TDW	Perinatal taurine depletion with water intake alone
Trop-T	Troponin T

1 Introduction

Taurine, 2-aminoethanesulfonic acid, is the major organic osmolyte in animal cells and plays many physiologic functions from prenatal to adult life (Sturman 1993). Prenatal and early postnatal taurine exposure is crucial to early development and can influence adult function and disease. Perinatal taurine depletion or excess can adversely affect adult offspring, but depletion appears to have more severe consequences (Roysommuti and Wyss 2014). Prenatal taurine depletion induces low birth weight newborns that are prone to developing cardiovascular disease and diabetes mellitus when they become adults. Further, perinatal taurine depletion increases cardiac ischemia and reperfusion (IR) injury and arterial pressure dysregulation in adult male rats, particularly when combined with high sugar intake after weaning (Kulthinee et al. 2010). High sugar intake also blunts baroreflex regulation of heart rate and renal nerve activity in both male (Roysommuti et al. 2009) and female (Thaeomor et al. 2010) rats that are perinatally depleted of taurine. In the female rats, the adverse effect of perinatal taurine depletion is reversed by short-term inhibition of the renin-angiotensin system, but not by estrogen receptor blockade (Thaeomor et al. 2010).

Sex differences underlie the incidence and progression of many diseases, particularly cardiovascular diseases (Ashraf and Vongpatanasin 2006; Hay 2016; Kim et al. 2014; Yanes and Reckelhoff 2011). The incidence of cardiovascular disease is low in premenopausal and high in menopausal women compared to age-matched men. However, high estrogen treatment and oral contraceptives are reported to increase arterial pressure in susceptible women and this can be abolished by inhibition of the RAS (Ashraf and Vongpatanasin 2006). Our previous experiments indicate that in adult male rats, cardiac IR markedly increases plasma cardiac injury markers but does not affect sympathetic nerve activity (Kulthinee et al. 2010), while in female rats, cardiac IR does not cause cardiac damage but increases sympathetic nerve activity, as estimated by arterial pressure variability (Kulthinee et al. 2015). Further, cardiac IR increases cardiac injury more in male rats perinatally depleted of taurine than in control rats and this adverse effect is exacerbated by high sugar intake after weaning (Kulthinee et al. 2010). Whether these adverse effects of perinatal taurine depletion also occur in female rats has not been tested.

In animals and humans, the adverse effects of cardiac IR include myocardial injury, decreased baroreflex sensitivity, increased sympathetic nerve activity, and increased renin-angiotensin system overactivity (Ando et al. 2002; de La Fuente et al. 2013; Huang et al. 2007; Jones et al. 2008). While the cardiac damage may result from increased cardiac oxidative stress and angiotensin II (Schaffer et al. 2014), baroreflex and autonomic dysfunction is related to central effects of the renin-angiotensin system (Huang et al. 2013). In addition, these changes are, at least in part, dependent on the severity of cardiac damage (Ando et al. 2002; Jones et al. 2008). Taurine exposure has many physiological interactions, including antioxidation, decreased sympathetic nerve activity, inhibition of the renin-angiotensin system activity, anti-apoptosis, anti-inflammation, and cardiac osmoregulation and calcium control (Roysommuti and Wyss 2014). Thus, taurine supplementation before or after cardiac IR are reported to prevent or decrease cardiac injury and dysfunction (Schaffer et al. 2014). Our previous experiments in adult female rats also support this beneficial effect of taurine and further indicate that taurine supplementation before and after cardiac IR abolishes baroreflex and autonomic dysfunction (Kulthinee et al. 2015). The present study tests the hypothesis that taurine supplementation ameliorates the adverse effects of perinatal taurine depletion followed by high sugar intake after weaning on myocardial injury and arterial pressure control after cardiac IR in adult female rats.

2 Methods

2.1 Animal Preparation

Sprague-Dawley rats were bred at the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand and maintained at constant humidity (60 ± 5%), temperature (24 ± 1 °C), and light cycle (06.00–18.00 h). Female

Sprague-Dawley rats were fed normal rat chow (C.P. Mice Feed 082) and drank water containing β-alanine from conception until weaning (taurine depletion, TD). After weaning, the female offspring were fed normal rat chow and drank water containing 5% glucose (TDG group) or water alone (TDW group) throughout the experiment. Starting a week before cardiac IR induction until the end of the experiment, half of the rats in each group were supplemented with 3% taurine in the drinking water (TDW with taurine, TDW + T; TDG with taurine, TDG + T). The female control rats were similarly treated with (CG group) or without (CW group) high sugar intake followed in some cases by taurine supplementation (CW + T and CG + T groups). The data for the control groups were previously published (Kulthinee et al. 2015) and their comparisons with the present data were also performed.

All experimental procedures were approved by the Khon Kaen University Animal Care and Use Committee (AEKKU 15/2557) and were conducted in accordance with the National Institutes of Health guidelines.

2.2 Experimental Protocol

At 7–8 weeks of age, all rats were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg, intraperitoneal). After catheterization of the femoral artery for arterial pulse measurements and blood sampling as well as that of the femoral vein for fluid or drug injection, their arterial pressures were continuously monitored (BIOPAC Systems, Goleta, CA, USA). Then, a tracheal tube was slowly and carefully inserted into the trachea. After 15–20 min of baseline recording, cardiac arrest was induced by direct tracheal tube clamping. Upon arrest of the heart and drop in mean arterial pressure to 50–60 mmHg, the clamp was released and cardiopulmonary resuscitation (CPR) was immediately performed by manual chest compression at 60 times/min. During CPR, animal ventilation was continuously controlled by a ventilator connected to the tracheal tube. Normalization of arterial pressure and self-automatic respiration were the indices of CPR success (Kulthinee et al. 2015).

Two days later in a conscious state, arterial pressure pulses were continuously recorded before and during infusion of phenylephrine (to increase arterial pressure) or sodium nitroprusside (to decrease arterial pressure) to measure baroreflex sensitivity control of heart rate.

After a 24-h recovery period, rats were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg, intraperitoneal), tracheotomized, and then arterial pressure was continuously recorded. Renal sympathetic nerve was then exposed and its activity was continuously recorded by using stainless steel electrodes (12 MΩ, 0.01 Taper, Model 5727; A-M System, Sequim, WA, USA) connected to DAM-80 amplifier (DAM 80; World Precision Instruments, Sarasota, FL, USA) and BIOPAC Systems, respectively. Multiunit recordings of renal nerve activity were conducted only on nerve units that responded to changes in arterial pressure following sodium nitroprusside or phenylephrine infusion. Body temperature was servo-controlled at 37 ± 0.5 °C by a rectal probe connected to a temperature regulator controlling an overhead heating lamp. At the end of experiment, blood volumes (about 5.0 mL)

were collected from abdominal aortas for determination of plasma sodium (Na^+), plasma potassium (K^+), plasma chloride (Cl^-), plasma bicarbonate (HCO_3^-), blood urea nitrogen (BUN), plasma creatinine (Cr), hematocrit (Hct), aspartate aminotransferase (AST), creatine kinase-MB (CK-MB), cardiac troponin T (Trop-T), and N-terminal prohormone brain natriuretic peptide (NT-proBNP). Finally, all animals were terminated by a high dose of anesthesia and heart weights were collected.

2.3 Data Analyses

Mean arterial pressure, heart rate, and arterial pressure variability were analyzed from recorded arterial pressure using Acknowledge software (BIOPAC Systems). The power spectral densities of arterial pressure wave were analyzed using fast Fourier transformation (Hanning window, pad with zeros, remove mean, remove trend, and linear magnitude) to indirectly estimate the sympathetic (low frequency 0.3–0.5 Hz) and the parasympathetic nerve activities (high frequency 0.5–4.0 Hz) (Acknowledge software; BIOPAC Systems). Each power spectral density was normalized to the percent of total power spectral density (summation of the two power spectral densities).

Changes in renal nerve activity and heart rate per changes in arterial pressure following either sodium nitroprusside or phenylephrine infusion were used to measure baroreceptor reflex control of renal nerve activity (BSRA) and heart rate (BSHR). In addition, all blood chemical parameters were specifically measured by the Srinagarind Hospital Laboratory Unit (Faculty of Medicine, Khon Kaen University).

2.4 Statistical Analysis

All data are expressed as mean ± SEM. Statistical comparisons among the groups were performed by using one-way ANOVA followed by the *post hoc* Tukey's test (StatMost32 version 3.6, Dataxiom, CA, USA). The significant criterion was $p < 0.05$.

3 Results

3.1 General Characteristics

At 7–8 weeks of age, body and heart weights were not significantly different among the four groups. At the end of the experiment, all groups displayed similar values of plasma electrolytes, blood urea nitrogen, plasma creatinine, and hematocrit (Table 1). In addition, these parameters were not significantly different from the control rats of same treatment (CW, CG, CW + T, and CG + T groups) (Kulthinee et al. 2015).

Table 1 Body weight (BW), heart weight (HW), plasma electrolytes (Na^+, K^+, Cl^-, and HCO_3^-), blood urea nitrogen (BUN), plasma creatinine (Cr), and hematocrit (Hct) after cardiac ischemia/reperfusion

Treatment	BW (g)	HW (g)	Na^+ (mEq/L)	K^+ (mEq/L)	Cl^- (mEq/L)	HCO_3^- (mEq/L)	BUN (mg/dL)	Cr (mg/dL)	Hct (%)
TDW	190 ± 1	0.79 ± 0.01	142.5 ± 0.9	4.5 ± 0.1	97.5 ± 0.7	25.6 ± 0.6	14.7 ± 0.7	0.5 ± 0.1	37.7 ± 0.7
TDW + T	191 ± 1	0.79 ± 0.01	142.7 ± 0.5	4.5 ± 0.1	98.3 ± 0.8	24.3 ± 0.6	15.4 ± 0.5	0.6 ± 0.1	37.5 ± 0.7
TDG	191 ± 1	0.80 ± 0.01	143.8 ± 0.5	4.4 ± 0.1	98.7 ± 1.0	24.6 ± 0.6	14.6 ± 0.4	0.6 ± 0.1	37.8 ± 0.5
TDG + T	191 ± 1	0.80 ± 0.01	143.0 ± 0.7	4.5 ± 0.1	99.3 ± 0.5	24.4 ± 0.6	15.5 ± 0.8	0.6 ± 0.1	37.8 ± 0.3

Values are means ± SEM. No significant differences were observed among the groups (TDW, perinatal taurine depletion with water intake alone; TDW + T, perinatal taurine depletion with water intake alone plus taurine; TDG, perinatal taurine depletion with high sugar intake; TDG + T, perinatal taurine depletion with high sugar intake plus taurine; n = 6–7 each group)

Table 2 Plasma cardiac injury markers in anesthetized animals

Treatment	AST (U/L)	CK-MB (U/L)	Trop-T (ng/mL)	NT-proBNP (pg/mL)
TDW	159.2 ± 0.8	330.2 ± 6.0	<0.01	<5.0
TDW + T	152.3 ± 0.8$^{\alpha}$	318.2 ± 7.3	<0.01	<5.0
TDG	157.7 ± 3.3	367.3 ± 2.6$^{\alpha,\beta}$	0.9 ± 0.1$^{\alpha,\beta}$	8.8 ± 0.3$^{\alpha,\beta}$
TDG + T	153.7 ± 0.3	334.2 ± 7.9$^{\delta}$	0.4 ± 0.1$^{\alpha,\beta,\delta}$	6.3 ± 0.2$^{\alpha,\beta,\delta}$

Values are means ± SEM ($^{\alpha,\beta,\delta}P < 0.05$ compared to TDW, TDW + T, and TDG, respectively; TDW, perinatal taurine depletion with water intake alone; TDW + T, perinatal taurine depletion with water intake alone plus taurine; TDG, perinatal taurine depletion with high sugar intake; TDG + T, perinatal taurine depletion with high sugar intake plus taurine; AST, aspartate aminotransferase; CK-MB, creatinine kinase-MB; Trop-T, troponin T; NT-proBNP, N-terminal prohormone brain natriuretic peptide; n = 6 each group)

3.2 Plasma Cardiac Injury Markers

Three days after cardiac IR induction, plasma AST, CK-MB, Trop-T, and NT-proBNP levels of TDW (Table 2) were not significantly different from those of the CW group, as previously published (TDW versus CW; ASP, 159.2 ± 0.8 U/L versus 157.0 ± 7.0 U/L, CK-MB, 330.2 ± 6.0 U/L versus 299.8 ± 7.3 U/L; Trop-T, both below 0.01 ng/mL; NT-proBNT, both below 5.0 pg/mL; $p > 0.05$) (Kulthinee et al. 2015). High sugar intake after weaning did not affect AST levels but significantly increased plasma CK-MB, Trop-T, and NT-proBNP levels in TDG compared to TDW groups. Taurine supplementation completely restored plasma CK-MP and partially restored plasma Trop-T and NT-proBNP levels of TDG compared to those of the TDW and TDW + T groups. In addition, taurine supplementation significantly decreased plasma AST levels in TDW + T but not in TDG + T compared to the TDW and TDG groups.

3.3 Mean Arterial Pressure and Heart Rate

Before cardiac IR (anesthetized rats), mean arterial pressures were not significantly different among the perinatal taurine depletion groups (Fig. 1 upper, Before) and control groups (Kulthinee et al. 2015). After cardiac IR, unconscious mean arterial pressures significantly increased in all groups compared to their values prior to IR (Fig. 1 upper, After-1) and these values were not significantly different among the four groups. Although the mean arterial pressures after cardiac IR were higher in conscious (Fig. 1 upper, After-2) than unconscious rats, these values also were not significantly different among the four groups.

Similar to the mean arterial pressures, before cardiac IR heart rates were not significantly different among the perinatal taurine depletion groups (Fig. 1 lower, Before) and control groups (Kulthinee et al. 2015). The heart rates were slightly and

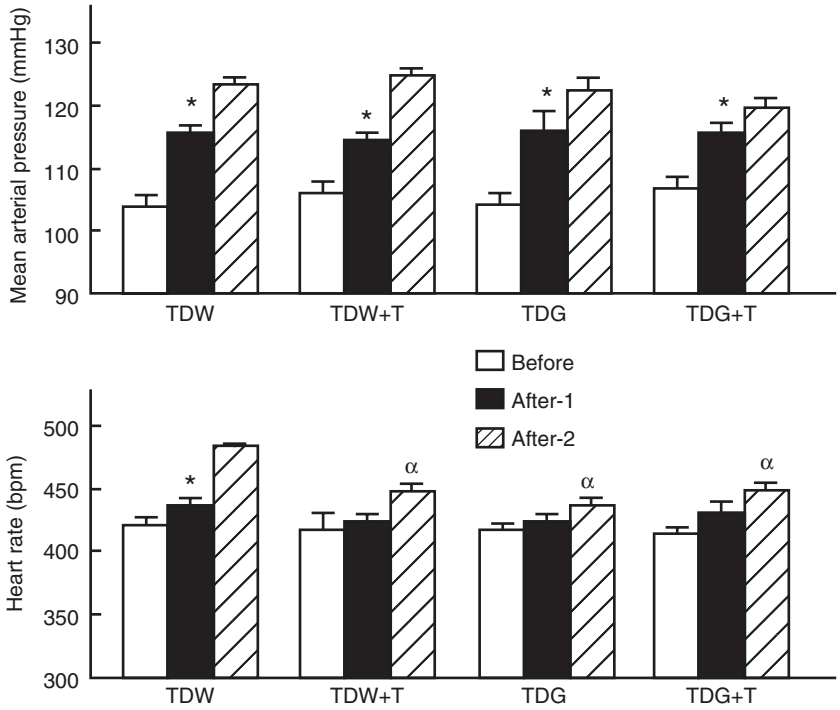

Fig. 1 Mean arterial pressures (*upper*) and heart rates (*lower*) before (anesthetized rats) and after (After-1, anesthetized rats; After-2, conscious rats) cardiac ischemia/reperfusion ($^{*,\alpha}P < 0.05$ compared to Before of the same group and After-2 of TDW, respectively; TDW, perinatal taurine depletion with water intake alone; TDW + T, perinatal taurine depletion with water intake alone plus taurine; TDG, perinatal taurine depletion with high sugar intake; TDG + T, perinatal taurine depletion with high sugar intake plus taurine; n = 6–7 each group)

significantly increased after cardiac IR induction only in anesthetized TDW rats, but these post IR heart rates were not significantly different among the four groups (Fig. 1 lower, After-1). However, in conscious, freely moving rats, the post IR heart rates were significantly increased in TDW compared to the other three groups (Fig. 1 lower, After-2).

3.4 Autonomic Control of Arterial Pressure

Power spectral analysis of arterial pressure waves indicated that sympathetic and parasympathetic nerve activities before and after cardiac IR were not significantly different among the TDW, TDW + T, and TDG + T groups, irrespective of consciousness (Fig. 2). In the TDG group, the sympathetic activity significantly

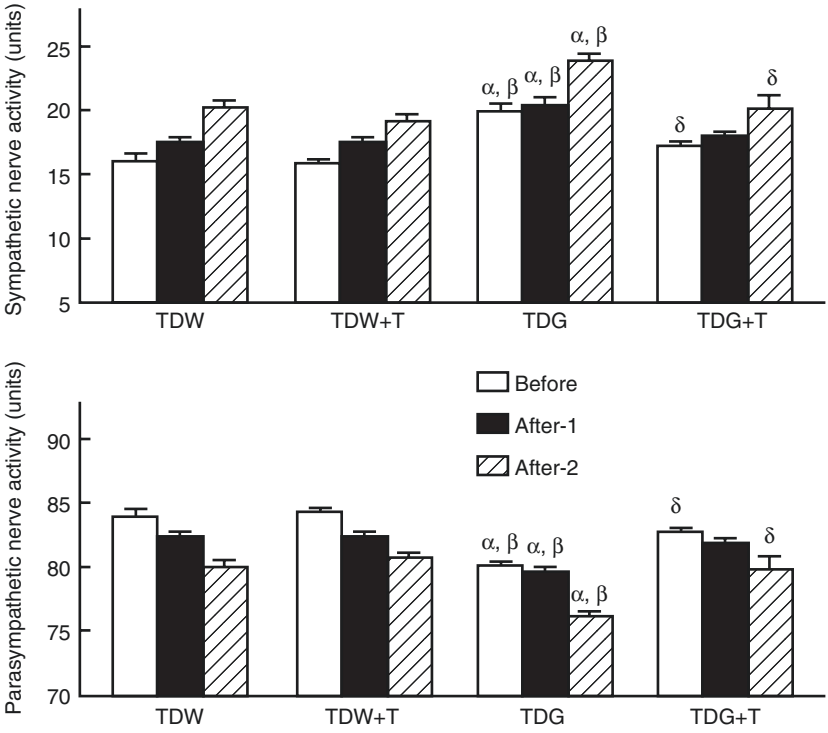

Fig. 2 Sympathetic (*upper*) and parasympathetic nerve activity (*lower*) estimated by power spectral analysis of arterial pressure ($^{\alpha,\beta,\delta}P < 0.05$ compared to corresponding TDW, TDW + T, and TDG, respectively; TDW, perinatal taurine depletion with water intake alone; TDW + T, perinatal taurine depletion with water intake alone plus taurine; TDG, perinatal taurine depletion with high sugar intake; TDG + T, perinatal taurine depletion with high sugar intake plus taurine; n = 6–7 each group)

increased and the parasympathetic activity significantly decreased compared to those of the other three groups. These autonomic functions were similar to those reported in control rats (Kulthinee et al. 2015).

3.5 Baroreflex Function

The BSHRs tested by phenylephrine infusion before and after cardiac IR were not significantly different (Fig. 3 upper), while those tested by sodium nitroprusside infusion significantly increased after IR in the TDW + T group (Fig. 3 lower). The latter value also was higher than those of the other three groups. High sugar intake

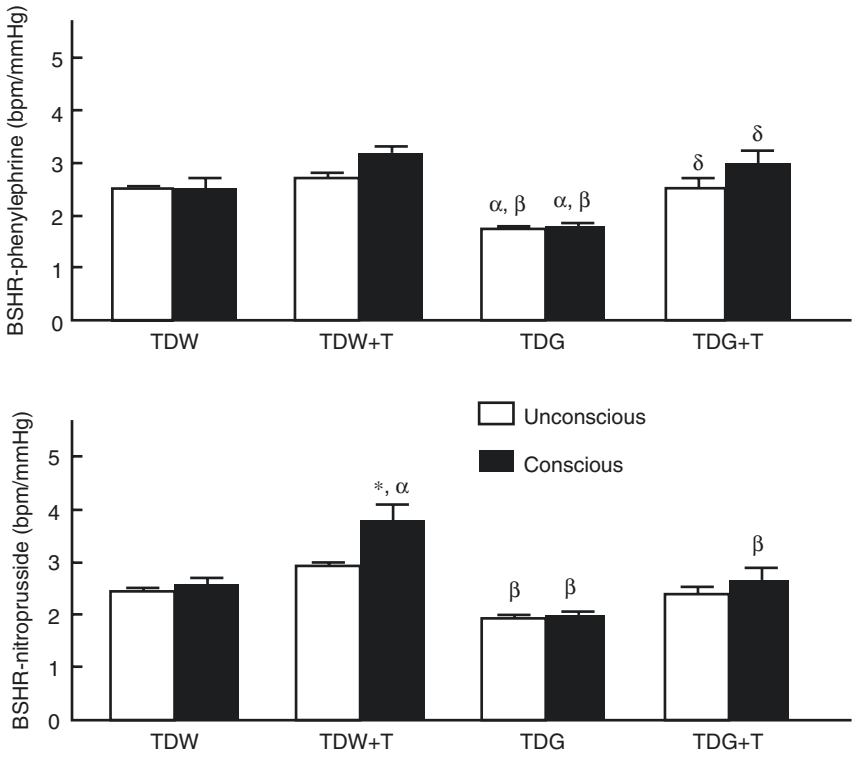

Fig. 3 Baroreflex sensitivity control of heart rate measured by phenylephrine infusion (BSHR-phenylephrine; upper) and sodium nitroprusside infusion (BSHR-nitroprusside; lower) in unconscious (white bars) and conscious animals (black bars) ($^{*,\alpha,\beta,\delta}P < 0.05$ compared to corresponding TDW + T (unconscious), TDW, TDW + T, and TDG, respectively; TDW, perinatal taurine depletion with water intake alone; TDW + T, perinatal taurine depletion with water alone intake plus taurine; TDG, perinatal taurine depletion with high sugar intake; TDG + T, perinatal taurine depletion with high sugar intake plus taurine; n = 6–7 each group)

after weaning significantly depressed BSHR in TDG compared to the TDW and/or TDW + T and this depression was restored to values for the TDW and TDW + T groups by short-term taurine supplementation (TDG + T group).

In anesthetized animals, the BSRAs tested by phenylephrine infusion were not significantly different among the four groups (Fig. 4 upper), while those tested by sodium nitroprusside significantly decreased in TDG compared to those of the other three groups (Fig. 4 lower). This depression was completely abolished by taurine supplementation.

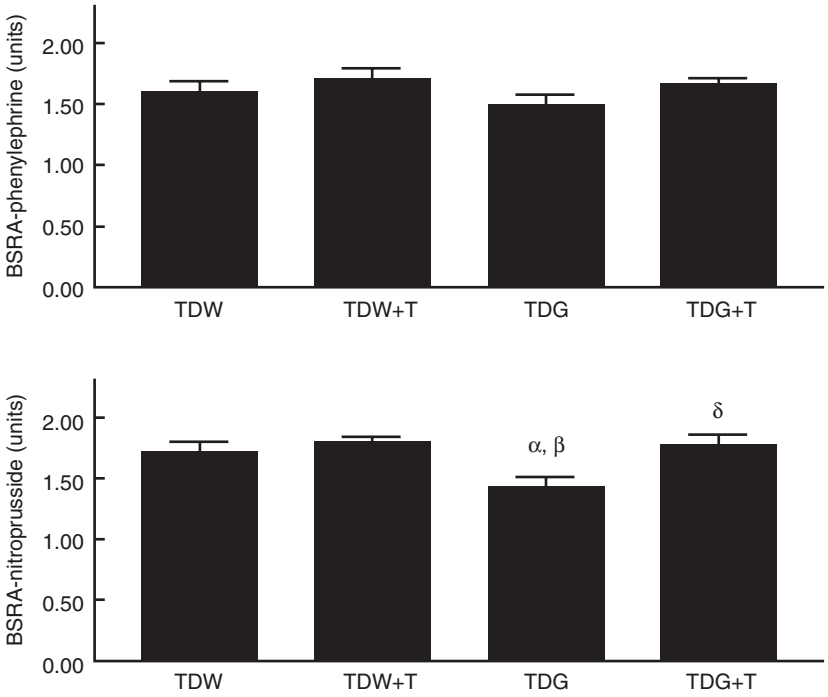

Fig. 4 Baroreflex sensitivity control of renal nerve activity measured by phenylephrine infusion (BSRA-phenylephrine; *upper*) and sodium nitroprusside infusion (BSRA-nitroprusside; *lower*) in unconscious animals ($^{\alpha,\beta,\delta}P < 0.05$ compared to corresponding TDW, TDW + T, and TDG, respectively; TDW, perinatal taurine depletion with water intake alone; TDW + T, perinatal taurine depletion with water intake alone plus taurine; TDG, perinatal taurine depletion with high sugar intake; TDG + T, perinatal taurine depletion with high sugar intake plus taurine; n = 6–7 each group)

4 Discussion

In male rats, perinatal taurine depletion increases cardiac injury, mean arterial pressure, and sympathetic nerve activity but decreases baroreflex sensitivity and parasympathetic nerve activity after cardiac IR in adults. These adverse effects of perinatal taurine are markedly exacerbated by high sugar intake after weaning (Kulthinee et al. 2010). The present study indicates that in female rats, perinatal taurine depletion alone did not alter cardiac injury and autonomic nerve activity but slightly depressed baroreflex function after cardiac IR in adults compared to adult control (CW) rats. High sugar intake after weaning increased cardiac IR-induced cardiac injury, elevated sympathetic nerve activity, decreased parasympathetic activity, and blunted baroreflex sensitivity in adult female TDG rats. In addition,

these adverse effects, particularly the autonomic and baroreflex dysfunction, can be prevented by taurine supplementation starting a week before IR induction. Together, these data suggest that a combination of perinatal taurine depletion and high sugar intake after weaning exacerbates myocardial dysfunction and arterial pressure dysregulation after cardiac IR in a sex different manner. Moreover, the present study indicates that taurine supplementation can ameliorate post cardiac IR injury and arterial pressure dysregulation, at least in adult female TDG rats.

Alterations in baroreflex sensitivity and autonomic nerve activity provide a useful index of neural regulation of arterial pressure that can predict the pathogenesis and progression of cardiovascular disease (Fernandez et al. 2015; La Rovere and Christensen 2015). It is well known that acute cardiac IR induces depressed baroreflex sensitivity and heightened sympathetic nerve and renin-angiotensin system activities (Ando et al. 2002; Huang et al. 2007; Jones et al. 2008) though the mechanisms of these alterations have not been fully clarified. Some investigators report the interplay of cardiac afferent fibers that are sensitized by some chemicals released from the infarct site may play a prominent role (Longhurst et al. 2001). The severity of baroreflex and autonomic dysfunction is directly related to the infarct size or the severity of myocardial injury (Jones et al. 2008). However, the infarct size is in turn increased by sympathetic nerve overactivity and decreased by cardiac cholinergic stimulation (de La Fuente et al. 2013). The present study partially supports this line of evidence. While taurine supplementation completely abolished the adverse baroreflex and autonomic effects of perinatal taurine depletion followed by high sugar intake after weaning, it also partially improved cardiac injury after cardiac IR. Further, the normalized plasma CK-MB and partial restoration of plasma Trop-T and NT-proBNP by the taurine supplementation in these rats suggests the permanent damage of ventricle or the interplay of other taurine-insensitive factors. The plasma NT-proBNP is mainly released from damaged ventricular cells, while CK-MB and Trop-T may reflect skeletal muscle damage (Kost et al. 1998). The present study induces cardiac IR injury by a brief tracheal obstruction that results in systemic hypoxemia and hypotension; thus, other organ damage cannot be excluded. In addition, perinatal taurine deficiency can cause damage to several organs in adults including heart, liver, brain, and skeletal muscle (Sturman 1993).

Renin-angiotensin system overactivity is reported to underlie baroreflex and autonomic dysfunction after cardiac IR. Central and systemic inhibition of the renin-angiotensin system improves cardiac injury, baroreflex sensitivity, and autonomic dysfunction after cardiac IR (Huang et al. 2007; Huang et al. 2013). Angiotensin type 1 receptors in the paraventricular nucleus (Huang et al. 2014) and rostral ventrolateral medulla (Shi et al. 2009) seem to play a major role in this case. The renin-angiotensin system may be activated by increased renal sympathetic nerve activity during cardiac IR, as well as induction due to hypoxemia and hypotension. Angiotensin II then acts centrally to depress baroreflex sensitivity, increase sympathetic nerve activity (Johns 2005) and directly increase cardiac injury and remodeling (Schaffer et al. 2014), but a role for activation of cardiac and brain renin-angiotensin system by the factors related to cardiac IR cannot be excluded. Previously, we reported that perinatal taurine depletion followed by high sugar intake depresses

baroreflex control of both heart rate and renal nerve activity (Roysommuti et al. 2009; Thaeomor et al. 2010) and elevates sympathetic nerve activity (unpublished data) in adult female rats, while high sugar intake or taurine depletion alone does not induce these adverse effects. Further, these baroreflex and autonomic dysfunctions are abolished by inhibition of angiotensin converting enzyme (Thaeomor et al. 2010). Thus, the combination of cardiac IR and perinatal taurine depletion followed by high sugar intake after weaning might amplify the adverse effect of angiotensin II in the TDG rats. These data suggest that the beneficial effects of taurine supplementation in the present study may be due to its inhibition of angiotensin II action on the heart and/or the central nervous system (Roysommuti and Wyss 2014). However, taurine can also blunt cardiac injury by its anti-oxidant activity, independent of the renin-angiotensin system (Ueno et al. 2007).

Sympathetic nervous system overactivity after cardiac IR may be a consequence of decreased baroreflex sensitivity, such that decreased baroreceptor signaling due to hypotension or depressed baroreceptor sensitivity of the brain cardiovascular control centers causing a rise in peripheral sympathetic outflow (Lohmeier and Iliescu 2015). However, this does not appear to be the case in the present study, since mean arterial pressures were similar before IR and increases were similar after cardiac IR in all groups, as well as in control rats (Kulthinee et al. 2015), despite cardiac, baroreflex, and autonomic dysfunctions in TDG groups. Further, although taurine supplementation ameliorated or abolished the adverse effects of cardiac IR on cardiac injury, baroreflex sensitivity, and sympathetic nerve activity, it did not affect mean arterial pressure and heart rate. These data suggest that the high arterial pressure after cardiac IR may be maintained by a rise in total peripheral resistance independent of sympathetic nerve activity, taurine, and probably renin-angiotensin system. Local vascular effects of cardiac IR need to be studied further. In dogs, the blunted baroreflex sensitivity and increased sympathetic activity after cardiac IR appears to result from sensitization of cardiac afferent fibers, while arterial baroreceptor sensitivity seems to be preserved (Jones et al. 2008).

Estrogen plays a protective role for many organs and in disease development, including cardiac IR injury (Ashraf and Vongpatanasin 2006; Hay 2016; Metcalfe and Meldrum 2006). Estrogen treatment can directly decrease myocardial infarct size and cardiac arrhythmias induced by IR (Hale et al. 1997), by acting on cardiac estrogen receptors (Deschamps et al. 2010). Estrogen action is, at least in part, related to decreased cardiac oxidative stress (Deschamps et al. 2010) and β_1- adrenergic receptor activity (Kam et al. 2004). However, the beneficial effect of estrogen may be acute, while the chronic effects of estrogen treatment on ischemic heart disease are rather complicated. Although estrogen may decrease sympathetic nervous system activity and subsequently decrease renin-angiotensin system activity, it may increase plasma renin activity and angiotensin II levels by directly stimulating hepatic angiotensinogen synthesis (Oelkers 1996). The present study also supports the protective role of estrogen on cardiac IR injury and neural control of arterial pressure after acute IR, as indicated by the lower severity of myocardial damage, post IR hypertension, blunted baroreflex sensitivity, and heightened sympathetic nerve activity in female than previously tested male rats (Kulthinee et al. 2010;

Kulthinee et al. 2015). Further, plasma electrolytes and blood chemical parameters were normal in the present female rat groups, but they were abnormal in previously tested male rats (Kulthinee et al. 2010). Estrogen alone cannot completely prevent the adverse effect of perinatal taurine depletion followed by high sugar intake after cardiac IR in adult female rats. Taurine supplementation in adults may offer a valuable adjuvant to other therapy to protect at risk female rats and even humans from IR damage.

5 Conclusion

Acute cardiac IR induces myocardial injury, depresses baroreflex sensitivity, increases sympathetic nerve activity, and increases renin-angiotensin system activity. These adverse effects are exacerbated by a high sugar diet, particularly in adult male rats that are perinatally depleted of taurine. Taurine is known to decrease cardiac IR injury, particularly in animal models. In adult female rats, short-term taurine supplementation in adults ameliorates IR-induced cardiac injury and the adverse effects of high sugar intake on baroreflex and autonomic function. The present study further indicates that in adult female rats, perinatal taurine depletion followed by high sugar intake after weaning exacerbates cardiac IR injury and arterial pressure dysregulation and these adverse effects can be prevented, at least in part, by taurine supplementation.

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Perinatal Taurine Supplementation Alters Renal Function via Renin-Angiotensin System Overactivity in Adult Female Rats

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Abstract This study tests the hypothesis that perinatal taurine supplementation followed by a high sugar diet since weaning impairs renal function via renin-angiotensin system (RAS) overactivity in adult female rats. Female Sprague-Dawley rats were fed normal rat chow and given water alone or water containing 3% taurine from conception until weaning. After weaning, the female rats received normal rat chow and water with (CG, TSG) or without (CW, TSW) 5% glucose throughout the experiment. At 7–8 weeks of age, renal function at rest and after an acute saline load was tested in conscious female rats after a week of captopril treatment. Body, heart, and kidney weights were not significantly different among the eight groups. Mean arterial pressures and heart rates were also not different among the groups. While effective renal blood flow did not significantly differ among the eight groups, TSG displayed higher renal vascular resistance compared to CW, CG, and TSW groups. Glomerular filtration rate, filtration fraction, and water and sodium excretion did not significantly differ among the groups. Compared to CW, the saline load significantly depressed fractional water excretion in CG and TSW and fractional sodium excretion in CG, TSW, and TSG groups. Captopril treatment abolished these differences but significantly decreased potassium excretion in CG, TSW, and TSG compared to CW and abolished the increased fractional potassium excretion in TSG compared to CG and TSW groups. These data strongly suggest that in adult female rats, perinatal taurine supplementation, particularly followed by high sugar intake, alters renal function via altered RAS activity.

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Abbreviations

ACE	Angiotensin converting enzyme
CG + Cap	CG plus captopril treatment
CG	Control with high sugar intake
CW + Cap	CW plus captopril treatment
CW	Control with water intake alone
ERBF	Effective renal blood flow
ERVR	Effective renal vascular resistance
FE_{H2O}	Fractional water excretion
FE_{K}	Fractional potassium excretion
FE_{Na}	Fractional sodium excretion
GFR	Glomerular filtration rate
KW	Kidney weight
RAS	Renin-angiotensin system
TDG	Perinatal taurine depletion with high sugar intake
TSG + Cap	TSG plus captopril treatment
TSG	Perinatal taurine supplementation with high sugar intake
TSW + Cap	TSW plus captopril treatment
TSW	Perinatal taurine supplementation with water intake alone

1 Introduction

Taurine (2-aminoethanesulfonic acid) plays several physiological roles in animals and humans from prenatal through adult life. Body taurine content is highest after birth and then declines with advancing age and is proposed to underlie renal damage that can be attenuated by daily taurine supplementation (Cruz et al. 2000). In addition, daily taurine administration to patients suffering from chronic heart disease improves their cardiac output and key symptoms (Ito et al. 2014).

During perinatal life, fetuses and newborns require taurine from their mothers via placenta and breast milk, because of their limited de novo taurine biosynthetic capacity and high taurine requirement (Sturman 1993). Insufficient taurine availability results in abnormal growth and development of the offspring and a high risk of developing cardiovascular and renal disease in adulthoods (Roysommuti and Wyss 2014). Thus, many maternal and neonatal foods contain-

ing taurine supplements have been developed. However, perinatal taurine excess can also affect adult organ function and disease. Prenatal taurine supplementation increases postnatal growth and risk of diabetes mellitus in adult rat offspring (Hultman et al. 2007). Further, prenatal and early postnatal taurine supplementation induces renal dysfunction in adult male and female rats, and these effects are exacerbated by high sugar intake (Roysommuti et al. 2009a, 2010). Our previous experiments demonstrate that taurine supplementation from conception until weaning followed by the high sugar intake significantly decreases baroreflex-mediated renal nerve activity in adult female rats (Thaeomor et al. 2013). In addition, this blunted baroreflex sensitivity is abolished by estrogen receptor blockade with tamoxifen, but not by angiotensin converting enzyme inhibition with captopril.

While systemic renin angiotensin system (RAS) is mainly regulated by renin release from renal juxtaglomerular cells, local RAS in most organs are able to locally control organ specific functions (Campbell 2014; De Mello and Frohlich 2014; Prieto et al. 2013). Renal function is regulated by both the systemic and intrarenal RAS (Prieto et al. 2013). For example, the RAS is found in proximal tubular cells and can respond to high glucose and taurine exposure *in vitro* (Hsieh et al. 2002). Previously, we have reported that perinatal taurine depletion followed by high sugar intake since weaning blunts the baroreflex control of heart rate and renal nerve activity (Thaeomor et al. 2010) and decreases fractional water and sodium excretion after an acute saline load in adult female rats (Lerdweeraphon et al. 2015). Further, captopril treatment abolishes baroreflex dysfunction (Thaeomor et al. 2010) but not renal tubular dysfunction (Lerdweeraphon et al. 2015) in these rats. These data indicate that the adverse effect of perinatal taurine depletion followed by high sugar intake since weaning may be organ specific. This suggests that the adverse renal effect of perinatal taurine excess followed by a high sugar diet since weaning might differ from the mechanisms altering baroreflex sensitivity in this model (Thaeomor et al. 2013). This study tests the hypothesis that in female rats, perinatal taurine supplementation followed by a high sugar diet since weaning impairs adult renal function via RAS overactivity.

2 Methods

2.1 Animal Preparation

Sprague-Dawley rats were bred from the Northeast Laboratory Animal Center, Khon Kaen University. After conception, each female rat was caged individually, fed normal rat chow (C.P. Mice Feed 082), and supplemented with (taurine supplementation, TS) or without 3% taurine in tap water (control, C) from conception until

weaning. After weaning, the female offspring were fed normal rat chow and drank either 5% glucose in tap water (TSG and CG group) or tap water alone (TSW and CW groups) throughout the experiment. At 6–7 weeks of age, each treatment group drank tap water containing captopril (an angiotensin converting enzyme inhibitor; 400 mg/L) (CW + Cap (n = 7), CG + Cap (n = 7), TSW + Cap (n = 9), and TSG + Cap (n = 8) groups) or no captopril (CW (n = 6), CG (n = 6), TSW (n = 7), and TSG (n = 8) groups) until animal termination.

All experiments were approved by the Khon Kaen University Animal Care and Use Committee (AEKKU 7/2555) and followed the National Institutes of Health guidelines.

2.2 Renal Function Study

The renal function study in the present experiment was similar to our previous report (Lerdweeraphon et al. 2015). In brief, renal function was studied in conscious rats at 7–8 weeks of age. Arterial pressure, heart rate, and renal parameter measurements were measured before, during, and after an intravenous isotonic saline infusion (5% of body weight). Finally, all animals were sacrificed followed by heart and kidney weights collection.

Data analyses and renal function parameters were also similar to our previous experiments (Lerdweeraphon et al. 2015). The present data are expressed as mean ± SEM. One-way ANOVA and the *post hoc* Tukey's test were used to explore the significant differences among groups ($P < 0.05$).

3 Results

3.1 Weights and Hemodynamics

At 7–8 weeks of age, body, heart, and kidney weights were not significantly different among the eight groups (data not shown). Mean arterial pressure and heart rate (both at rest and after a saline load) were not significantly different among the eight groups (Fig. 1).

ERBF before and after captopril treatment were not significantly different among the eight groups (Fig. 2, top two panels). Without captopril treatment, TSG displayed higher ERVR at rest and 15 min after isotonic saline load than the other groups did. These differences were abolished by the captopril treatment (Fig. 2, bottom two panels).

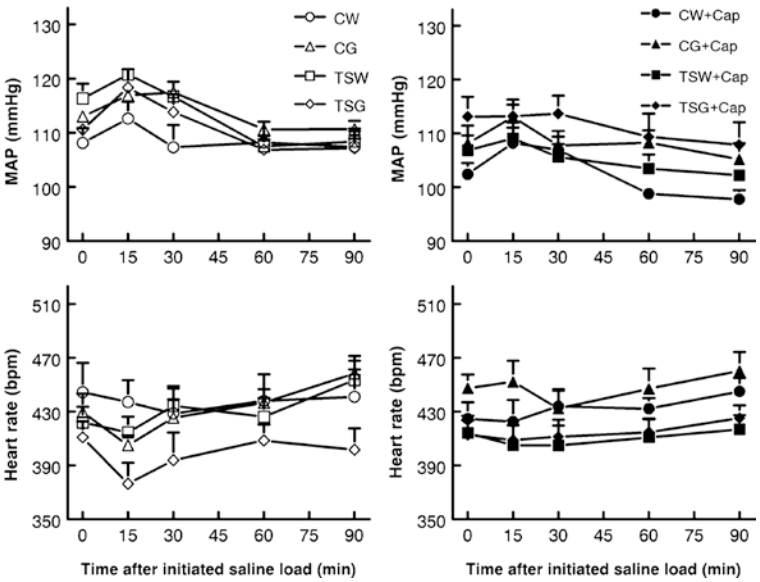

Fig. 1 Mean arterial pressure (MAP; upper two) and heart rate (lower two) before (0 min) and after a saline load (15–90 min) in experimental groups with (right two) or without captopril treatment (left two) (n = 6–8 each group; see Abbreviations for group details)

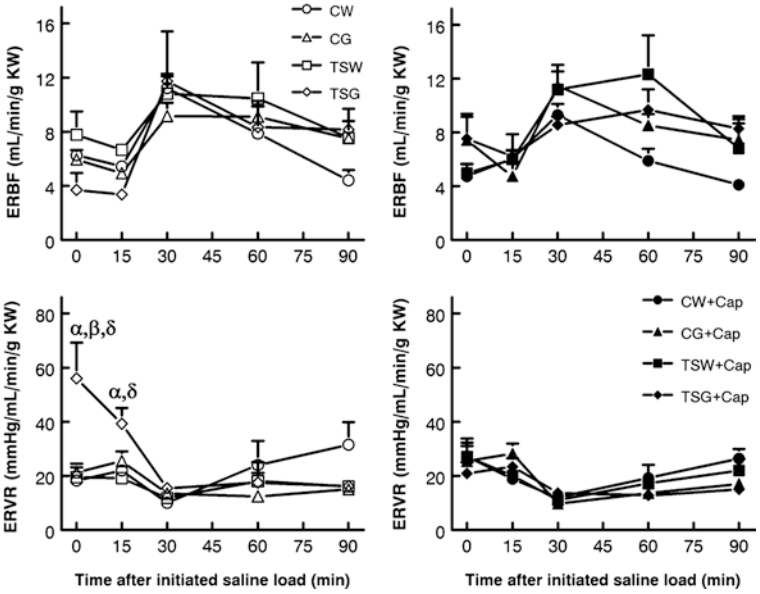

Fig. 2 Effective renal blood flow (ERBF; upper two) and effective renal vascular resistance (ERVR; lower two) before (0 min) and after a saline load (15–90 min) in experimental groups with (right two) or without captopril treatment (left two) ($^{\alpha,\beta,\delta}P < 0.05$ compared to CW, CG, and TSW, respectively; n = 6–8 each group; see Abbreviations for group details)

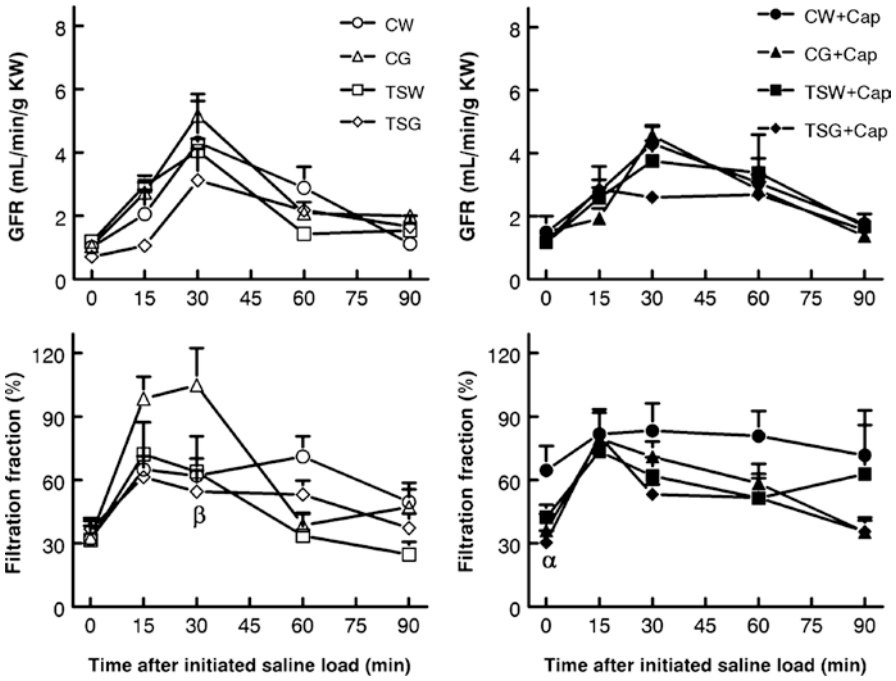

Fig. 3 Glomerular filtration rate (GFR; upper two) and filtration fraction (lower two) before (0 min) and after a saline load (15–90 min) in experimental groups with (right two) or without captopril treatment (left two) ($^{\alpha,\beta}P < 0.05$ compared to CW and CG, respectively; n = 6–8 each group; see Abbreviations for group details)

3.2 Glomerular Filtration

GFR significantly increased in all groups after a saline load and reached a peak 30 min post saline load (about five times of resting values; Fig. 3, top two panels). These changes and the resting GFR were not significantly different among the groups. Filtration fractions increased after the saline load in all groups; however, significant differences were observed between CG and TSG at 30 min post saline load and TSG + Cap and CW + Cap at rest (Fig. 3, bottom two panels).

3.3 Water Excretion

Similar to glomerular filtration rates, water excretion significantly increased in all groups after a saline load and reached a peak 30 min post saline load (about five times of resting values; Fig. 4, top two panels). These changes and also the resting water excretion were not significantly different among the eight groups, irrespective of high sugar intake or captopril treatment.

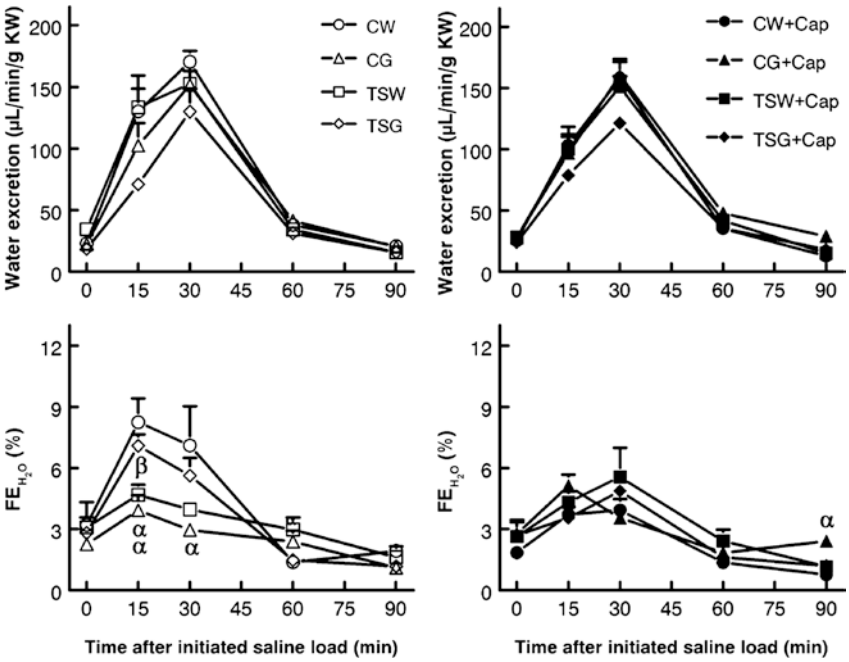

Fig. 4 Water excretion (upper two) and fractional water excretion (FE_{H2O}; lower two) before (0 min) and after a saline load (15–90 min) in experimental groups with (right two) or without captopril treatment (left two) ($^{\alpha,\beta}P < 0.05$ compared to CW and CG, respectively; n = 6–8 each group; see Abbreviations for group details)

Resting fractional water excretions did not significantly differ among the eight groups. After a saline load, fractional water excretion significantly increased in CW, reached a peak (about three times of resting) 15–30 min post saline load and returned back to baseline 60 min post saline load. Compared to CW, these changes were significantly attenuated in CG and TSW, but not in TSG (Fig. 4, bottom two panels). Although these attenuated responses were not significantly affected by short-term captopril treatment, the fractional water excretion of CG + Cap were slightly and significantly higher than that of the CW + Cap groups 90 min after the saline load. In addition, the fractional water excretion between 0 and 60 min were similar among the groups after the captopril treatment.

3.4 Sodium Excretion

Similar to glomerular filtration and water excretion rates, sodium excretion significantly increased in all groups after a saline load and reached a peak at 30 min post saline load (about 30 times of resting values; Fig. 5, top two panels). These changes as well as resting sodium excretion were not significantly different among the eight groups.

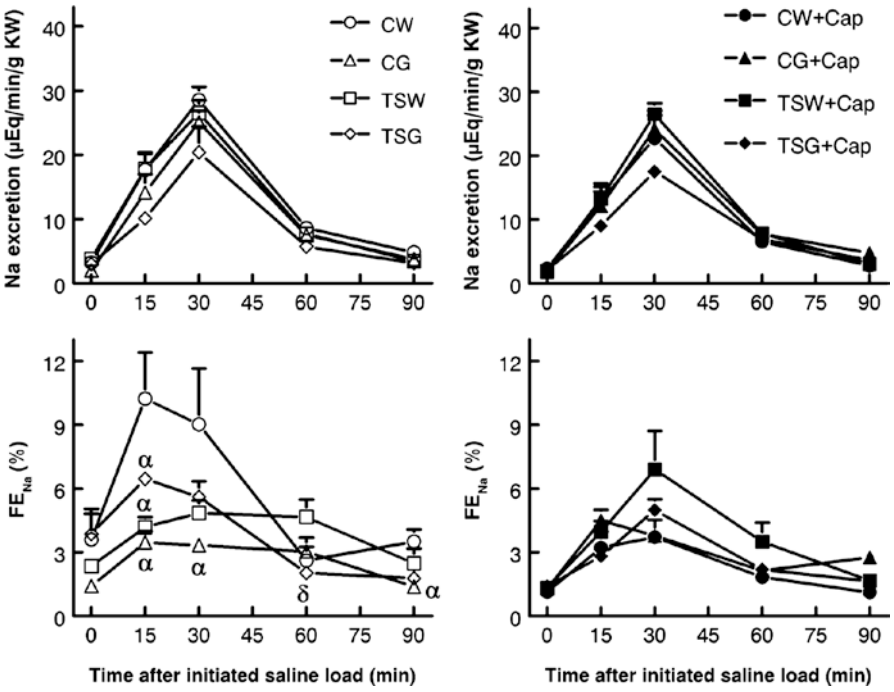

Fig. 5 Sodium excretion (upper two) and fractional sodium excretion (FE_{Na}; lower two) before (0 min) and after saline load (15–90 min) in experimental groups with (right two) or without captopril treatment (left two) ($^{\alpha}P < 0.05$ compared to CW; n = 6–8 each group; see Abbreviations for group details)

While resting fractional sodium excretion was not significantly different among the eight groups, it significantly increased in CW, reaching a peak at 15–30 min post saline load (about three times of resting), and returning back to baseline at 60 min post saline load. Although fractional sodium excretion after a saline load was significantly attenuated in CG, TSW, and TSG compared to the CW groups (Fig. 5, bottom two panels), the decrease in TSG was less than that of CG and TSW. Further, 60 min post saline load, the fractional sodium excretion of TSG was significantly lower than that of TSW, while at 90 min, those of CG were lower than CW. These attenuated responses were not significantly affected by short-term captopril treatment throughout the study. In addition, captopril treatment significantly decreased fractional sodium excretion of CW to the attenuated levels of the other groups.

3.5 Potassium Excretion

Potassium excretion before and after a saline load were not significantly different among the untreated groups, but short-term captopril treatment significantly decreased these measures in CG + Cap at rest and TSW + Cap and TSG + Cap at 15 min post saline load compared to CW + Cap groups (Fig. 6, top two panels).

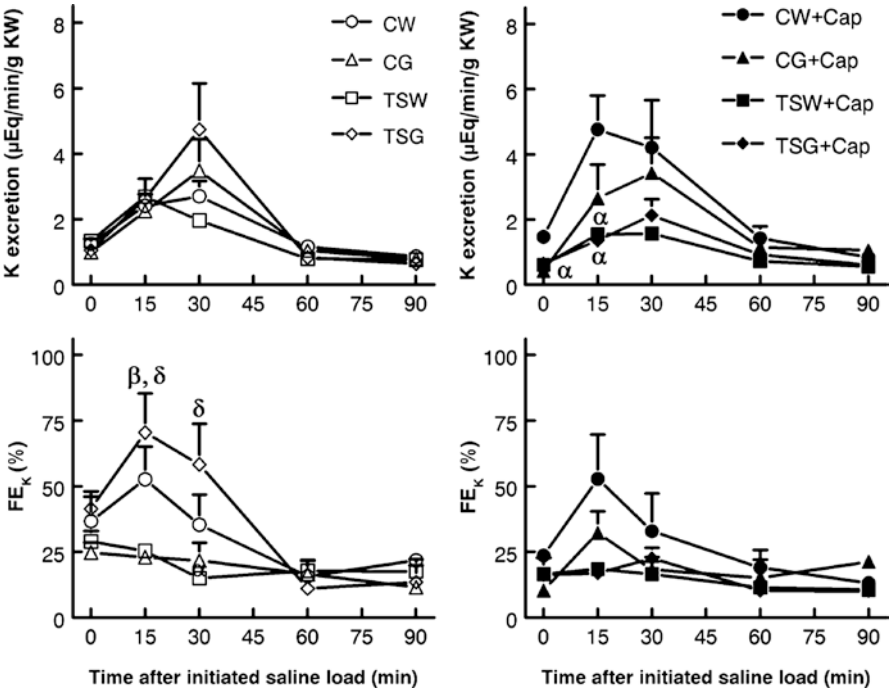

Fig. 6 Potassium excretion (upper two) and fractional potassium excretion (FE_K; lower two) before (0 min) and after saline load (15–90 min) in experimental groups with (right two) or without captopril treatment (left two) ($^{\alpha,\beta,\delta}P < 0.05$ compared to CW, CG, and TSW, respectively; n = 6–8 each group; see Abbreviations for group details)

Compared to CG and TSW groups, TSG significantly increased fractional potassium excretions after a saline load, but not at rest. These changes were abolished by captopril treatment (Fig. 6, bottom two panels).

4 Discussion

During pregnancy and lactation, imbalance of nutrition and hormones can lead to alterations of renal function (Kett and Denton 2011) and arterial pressure control in adult offspring (Roysommuti and Wyss 2014). Previous studies reported that the perinatal taurine imbalance affects adult renal function in a sex-specific manner (Roysommuti et al. 2009b). In female rats, either perinatal taurine depletion (TDG) (Thaeomor et al. 2010) or supplementation (Thaeomor et al. 2013) followed by high sugar intake from weaning onward (TSG) depresses baroreceptor reflex sensitivity. Further, inhibition of RAS by captopril normalizes baroreflex function in TDG (Thaeomor et al. 2010) but not TSG rats (Thaeomor et al. 2013). In contrast, inhibition of estrogen activity by the estrogen receptor blocker tamoxifen normalizes baroreflex function in TSG (Thaeomor et al. 2013) but not in TDG rats (unpublished

data). The present experiment demonstrates that in adult female rats, RAS overactivity underlies the adverse effects of perinatal taurine supplementation on adult renal vasculature and tubular function, particularly followed by a post-weaning high sugar diet. Altogether, these data suggest that these adverse effects are system specific, at least between the cardiovascular and renal systems.

Tubular water and salt reabsorption depends on glomerular filtration or filtered load; a mechanism known as "glomerulotubular balance" (Thomson and Blantz 2008). Thus, in CG and TSW, increased activity of tubular water and sodium reabsorption is reflected in the depressed fractional water and sodium excretion despite similar glomerular filtration and water and sodium excretion. Normal natriuretic/diuretic responses to acute isotonic volume expansion involve several factors, particularly decreased renal sympathetic nerve activity (Rodriguez-Martinez and Brito-Orta 1999), decreased renin-angiotensin system activity, and decreased plasma aldosterone (Jensen et al. 2013). In the present study, captopril treatment depresses CW fractional water and sodium excretion to the level of CG and TSW without affecting the levels of fractional water and sodium excretion of CG and TSW groups. These data suggest that in female rats, the natriuretic/diuretic responses to a saline load of CG and TSW are independent of the RAS. Thus, it appears that high sugar intake and/or perinatal taurine excess depresses the role of RAS in pressure-natriuretic/diuretic control. This may relate to RAS modification of renal sympathetic nerve activity, independent of renin release, but that hypothesis requires further assessment.

Surprisingly, the combination of perinatal taurine supplementation followed by high sugar intake since weaning (TSG group) completely preserved fractional water excretion and partially preserved fractional sodium excretion in response to a saline load. The role of RAS activity on pressure-diuretic/natriuretic mechanisms seems to be preserved, at least as measured by their similar depressions after captopril treatment compared to CW. TSG did display higher ERVR compared to CW, CG, and TSW groups, but the increased TSG's ERVR was normalized by captopril treatment. These data suggest that perinatal taurine excess followed by a high sugar diet since weaning increases RAS activity in adult female rats. This renal effect differs from the blunted baroreflex effect that depends on estrogen rather than the RAS (Thaeomor et al. 2013). It is likely that intrarenal RAS plays a dominant role in the present study. If systemic RAS played a major role in this case, both baroreflex and renal dysfunction would be restored to CW levels by the captopril treatment.

Although this study indicates an increase in tubular reabsorption ability in TSW and TSG groups, the severity was partially offset by a marked rise in glomerular filtration rate (about five times above resting in all groups) after a saline load. This explains why the diuretic and natriuretic responses to a saline load were not significantly different among the eight groups. Further, renal vascular resistance in TSG increased at rest and declined after a saline load due to RAS activity being increased at rest and declining after a saline load (Jensen et al. 2013), but not sufficiently to affect renal blood flow. In addition, the perinatal taurine excess had minimal effects on renal potassium excretion. These data suggest that in young adult female rats, renal dysfunction induced by perinatal taurine supplementation with or without

high sugar intake since weaning may be an early process. Studies in older animals are necessary to explore more adverse effects. The perinatal programming effect of taurine might potentiate the decreased renal excretory function as increasing age.

5 Conclusion

The function of kidneys, like other organs, can be altered by their perinatal environments leading to abnormal adult function. Although taurine exposure is reported to play a critical role in growth and development of the kidney, the adverse effect of perinatal taurine imbalance on adult renal function is sex-specific. Both estrogen and RAS are reported to contribute importantly to this effect. The present study demonstrates that in young adult female rats, altered RAS activity underlies the effects of perinatal taurine supplementation on renal function (vascular and tubular), particularly if the animals tested have been on a high sugar diet.

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Perinatal Taurine Imbalance Followed by High Sugar Intake Alters the Effects of Estrogen on Renal Excretory Function in Adult Female Rats

Sanya Roysommuti, Wichaporn Lerdweeraphon, and J. Michael Wyss

Abstract This study tests the hypothesis that perinatal taurine imbalance impairs renal function in adult female rats via alterations in estrogen activity. Female Sprague-Dawley rats were fed normal rat chow and water containing 3% beta-alanine (TD), 3% taurine (TS) or water alone (C) from conception until weaning. Then, female offspring received normal rat chow and water with (CG, TDG, TSG) or without (CW, TDW, TSW) 5% glucose. At 7–8 weeks of age, renal function at rest and after acute saline load was tested in conscious, restrained female rats treated with non-selective estrogen receptor blocker tamoxifen for a week. Compared to control, TD or TS did not affect mean arterial pressure (MAP). Tamoxifen significantly increased resting MAP only in TDG compared to TDW groups. Although renal blood flow did not significantly differ among the groups, renal vascular resistance increased in TSG compared to CW, CG, and TSW groups. Glomerular filtration rate and water and sodium excretion were not significantly different among the groups. Compared to CW, saline load significantly depressed fractional water excretion in CG, TDW, TDG, and TSW, and fractional sodium excretion in CG, TDW, TDG, TSW, and the TSG groups. Potassium excretion was not significantly different among the corresponding groups. Fractional potassium excretion significantly increased in TDW compared to CG and in TSG compared to CG and TSW groups. These differences were abolished by tamoxifen treatment. These data indicate that in adult female rats, perinatal taurine imbalance, particularly followed by high sugar intake, alters renal function via an estrogenic mechanism.

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Abbreviations

CG + Tam	CG plus tamoxifen treatment
CG	Control with high sugar intake
CW + Tam	CW plus tamoxifen treatment
CW	Control with water intake alone
eNOS	Endothelial nitric oxide synthase
ERBF	Effective renal blood flow
ERVR	Effective renal vascular resistance
FE_{H2O}	Fractional water excretion
FE_K	Fractional potassium excretion
FE_{Na}	Fractional sodium excretion
GFR	Glomerular filtration rate
KW	Kidney weight
RAS	Renin-angiotensin system
TDG + Tam	TDG plus tamoxifen treatment
TDG	Perinatal taurine depletion with high sugar intake
TDW + Tam	TDW plus tamoxifen treatment
TDW	Perinatal taurine depletion with water intake alone
TSG + Tam	TSG plus tamoxifen treatment
TSG	Perinatal taurine supplementation with high sugar intake
TSW + Tam	TSW plus tamoxifen treatment
TSW	Perinatal taurine supplementation with water intake alone

1 Introduction

The incidence of hypertension is higher in post-menopausal than pre-menopausal women and this can be prevented by estrogen replacement therapy (Hay et al. 2014; Kim et al. 2014; Yanes and Reckelhoff 2011). Hypertension can be induced in female rats by bilateral ovariectomy, to which can be prevented by diets high in phytoestrogens or estrogen replacement (Carlson et al. 2008). Estrogen may act directly or indirectly via inhibition of the renin-angiotensin system (RAS) and sympathetic nervous system (Ashraf and Vongpatanasin 2006). However, the effects of estrogen on cardiovascular control are rather complicated. Estrogen itself possesses vasoprotective and anti-oxidative activity (Holm and Nilsson 2013); however, high estrogen levels during pregnancy increases water and salt retention and arterial

pressure by direct and indirect increases in angiotensin II and aldosterone (Oelkers 1996). Further, oral contraceptives are reported to increase arterial pressure in susceptible women, and this can be abolished by inhibition of the RAS (Ashraf and Vongpatanasin 2006). In addition, a low incidence of hypertension in vegetarians is related to a high phytoestrogens in their normal diets that regulate renal sodium excretion (Garay et al. 1998).

A major factor underlying these estrogenic effects on blood pressure may be estrogen's ability to modify renal pressure-diuresis/natriuresis mechanisms (Kim et al. 2014). An increase in blood pressure and a reduction in fractional sodium excretion occur in female rats in persistent estrus (Rocha Gontijo et al. 2010). Further, estrogen treatment in rats increases prostaglandin E_2, eNOS expression, and angiotensin II type 1 and type 2 receptors expression in the kidney (Baiardi et al. 2005). The cyclical changes in renal function correlates with estradiol but not nitric oxide levels in the virgin female rat (Santmyire et al. 2010). However, hormonal levels may not indicate their actual actions on the kidney. In humans, renal hormone sensitivity decreases in the low-hormone menstrual phase following sodium/fluid ingestion (Sims et al. 2008). In addition, estrogen deficiency increases the severity of kidney disease in both animals and humans and that estrogen replacement is renoprotective (Cobo et al. 2016; Ramesh et al. 2015).

Several studies report that taurine exposure affects renal function and progression of kidney disease (Han and Chesney 2012). Body taurine content declines and renal damage increases with advancing age, while taurine supplementation attenuates this age-related renal damage (Cruz et al. 2000). During pregnancy and lactation, taurine exposure is essential for growth and development (Sturman 1993) and programs renal function and disease of adult offspring (Lerdweeraphon et al. 2013; Roysommuti and Wyss 2014). It is well known, particularly in animal models, that perinatal taurine deficiency retards fetal growth, causes low birth weight newborns, and results in adult offspring being at high risk for cardiovascular and renal disease (Roysommuti and Wyss 2014). Although perinatal taurine excess seems to have less adverse effects, particularly in humans, taurine supplementation in late pregnant rats induces obesity and diabetes mellitus in adults (Hultman et al. 2007). Thus, perinatal taurine imbalance can seriously alter adult renal function.

Perinatal taurine deficiency impairs renal and autonomic nervous system function in adult male rats (Roysommuti and Wyss 2014). Further, high sugar diets since weaning increase sympathetic nerve activity (Roysommuti et al. 2009) and blunt baroreflex sensitivity for both heart rate and renal nerve activity (Roysommuti et al. 2009; Thaeomor et al. 2010). These abnormalities of adult female offspring can be restored by short-term treatment with angiotensin converting enzyme inhibitor captopril (Thaeomor et al. 2010), but not by estrogen receptor blocker tamoxifen (unpublished data). In contrast, the blunted baroreflex control of renal nerve activity in adult rats exposed to perinatal taurine excess can be abolished by estrogen receptor blocker tamoxifen, but not by captopril (Thaeomor et al. 2013). These effects of perinatal taurine imbalance seem to be system specific. While perinatal taurine supplementation followed by a high sugar diet since weaning depresses renal func-

tion in adult rats, the depression can be abolished by captopril (Lerdweeraphon et al. 2017). In contrast, the renal effect of perinatal taurine depletion is not reversed by captopril treatment irrespective of dietary sugar (Lerdweeraphon et al. 2015). Whether the renal effect of perinatal taurine imbalance in females is dependent on estrogen action has not been tested. This study tests the hypothesis that perinatal taurine imbalance impairs renal excretory function in adult female rats via estrogen activity.

2 Methods

2.1 Animal Preparation

Sprague-Dawley rats were bred from the Northeast Laboratory Animal Center, Khon Kaen University. After conception was confirmed, each female rat was caged individually. These rats were fed normal rat chow (C.P. Mice Feed 082) and treated with either 3% β-alanine in tap water (taurine depletion, TD), 3% taurine in tap water (taurine supplementation, TS) or tap water alone (control, C) from conception continuously through weaning. Further, their female offspring were fed the normal rat chow and drank either 5% glucose in tap water (CG, TDG, and TSG groups) or tap water alone (CW, TDW, and TSW groups) throughout the experiment. At 6–7 weeks of age, each group drank tap water containing either tamoxifen (a non-selective estrogen receptor antagonist; 100 mg/L) (CW + tam (n = 10), CG + tam (n = 8), TDW + tam (n = 8), TDG + tam (n = 8), TSW + tam (n = 9), and TSG + tam (n = 7) groups) or no tamoxifen (CW (n = 6), CG (n = 6), TDW (n = 9), TDG (n = 8), TSW (n = 7), and TSG (n = 8) groups) until the end of experiment.

This study was approved by the Khon Kaen University Animal Care and Use Committee (AEKKU 7/2555) and was performed in accordance with the National Institutes of Health guidelines.

2.2 Renal Study Protocol

At 6–7 weeks of age, all rats were acclimated to the rat restrainer used for a renal function study (3 h per day for a week). Then, they were anesthetized with sodium pentobarbital or Nembutal® (50 mg/kg of body weight, intraperitoneal) and inserted with femoral arterial, femoral venous, and bladder catheters. Two days later, their renal functions were studied before, during, and after an acutely intravenous saline load (5% of body weight, 0.5 mL/min) in a conscious, restrained condition, as

previously described (Lerdweeraphon et al. 2015). After 24-h recovery, all rats were weighed and anesthetized with sodium pentobarbital. Then, their blood volumes (at least 2 mL each rat) were drawn from the abdominal aorta and then a high dose of anesthesia was infused to sacrifice the animals. Finally, the heart and kidneys were removed and weighed.

Mean arterial pressure, heart rate, and renal function parameters were analyzed as previously reported (Lerdweeraphon et al. 2015). The present data are expressed as means ± SEM. Roles of estrogen on renal effects of perinatal taurine depletion and supplementation were statistically compared among the corresponding TD (CW, CG, TDW, TDG, CW + Tam, CG + Tam, TDW + Tam, and TDG + Tam) or TS (CW, CG, TSW, TSG, CW + Tam, CG + Tam, TSW + Tam, and TSG + Tam) groups by one-way ANOVA and the *post hoc* Tukey's test ($P < 0.05$).

3 Results

3.1 General Characteristics

The data for the CW, CG, TDW, TDG, TSW, and TSG groups were previously reported in relation to the study of Lerdweeraphon and colleagues (Lerdweeraphon et al. 2015, 2017) which focused on the RAS. The tamoxifen study animals were not reported in that study but were run simultaneously. Therefore, the data for these groups are included as a control comparison in this article.

Body, heart, and kidney weights were similar among the 12 groups (data not shown). Further, mean arterial pressure at rest and after saline load did not significantly differ among the CW, CG, TDW, and TDG groups (Fig. 1, upper left panel). Compared to the untreated groups, tamoxifen treatment had no effect on mean arterial pressures both at rest and after the saline load, except for TDG + Tam, which was slightly higher than that of the TDW + Tam group (Fig. 1, lower left panel). In addition, perinatal taurine supplementation with or without high sugar intake did not significantly affect mean arterial pressure both at rest and after saline load, irrespective of tamoxifen treatment (Fig. 1, two right panels).

3.2 Effective Renal Blood Flow and Vascular Resistance

Perinatal taurine depletion and supplementation with or without high sugar intake since weaning did not significantly alter effective renal blood flow before or after a saline load (Fig. 2). Compared between corresponding treated

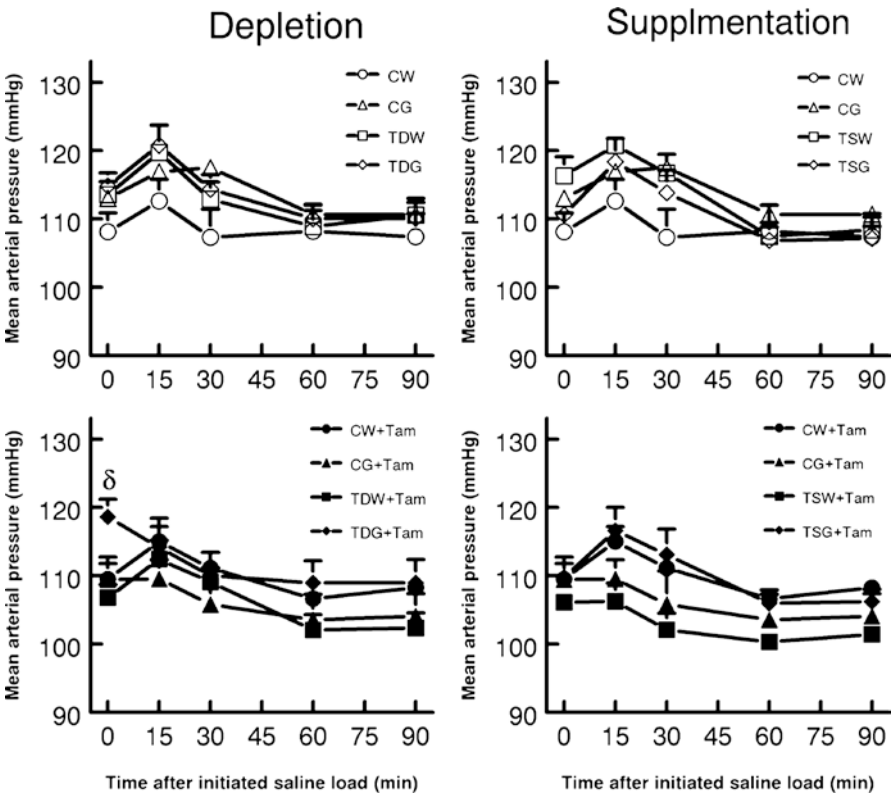

Fig. 1 Mean arterial pressure before (0 min) and after saline load (15–90 min) in conscious, female rats ($^{\delta}P < 0.05$ compared to TDW + Tam; n = 6–10 each group; see Abbreviations for group details)

and untreated TD and TS groups, tamoxifen treatment did not significantly alter effective renal blood flow; however, the value of TDG + Tam (but not TDW + Tam) was significantly lower than that of the CG + Tam group at 60 min post saline load.

Effective renal vascular resistance before and after a saline load did not significantly differ among the corresponding TD groups, irrespective of high sugar intake or tamoxifen treatment (Fig. 3, two left panels). While TSW did not significantly

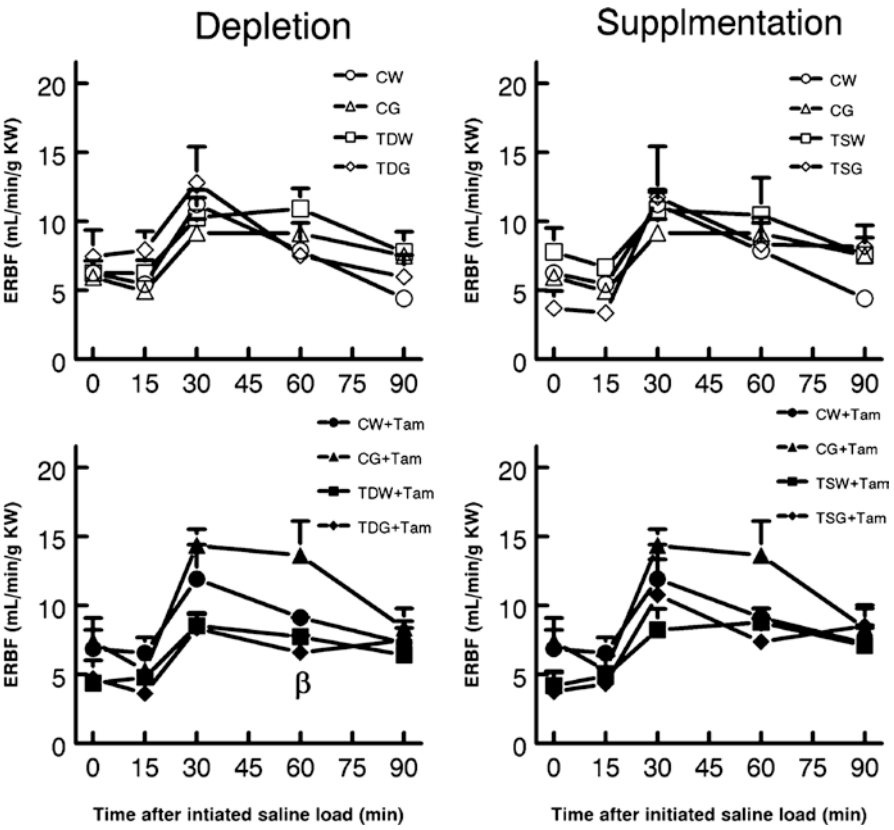

Fig. 2 Effective renal blood flow (ERBF) before (0 min) and after saline load (15–90 min) in conscious, female rats ($^{\beta}P < 0.05$ compared to CG + Tam; n = 6–10 each group; see Abbreviations for group details)

alter effective renal vascular resistance compared to the CW and CG groups, TSG significantly increased the effective renal vascular resistances before and after the saline load compared to CW, CG, and TSW groups (Fig. 3, upper right panel). Tamoxifen treatment eliminated these differences in effective renal vascular resistance, by raising the values of CW + Tam, CG + Tam, and TSW + Tam to those of TSG + Tam.

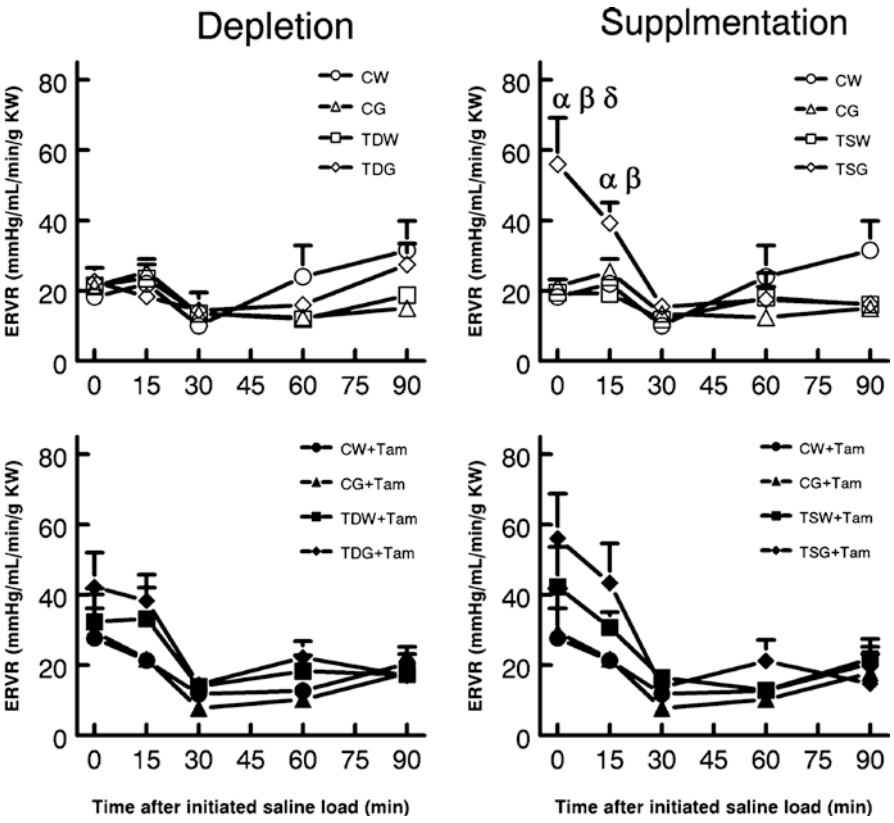

Fig. 3 Effective renal vascular resistance (ERVR) before (0 min) and after a saline load (15–90 min) in conscious, female rats ($^{\alpha,\beta,\delta}P < 0.05$ compared to CW, CG, and TSW, respectively; n = 6–10 each group; see Abbreviations for group details)

3.3 Glomerular Filtration

Glomerular filtration rates significantly increased in all groups after a saline load and reached a peak at 30 min post saline load (about five times of resting values; Fig. 4). These changes and resting glomerular filtration rates were not significantly different among the corresponding TD or TS groups, irrespective of high sugar intake or tamoxifen treatment.

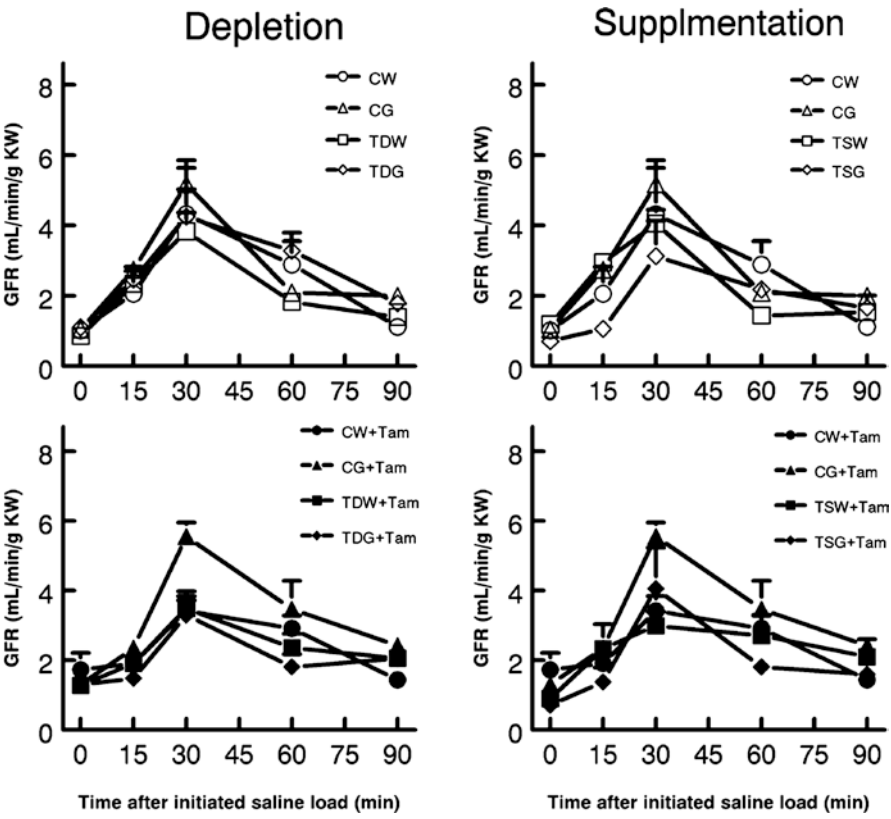

Fig. 4 Glomerular filtration rate (GFR) before (0 min) and after a saline load (15–90 min) in conscious, female rats (n = 6–10 each group; see Abbreviations for group details)

3.4 Water Excretion

Water excretion before and after saline load were not significantly different among the corresponding TD or TS groups, irrespective of high sugar intake and tamoxifen treatment. Water excretion significantly increased in all groups after the saline load, reached a peak at 30 min, and returned back to baselines at 90 min post saline load (Fig. 5).

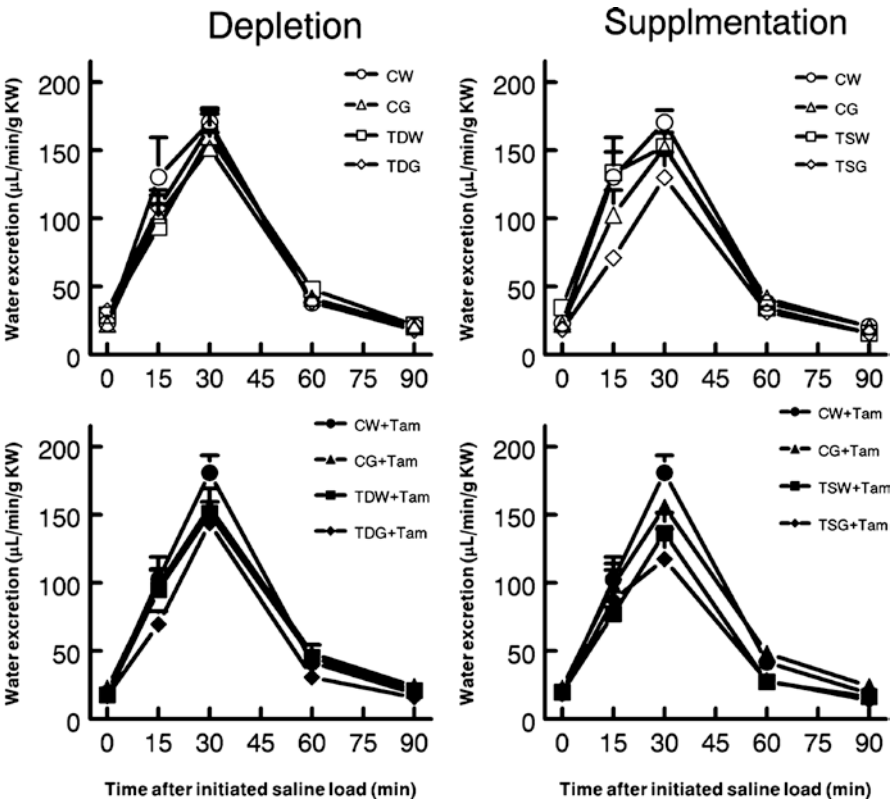

Fig. 5 Water excretion before (0 min) and after a saline load (15–90 min) in conscious, female rats (n = 6–10 each group; see Abbreviations for group details)

Resting fractional water excretions did not significantly differ among the corresponding TD or TS groups, irrespective of high sugar intake and tamoxifen treatment (Fig. 6). After saline load, fractional water excretions significantly increased in CW group and reached a peak at 15–30 min post saline load. Compared to CW, the fractional water excretions were significantly attenuated in CG, TDW, TDG, and TSW, but not in TSG groups (Fig. 6, two upper panels). Those attenuated responses were not significantly affected by tamoxifen treatment (Fig. 6, two lower panels). In contrast, the tamoxifen treatment significantly decreased fractional water excretion of CW to the attenuated levels of the other groups. It is noted worthy that the fractional water excretions were not significantly different among the tamoxifen-treated TD and TS groups.

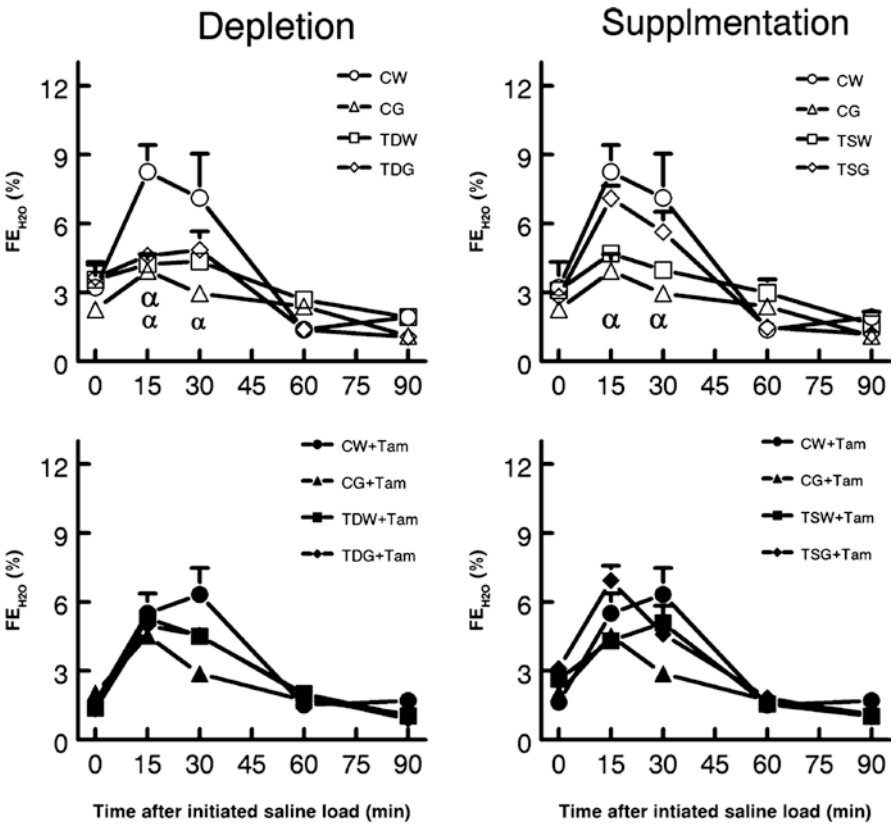

Fig. 6 Fractional water excretion (FE_{H2O}) before (0 min) and after a saline load (15–90 min) in conscious, female rats ($^{\alpha}P < 0.05$ compared to CW; n = 6–10 each group; see Abbreviations for group details)

3.5 Sodium Excretion

Like water excretion, sodium excretion was not significantly different among the corresponding TD or TS groups, and significantly increased after a saline load, reached a peak at 30 min and returned back to the baseline 90 min post saline load (Fig. 7).

Resting fractional sodium excretion was not significantly different among the corresponding TD or TS groups, irrespective of high sugar intake and tamoxifen treat-

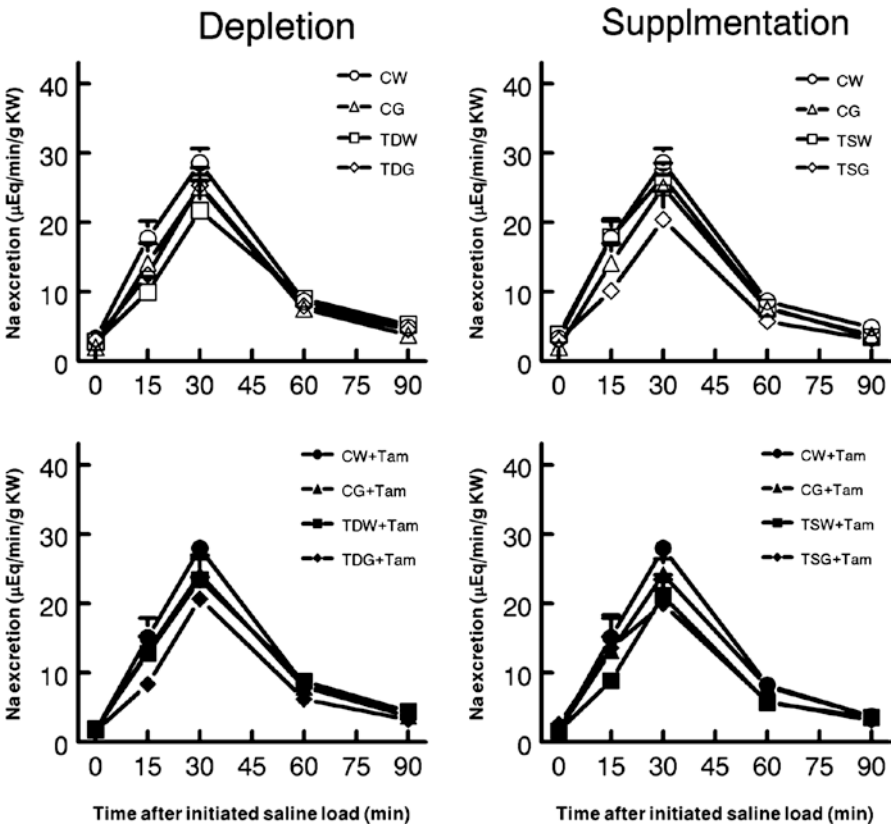

Fig. 7 Sodium excretion before (0 min) and after a saline load (15–90 min) in conscious, female rats (n = 6–10 each group; see Abbreviations for group details)

ment (Fig. 8). After a saline load, fractional sodium excretion significantly increased in the CW group and reached a peak at 15–30 min post saline load. Compared to CW, these changes were significantly attenuated in CG, TDW, TDG, TSW, and TSG groups (Fig. 8, two upper panels). These attenuated responses were not significantly affected by tamoxifen treatment, but tamoxifen treatment significantly decreased

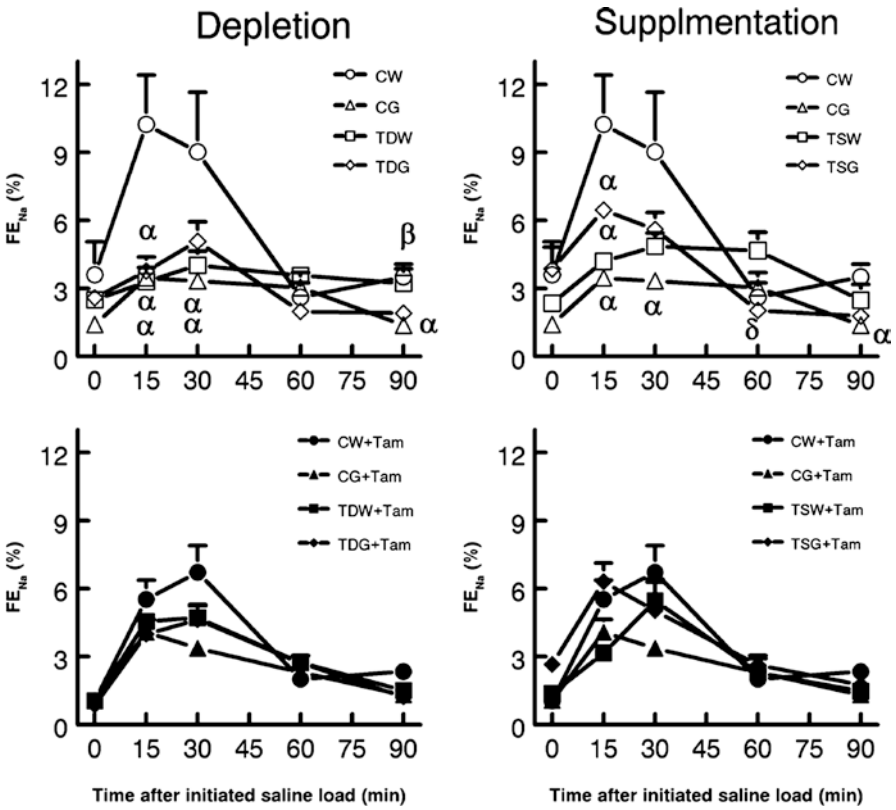

Fig. 8 Fractional sodium excretion (FE_{Na}) before (0 min) and after a saline load (15–90 min) in conscious, female rats ($^{\alpha,\beta}P < 0.05$ compared to CW and CG, respectively; n = 6–10 each group; see Abbreviations for group details)

fractional sodium excretions of CW to the attenuated levels of the other groups (Fig. 8, two lower panels). Fractional sodium excretion was significantly lower in TSG compared to TSW at 60 min and in CG compared to CW and TDW at 90 min post saline load. Like fractional water excretion, fractional sodium excretion was not significantly different among the tamoxifen-treated TD and TS groups.

3.6 Potassium Excretion

Similar to water and sodium excretion, potassium excretion before and after a saline load was not significantly different among the corresponding TD or TS groups (Fig. 9). However, potassium excretion before and after the saline load tended to decrease in tamoxifen-treated compared to untreated groups ($P > 0.05$).

In perinatal taurine depleted rats, changes in fractional potassium excretion before and after a saline load were similar to those of potassium excretion, except

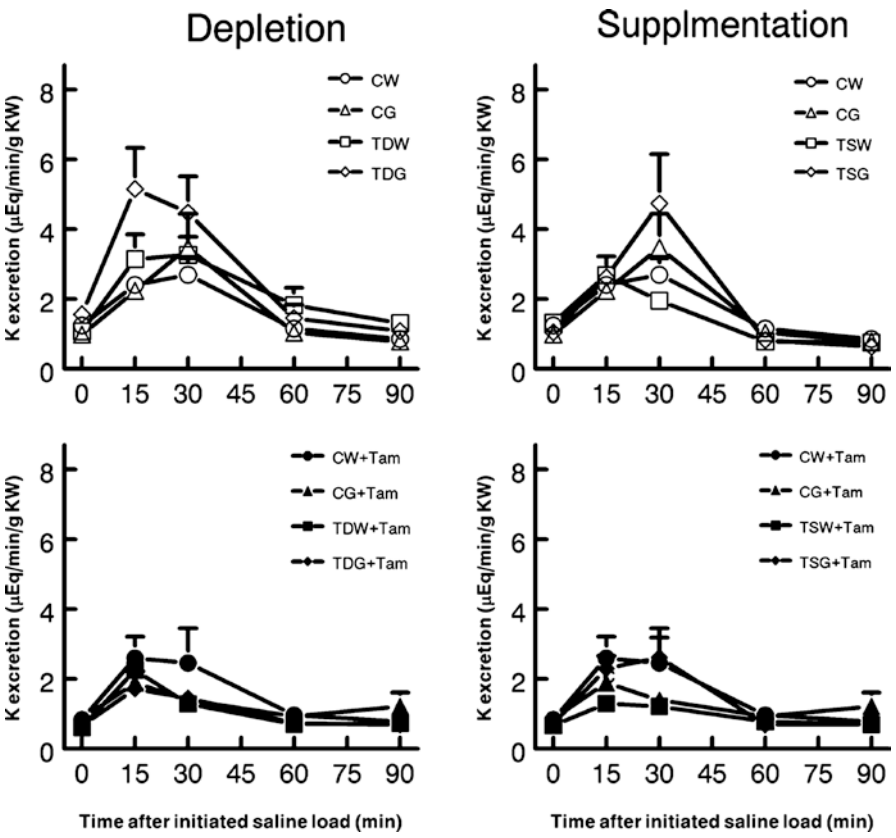

Fig. 9 Potassium excretion before (0 min) and after a saline load (15–90 min) in conscious, female rats (n = 6–10 each group; see Abbreviations for group details)

that the value of TDW at 90 min post saline load was significantly higher than that of the CG group (Fig. 10, upper left panel). Compared to the CG and TSW groups, TSG's fractional potassium excretion significantly increased after a saline load, but not at rest (Fig. 10, upper right panel). These changes were abolished by tamoxifen treatment (Fig. 10, two lower panels). Like fractional water and sodium excretion, fractional potassium excretion was not significantly different among the tamoxifen-treated TD and TS groups.

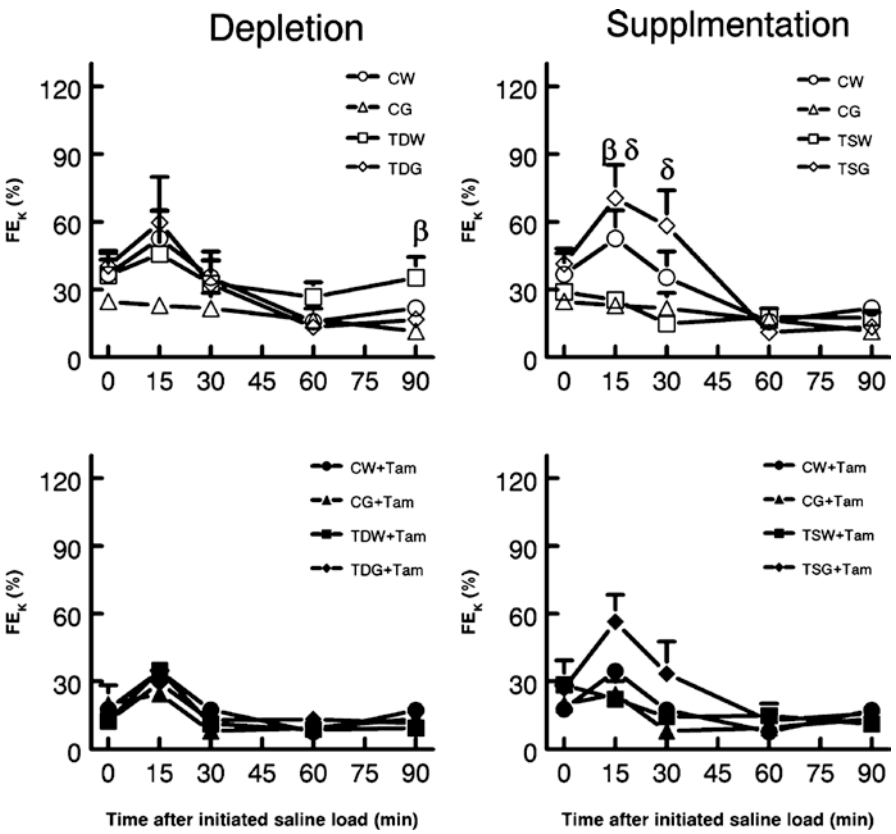

Fig. 10 Fractional potassium excretion (FE_K) before (0 min) and after a saline load (15–90 min) in conscious, female rats ($^{\beta,\delta}P < 0.05$ compared to CG and TSW, respectively; n = 6–10 each group; see Abbreviations for group details)

4 Discussion

Sex hormones (Gilbert and Nijland 2008), RAS (Harris and Gomez 1997; Johnston et al. 1993; Wolf and Neilson 1993), and taurine (Roysommuti and Wyss 2014) affect renal growth and function from prenatal through adult life. Further, high sugar intake since weaning (Roysommuti et al. 2002) and perinatal taurine imbalance (Roysommuti and Wyss 2014) alter adult renal function in a sex-dependent manner. In adult female rats, perinatal taurine depletion alters pressure-diuresis/natriuresis mechanisms by depressing RAS action after acute saline load, irrespective of high sugar intake since weaning (Lerdweeraphon et al. 2015), while perinatal taurine supplementation, particularly followed by high sugar intake since weaning, does by increasing RAS activity (Lerdweeraphon et al. 2017). The present study indicates that the renal effects of estrogen are depressed in adult female rats perinatally depleted of taurine, irrespective of high sugar intake, but estrogen, like the RAS, underlie the renal effects of perinatal taurine supplementation, especially if the animals were treated with a high sugar diet.

Estrogen receptors are found in many parts of the kidney and possesses antinatriuretic activity that play an important role in water and salt retention during pregnancy (Christy and Shaver 1974). Although estrogen may directly affect renal function, it also indirectly acts via the RAS, aldosterone, and atrial natriuretic hormone (Metcalfe and Meldrum 2006). Diuresis and natriuresis following an acute saline load has been shown to depend on several factors, including RAS activity and renal nerve activity (Drummer et al. 1992; Jensen et al. 2013). In the present study, estrogen receptor blockade depressed fractional water and sodium excretions after a saline load, as did inhibition of the RAS in other experiments (Lerdweeraphon et al. 2015; Lerdweeraphon et al. 2017). In addition, inhibition of either factor causes similar effects on renal function in rats that were depleted of or supplemented with taurine followed by high sugar intake since weaning. Thus, it is likely that the role of the RAS on renal function in the present rats is mainly regulated by estrogen. However, the effect of the RAS on renal function in these rats after ovariectomy has to be further studied. In addition, the estrogen effects on other factors, e.g., atrial natriuretic peptide and aldosterone, have to be further clarified in these rats.

Like water and sodium excretion, potassium excretion is not affected by perinatal taurine imbalance, in agreement with previous studies (Roysommuti et al. 2010). Estrogen receptor blockade slightly depresses potassium excretion both at rest and after a saline load, but these changes were similar among the corresponding TD or TS groups, suggesting that estrogen has minimal effects on potassium excretion in rats perinatally taurine imbalanced with or without a high sugar diet. In addition, these changes in renal potassium excretion are different from those observed in captopril-treated rats (Lerdweeraphon et al. 2015, 2017). These data suggest that the RAS may serve as a more important regulator of renal potassium excretion in these rats than does estrogen. However, this difference may not be due to a difference in tubular potassium transport since blockade of either factor displayed similar fractional potassium excretion at rest and after a saline load, irrespective of their differ-

ent baseline values. The minimal effect of estrogen on renal potassium excretion is also supported by other studies (Christy and Shaver 1974).

Gender and sex hormones affect the RAS, arterial pressure, and renal function (Ashraf and Vongpatanasin 2006; Oelkers 1996). Hypertension is more common in men than in age-matched, pre-menopausal women, but this begins to reverse after women reach menopause. In animal models, sex differences are also apparent, as males generally display greater hypertension than females. In young adult SHR, mean arterial pressure is higher in males than in females, and inhibition of the RAS eliminates this sex difference (Yanes et al. 2006). In addition, renal angiotensin II is higher in older females than in age-matched males. However, it is well known that high plasma estrogen levels (e.g., after injection or during pregnancy) increase plasma angiotensin II and aldosterone levels, while plasma renin levels are low or normal (Oelkers 1996). The increase in angiotensin II level is an indirect process since estrogen increases hepatic angiotensinogen synthesis. The high angiotensinogen content leads to accelerated enzymatic conversion of angiotensinogen to angiotensin I by renin, and subsequent conversion of angiotensin I to angiotensin II by the angiotensin converting enzyme. The low or normal plasma renin level in high estrogen stages may represent a negative feedback response to high angiotensin II levels (Oelkers 1996) or low renal sympathetic nerve activity. Further, estrogen can act centrally to decrease sympathetic nerve activity that decreases renal renin release (Hay 2016).

Tamoxifen is widely used to treat breast cancer due to its effect on estrogen receptors. It decreases the hexose monophosphate shunt and thereby increases oxidative stress in cells (Ryden et al. 2016; Spring et al. 2016). The oxidative stress induced by tamoxifen is reported to cause nephrotoxicity that can be prevented by taurine supplementation (Tabassum et al. 2007). Taurine is an antioxidant that converts hypochlorous acid to taurochloramine, which possesses lesser oxidative stress activity than the former (Roysommuti and Wyss 2014). In the present study, rats were treated with tamoxifen for one week before the renal studies. The major renal functions, i.e., renal blood flow, glomerular filtration, and water and electrolyte excretion, were not significantly different from the untreated control rats. Thus, tamoxifen toxicity is unlikely to underlie the present data.

5 Conclusion

Both estrogen and the RAS play an important role in renal and vascular function in the adult rat, and the current data reiterate that early environmental challenges, such as taurine depletion or supplementation, can alter this role. In female rats, both perinatal taurine alterations and glucose excess lead to impaired renal function. Estrogen appears to mediate normal responses in the control rats, but perinatal taurine imbalance or high glucose intake eliminates most of that control. This indicates that there is a complex interaction between perinatal taurine, dietary glucose, and circulating estrogen that modulates renal function.

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Taurine Inhibits Kupffer Cells Activation Induced by Lipopolysaccharide in Alcoholic Liver Damaged Rats

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Abstract Taurine, a β free amino-acid, takes various biological functions including maintain the normal hepatic structure and function. In this study, the regulation mechanism of taurine on lipopolysaccharide (LPS) induced activation of Kupffer cells (KC) in the liver of rats with alcoholic liver disease (ALD) were explored. Male wistar rats were intragastrically administered with alcohol and pyrazole, and ate high-fat diet in order to establish ALD model. Taurine were administered in drinking water simultaneous with and after ALD model establishment. The preventive trial was lasted for 12 weeks, while the curative trial was lasted for 4 weeks. Finally, blood and liver were collected in order to detect the concentrations of plasma LPS and hepatic tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6). Hepatic total RNA were extracted, gene expressions of *LPS binding protein (LBP)*, *leukocyte differentiation antigen 14 (CD14)*, *toll-like receptors (TLR4)*, *nuclear transcription factor (NF-κB)* and *TNF-α* were detected by semi-quantitative RT-PCR. The results showed significant elevated levels of plasma LPS, hepatic TNF-α, IL-1β and IL-6 in ALD rats ($P < 0.05$), and heightened gene expressions of *LBP*, *CD14*, *TLR4*, *NF-κB* and *TNF-α* ($P < 0.05$); Taurine no matter administered preventively or curatively can reduce the levels of plasma LPS, hepatic TNF-α, IL-1β, IL-6, and down-regulate the gene expressions of *LBP*, *CD14*, *TLR4*, *NF-κB* and *TNF-α*. The results demonstrated that taurine can prevent and cure ALD by reducing the production and transformation of LPS as well as inhibiting the opening and the transmission of LPS induced KC activation and the downstream signaling pathway.

Keywords Taurine • Kupffer cell • Lipopolysaccharide • Alcoholic liver disease • Rat

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Abbreviations

ALD	Alcoholic liver disease
CD14	Leukocyte differentiation antigen 14
IL-1β	Interleukin-1β
IL-6	Interleukin-6
LBP	LPS binding protein
LPS	Lipopolysaccharide
NF-κB	Nuclear transcription factor
TLR4	Toll-like receptors
TNF-α	Tumor necrosis factor alpha

1 Introduction

Alcoholic liver disease (ALD), consisting of alcoholic fatty liver disease (AFLD), alcoholic hepatitis (AH), alcoholic fibrosis (AF), and hepatocellular carcinoma (HCC), has become the second liver disease after viral hepatitis around the world and induces an increase in the morbidity and mortality. Currently, it is believed that alcohol abuse is one of the important reasons accounting for chronic liver disease all over the world (Schwartz and Reinus 2012; Hsieh et al. 2014), among which innate immunity plays a central role. In the recent few years, researchers made significant progress trying to understand the molecular mechanism of alcohol-dependent activation relating to innate immunity and inflammation. Many reports manifested that excessive alcohol intake causes dysbiosis and overgrowth of intestinal microflora (Rao 2009), which increased the gut permeability, indicating the disruption of intestinal barrier function is an important pathogenesis of ALD (Forsyth et al. 2014). Consequently, a great deal of lipopolysaccharide (LPS) release through the intestinal wall into the bloodstream, inducing the elevation of LPS in the portal circulation. Researches manifested that both alcohol and acetaldehyde can increase intestinal permeability and lead to the accumulation of LPS that induce the pathogenesis of ALD (Elamin et al. 2013). LPS, translocating from the intestinal tract into the liver, activates the Kupffer Cells (KC), which then generate a large amount of cytokines that initiate inflammation reaction and injure the liver cells (Petrasek et al. 2015). It was reported that AH patients with rising serum tumor necrosis factor (TNF) levels are more severe and have unfavourable prognosis (McClain et al. 2005). While the development of experimental ALD could be prevented by the inhibition of TNF production (Tilg et al. 2003), which further stressed that LPS induced KC activation and the downstream signaling pathway takes vital effects in the progressing of ALD.

Taurine, a kind of β free amino-acid, widely existing in the tissues of many mammalian, especially in the liver, has a large variety of biological functions including maintain the normal hepatic structure and function. Taurine deficient liver has been observed to have abnormal morphology and incomplete development (Miyazaki and Matsuzaki 2014). This cytoprotective function of taurine was mainly considered to be due to its antioxidant and osmoregulatory capabilities (Schaffer et al. 2014). All these evidence indicated significant protective effects of taurine on the liver. Our previous research also manifested that taurine can protect hepatic cells from damage induced by excessive alcohol drinking (Wu et al. 2009, 2015). In this study, the inhibitory action of taurine on LPS-induced liver injury of ALD rats was explored to reveal the mechanism of taurine on ALD.

2 Materials and Methods

2.1 Animal Models of ALD and Experimental Design

Animal feeding and experimental procedures were complied with protocols approved by the Animal Care and Use Committee of Shenyang Agricultural University.

Male SPF Wistar rats (120 ± 20 g) obtained from Liaoning Chang Sheng Biotechnology Co. Ltd., China were given diet and water ad libitum and maintained at the temperature of 22 ± 2 °C with 12 h light and dark cycle. Rats in ALD model group (M) were intragastrical administration of alcohol and pyrazole, and ate high fat diet. The whole experiment includes preventive trail and curative trail. In the preventive trial, taurine preventive rats (P) drank water containing 2% taurine simultaneous with ALD model establishment, the normal control rats (N) were intragastrical administration of water, and drank tap water, while the taurine control rats (C) received the same treatment as the normal control rats, and drank 2% taurine added in drinking water. In the curative trial, after ALD model was successfully established, taurine treatment rats (T) were given 2% taurine in drinking water, while the automatically recovery rats (A) drank tap water. The preventive trial was lasted for 12 weeks, while the curative trial was lasted for 4 weeks.

2.2 Biochemical Analysis

Plasma concentration of LPS and hepatic TNF-α, interleukin-1β (IL-1β) and interleukin-6 (IL-6) levels were assayed using ELISA Kits (R&D system, USA) as per the protocol from the manufacturer.

2.3 Semi-quantitive RT-PCR

Hepatic total RNA was extracted using RNAiso Plus (TaKaRa, China). The purity and quality of RNA were assessed at 260 and 280 nm by spectrophotometry. After cDNA was synthesized (Sangon, China), the PCR began with an initial denaturation at 94 °C for 3 min, 34 cycles of denaturation at 94 °C for 30 s, and annealing at 58 °C, 60 °C, 57.8 °C, 55.8 °C, 55 °C, 55.5 °C, 55.5 °C respectively for *LPS-binding Protein (LBP), cell differentiation antigen (CD14), Toll-like receptor 4 (TLR4), nuclear transcription factor-κB (NF-κB), TNF-α, GAPDH* and *β-actin* for 30 s and extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. The primers were designed as follows: *LBP* (CCGTGGGCAGTACGAGTTTCAT-forward and CTGGCAGAGTTTGGAGATAAGG-reverse), *CD14* (GAGGAAA GTGTCCGCTGCTA-forward and CAGTTCTGCGAGCCAGGTAT-reverse), *TLR4* (ATGAGGACTGGGTGAGAAAC-forward and TGCTAAGAA GGCGATACAAT-reverse), *TNF-α* (TCCCAACAAGGAGGAGAAGT-forward and TGGTATGAAATGGCAAATCG-reverse), *NF-κB* (TCTGTTTCCC CTCATCTTTC-forward and GTGCGTCTTAGTGGTATCTG-reverse), *GAPDH* (CAGTGCCAGCCTCGTCTCAT-forward and AGGGGCCATCCACAGTCTTC-reverse) and *β-actin* (TTGTAACCAACTGGGACG-forward and GATATTGATC TTCATGGTG-reverse). Three parallel experiments were conducted and the PCR products were separated using 1.5% agarose gel which was then stained with Goldview (Takara, China). Image J software was used to quantify the band intensity of RT-PCR products. Finally, the aim genes/*β-actin* or *GAPDH* ratios were calculated.

2.4 Statistic Analysis

All the data were represented as means ± SE. SPSS 16.0 software was used to determine the significant differences by the methods of one-way ANOVA and Duncan's multiple range tests. $p < 0.05$ was considered as significant difference.

3 Results

3.1 Effects of Taurine on Plasma LPS and Hepatic Cytokines

The results showed that plasma LPS in ALD model rats was significantly higher than the normal rats ($P < 0.01$), while taurine administration both preventively or curatively obviously decreased plasma LPS compared with the model and the automatically recovery rats ($P < 0.05$) (Fig. 1).

Hepatic concentrations of TNF-α, IL-1β and IL-6 in ALD model rats increased compared with rats in the normal control and taurine control groups ($P < 0.01$), but

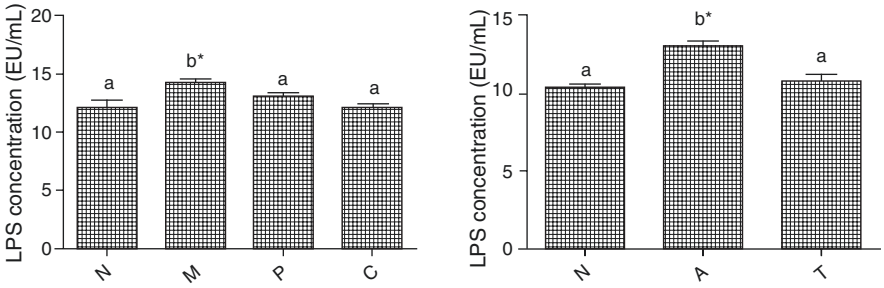

Fig. 1 Taurine induces a decrease in the concentration of plasma LPS in rats with ALD. Taurine (2%) was supplemented in drinking water simultaneous with or after ALD establishment. N-C: preventive trial, N-T: treatment trial. N, normal control group; M, ALD model group; P, taurine preventive group; C, taurine control group; A, automatically recovery group; T, taurine treatment group. Different lowercase on the column indicate $P < 0.05$, the same lowercase indicate $P > 0.05$. *$P < 0.01$ versus N group

the levels of these cytokines in taurine treated rats were significantly lower than the ALD model and automatically recovery rats ($P < 0.05$), and was similar to the normal rats ($P > 0.05$). All these parameters in rats only treated with taurine had no significant differences to the normal rats ($P > 0.05$) (Fig. 2).

3.2 mRNA Expressions of Factors Involved in the Pathway of LPS Induced KC Activation

LPS researching the liver from the gut first combined to LBP to form a LPS-LBP compound which then bond to CD14 expressed on the surface of KC, which could consequently combine to TLR4. The combination of these molecules causes the phosphorylation of IκB and expose the nuclear localization signal site. NF-κB enters the nucleus, triggers the consequent transcription of target genes and producing cytokines, which inducing inflammation and injury in the liver. The mRNA expressions of *LBP*, *CD14*, *TLR4*, *NF-κB*, *TNF-α* which were the main factors involved in the LPS induced KC activation signaling pathway were analyzed to elucidate the inhibition mechanism of taurine on this pathway.

The electrophoresis results of RT-PCR products showed that the specific bands of *LBP*, *CD14*, *TLR4*, *NF-κB* and *TNF-α* appeared in all the experimental groups, indicating all these genes were expressed in the rat hepatic tissues. As illustrated in Figs. 3, 4, 5, 6, and 7, mRNA expressions of *LBP*, *CD14*, *TLR4*, *NF-κB* and *TNF-α* in ALD model group increased extremely significant than the normal rats ($P < 0.01$), mRNA expressions of *LBP*, *CD14* and *TNF-α* of rats in taurine preventive group were extremely reduced than the ALD model rats ($P < 0.01$), and mRNA expressions of *TLR4* and *NF-κB* were significant lower than ALD model rats ($P < 0.05$), all the above genes have no significant differences between the normal and taurine control

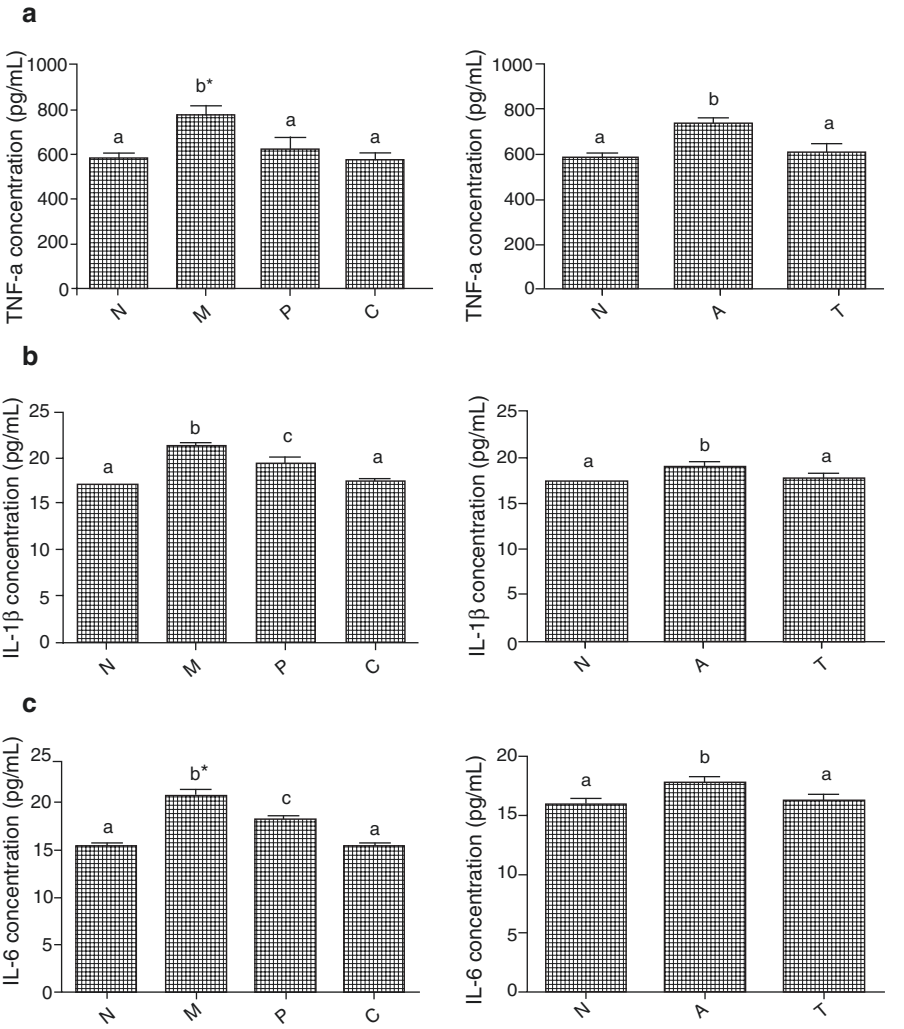

Fig. 2 Taurine decreases hepatic concentration of cytokines in rats with ALD. Taurine (2%) was supplemented in the drinking water simultaneous with or after ALD establishment. N-C: preventive trial, N-T: treatment trial. N, normal control group; M, ALD model group; P, taurine preventive group; C, taurine control group; A, automatically recovery group; T, taurine treatment group. Different lowercase on the column indicate $P < 0.05$, the same lowercase indicate $P > 0.05$. *$P < 0.01$ versus N group. (**a**) Concentrations of TNF-α in each group. (**b**) Concentrations of IL-1β in each group. (**c**) Concentraions of IL-6 in each group

rats ($P > 0.05$). In taurine treatment rats, mRNA expressions of *LBP*, *CD14*, *NF-κB* and *TNF-α* were reduced than the ALD rats ($P < 0.01$) and automatically recovery rats ($P < 0.05$), mRNA expression of *TLR4* was obviously lower than the ALD rats ($P < 0.05$), but had no obvious difference compared to the automatically recovery rats ($P > 0.05$).

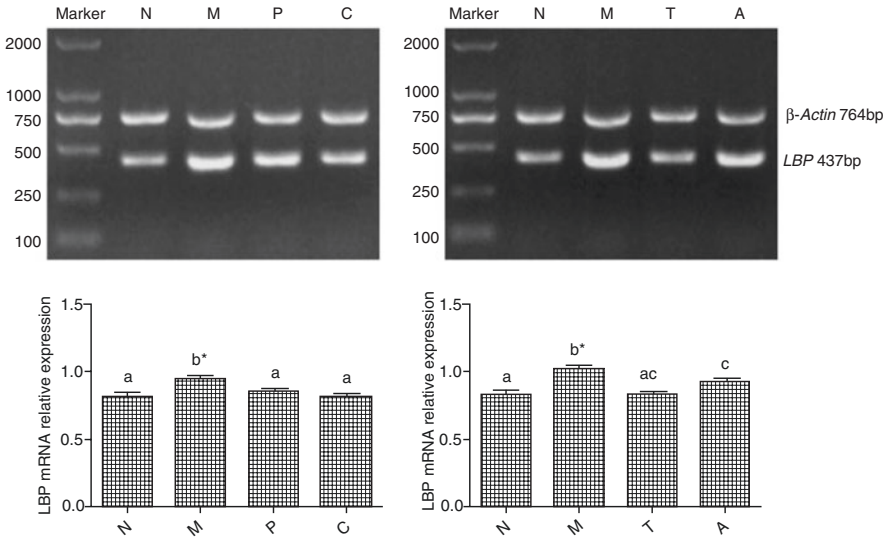

Fig. 3 Taurine down-regulates *LBP* mRNA expression in ALD rats. Taurine (2%) was supplemented in the drinking water simultaneous with or after ALD establishment. N-C: preventive trial, N-T: treatment trial. N, normal control group; M, ALD model group; P, taurine preventive group; C, taurine control group; A, automatically recovery group; T, taurine treatment group. Different lowercase on the column indicate $P < 0.05$, the same lowercase indicate $P > 0.05$. *$P < 0.01$ versus N group

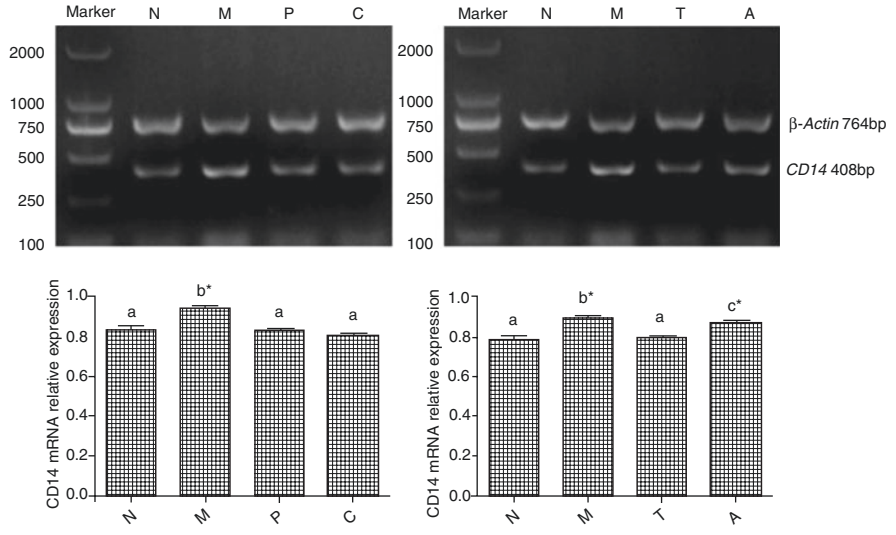

Fig. 4 Taurine down-regulates *CD14* mRNA expression in ALD rats. Taurine (2%) was supplemented in the drinking water simultaneous with or after ALD establishment. N-C: preventive trial, N-T: treatment trial. N, normal control group; M, ALD model group; P, taurine preventive group; C, taurine control group; A, automatically recovery group; T, taurine treatment group. Different lowercase on the column indicate $P < 0.05$, the same lowercase indicate $P > 0.05$. *$P < 0.01$ versus N group

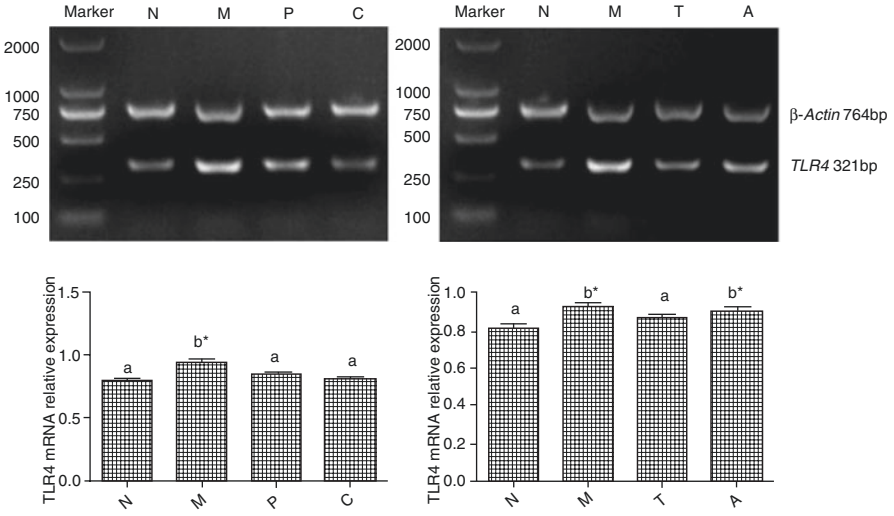

Fig. 5 Taurine down-regulates *TLR4* mRNA expression in ALD rats. Taurine (2%) was supplemented in the drinking water simultaneous with or after ALD establishment. N-C: preventive trial, N-T: treatment trial. N, normal control group; M, ALD model group; P, taurine preventive group; C, taurine control group; A, automatically recovery group; T, taurine treatment group. Different lowercase on the column indicate $P < 0.05$, the same lowercase indicate $P > 0.05$. *$P < 0.01$ versus N group

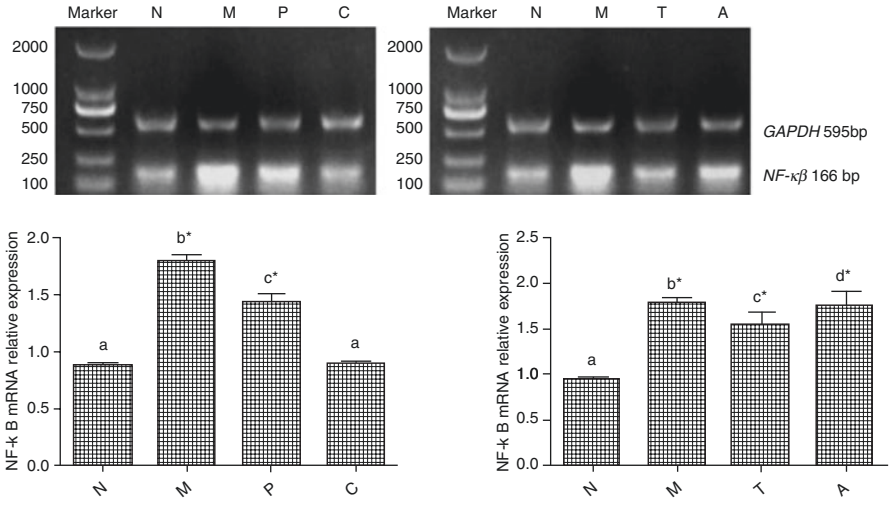

Fig. 6 Taurine down-regulates *NF-κB* mRNA expression in ALD rats. Taurine (2%) was supplemented in the drinking water simultaneous with or after ALD establishment. N-C: preventive trial, N-T: treatment trial. N, normal control group; M, ALD model group; P, taurine preventive group; C, taurine control group; A, automatically recovery group; T, taurine treatment group. Different lowercase on the column indicate $P < 0.05$, the same lowercase indicate $P > 0.05$. *$P < 0.01$ versus N group

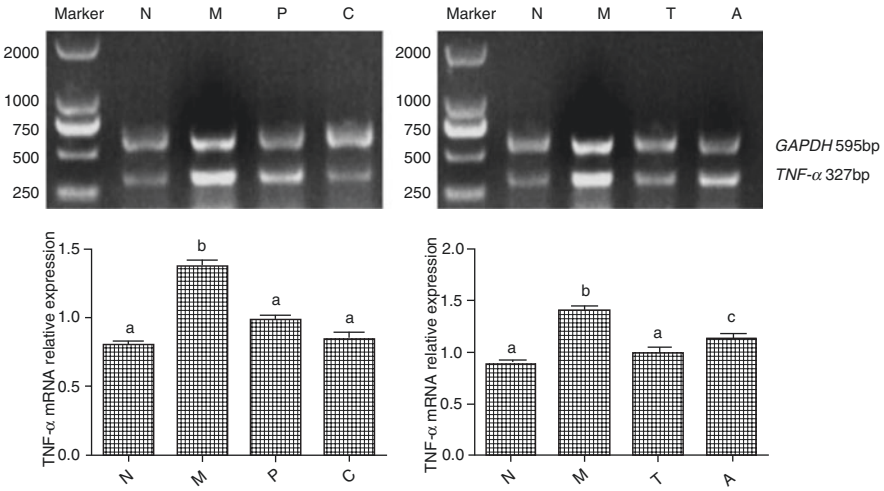

Fig. 7 Taurine down-regulates *TNF-α* mRNA expression in ALD rats. Taurine (2%) was supplemented in the drinking water simultaneous with or after ALD establishment. N-C: preventive trial, N-T: treatment trial. N, normal control group; M, ALD model group; P, taurine preventive group; C, taurine control group; A, automatically recovery group; T, taurine treatment group. Different lowercase on the column indicate $P < 0.05$, the same lowercase indicate $P > 0.05$

4 Discussion

LPS, a component of the gram negative cell envelope, released during the bacterial death or reproduction active periods, is considered as gut-derived microbial products that can stimulate the synthesis and release of a variety of endogenous bioactive factors in the cells. Under physiological conditions, only a small amount of LPS penetrate from the intestinal mucosa into the portal circulation, which could be identified and eliminated by KC and liver cells. But in chronic and excessive alcohol consumption, the intestinal barrier was disrupted, thus a larger amount of LPS released by intestinal flora passing the systemic and portal circulation and active the release of inflammatory cytokines which will further injure intestinal mucosa and the hepatic cells (Hartmann et al. 2012). It is reported that an increase of serum LPS was found in ALD animal models and human patients (Rao 2009; Parlesak et al. 2000), which are in accordance with the present result that plasma LPS in ALD model rats increased significantly, while taurine administration no matter at the same time or after ALD model establishment could obviously decrease plasma LPS, which is similar to the report from Yu et al. (2016) that serum LPS was decreased in mice administered with taurine. The results suggested that taurine could protect hepatic cells from injury of LPS by lowering LPS released from gut through maintenance of the intestinal permeability. Another reason maybe that taurine could inhibit the transference of LPS from the gut.

Under ALD condition, the binding of LPS to LBP facilitates the association between LPS and CD 14. Experiment from LBP knockout mice showed reduced pathological changes such as LPS concentrations, steatosis, inflammation and injury of hepatic cells, meanwhile, TNF-α expression was down-regulated, which manifested that LBP had significant effects in the process of ALD (Uesugi et al. 2002). In this study, *LBP* mRNA expression was significantly up-regulated by long-term alcohol consumption in ALD rats, while taurine could down-regulate *LBP* expression, reduce the combination of LPS and LBP, and consequently inhibit the activation of KC.

The LPS-LBP compound will then combine to CD14, the receptor of LPS on the membrane surface of KC, which mediates LPS signal transduction, and could trigger monocyte macrophage to play vital effects in a series of reactions caused by the immune system and defense system. It has been documented that severe alcohol-related liver damage and significant elevations of NF-κB and TNF-α were observed in CD14 knockout mice, indicating a essential role of CD14 in the progression of ALD (Yin et al. 2001). Further, chronic ethanol exposure triggers the activation of TLR4 and CD14 mediated by LPS, and up-regulation of protein expressions of TLR4 and CD14 (Nezi et al. 2013, Song et al. 2016), which is similar to the present results that chronic alcohol administration up-regulated the expression of *CD14*, which was significantly decreased by taurine administration, illustrating that taurine can inhibit the LPS-KC signal pathway by down-regulating *CD14* expression and its combination to LPS.

The CD14-LBP-LPS compound then stimulate TLR4, a specific receptor of LPS, to trigger a KCs signaling pathway that phosphorylate IκB proteins and subsequently activates the translocation of NF-κB, which controls the expressions of pro-inflammatory cytokines. Under normal circumstances, NF-κB exists in the cytoplasm without activity, but multiple external signals such as LPS and TNF-α can induce the phosphorylation of NF-κB to participate in cell differentiation, development, apoptosis, adhesion and inflammatory reaction. Research using TLR4 knockout mice displayed an alleviation of hepatic injury caused by alcohol, indicating TLR4 is essential for KC activation induced by LPS (Wu et al. 2014, Inokuchi et al. 2011). The current study found a up-regulation of *TLR4* and *NF-κB* expression in ALD model rats, which is similar to the in vitro and in vivo evidences (Petrasek et al. 2013, Hong et al. 2015). Taurine administration could obviously down-regulate the expressions of *TLR4* and *NF-κB*, indicating that taurine could inhibit KC activation by blocking the combination of CD14-LBP-LPS complex to TLR4 which was also decreased by taurine, thus decreasing the synthesis and release of cytokines, which would then further injure the hepatocytes.

TNF-α release, one of the initial activities in the process of hepatic damage, could stimulate the production of other cytokines. Under normal circumstances, only a small amount of TNF-α produced in the liver, but in ALD, KC activates NF-κB, producing large amount of TNF-α. On the one hand, TNF-α could induce cell death after combining with cell membrane receptors TNF-R1and TNF-R2. On the other hand, TNF-α could trigger the transcriptional activation of NF-κB, and

consequently increase the release and activities of inflammatory cytokines such as IL-1β, which will then motivate the synthesis of IL-6. All these reactions would aggravate the inflammation in the liver. Furthermore, TNF-α can directly increase intestinal permeability, and cause oxidant stress, which in turn aggravate the liver injury. In the ALD mice, alcohol co-administered with high-fat diet could elevate serum levels and expressions of pro-inflammatory cytokines (Hong et al. 2015; Bavia et al. 2016), the results is the same as the present study. While taurine could significantly down-regulate the expression of *TNF-a* and lower hepatic TNF-a, IL-1β, and IL-6 concentrations, indicating that taurine could protect the hepatocytes from alcohol injury by reducing inflammatory factors, which is the same as the results reported by Lin et al. (2015) in rats.

5 Conclusion

This study manifested that taurine can inhibit the LPS induced KC activation and its downstream signaling pathway in ALD rats by maintaining the intestinal osmotic pressure and normal structure, thus reducing LPS release from the gut. At the same time, taurine could reduce the mRNA expressions of *LBP*, *CD14*, *TLR4*, *NF-κB and TNF-a*, which consequently block the conduction of the pathway, and finally decrease the synthesis and release of inflammatory cytokines that is harmful to hepatocytes.

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Taurine Recovers Testicular Steroidogenesis and Spermatogenesis in Streptozotocin-Induced Diabetic Rats

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Abstract A great deal of investigations have verified that diabetic male reproductive impairment is associated with the dysfunction of testicular steroidogenesis and spermatogenesis resulted from insulin deficiency and hyperglycaemia-induced oxidative stress. It has been identified taurine is profitable for diabetes mellitus and diabetic implications through its insulin-like and islet cells protective activity. Furthermore, our previous studies found that taurine could increase testicular antioxidative ability, stimulate the endocrine activity of hypothalamic-pituitary-testicular axis, elevate testosterone level, raise sperm quality, suppress the deterioration of testicular function. Accordingly, we hypothesized that taurine may have beneficial effects on testicular dysfunction under diabetic mellitus status. Here, we investigated the effects of taurine on testicular steroidogenesis and spermatogenesis in streptozotocin (STZ)-induced type I diabetic rats. We observed that taurine treatment can markedly increase the body and testis weights, testicular SDH and G6PDH activities, decrease the serum fasting glucose concentration of diabetic rats. Serum contents of GnRH, LH, FSH, T, and testicular StAR, 3β-HSD, 17β-HSD mRNA expression levels were also obviously raised by taurine administration, indicating that taurine can improve testicular steroidogenesis in diabetic animals. Finally, we found taurine supplementation effectively elevated the sperm count and

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motility, reduced sperm abnormality, suggesting that taurine can increase the testicular spermatogenesis function of diabetic rat. In summary, the present data indicated that taurine can rescue the function of testicular steroidogenesis and spermatogenesis in STZ-induced type I diabetic rats possibly by increasing the endocrine activity of hypothalamic-pituitary-testicular axis.

Keywords Taurine • Antioxidant • Steroidogenesis • Spermatogenesis • Diabetic rats

Abbreviations

17β-HSD	17β-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
FSH	Follicle-stimulating hormone
G6PDH	Glucose-6-phosphate dehydrogenase
GnRH	Gonadotropin-releasing hormone
LH	Luteinizing hormone
SDH	Sorbitol dehydrogenase
StAR	Steroidogenic acute regulatory protein
STZ	Streptozotocin
T	Testosterone

1 Introduction

Diabetes mellitus is a kind of metabolic disease with the characteristic of hyperglycaemia which is the main factor of its chronic multi-organs damage and complicating diseases. Many studies have demonstrated that either diabetic human or animals showed male reproductive impairment (Rossi and Bestetti 1981; Ramalho-Santos et al. 2008). Diabetic male reproductive impairment is multiple levels mainly including hypothalamo-pituitary-gonads axis abnormalities (Distiller et al. 1975; Seethalakshmi et al. 1987), steroidogenesis diminution (Ballester et al. 2004), spermatogenesis decline (Amaral et al. 2008), infertility (Scarano et al. 2006), and sexual dysfunction (Morano 2003). Although the mechanism of male reproductive impairment in diabetes is usually multifactorial, increasing evidences suggested that the decline of Leydig cells, Sertoli cells and spermatogenic cells number resulting from insulin deficiency and hyperglycosemia induced oxidative stress action may be contribute to this problem (Rossi and Aeschlimann 1982; Anderson and Thliveris 1986; Brownlee 2001; Ballester et al. 2004; Mangoli et al. 2013).

Taurine, a conditionally essential amino acid of human and most animals, was demonstrated to be rich in lots of tissues and involve in several normal functions in human and animals (Huxtable 1992). A large number of human clinical and animal

model tests have demonstrated that taurine has beneficial effects against diabetes and diabetic complications mainly by two pathways. One is that taurine itself has insulin-like activity (Maturo and Kulakowski 1987). Taurine could decrease blood glucose by way of combining to the insulin receptor located on the cell, and under the condition of hyperglycaemia, it could accelerate the intake of glucose in target cells of skeleton muscles and cardiac muscle, promote the glycometabolism and the synthesis of hepatin (Fromageot 1964; Goodman and Shibabi 1990; Nandhini et al. 2004). The other one is taurine could directly protect pancreatic islet β-cells, repair damaged β-cells, promote the secretion and the sensitivity of insulin by its antioxidative, antiapoptotic and anti-inflammatic activity (Kyuang and Woojung 2000; Schaffer et al. 2009; Lin et al. 2013; Imae et al. 2014). Previous researches have identified that taurine can be synthesized in male gonad (Yang et al. 2010a, b) and mainly located in the Leydig cells (Lobo et al. 2000), suggesting that taurine might play an important role in testicular steroidogenesis and spermatogenesis. Our further studies found taurine supplement can stimulate testosterone secretion, elevate testicular antioxidation and sperm quality in rats, which is associated with hypothalamo-pituitary-testicular axis function enhancement (Yang et al. 2010a, b, 2013). Furthermore, our recent study also found that aged testicular steroidogenesis and spermatogenesis functions can be increased by taurine administration in rats through enhancing testicular antioxidase system activity, decreasing the lipid peroxidation, as well as its antiapoptotic activity (Yang et al. 2015). In addition, many researches have confirmed that taurine can protect testes from heavy metals and some drugs induced damage (Manna et al. 2008; Das et al. 2012; Aly and Khafagy 2014). Taking the above, we presumes that taurine may have beneficial effects on testicular steroidogenesis and spermatogenesis under diabetic mellitus status. Accordingly, the objective of this study was to assess the effects of taurine supplementation on steroidogenesis and spermatogenesis in streptozotocin-induced diabetic rats.

2 Materials and Methods

2.1 Animals and Treatments

Rats were performed in a pathogen free facility at 22 ± 2 °C under normal feeding and management. The experimental protocols were executed according as the criterion of Shenyang Agricultural University Ethical Committee. Male adult Sprague-Dawley rats (180–210 g) were intraperitoneally injected with STZ (Sigma, USA) (50 mg/kg body weight, i.p.) or vehicle (0.1 M citrate buffer, pH 4.6). The blood glucose meter test strips was used to assay the fasting blood glucose of 72 h after the treatment; and only animals with glucose levels above 16.7 mmol/L were considered as diabetes and used in the following experiment. Animals were divided into four groups: the control group (CON) received single injection of vehicle and drank tap water, the diabetic group (DM) received single injection of STZ and drank tap water, the diabetic + taurine group (DM + Tau) drank 2% taurine water after single injection of STZ, and the

taurine group (Tau) drank 2% taurine water after single injection of vehicle. All the rats were weighted and anesthetised sacrificed after 8 weeks treatment, blood and testes were acquired. Blood samples were utilized for serum biochemical assay, and testes were weighted, the left testis were homogenized in cold PBS and used for testicular biochemical analyzation, the right testis were stored at −80 °C till later analysis. The left cauda epididymides were minced for sperm quality assay.

2.2 Biochemical Assay

Serum fasting serum glucose, gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone (T) concentration, testicular sorbitol dehydrogenase (SDH) and glucose-6-phosphate dehydrogenase (G6PDH) levels were determined by the means of ELISA kits as per the manufacturer's introduction (Nanjing Jiancheng Bioengineering Institute, China).

2.3 Real-Time RT-PCR

After extracting testicular total RNA, RNA purity and quality were detected by spectrophotometry. cDNA was synthesized using AMV First Strand cDNA Synthesis Kit (Sangon, China). Quantitative PCR was carried out on a Bio-Rad iQTM5 system using SYBR Green PCR Master Mix (ABI). The primers were designed for the objective genes: steroidogenic acute regulatory protein (StAR) forward primer: 5′-AACCAGGAAGGCTGGAAGAA-3′, reverse primer: 5′-TCTGTCCATGGGCTGGTCTA-3′; 3β-hydroxysteroid dehydrogenase (3β-HSD) forward primer: 5′-TGTGCCAGCCTTCATCTAC-3′, reverse primer: 5′-CTTCTCGGCCATCCTTTT-3′, 17β-hydroxysteroid dehydrogenase (17β-HSD) forward primer: 5′-GACCGCCGATGAGTTTGT-3′, reverse primer: 5′-TTTGGGTGGTGCTGCTGT-3′; and β-actin forward primer: 5′-TCGTGCGTGACATTAAAGAG-3′, reverse primer: 5′-ATTGCCGATAGTGATGACCT-3′. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the expression of β-actin mRNA in the same sample (Schmittgen and Livak 2008). Data were expressed as relative fold change according to the control group.

2.4 Sperm Quality Evaluation

The cauda epididymides were used to prepare sperm suspension and used for sperm quality detection as previous report (Türk et al. 2008). Sperm count, motility, viability and abnormality were determined as sperm quality parameters.

2.5 Statistical Analysis

Statistical analysis was performed using SPSS 16.0 software. Results were presented as mean ± SE and analyzed by one-way ANOVA and Duncan's multiple range test with $p < 0.05$ as the significant difference.

3 Results

3.1 Body, Testis Weights and Fasting Serum Glucose Level

Compared with the control and Tau group, the final body and testis weights in diabetic model rats significantly decreased ($P < 0.05$), which could be remarkably evaluated by taurine administration ($P < 0.05$). However, the levels of fasting serum glucose increased in diabetic rats ($P < 0.01$), and were obviously reduced after taurine supplementation ($P < 0.01$, Fig. 1).

3.2 Activities of Testicular Marker Enzymes

Figure 2 showed that there were statistically decline in testicular G6PDH and SDH activities of diabetic model rats compared with the control rats ($P < 0.05$), but taurine treatment could notably elevate the testicular marker enzymes levels ($P < 0.05$).

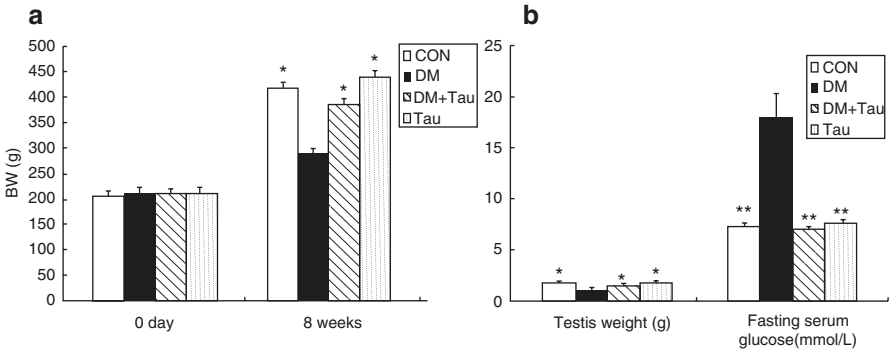

Fig. 1 Taurine increased the body and testis weight, the fasting serum glucose level. (**a**) effect of taurine on body weight (BW), (**b**) effects of taurine on the testis weight and fasting serum glucose. The values were presented as the means ± SE (n = 8). *P < 0.05 vs. DM, **P < 0.01 vs. DM. 0 day is the first day of the experiment

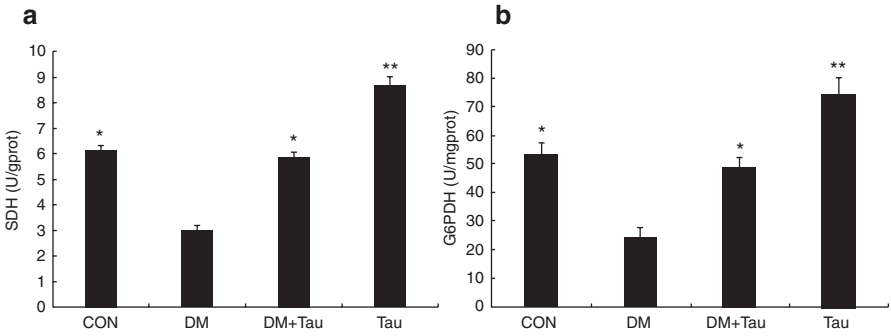

Fig. 2 Taurine elevated testicular SDH and G6PDH activities. (**a**) Effect of taurine on testicular SDH activity, (**b**) effect of taurine on testicular G6PDH activity. The values were presented as the means ± SE (n = 8). *P < 0.05 vs. DM, **P < 0.01 vs. DM

3.3 Levels of Serum Sex Hormones and Testicular Steroidogenic Genes Expression

As Fig. 3 illustrates, the concentrations of serum GnRH, LH, FSH and T in diabetic animals were less than the control rats ($P < 0.05$), whereas, taurine administration obviously prevented diabetes-induced reproductive hormones decline ($P < 0.05$).

Figure 4 shows the increased-effect of taurine supplementation on testicular steroidogenesis related gene expression levels of STZ-diabetic rats. StAR, 3β-HSD and 17β-HSD mRNA expression levels in diabetic animals were notably reduced compared to the control rats ($P < 0.05$), while taurine treatment remarkably increased mRNA expression of steroidogenesis ($P < 0.05$) to the similar levels of the controls. Moreover, testicular StAR, 3β-HSD and 17β-HSD mRNA expression levels were statistically higher in taurine group than that of the control ($P < 0.05$).

3.4 Sperm Quality

Figure 5 showed the elevated-effect of taurine on sperm quality in STZ-diabetic animals. The sperm count and motility were obviously raised ($P < 0.05$), while the sperm abnormality was remarkedly reduced in taurine group rats compared to the STZ-diabetic rats ($P < 0.05$). The sperm viability has no statistic differences among the control group, diabetic group and diabetic + taurine group ($P > 0.05$). But, the sperm count, viability and motility of the taurine group were notably higher than the control rats ($P < 0.05$).

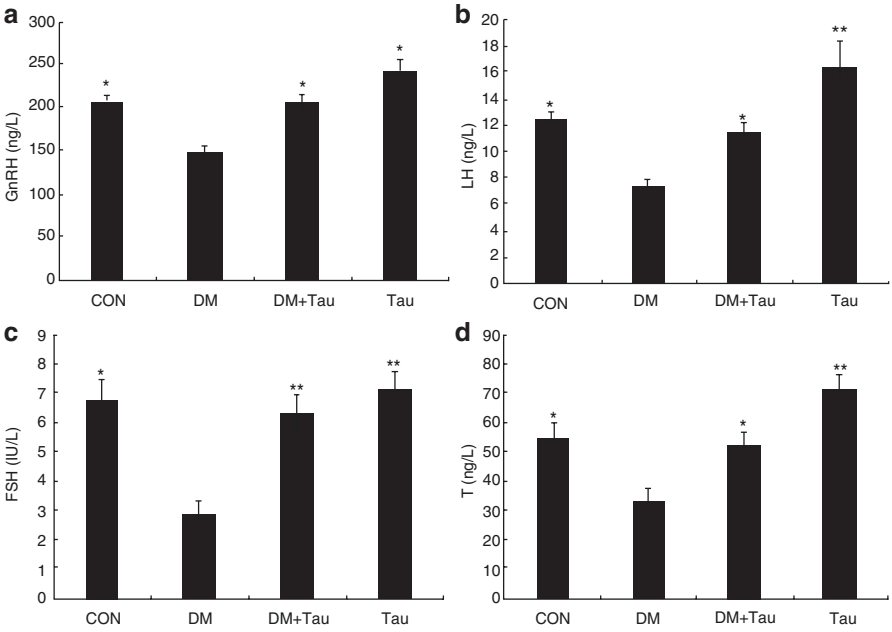

Fig. 3 Taurine increased serum sex hormone levels. (**a**) effect of taurine on serum GnRH level, (**b**) effect of taurine on serum LH level, (**c**) effect of taurine on serum FSH level, (**d**) effect of taurine on serum T level. The values were expressed as the means ± SE (n = 8). *P < 0.05 vs. DM, **P < 0.01 vs. DM

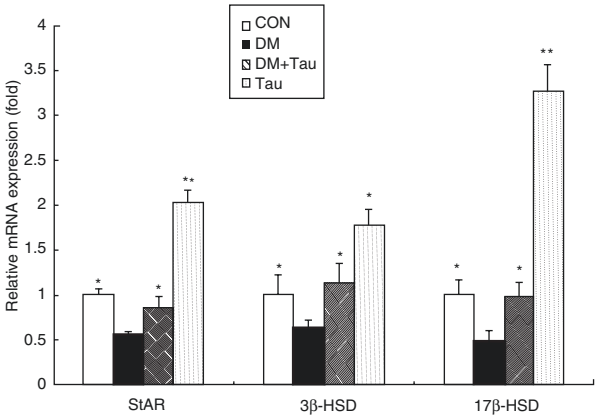

Fig. 4 Taurine raised steroidogenic genes expression levels in testes. The values were presented as the means ± SE (n = 8). *P < 0.05 vs. DM, **P < 0.01 vs. DM

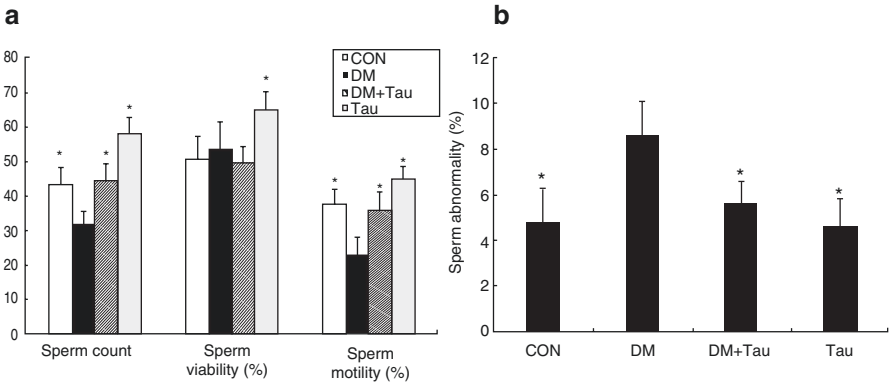

Fig. 5 Taurine increased the sperm quality of STZ-diabetic rats. (**a**) effect of taurine on the sperm count, viability and moltility; (**b**) effect of taurine on the sperm abnormality. The values were presented as means ± SE (n = 8). *P < 0.05 vs. DM, **P < 0.01 vs. DM

4 Discussion

A large number of tests have identified that taurine has hypoglycemic activity on diabetes mellitus, and can diminish the complications severity of among diabetic target organs, including brain, retina and kidney (Schaffer et al. 2009). Previous studies also found that taurine can stimulate hypothalamic-pituitary-testicular axis endocrine activity (Yang et al. 2013), elevate the sperm quality (Yang et al. 2010a, b), inhibit many chemical substances induced testes damage (Aly and Khafagy 2014), and increase aged rat testicular function (Yang et al. 2015). However, until now, we have no enough available advices about whether taurine supplementation can improve diabetic testicular dysfunction. To examine the effect of taurine treatment on the testicular function under diabetes, body and testis weights, fasting serum glucose, testicular function marker enzymes (SDH and G6PDH) activities in STZ-induced type I diabetic rats were analyzed. Similarly to the previous findings (Nandhini et al.; 2004; Das et al. 2012; Askwith 2014), our results showed taurine treatment could effectively ameliorate diabetic-induced body weight loss and hyperglycemia. STZ-diabetic testicular impairment resulted in a testosterone production decline, which may be associated with testicular Leydig cells, Sertoli cells and spermatogenic cells numbers reduction (Anderson and Thliveris 1986; Brownlee 2001; Hurtado de Catalfo and Nelva 1998; Mangoli et al. 2013). The present data showed that the testes weight, SDH and G6PDH activities, testicular function marker enzymes were statistically increased by taurine. The results demonstrated that taurine can restrain testicular atrophy and increase testicular function of STZ-induced diabetic rats, combined with the previous researches on taurine and aged testicular dysfunction (Aly and Khafagy 2014; Yang et al. 2015), the mechanism may be due to its antioxidative and antiapoptotic activity.

The testes have two primary functions in male reproduction, steroidogenesis and spermatogenesis. Both processes are regulated by hypothalamic-pituitary gonado-

tropins, GnRH, LH and FSH. To examine whether taurine can increase steroidogenesis in diabetic animals, we further assessed the action of taurine intake on the hypothalamic-pituitary-testicular axis reproductive hormones secretion and mRNA expressions of testicular steroidogenesis key enzymes (StAR, 3β-HSD and 17β-HSD). We found that taurine markedly increased serum GnRH, LH, FSH and T contents, and testicular StAR, 3β-HSD and 17β-HSD mRNA expression levels in STZ-diabetic rats. Male testicular steroidogenesis is mainly regulated by LH, namely that LH can stimulates the Leydig cells to synthesize and release androgen, and is involved in several factors/enzymes such as StAR, 17α-hydroxylase, 3β-HSD and 17β-HSD etc. Moreover, FSH also participates the adjustment of Leydig cells function by stimulating the Sertoli cells to secret various factors. The present data suggested that taurine can elevate diabetic steroidogenesis by improving hypothalamic-pituitary axis gonadotropins secretion and androgen biosynthesized rhythm. The present findings were also in consistent with the previous results, in spite of the difference of experimental animals (Aly and Khafagy 2014; Das et al. 2012; Yang et al. 2015).

Finally, the action of taurine on diabetic spermatogenesis was determined. The present study demonstrated that the sperm count and motility obviously reduced, sperm abnormality markedly elevated in STZ-induced diabetic rats, while taurine treatment could effectively increased sperm quality. In male, spermatogenesis is a complex cyclical process with a nearly 52 days cycle including mitotic cell division, meiosis and spermiogenesis within the seminiferous tubules of both testes, the process is considered critically depend on high intratesticular testosterone levels induced by the two pituitary gonadotropins, LH and FSH. It has been demonstrated that diabetic-induced testosterone synthesis disorder influences spermatogenesis in testicular seminiferous tubules, resulting in lower sperm counts and sperm motility (Amaral et al. 2008). These data indicated that taurine can promote spermatogenesis ability through stimulating hypothalamic-pituitary-testicular endocrine. It has identified that taurine plays a crucial role in spermatogenesis through protecting germ cells (Higuchi et al. 2012). In addition, there have been several reports suggesting that taurine has beneficial effects on spermatozoa, it may act as a sperm lipid peroxidation inhibitor (Alvarez and Storey 1983), membrane stabilizer (Mrsny and Meizel 1985), capacitating factor (Meizel et al. 1980) and motility agent (Fraser 1986).

5 Conclusion

The present results showed that taurine administration can recover testicular steroidogenesis and spermatogenesis in streptozotocin-induced type I diabetic animals possibly through increasing hypothalamic-pituitary-testicular endocrine activity.

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Effects of Taurine and L-Arginine on the Apoptosis of Vascular Smooth Muscle Cells in Insulin Resistance Hypertensive Rats

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Abstract *Objective*: In order to find the effects and mechanism of taurine and L-arginine on the apoptosis of VSMCs in insulin resistance hypertensive rats. *Methods*: 25% fructose were administered in the drinking water to Wistar rats for 12 weeks to induce the insulin resistance hypertensive model. Apoptosis of VSMCs was identified by TUNEL. The expressions of Bax protein and Bcl-2 protein were examined by immunohistochemistry. *Results*: High level of fructose significantly suppressed the apoptosis of VSMCs. Taurine and L-arginine promoted apoptosis of VSMCs via increasing the Bax protein expression and decreasing the Bcl-2 protein expression. *Conclusions*: Taurine and L-arginine have obvious anti-hypertensive effects in insulin resistance hypertensive rats, and its mechanism might partially be associated with attenuating vascular remodeling by promoting apoptosis in VSMCs. In addition, the combined medication of taurine and L-arginine was more effective than single medication.

Keywords Taurine • L-Arginine • Apoptosis • Hypertension

Abbreviations

IOD	Integrated optical density
PASMC	Pulmonary artery smooth muscle cell
SBP	Systolic blood pressure
TUNEL	*In situ* TdT-mediated dUTP nick end labeling
VSMCs	Vascular smooth muscle cells

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1 Introduction

Hypertension is a chronic cardiovascular disease with high incidence among people throughout the world, and it is also a key risk progenitor of the development of atherosclerosis, coronary heart disease, cerebrovascular disease, and so on. Insulin resistance is one of the risk factors for essential hypertension and its mechanism may be water-sodium retention, increased sympathetic activity, imbalance of vasoactive substances, dysfunction of vascular endothelial cells, atherosclerosis and other factors (Folkow 1982; Heagerty et al. 1993). Developing new antihypertensive drugs of improving insulin resistance will be a trend in research of new drugs in the future.

Taurine (Tau) is rich in the mammalian cells and tissues, especially in muscle and nerve tissues. Taurine has diverse biological functions, such as promoting neural development, treating and preventing cardiovascular diseases, improving the endocrine, enhancing memory, and maintaining normal reproductive function (Sturman 1993). It has been confirmed that taurine has a very good antihypertensive effect in different hypertensive models (Yamamoto et al. 1985; Fujita and Sato 1986; Anuradha and Balakrishman 1999; Yu et al. 2000; Zheng et al. 2003).

L-arginine (L-arg), an essential amino acid, is the principal precursor of nitric oxide, which lowers SBP through the expansion of blood vessels. Nitric oxide is of vital importance in the nervous system, cardiovascular system and immune system. Many studies showed that L-arginine has obvious antihypertensive effect, and is suitable for various types of hypertension (Gokce 2004). Sumou IK has demonstrated that L-arginine is crucial to attenuate vascular structural remodeling by upregulating Fas expression in PASMC, therefore accelerating the apoptosis (Lee et al. 1995; Sumou et al. 2006).

Our research has confirmed the anti-hypertension effects of co-administration of taurine and L-arginine (Feng et al. 2013). We aimed to further explore their effects on VSMCs apoptosis in rat models of insulin resistance.

2 Methods

2.1 Animals and Experimental Design

Wistar rats were provided by Beijing Vital River Laboratories Animal Technology Co., Ltd., and were free of rat food and filtered tap water.

Six-week-old Wistar rats were randomly divided into control group (the rats were given purified-water) and model group (the rats were given purified-water containing 25% fructose). After 12 weeks, 42 model rats with SBP higher than 150 mmHg were randomly divided into six groups. The rats were administered with purified-water, 3% Tau, 3% L-arg, 2.7% Tau +0.3% L-arg, 2.1% Tau +0.9% L-arg, 1.5% Tau +1.5% L-arg in drinking water respectively for 4 weeks.

2.2 Determination of the Apoptosis Index of VSMCs

The formalin-fixed thoracic aortas were embedded in paraffin wax. Cryostat-cut sections (5 μm) were picked up onto gelatin-coated glass slides. The apoptosis index of VSMCs was detected by TUNEL. Experimental procedures were conducted according to the manufacturers instructions. Tissue sections from each specimen were examined microscopically randomly four fields at 400× magnification. The apoptotic nucleus manifested brown and looked like positive cells. On the contrary, the normal nucleus manifested indigo and looked like negative cells. The apoptosis index was calculated with the average percentage ratio of the positive cells to the total VSMCs.

2.3 Examination of the Protein Expressions of the Bax and Bcl-2

The protein expressions of Bax and Bcl-2 were determined by immunohistochemistry staining. Bax antibody, Bcl-2 antibody were provided by Boster Biological Engineering Co., Ltd. Experimental procedures were conducted according to the manufacturers' instructions. The expressions of Bax protein and Bcl-2 protein were visualized as brown color in cytoplasma. Integrated optical density (IOD) was measured with computerized image analysis system.

2.4 Statistical Analysis

All values were presented as mean ± SD. Comparisons were performed by Duncan's multiple range tests using SPSS 16.0 statistical analysis software. Statistical significance was accepted at $P < 0.05$.

3 Results

3.1 Taurine and L-Arginine Increased the Apoptosis Index

The morphological change of vascular wall was investigated by the microscopy (Fig. 1). We observed endothelial injury, matrix alteration, migration and proliferation of VSMCs in the model group (Fig. 1b). The degree of VSMCs proliferation and the thickness of the arterial wall were diminished in all the treatment groups. The effects of combination of taurine and L-arginine on apoptosis index were presented in Fig. 2. The apoptosis rate of model group was obviously decreased as

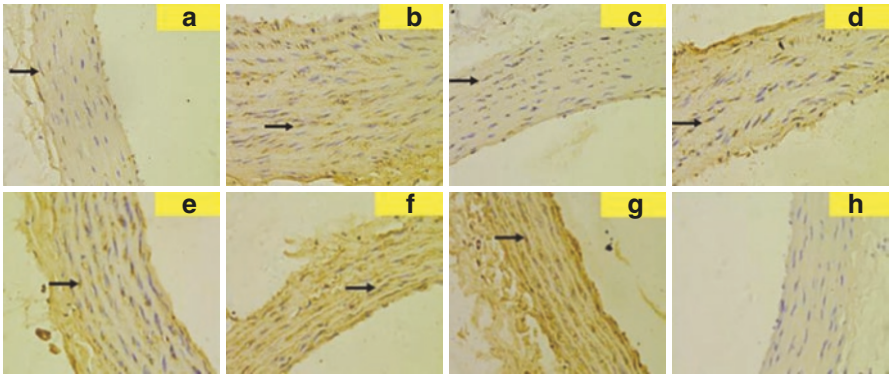

Fig. 1 Morphology change of VSMCs. (**a**) Control group. (**b**) Model group. (**c**) 3% Tau alone group. (**d**) 3% L-arg alone group. (**e**) 2.7% Tau and 0.3% L-arg group. (**f**) 2.1% Tau and 0.9% L-arg group. (**g**) 1.5% Tau and 1.5% L-arg group. (**h**) Negative control. Images were acquired with 400× magnification. Indicated by the arrow in figures is apoptotic cell

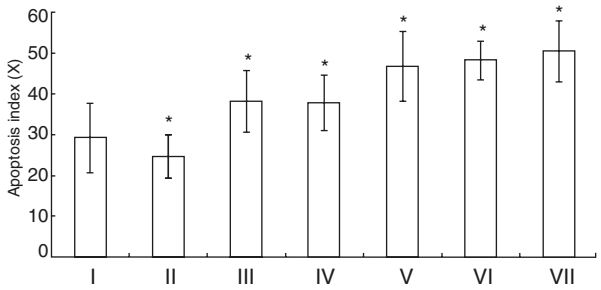

Fig. 2 Effects of taurine and L-arginine on the apoptosis index of VSMCs. *I*: Control group. *II*: Model group. *III*: 3% Tau alone group. *IV*: 3% L-arg alone group. *V*: 2.7% Tau and 0.3% L-arg group. *VI*: 2.1% Tau and 0.9% L-arg group. *VII*: 1.5% Tau and 1.5% L-arg group. Values with asterisk are significantly different ($P < 0.05$ vs. control group)

compared with control group. The apoptosis rates in all treatment groups were extremely higher than model groups. Furthermore, co-treatment of taurine and L-arginine has more apoptosis inducing effect than single treatment, which is consistent with the morphological changes by immunohistochemical staining.

3.2 Taurine and L-Arginine Up-Regulate Bax Expression and Down-Regulate Bcl-2 Expression

As shown in Fig. 3, there is a strong negative correlation between the Bax protein expression and the Bcl-2 protein expression. In the model group, relatively low level of Bax and high level of Bcl-2 were detected. The IOD value of bax in the model

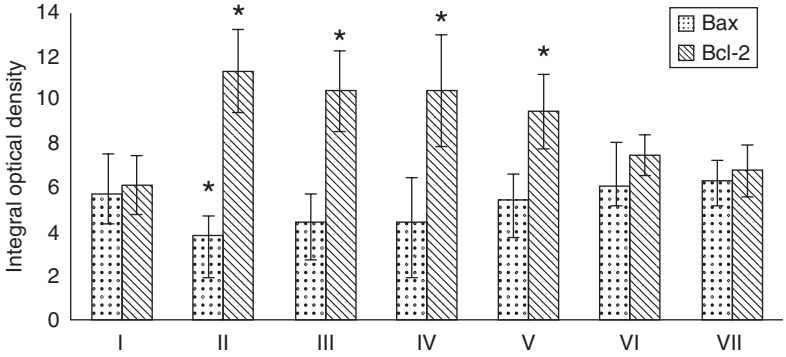

Fig. 3 Effects of taurine and L-arginine on the protein expressions of Bax and Bcl-2 in VSMCs. *I*: Control group. *II*: Model group. *III*: 3% Tau alone group. *IV*: 3% L-arg alone group. *V*: 2.7% Tau and 0.3% L-arg group. *VI*: 2.1% Tau and 0.9% L-arg group. *VII*: 1.5% Tau and 1.5% L-arg group. Values with asterisk are significantly different ($P < 0.05$ vs. control group)

group was greatly decreased, however, there were no significant differences between control group and treatment groups. On the contrary, the IOD value of bcl-2 in the model group was remarkably increased compared with the control group, and there were no significant differences among group I, VI, and VII.

4 Discussion

In our study, insulin resistance hypertensive models were successfully established by administering 25% fructose to rats for 12 weeks. Our previous experiments have confirmed that co-administration of taurine and L-arginine showed a better anti-hypertension effect and the mechanism may be due to correct the disturbance in the lipid and glucose metabolism, restore vascular endothelial activity, and inhibit renin-angiotensin-aldosterone system (Feng et al. 2013).

The excessive proliferation and the blocked apoptosis play key role in atherosclerosis. The block of apoptosis, above all, may be one of the mechanism of macroangiopathy in type 2 diabetes patients. Several studies have concluded that vascular remodeling is main pathological basis of hypertension-related diseases, and is due to the inhibition of normal apoptosis process, which caused the imbalance of proliferation and apoptosis of VSMCs (Hadrava et al. 1991; Hamet et al. 1995; Deblois et al. 1997). Apoptosis is programmed cell death, and is regulated by genes. Apoptosis can be activated or inhibited under pathological conditions. Wu et al.(2001) has demonstrated that the proliferation of VSMCs can be suppressed by taurine. It has been reported that restenosis after angioplasty is related to inhibition of apoptosis in VSMCs (Isner et al. 1995). The Bcl-2 family proteins are crucial regulators of apoptosis, which includes the death antagonists Bcl-2 and death agonists Bax (Diez et al. 1997). The ratio between Bcl-2 to Bax regulates cell survival

or death after apoptotic stimuli. Bax has proapoptotic activity, by binding and antagonizing the antiapoptotic Bcl-2, thereby accelerating apoptosis (Korsmeyer et al. 1993; Li et al. 2005).

In this study, high fructose induced the proliferation and inhibited the apoptosis of VSMCs in insulin resistance hypertensive rats. We investigated the apoptosis and the expression of pro- and anti-apoptotic proteins in VSMCs. The results show that taurine and L-arginine attenuated the progression of vessel wall injury, and promoted the apoptosis of VSMCs via up-regulation of Bax protein and down-regulation of Bcl-2 protein. Therefore, we propose that taurine and L-arginine have obvious anti-hypertensive effects partly due to enhancing apoptosis in VSMCs.

5 Conclusion

In summary, taurine and L-arginine can effectively attenuate high-fructose induced hypertension when given either singly or in combination. And its mechanism might partially be associated with attenuating vascular remodeling by promoting apoptosis in VSMCs. Moreover, co-treatment of taurine with L-arginine was more effective than taurine or L-arginine single treatment.

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Taurine Prevents the Electrical Remodeling in Ach-$CaCl_2$ Induced Atrial Fibrillation in Rats

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Abstract *Objective*: To study the preventive actions and mechanism of taurine on the electrical remodeling in atrial fibrillation (AF) rats. *Methods*: Male Wistar rats were injected with the mixture of acetylcholine (Ach) (66 μg/mL)-$CaCl_2$ (10 mg/mL) (i.v.) for 7 days to establish AF model. Taurine was administered in drinking water 1 week before or at the same time of AF model establishment. The duration of AF was monitored by recording ECG of rats during the model establishment. At the end of the experiment, left atrial appendages were cut down to measure the effective refractory period (ERP) by S1-S2 double stimulation method; atrial tissues were collected in order to detect the concentration of K^+ and taurine by flame atomic absorption spectrometry and ELISA respectively; total RNA were extracted from the atrium, gene expressions of Kv1.5, Kv4.3, Kir2.1, Kir3.4 were detected by semi-quantitative RT-PCR. *Results*: Taurine administration effectively shortened the AF duration of rats and prolonged atrial ERP than the model and taurine depleted rats. In addition, atrial K^+ level in taurine treated groups was significantly reduced nearly to the normal level. Moreover, the mRNA expression levels of Kir3.4 and Kv1.5 were significantly increased in the taurine preventive treated groups. *Conclusions*: Taurine can prevent the atrial electrical remodeling and decrease the duration of AF in rats by reducing the atrial K^+ concentration and up-regulating mRNA expression levels of Kir3.4 and Kv1.5.

Keywords Taurine • Atrial fibrillation • Atrial effective refractory period • K^+ concentration • mRNA expression levels of K^+ channels

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Abbreviations

AF	Atrial fibrillation
ECG	Electrocardiogram
ERP	Effective refractory period
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Kir2.1	Gene encoding part of the inward rectifier potassium current I_{K1}
Kir3.4	Gene encoding part of the I_{KAch}
Kv1.5	Gene encoding the I_{Kur}
Kv4.3	Gene encoding calcium independent transient outward current I_{to}

1 Introduction

Taurine presents a particularly high concentration in cardiac. It can be synthesized in body, but the biosynthesis of taurine is not enough for human. Additional supplementation taurine was first reported to have anti-arrhythmic properties in human in 1969 (Novelli et al. 1969). After that, the anti-arrhythmic mechanisms of taurine were discovered gradually. Experimental evidences had indicated that taurine protects heart against arrhythmia by modulating the potassium and calcium ions handling in *vitr*o and in *vivo* (Shustova et al. 1986), modifying membrane permeability to potassium (Chazov et al. 1974) and scavenging of free radicals against reperfusion-induced ventricular fibrillation (Hanna et al. 2004) and in paroxysmal atrial fibrillation patients (Takano et al. 2016). Besides, Sada et al. (1996) reported taurine could maintain stable action potential (AP) duration in ventricular myocytes.

As one of the common sustained cardiac arrhythmia, AF is affecting the live quality of more and more people. Atrial electrical remodeling has emerged as crucial in the onset or persistence of AF. An obvious feature of the electrical remodeling related to AF is abbreviation of ERP and action potential duration (APD) (Iwasaki et al. 2011). These alterations in atrial electrical properties are closely linked to the derangements in the ion channel. It is widely recognized that potassium current plays a critical role in repolarizing and keeping the resting membrane potential of cardiac action potential. In this experiment, the inhibit action of taurine on the atrial electrical remodeling of AF rats was investigated to gain some insight into the preventive mechanism of taurine on AF.

2 Material and Methods

2.1 Animal Models of AF and Experimental Design

Fifty male Wistar rats (210 ± 10 g) were obtained from Liaoning (Certificate Scxk (liao) 2010-0001). Rats were free to obtain water and commercial food with a controlled temperature at 23 ± 2 °C and light (12-h light-dark cycle). Rats were given 7

days to acclimate the conditions. Forty rats with normal ECG were randomly divided five groups (n = 8). Rats in taurine preventive groups were given tap water containing 1% or 2% taurine respectively (PI, PII). The endogenous taurine depletion was induced by dinking tap water containing 1% β-alanine (β-Ala). Rats in control group (C) or in AF model group (M) were maintained on tap water. All the administration last for 2 weeks. From day 8 to day 14, saline was given to the control rats through the caudal vein (1 mL/kg, i.v.) after rats were anesthetized (3 mL/kg, i.p.) with 10% chloral hydrate every morning, while the other rats were given intravenous injection with a mixture of Ach (66 μg/mL) and $CaCl_2$ (10 mg/mL) to establish the AF model according to Chen et al. (2009).

2.2 Measurement the Duration of AF

The duration of AF was detected by recording the ECG of rats on the morning in all groups from the 8th day to the 14th day. The Lead II surface ECG was taken via a pair of electrodes that were connected to a data acquisition and analysis system (BL-420F, Cheng Du TaiMeng, China) from rat anesthetized to administration finishing.

2.3 Recording of the Atrial ERP

On the 15th day, left atrial appendages were separated and the atrial ERP was measured according to Tang et al. (2010).

2.4 Biochemical Analysis

On the 15th day, after the vein blood was collected and sera were separated by centrifuging 1368g at 3000 rpm for 15 min at 4 °C. Meanwhile, after left atrial appendages were excised, hearts were washed immediately in de-ionized water and atrial tissues were rapidly frozen in liquid nitrogen. All the samples were stored at −70 °C for subsequent experiments. The K^+ concentrations in atrial tissue and serum were measured by flame atomic absorption spectrometry (Hitachi180-80, Japan). The level of taurine was detected by ELISA and the reagent kit was purchased from Shanghai Jinma Bioengineering Institute (China).

2.5 Semi-quantitative RT-PCR Assay

Total RNA was extracted from atrium using RNAiso Plus according to the instructions (TaKaRa, China). After RNA quality was detected, reverse transcription was performed using AMV First Strand cDNA Synthesis Kit (Sangon, China). The PCR

Table 1 Sequences of PCR primers

Gene	Primer sequence(5′ → 3′)	Product size (bp)
KCNA5(Kv1.5)	S—TTCATTGGAGTCATCCTCTTCTC	300
	A—GTTGCCTTGTTCTTCCTTCAG	
KCND3(Kv4.3)	S—TTGGCTCCATCTGCTCCCTAA	254
	A—CTTGCCCATGTGCTCCTCTTC	
KCNJ2(Kir2.1)	S—TGCCCGATTGCTGTTTTC	373
	A—GGCTGTCTTCGTCTATTT	
KCNJ5(Kir3.4)	S—CCCTTGAACCAGACCGACA	208
	A—TCCGTGCTTGGCAAGTCAT	
GAPDH	S—CAGTGCCAGCCTCGTCTCAT	595
	A—AGGGGCCATCCACAGTCTTC	

conditions for the mRNA expression levels of potassium channels subunits were as follows: Kv1.5 and Kir3.4 (95 °C, 3 min; 95 °C, 30 s; 57 °C, 30 s; 72 °C, 1 min; 72 °C, 10 min; for 32 cycles), Kir2.1 (94 °C, 3 min; 94 °C, 30 s; 53 °C, 30 s; 72 °C, 1 min; 72 °C, 5 min; for 32 cycles), Kv4.3 (95 °C, 3 min; 95 °C, 30 s; 57 °C, 30 s; 72 °C, 1 min; 72 °C, 10 min; for 35 cycles). GAPDH was used as an endogenous standard. Specific primers of Kv1.5, Kv4.3, Kir2.1, Kir3.4 and GAPDH are presented in Table 1. The intensity of RT-PCR produced band was measured using Image J software. Finally, the aim genes/GAPDH ratios were calculated.

2.6 Statistical Analysis

Data were expressed as means ± SEM. One-way analysis of variance (ANOVA) was performed using SPSS 16.0 software. A difference was considered significant when p value <0.05.

3 Results

3.1 Taurine Reduced the Duration of AF in Rats

A typical AF ECG with the disappeared P wave, appeared f wave and prolonged R-R interval. The results of Table 2 showed that the duration of AF in rats was obviously increased with the treatment of Ach-$CaCl_2$ for 7 consecutive days. At the day 14^{th}, 1 and 2% taurine significantly prevented the increase of AF duration relative to model group ($p < 0.05$, $p < 0.01$). While in β-Ala group, the duration of AF in rats was similar to model group, but much longer than 2% taurine treatment group ($p < 0.01$).

Table 2 Effect of taurine on the duration of AF in rats (Time (ms))

Groups	8^{th} day	9^{th} day	10^{th} day	11^{th} day	12^{th} day	13^{th} day	14^{th} day
C	0	0	0	0	0	0	0
M	9.51 ± 1.86^{a}	10.67 ± 0.30	11.28 ± 0.66	12.08 ± 1.21	14.37 ± 0.23	18.42 ± 1.45	21.25 ± 0.60^{aA}
PI	9.02 ± 1.32^{a}	8.54 ± 0.24	9.06 ± 0.90	10.5 ± 1.10	11.83 ± 0.41	13.2 ± 0.45	16.32 ± 1.26^{bAB}
PII	8.72 ± 1.68^{a}	8.29 ± 0.16	8.92 ± 0.65	11.24 ± 0.36	9.71 ± 0.70	10.74 ± 0.55	12.19 ± 0.81^{cB}
β-Ala	8.73 ± 1.04^{a}	11.98 ± 0.64	12.33 ± 1.61	12.45 ± 1.11	14.25 ± 0.92	18.7 ± 1.37	19.13 ± 1.67^{abA}

Results are presented as mean ± SEM (n = 6). The values in same column with different lowercase letters represent $p < 0.05$, with different capital letters represent $P < 0.01$

Table 3 Effect of taurine on the atrial ERP in rats

Groups	C	M	PI	PII	β-Ala
AERP (ms)	75.11 ± 0.77^{aA}	60.77 ± 1.06^{cC}	75.00 ± 0.71^{aA}	73.22 ± 0.60^{aAB}	69.56 ± 0.67^{bB}

Results are presented as mean ± SEM (n = 8). The values in same line with different lowercase letters represent $p<0.05$, with different capitals represent $P<0.01$

3.2 Taurine Restored the Atrial ERP of AF Rats

The atrial ERP of model group rats showed significantly reduction in contrast with control group ($P < 0.01$) (Table 3). While in contrast with the model group, atrial ERP of AF rats treated with taurine was prolonged by 23.42% ($P < 0.01$) (group PI) and 20.49 ($P < 0.01$) (group PII) respectively. However difference in atrial ERP between control and taurine treatment groups were not statistically significant. While in the β-Ala group, the atrial EPR of rats was prolonged compared with model group ($P < 0.01$), but significantly reduced as compared with taurine treatment groups ($P < 0.01$ vs. PI group; $P < 0.05$ vs. PII group).

3.3 The Concentrations of K^+ and Taurine in Serum and Atrial Tissue

The K^+ concentration in atrium was significantly elevated by Ach-$CaCl_2$ treatment in contrast with control group ($P < 0.01$) (Table 4). However the rats pre-treated with taurine exhibited much lower K^+ concentration by contrast with the model group ($P < 0.01$, $P < 0.01$), and the values were accessed to the value of control group. While in the β-Ala group, the K^+ level in atrium showed an obvious increase compared with the control group ($P < 0.05$), but it was decreased obviously compared with model group ($P < 0.05$). It is interesting that β-Ala administration did not increase the K^+ level in atrium relative to the model group. However, differences in serum K^+ level were not statistically significant in different groups.

Table 4 Effects of taurine on the concentration of K^+ and taurine

Groups	K^+		Taurine	
	Atrium (mg/kg)	Serum (mg/L)	Atrium (pg/mgprot)	Serum (pg/ml)
C	1973.68 ± 71.13^{aA}	229.35 ± 4.57^{a}	81.30 ± 1.20^{ab}	0.12 ± 0.01^{a}
M	2303.30 ± 32.80^{dB}	239.04 ± 9.90^{a}	83.51 ± 6.16^{ab}	0.11 ± 0.01^{a}
PI	1981.85 ± 54.48^{abA}	248.46 ± 10.16^{a}	88.85 ± 3.00^{b}	0.12 ± 0.02^{a}
PII	1988.79 ± 57.58^{abA}	225.20 ± 4.03^{a}	90.56 ± 4.63^{b}	0.12 ± 0.01^{a}
β-Ala	2136.14 ± 30.66^{bcAB}	255.26 ± 14.43^{a}	74.19 ± 4.96^{a}	0.12 ± 0.00^{a}

Results are presented as mean ± SEM (n = 5). The values in same column with different lowercase letters represent $p < 0.05$, with different capitals represent $P < 0.01$

The values of Table 4 also showed that rats pre-treated with taurine had much higher taurine levels by contrast with the control group and model group. Meanwhile the taurine level in the β-Ala treated group was significantly decreased compared with taurine treatment groups. Furthermore, serum taurine level between the groups did not show statistical difference.

3.4 Potassium ion Channel Subunit mRNA Expression

Rats in model group exhibited significant lower mRNA expression levels of Kir3.4 ($P < 0.01$) and Kv1.5 ($P < 0.01$) than that in control. Rats pre-treated with taurine exhibited significant higher mRNA expression levels of kir3.4 and kv1.5 by contrast with the model group. While the rats pre-treated with β-Ala, the mRNA expression levels of kir3.4 and kv1.5 were both decreased remarkably compared with the control group or taurine treated groups, and the value is similar to the model group. However, the mRNA expression levels of Kv4.3 and Kir2.1 in different groups did not show significant differences (Figs. 1 and 2).

4 Discussion

The major findings of our study are as follows: (1) Taurine could decrease the duration of AF and restore the atrial ERP to the normal level in AF model rats induced by Ach-$CaCl_2$. (2) Restoring the atrial ERP by taurine partly due to reduce the atriai K^+ concentration and increase the atrial Kv1.5, Kir3.4 mRNA expression levels.

It is widely recognized that acetylcholine (Ach) could promote the atrial fibrillation through activating Ach-activated potassium channels (I_{KAch}). Furthermore, a rapid increase of $CaCl_2$ concentration could lead to severe hypercalcemia causing an influx of calcium. Combination with $CaCl_2$ and Ach could induce AF in animals. On this AF model, taurine supplementation (PI and PII) significantly reduced the AF duration and prolonged the atrial ERP in AF rats. Besides, the value of atrial

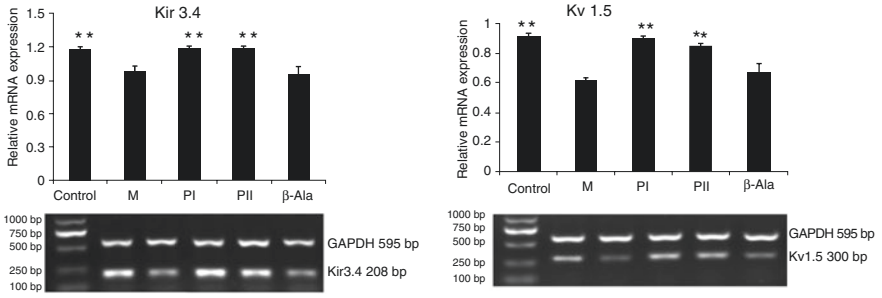

Fig. 1 The mRNA relative expression levels of Kir3.4 and Kv1.5 in atrial tissue. Data are the mean ± SEM (n = 3). *p < 0.05 vs. the model group; **p < 0.01 vs. the model group

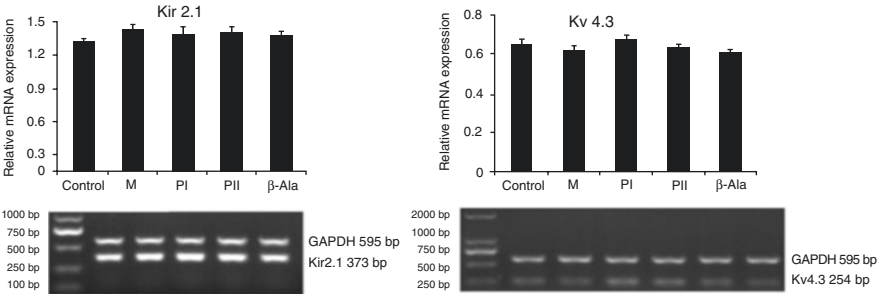

Fig. 2 The mRNA relative expression levels of Kir2.1 and Kv4.3 in atrial tissue. Data are the mean ± SEM (n = 3). *p < 0.05 vs. the model group; **p < 0.01 vs. the model group

ERP in taurine treated groups was prolonged to the normal level. This suggested that taurine could recover the atrial ERP of AF rats to a normal level and not cause other arrhythmias.

Taurine exhibits a particularly high concentration in cardiac tissues. Cardiac dysfunction had been found in taurine transporter knock out mice (Ito et al. 2008) or in cats fed with taurine deficient diet (Pion et al. 1987). In this experiment, additional supplementation taurine increased the taurine concentration in atrium but had no effect on the serum taurine level. Furthermore, the potassium ion level in taurine pre-treated groups (PI and PII) was significantly decreased respectively relative to model group, but there was not obvious difference between the PI and PII.

Researches had demonstrated the initiation and perpetuation of AF associated with the cardiomyocyte ion-channel remodeling (Nattel et al. 2002; Wakili et al. 2011). Potassium current takes effects on maintaining atrial action potentials. Particularly important potassium currents for the atria are I_{Kur} (ultra-rapid delayed rectifier current, I_{Kur}) and I_{KAch} (acetylcholine-sensitive potassium currents, I_{KAch}).

As an atrial specific potassium current, constitutively active I_{KAch} has been reported in patients with persistent AF (Dobrev et al. 2005). Kir3.1/Kir3.4 proteins are the components of endogenous I_{KAch} channels encoded by KCNJ3 and KCNJ5 genes. Researches in several animal models indicated that AF is linked with the increased

activity of Kir3.4 (Kovoor et al. 2001; Cha et al. 2006). Especially Kir3.4 deficient mice showed a resistance to AF induced by vagal stimulation (Kovoor et al. 2001). In this experiment, we found that the Kir3.4 mRNA expression level in model group exhibited significant reduction, which was consisted with previous studies (Dobrev et al. 2001; Brundel et al. 2001; Gaborit et al. 2005). Meanwhile these studies indicated that there was agreement between reduced channel subunit expression at the mRNA and protein and the lower activation of I_{KAch} in AF. Dobrev et al. (2005) reported that M-receptor-mediated activation of I_{KAch} is reduced in chronic AF patients. The reduction in mRNA level may be a self-adaption of the atrium which could prevent further shortening of the action potential duration during AF. While in the taurine treatment groups, the Kir3.4 mRNA expression level was significantly increased and the value was similar to the normal level. The mechanism need to be further studied.

I_{Kur} (ultra-rapid delayed rectifier current, I_{Kur}) mediates a major part of repolarization in an action potential (AP) in atrial cardiomyocytes in humans (Ehrlich and Nattel 2009). It is not only plays a role in the early repolarization but also during all phase 1–3. I_{Kur} is conducted by Kv1.5 potassium channel, and silencing Kv1.5 in human atrial myocytes leads to a major decrease of the I_{Kur} current (Feng et al. 1998; Ravens and Wettwer 2011). Kv1.5 was coded by KCNA5 gene, it has been demonstrated KCNA5 mutation is linked to familiar atrial fibrillation (Olson et al. 2006) and early-onset lone atrial fibrillation (Christophersen et al. 2013). In our study, the Kv1.5 mRNA expression level in model group exhibited significantly decrease, which is consisted with the former reports (Caballero et al. 2010; TANG et al. 2011; Gu et al. 2014). Evidence has shown that in tissues from patients with disease related AF, protein levels of potassium channels, including Kv1.5 were decreased (Nattel et al. 2010). However, other studies indicated that I_{Kur} was increased (Lammers et al. 2012) or unchanged (Bosch et al. 1999). The down-regulated mRNA or protein expression level of Kv1.5 contributes the decline of I_{Kur}, which may not explain the shorting of atrial ERP. One possible hypothesis is that the reduction in mRNA or protein expression level of potassium channels is attributed to the self-adaption of the atrium during pathological process (Wakili et al. 2011; Greiser and Schotten 2013). While in taurine pre-treated group, the Kv1.5 mRNA expression level was significantly increased and the value was similar to the normal level.

Although the transient outward potassium channels (I_{to}) and inward rectifier potassium current are also involved in the onset or persistence of AF, the mRNA expression levels of Kv4.3 and Kir2.1 did not show significant differences between groups in our study. This may be explained by the fact that different models of species were used in different experiments.

5 Conclusion

In summary, taurine with preventive supplementation effectively decreased duration of AF and inhibited atrial electrical remodeling (AER) in AF model rats induced by Ach-$CaCl_2$ partly through decreasing the atrial potassium ion K^+ concentration and restoring the mRNA expression levels of Kir3.4 and Kv1.5.

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Taurine Reverses Atrial Structural Remodeling in Ach-Cacl$_2$ Induced Atrial Fibrillation Rats

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Abstract Taurine has been reported to have anti-arrhythmia effects, but the anti-atrial fibrillation (AF) effects and its mechanism remain incompletely understood. In the present study, the therapy effects and partly mechanisms were investigated. AF animal model was established by intravenous administered with the mixture of acetylcholine (Ach) and $CaCl_2$ (66 μg/mL + 10 mg/mL) (i.v.) for 7 days. The actions of taurine (99 mg/kg•d, introgastric administration) on the levels of Hs-CRP, IL-6, TNF-α, MMP-9, AngII, the extent of the fibrosis and ultrastructural changes in left atrial were studied. The data showed that the serum levels of TNF-α, IL-6, AngII and the plasma levels of Hs-CRP and MMP-9 were significantly elevated in automatic recovery group relative to the control group ($p < 0.01$), which were all decreased by taurine administration ($p < 0.01$) similar to Verapamil treatment. Masson's trichrome staining of the left atrial tissue showed an obvious interstitial fibrosis in rats of automatic recovery group. The alteration could be reversed by additional taurine. Electron microscopy revealed that taurine administration could significantly alleviate the ultrastructural damage of atrial cells, and the effects were similar to the Verapamil treatment. In conclusion, the results suggested that taurine could inhibit the structural remodeling of AF in rats partly by decreasing the levels of inflammatory factors and profibrotic molecules, attenuating the extent of myocardial fibrosis and protecting the integrity of myocardial ultrastructure.

Keywords Taurine • Atrial fibrillation • Inflammatory factors • Fibrosis • Ultrastructure

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Abbreviations

Ach	Acetylcholine
AF	Atrial fibrillation
AngII	Angiotensin II
Hs-CRP	High-sensitive C-reactive protein
IL-6	Interleukin-6
MMP-9	Matrix metallaproteinase-9
MMPs	Matrix metalloproteinases
TNF-α	Tumor necrosis factor-α

1 Introduction

In the mammalian heart, taurine has extremely high proportion of the total amino acid pool (Lombardini 1996). Ito et al. (2008) has reported that taurine deficiency is related with the cardiac dysfunction. As a conditionally-essential amino acid, oral supplementation is effective to animals and patients with cardiac arrhythmias (Eby and Halcomb 2006), heart failure (McCarty 2010) and ischemic heart disease (Xu et al. 2008).

As one of common sustained cardiac arrhythmia, Atrial fibrillation (AF) had an occurrence of 1% in the general population (Go et al. 2001). Future projections indicate that its prevalence will at least double in the next five decades with the ageing population (Pamukcu and Lip 2011). Taurine had anti-arrhythmic effects in dog was reported firstly by Read and Welty (1963). After that, some studies also had shown that taurine has the effects (Chazov et al. 1974; Eby and Halcomb 2006; Zhao et al. 2013). But the relevant researches about taurine and atrial structural remodeling in AF are few and the mechanism is not clear. In this experiment, the effects of taurine on the levels of Hs-CRP, TNF-α, IL-6, MMP-9, AngII, the extent of the fibrosis and ultrastructural changes in left atrium were studied.

2 Methods

2.1 Experimental Animals

Fifty male Wistar rats (weighed 250 ± 30 g) were bought from Beijing HFK Bio-Technology CO., LTD (Certificate SKxk (jing) 260P-0004). Rats were maintained in controlled light (12 h of light, 12 h of dark) and temperature (23 ± 2 °C), with free to obtain water and commercial food. The animals were given 3 days to acclimate the conditions. On the day 4, 40 rats were randomly selected to establish the AF model.

2.2 Experimental Design

AF model in rats was established through intravenous injection with the mixture of acetylcholine (Ach) and $CaCl_2$ (66 μg/mL + 10 mg/mL) (i.v.) for 7 days. After the rats were anesthetized (3 mL/kg, i.p.) with 10% chloral hydrate (Xinguang Chemical Group, Shenyang, China) once a day for 7 days. Saline (1 mL/kg) was given to the rats in control group (C) according to Chen et al. (2009). The electrocardiogram (ECG) recording was maintained from rat anesthetized to administration finishing every day. Typical AF electrocardiogram was charactered by appearance of the f wave and disappearance of the P wave. On the day 8, 30 AF model rats were randomly divided into three groups (n = 10). Saline (8 mL/kg•d) were given to the rats in automatic recovery group (A) by intragastric administration, while rats in the taurine administration group (T) and Verapamil treatment group (V) were administered with 99 mg/kg•d Tau and 100 mg/kg•d Verapamil by intragastric respectively. In control group (C), rats were given of administration of saline (8 mL/kg•d) by intragastric. The treatment was lasted for 4 weeks.

2.3 Chemicals and Reagents

Taurine was bought from Sigma (St. Louis, MO, USA). Verapamil was purchased from Northeast General Pharmaceutical Factory (China) (Lot.: 20050322). ELISA reagent kits of high-sensitive C-reactive protein (hs-CRP), IL-6, TNF-α, matrix metallaproteinase-9 (MMP-9), angiotensin II (AngII) were bought from Nanjing Jiancheng Bioengineering Institute (China).

2.4 Sample Preparation and Biochemical Analysis

On the 29th day, left atrial blood samples were collected by blood taking needle after the rats were anesthetized (3 mL/kg, i.p.) with 10% chloral hydrate. Sera were separated from blood samples by centrifugation and then stored at −80 °C. The serum concentrations of IL-6, TNF-α, AngII and the plasma Hs-CRP and MMP-9 were detected using ELISA kits (R&D system, USA).

2.5 The Masson's Trichrome Staining for Fibrosis

After fixed in neutral 4% paraformaldehyde buffered at room temperature for 24 h, the dehydration of left atrial tissues were carried out in a serious of sucrose buffer 100, 200, and 300 g/L. After that, tissues were embedded with OCT and frozen in

liquid nitrogen vapours. Cryostat-cut sections (7 μm) were selected and the degree of atrial fibrosis was evaluated by Masson's trichrome method.

2.6 Electron Microscope Examination

After fixed with 2.5% glutaraldehyde solution (0.1 MPBS) for 24 h at 4 °C and postosmicated in 2% osmium tetroxide solution, the atrial tissues were dehydrated using graded ethanol and embedded in epoxy resin. Each Section was viewed under JEM-1200EX (JEOL, Japan).

2.7 Statistic Analysis

Each value was expressed as the mean ± SEM. The values were analyzed by using one-way analysis of variance (ANOVA), followed by Turkey's post hoc test. A difference was considered significant when $p < 0.05$.

3 Results

3.1 Serum IL-6, TNF-α and AngII

As shown in Fig. 1, the rats in automatic recovery group (A) exhibited significant increase in serum TNF-α ($p < 0.01$), IL-6 ($p < 0.01$), and Ang II ($p < 0.01$) compared with the control group, but those were significantly decreased by administration of 99 mg/kg·d or 66 mg/kg·d Verapamil. Furthermore, the values of these indexes in taurine group were similar to that in Verapamil treatment group.

3.2 The Concentrations of MMP-9 and Hs-CRP

The results of Fig. 2 showed that plasma concentrations of Hs-CRP and MMP-9 in automatic recovery group (A) were elevated significantly by contrast with the control group ($p < 0.01$, $p < 0.01$), but taurine treatment significantly inhibited the increase of MMP-9 ($p < 0.01$) and Hs-CRP ($p < 0.01$), and the values in taurine group were similar to that in Verapamil group.

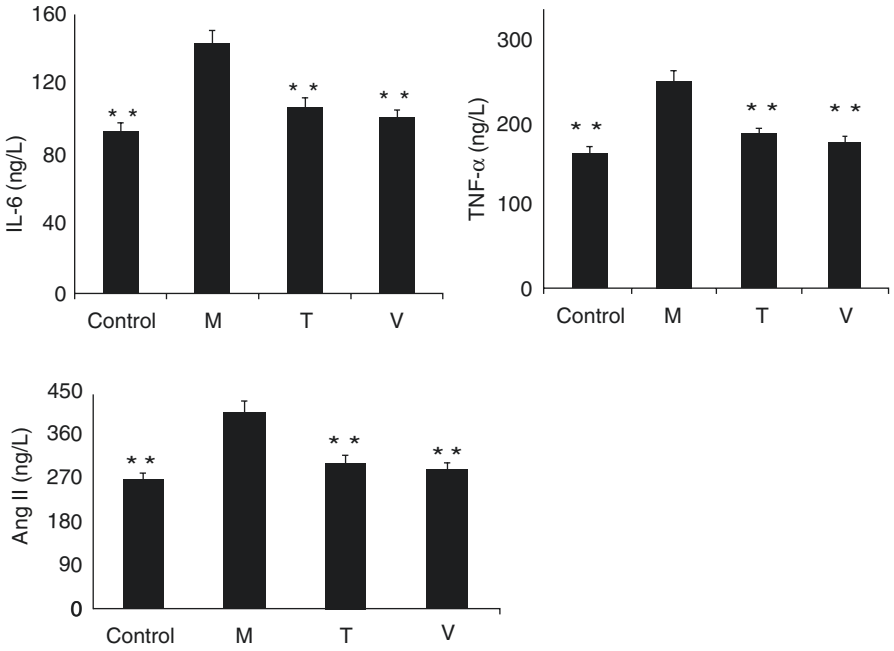

Fig. 1 The levels of serum IL-6, TNF-α and Ang II in rats. Data are the mean ± SEM (n = 5). Differences were statistically significant vs. model group at *p < 0.05 and **(p < 0.01)

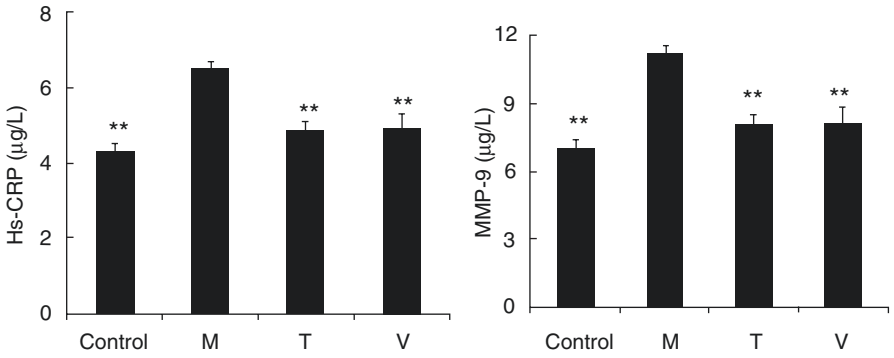

Fig. 2 The concentrations of plasma MMP-9 and Hs-CRP in rats. Data are the mean ± SEM (n = 5). Differences were statistically significant vs. model group at *p < 0.05 and ** (p < 0.01)

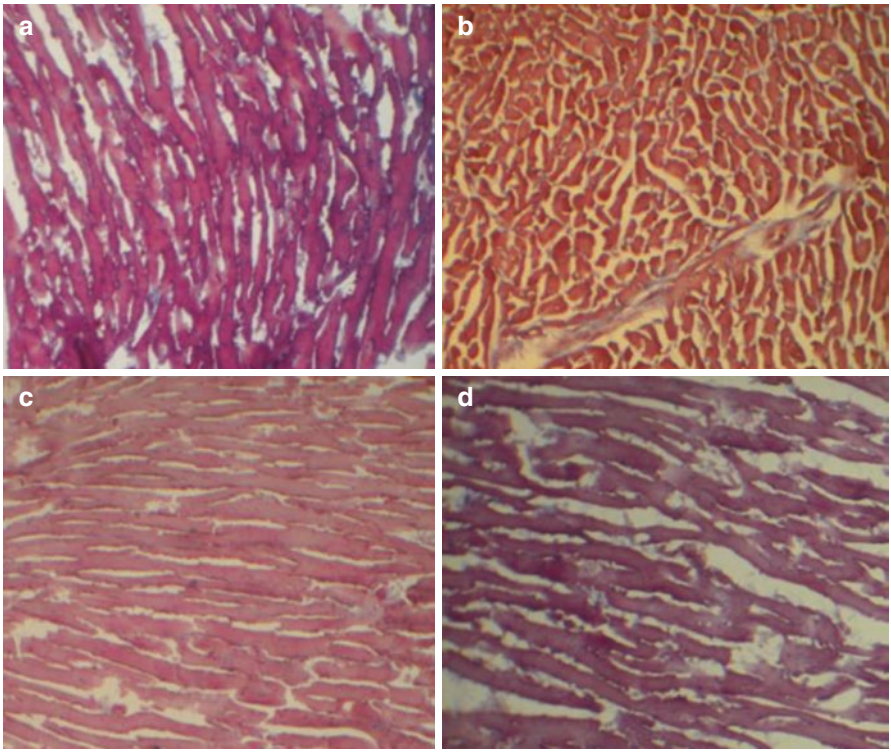

Fig. 3 The myocardial fibrosis of left atrial tissues. (**a**) C group. (**b**) A group. (**c**) T group. (**d**) V group. The collagen fibers are stained blue and the background is stained red (Magnification ×100)

3.3 Results of Masson's Trichrome Staining

The Masson's trichrome staining results were shown in Fig. 3. In the left atrial tissues, obvious interstitial fibrosis was observed in automatic recovery group (A), even with thicker fibrotic septa (Fig. 3b). However fibrosis extent could be reserved obviously by taurine (Fig. 3c) or Verapamil (Fig. 3d) supplementation respectively, and the treatment effect of taurine is similar to that of Verapamil.

3.4 Electron Microscope Results

The ultrastructural alterations of left atrial cells are shown in Fig. 4. Group control showed slightly contracted myofibrils, well-arranged Z disk, an intact sarcolemma, and mitochondria with tightly packed cristae (Fig. 4a, e). The structure changes in

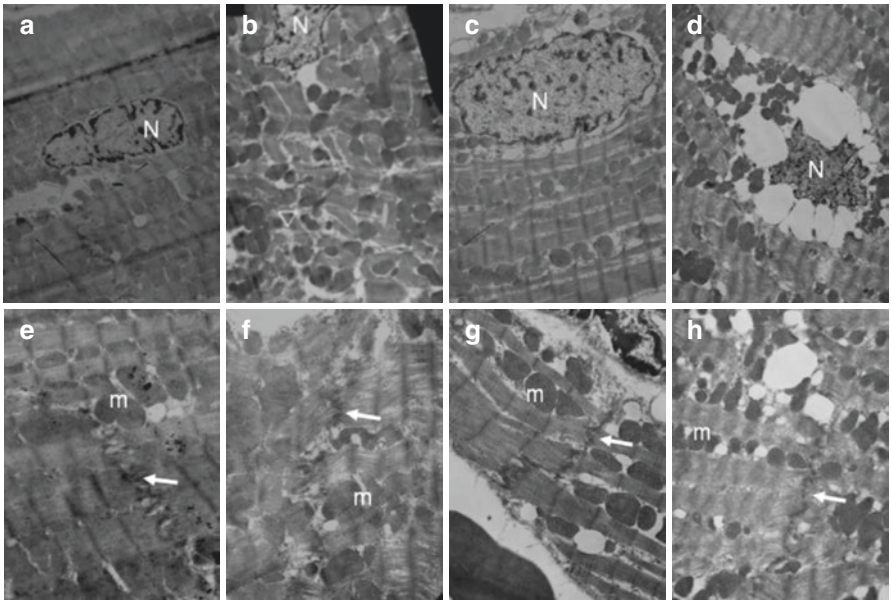

Fig. 4 The ultrastructural changes of left atrial cardiomyocyte. (**a**, **e**) C group. (**b**, **f**) A group. (**c**, **g**) T group. (**d**, **h**) V group. Magnification (as indicated): (**a**, **b**, **c**, **d**) ×5000; (**e**, **f**, **g**, **h**) ×8000. *M* mitochondrion, *n* nuclear, *arrows* intercalated disk, *triangle* myofilament breakdown

A group are as follows (Fig. 4b, f): edema of cardiomyocyte, enlargement of the gap among the filaments, disarrayed myofibrils with fragmentation and dissolution; swollen mitochondria; ruptured sarcolemma; unclear intercalated disk and the decrease of filaments related to the intercalated disk. The cardiomyocyte in T group showed a lesser extent injury (Fig. 4c, g). Compared with A group, there was no obvious interstitial edema and myofibrils disarray, the structure of intercalated disks was clear. The cardiomyocyte structures in V group (Fig. 4d, h) also showed lesser extent injury, which is similar to those described in T group.

4 Discussion

Paroxysmal AF (self-terminating episodes) may eventually turn into persistent (>7 days) and perhaps even permanent AF. This indicated atrial structural remodeling may be involved when paroxysmal AF occurs for continuous 7 days. This study aims toward researching the therapy action of taurine on atrial structural remodeling in AF model rats.

Researches in clinical AF pointed that the hallmark feature of arrhythmogenic structural remodeling is atrial fibrosis (Kostin et al. 2002) and the perpetuation of AF mainly attributed to the structural remodeling (Allessie et al. 2002; Burstein and

Nattel 2008). AngII, a well-characterized profibrotic molecule, evidences had shown that it could induce inflammation, leading to cardiac remodeling (Jia et al. 2012; Gu et al. 2014). Moreover, matrix metalloproteinases (MMPs) are zinc- and calcium-dependent proteases participating in the degradation of the extracellular matrix (ECM) molecules. Cardiomyocytes and leukocytes could generate and release MMPs, which could injury the function of connective tissues by degrading ECM proteins on activation. The activity and mRNA expression of profibrotic MMP-9 has been increased in a porcine AF model (Chen et al. 2008). Similarly, Friedrichs et al. (2011) reported that MMP-2 and-9 caused fibrinolysis and tissue remodeling, which resulted in the progression of atrial fibrosis. In particular, MMP-8 and MMP-9 are released by leukocytes.

In this experiment, the obvious increase of Ang II and MMP-9 were decreased by taurine or Verapamil treatment respectively; histological examination of the left atria revealed that the blue collagen fibrils distributed mainly in the interstitium around the red myocardial cells were obviously increased in the rats of automatic recovery group (Fig. 3b), but taurine treatment inhibited the fibrosis degree (Fig. 3c), and the treatment effect of taurine is similar to that of Verapamil. The data of the experiment suggested that taurine could inhibit the fibrosis by decreasing the level of AngII and MMP-9.

Ultrastructural alteration is another hallmark of structural remodeling. Atrial myocyte structural alterations concluded the following (Fig. 4b, f): disorganization and dissolution of myofibrils, enlargement of the gap among the filaments; especially the intercalated disk was unclear and the filaments related to the intercalated disk were decreased. However, taurine treatment could reverse the gap junction alterations and preserve the myocardial ultrastructure integrity. It has been demonstrated that changed expression of connexins are related to the inflammatory processes (Patel et al. 2010). Friedrichs et al. (2011) pointed out inflammatory mediators could disturb the integrity of connexin, which promotes AF.

Researches had shown that inflammation is involved in the initiation and perpetuation of AF (Issac et al. 2007; Kourliouros et al. 2009). As circulating inflammatory marker, plasma hs-CRP levers were increased in patients with paroxysmal AF or persistent AF (Zheng et al. 2016). Some cytokines have also contributed to AF (Henningsen et al. 2009; Marcus et al. 2010). Several studies suggested TNF-α not only enhances atrial collagen deposition (Saba et al. 2005), but also induces MMP-9 expression (Balasubramanian et al. 2011).

In this study, the rats treated with $CaCl_2$ and Ach showed significantly increase in the systemic inflammatory markers hs-CRP, inflammatory mediators TNF-α and IL-6. However, taurine administration completely abolished the significant increase of hs-CRP, cytocine TNF-α and IL-6 compared with the automatic recovery group, indicating that taurine could abate AF by anti-inflammatory effect.

Recently, accumulating evidences appear that taurine has anti–inflammatory effects under various pathological process (Chou et al. 2012; Latchoumycandane et al. 2014). Taurine has very high concentration in leukocytes (20–50 mM) (Learn et al. 1996), it is a major scavenger of hypochlorous acid (HOCl) from neutrophils. It is known that taurine chloramine (TauCl) was released from apoptotic neutrophils and had anti-inflammatory actions. Schuller-Levis et al. (2009) and Wu et al. (2013) both

reported that taurine administration could decrease the TNF-α level during inflammatory response. This study also demonstrated that taurine could decrease the inflammatory product of hs-CRP in AF model rats, via reversing the elevation of proinflammatory mediators TNF-α and IL-6, but the specific mechanism needs further research.

In addition, the MMPs are induced by TNF-α as a consequence of response to inflammation has been demonstrated by Fernandes et al. (2002). Balasubramanian et al. (2011) reported that TNF-α induces MMP-9 expression. The results of this experiment suggest that taurine could inhibit the atrial fibrosis and preserve the integrity of myocardial ultrastructure, the mechanism of which may partly through the anti-inflammation effect of taurine.

5 Conclusion

In summary, taurine can inhibit atrial structure remodeling of AF model rats by decreasing the concentrations of inflammatory factors and fibrogenic mediators.

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Taurine Normalizes the Levels of Se, Cu, Fe in Mouse Liver and Kidney Exposed to Arsenic Subchronically

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Abstract To evaluate the benefits of taurine on the homeostasis of trace elements induced by toxic metals, we investigated the concentration of Selenium (Se), Copper (Cu), Iron (Fe) and Manganese (Mn) in mouse liver and kidney after arsenic exposure for 2 months. The experimental animals were divided into control group, arsenic exposure group (1, 2, 4 ppm) and taurine protective group randomly. Concentrations of serum, liver and kidney trace elements such as Se, Cu, Fe, Mn were measured by Inductively Coupled Plasma-Mass Spectrometry. Our results showed that the concentration of Cu was higher, however, the concentration of Se and Fe was lower in mice liver and kidney exposed to arsenic. The levels of Se, Cu, Fe were alleviated by co-administered with taurine. Furthermore, there was no difference in the concentration of Mn between the three groups. Our finding suggests that taurine may relieve the disturbed levels of Se, Cu and Fe in liver and kidney induced by arsenic.

Keywords Taurine • Arsenic • Trace elements

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Abbreviations

As	Arsenic
Cu	Copper
Fe	Iron
Mn	Manganese
Se	Selenium

1 Introduction

Arsenic (As) is a kind of toxic metals, which has been considered as a natural environmental pollutant. Drinking water contaminated by As has received increasing attention, as it seriously affects the health of people in high As areas around the world, and known as a public health problem (Abernathy et al. 1999). Chronically exposure to As may cause kinds of diseases including respiratory gastrointestinal, cardiovascular, genitourinary, hematopoietic, endocrine, neurological system and skin (Duker et al. 2005; Ratnaike 2003; Rios et al. 2009). Epidemiology studies and rodent studies demonstrated that As may lead to degenerative changes in liver including abnormal liver function, hepatomegaly, liver fibrosis and cirrhosis (Singh et al. 2011). Chronic As exposure can also induce nephrotoxicity, including vacuolation of tubular cell, glomerular swelling, and interstitial nephritis (Liu et al. 2000).

Liver is essential in the regulation of the metabolic pathway and trace elements transport, and controls their tissue distribution, bioavailability and eventual toxicity. The kidney also regulates trace elements homeostasis in an animal body. Although the amount of trace elements is relatively small in body tissues, they are important in many vital processes including oxygen metabolism, free radicals scavenging (Ceylan et al. 2011). In recent years, a number of chronic diseases such as kidney disease, cancer and neurological diseases were found to be associated with trace element abnormal. Essential trace elements have also been implicated in the pathogenesis of hepatic disease (Webb and Twedt 2008). Iron (Fe), Selenium (Se), manganese (Mn), and copper (Cu) required for the synthesis of RNA, DNA, and proteins as a cofactor in the metalloprotein, as well as in nitric oxide metabolism, oxygen transport, oxidative phosphorylation, and antioxidant defense. It was well known that essential elements could be competed or interfered with toxic elements. The interaction of toxic metals and essential metals, may cause the disturbances in essential elements homeostasis, which can be a foundation of their toxicity actually (Molin et al. 2008; Liu et al. 1992). Literatures reported that interaction between As and many essential trace minerals might be involved in the toxic effects of As (Shibata et al. 1992; Wang et al. 2006). Cui studied out As could widely distributed and significantly accumulated in various organs and influence on other trace elements (Cui and Okayasu 2008). Our earlier study discovered that As exposure might change the levels of trace elements such as Cu, Fe, Se, and Cr in mouse brain (Wang et al. 2013). However,

there are few studies about the influence on essential trace elements in liver and kidney subchronic exposure to As. Taurine is a sulfur-containing amino acid, and exist abundantly in human and animal organs. Recently, scientific studies showed that taurine could be effective in diminishing the symptoms of toxicity induced by lead, cadmium, copper in experimental animals. Taurine has hepatoprotective (Balkan et al. 2005) and renoprotective (Acharya and Lau-Cam 2010; Das et al. 2010) properties in maintaining the homeostasis of trace elements (Park et al. 2009). As is an important toxicants in environment. Therefore, it prompted us to explore the protective effect of taurine on the As toxicity. To make clear weather the essential trace elements are interfered by As exposure, and the protection effect of taurine in liver and kidney on them disturbed levels. In this study, the levels of Se, Cu, Fe and Mn as important essential trace elements were determined in mouse liver and kidney after exposure to As subchronically alone or both of As and taurine by ICP-MS.

2 Methods

2.1 Chemicals

Arsenic trioxide (As_2O_3), H_2O_2 andHNO_3were purchased from Sigma Chemical Company (St. Louis, USA).These solution were ultra-pure. All standard solutions involving As, Cu, Fe, Se and Mn were provided by the development of national standard materials center.

2.2 Animals Model

All procedures involving animal were performed in accordance with the procedures outlined in the "Dalian Medical University Animal Guideline" and in agreement with the "Dalian Medical University Ethical Committee". One hundred mature, healthy SPF mice (half male and half female) were obtained from the animal center of Dalian Medical University. All mice were kept in room with a light-dark cycle for each day, (humidity: 50%, temperature: 18–22 °C). We assigned these mice to control group, arsenic exposure group (1, 2, 4 ppm) and taurine protective group randomly (each 20). The animals in control group were feed on drinking water alone. Mice in experimental groups were administered with drinking water containing 1 ppm, 2 ppm, and 4 ppm As_2O_3, respectively. Mice in protective group received 4 ppm As_2O_3 with 150 mg/kg taurine. As_2O_3 was provided through the drinking water ad libitum for a total of 2 months. Taurine was administrated by forced feeding twice a week. At the end of administration, all of the mice were weighed. Blood samples were collected and taken into EP tubes, then centrifuged. Serum was extracted and kept at −20 °C for measurement of As level. Animals were euthanized by decapitation. Their liver and kidney were obtained and frozen at −80 °C.

2.3 Preparations of Test Samples

Samples of Liver tissue (0.300 g, wet weight) and kidney tissue (0.200 g, wet weight) were prepared by microbalance (20 samples for each group). The samples were digestive in Microwave digestion furnace system (MLS1200PYRO, Italy Milestone Corporation) after soaking for 30 min according our previous procedure (Wang et al. 2013). The measurement of these digested samples should in room temperature.

2.4 Analysis of Trace Elements

Trace elements Concentrations of As, Cu, Fe, Se, and Mn were analyzed by ICP-MS (7500CE, Agilent Technologies). The ICP-MS measurements were ran according to the conditions we used before (Wang et al. 2013). The calibration range of As is 0–20 μg/L, and four essential trace elements as follows: Cu: 0–300 μg/L, Fe: 0–350 μg/L, Se: 0–20 μg/L, Mn: 0–35 μg/L. 1.

2.5 Statistical Analysis

All test data were converted and manipulated by using SPSS 17.0 for windows. The data are expressed as mean ± standard deviations, significant differences between concentrations in liver and kidney were determined by one-way analysis of variance (ANOVA) followed by the Scheffe's test. All test data were converted and manipulated by using SPSS 17.0 for windows. All experiments were done at least in three times and the P value < 0.05 considered as statistical significance.

3 Results

3.1 The Role of Taurine on As Concentration in the Serum, Liver and Kidney

The As concentration in mouse serum, liver and kidney is shown in Table 1. In control group, As concentration in serum was 30.81 ng/g. The As concentration in the mouse serum was 43.23 ng/g, 56.43 ng/g and 71.59 ng/g received 1 ppm, 2 ppm and 4 ppm As_2O_3, respectively. The As concentration in the experimental groups was obviously

Table 1 As concentration in serum, liver and kidney of mice

Groups	Concentration of As in tissues (ng/g)		
	Serum	Liver	Kidney
Control	30.81 ± 1.52	124.35 ± 36.56	101.38 ± 22.49
1 ppm As_2O_3	43.23 ± 3.53[a]	140.40 ± 20.39	143.09 ± 27.20
2 ppm As_2O_3	56.43 ± 6.37[ab]	171.67 ± 29.47[ab]	211.22 ± 39.02[ab]
4 ppm As_2O_3	71.59 ± 2.29[abc]	200.80 ± 32.45[abc]	263.69 ± 46.76[abc]

[a]$P < 0.05$, [b]$P < 0.05$, [c]$P < 0.05$ are represent As concentration in the experimental groups significantly different compared with the control group, 1 ppm As_2O_3-treated group, 2 ppm As_2O_3-treated group, respectively

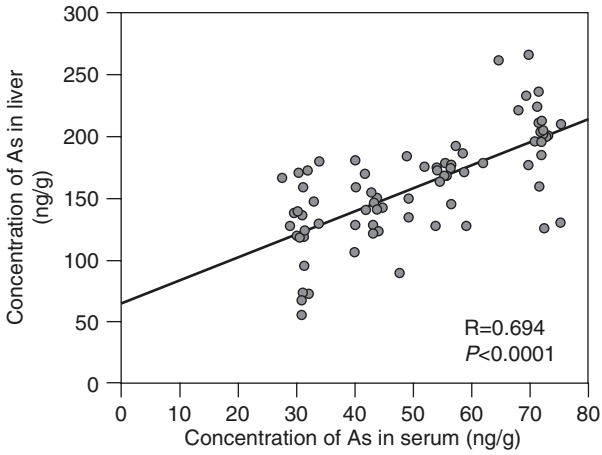

Fig. 1 Correlation analysis in As concentration between serum and liver. Independent variable is x (As concentration in serum), dependent variable is y (As concentration in liver), R = 0.694, $P < 0.0001$

higher than that in the control group ($P < 0.05$) and concentration-dependent. The As concentration in mouse liver and kidney was 124.35 and 101.38 ng/g in control group. In the experimental groups, As concentration in mouse liver and kidney was 140.40 ng/g, 171.67 ng/g and 200.80 ng/g, and 143.09 ng/g, 211.22 ng/g and 263.69 ng/g, respectively. The As concentration in mouse liver and kidney significantly increased in a dose-response manner in these experimental groups also ($P < 0.05$).

Correlation of As concentration between serum and liver is shown in Fig. 1. As concentration in serum was a correlate of the As level in liver ($P < 0.001$). Figure 2 showed the relation of As concentration between serum and kidney. As concentration in serum was a correlate of the As level in kidney ($P < 0.001$). Co-administration of taurine affected the level of As in these tissues after As exposure. The As concentration was lower in serum, liver and kidney co-administered with taurine than that in mice given with As (Fig. 3).

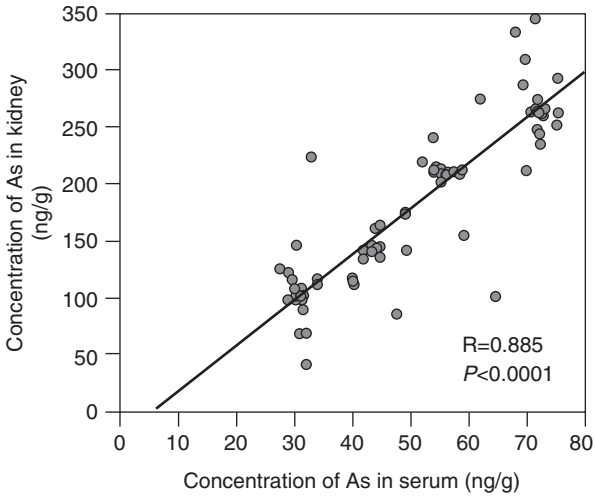

Fig. 2 Correlation analysis in As concentration between serum and kidney. Independent variable is x (As concentration in serum), dependent variable is y (As concentration in kidney), R = 0.885, $P < 0.0001$

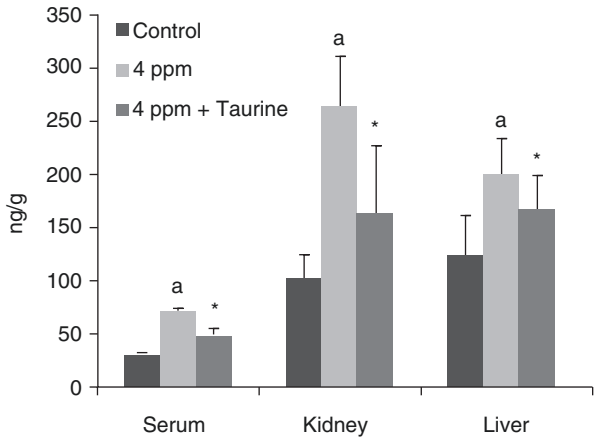

Fig. 3 The As concentration in multi tissues co-administered with taurine. $^{a}P < 0.05$ significantly different compared with the control group. $^{*}P < 0.05$ vs. 4 ppm As_2O_3-treated group

3.2 The Role of Taurine on Trace Elements Concentration in Mouse Liver and Kidney

Figures 4, 5, 6 and 7 showed the levels of Se, Fe, Cu and Mn in liver and kidney of mice, respectively. In Figs. 4 and 5 the concentration of Se and Fe in liver and kidney in the group administrated by 2 or 4 ppm As_2O_3 was obviously lower ($P < 0.05$). And the levels of Se and Fe in liver and kidney of mice that received As_2O_3 with taurine was clearly increased ($P < 0.05$).

In Fig. 6 the Cu concentration in liver and kidney was significantly higher in the group received 4 ppm As_2O_3 ($P < 0.05$). The Cu concentration in kidney was significantly lower in the group received 2 ppm As_2O_3 ($P < 0.05$). Cu concentration significantly increased in mouse liver and kidney co-administered with taurine ($P < 0.05$).

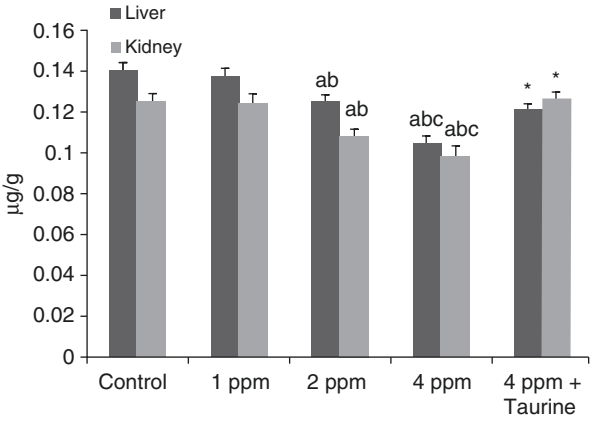

Fig. 4 The Se concentration in mouse liver and kidney or co-administered with taurine. Graphs show mean ± SD. Different from control group: [a]$P < 0.05$, Different from 1 ppm As_2O_3 group: [b]$P < 0.05$, Different from 2 ppm As_2O_3 group: [c]$P < 0.05$, Different from 4 ppm As_2O_3 group: *$P < 0.05$

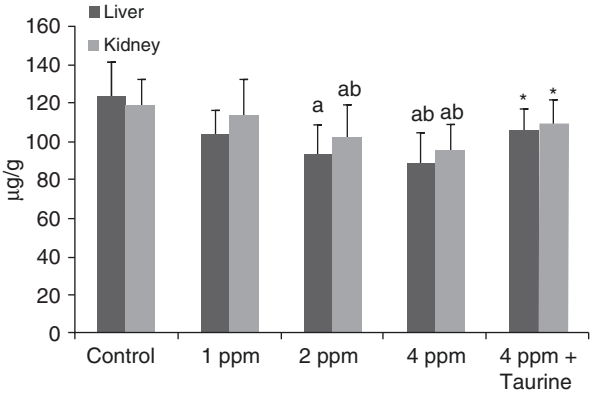

Fig. 5 The concentrations of Fe in liver and kidney of mice or co-administered with taurine. Graphs show mean ± SD. Different from control group: [a]$P < 0.05$, Different from 1 ppm As_2O_3 group: [b]$P < 0.05$, Different from 2 ppm As_2O_3 group: [c]$P < 0.05$, Different from 4 ppm As_2O_3 group: *$P < 0.05$

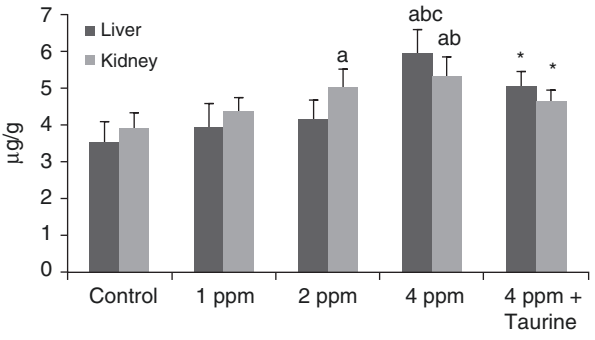

Fig. 6 The concentrations of Cu in liver and kidney of mice or co-administered with taurine. Graphs show mean ± SD. Different from control group: [a]$P < 0.05$, Different from 1 ppm As_2O_3 group: [b]$P < 0.05$, Different from 2 ppm As_2O_3 group: [c]$P < 0.05$, Different from 4 ppm As_2O_3 group: *$P < 0.05$

However, among the three As exposure groups, protective group and control group, Mn concentration was no significant difference in mouse liver and kidney exposure to As, shown in Fig. 7.

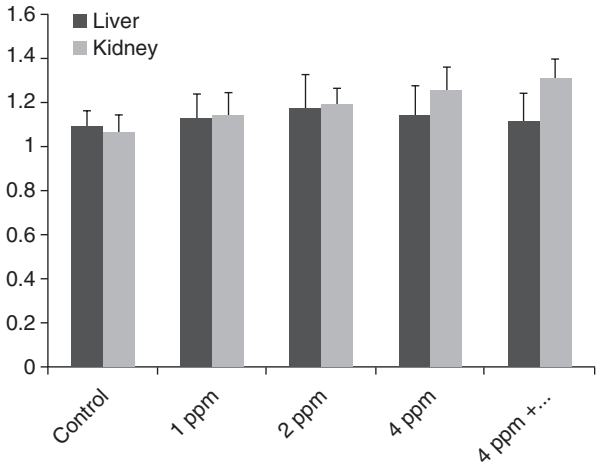

Fig. 7 The concentrations of Mn in liver and kidney of mice or co-administered with taurine. Graphs show mean ± SD

4 Discussion

Arsenic (As) is distributed widely in the environment, which enters the body via inhalation, ingestion or dermal exposure. Consumption of As contaminated drinking water usually cause As related illness though the gastrointestinal tract (Gonzalez et al. 1995). Once absorbed, As redistributes in many organs including lung, spleen, liver and kidney. In our body, As has toxicity on nearly entire organ systems (Ratnaike 2003). Literatures reported that As accumulated in various organs dose-dependently (Cui and Okayasu 2008). In this study, the As concentration in mice were significantly higher in serum, liver and kidney after As exposure. Moreover, correlation in As concentration between serum and liver or kidney was positive correlation. Our results and literatures indicate that As can accumulate in liver and kidney, which concentration increased depending on the concentration of serum As. Meanwhile, As concentration was significantly decreased in mouse serum, liver and kidney exposed to As with taurine than treated by As alone. It indicates that taurine could significantly decrease the accumulated As. Taurine composed of some functional groups such as amino group and sulfonate group, which might stimulate the excretion of heavy metals by bind with such compounds (Hwang and Wang 2001; Yeh et al. 2011). The decreased level of As may be related to the excretion of As, which promoted by taurine.

A series of studies have been reported that the role of toxic elements in essential trace elements, and the homeostasis of essential elements may be disturbed by the toxic interaction between metal and the necessary trace metal (Liu et al. 1992). The disturbed homeostasis of essential elements may be involved in development of various diseases induced by toxic metals. Our earlier study discovered that subchronic As exposure disturbed the levels of multi essential trace elements in mouse central nervous system. Therefore, we are interested in whether the levels of various essential trace elements changed after As exposure in mouse kidney and liver. In this study, the Cu concentration was significantly increased, whereas the concentration of Fe and Se was significantly decreased in liver and kidney exposed to As than that

in control group, being accordant to our previous study (Wang et al. 2013). In general, Cu is an essential element, and absorbed into the circulation from the gastrointestinal tract. Cu plays a role in many metabolic procedures. The liver and kidney are main target organs of Cu. However, Cu can cause toxicity to our body in excess amount. The physiological needs of body Cu is regulated by liver, which removes excess Cu via bile. It was reported that biliary excretion of Cu could be decreased by high dietary As. Oxidative stress and cellular death may involve in the free Cu in the cytosol, which leads to oxidative stress mostly to be the result of reactive oxygen species. Numbers of the enzymes and oxygen-transporting proteins has the key catalytic site of Iron in cells. Fe is possibly the element that has received most attention and Fe- induced hepatic and renal toxicity is thought the formation of oxidant damage by free radical-generated. Numbers of toxic metals such as aluminum and cadmium can disturb Iron homeostasis. Jurczuk et al. carried out that the Fe concentration down-regulated in liver and kidney after Cd exposure (Jurczuk et al. 2004). Se is a trace element and involved in the oxidation–reduction processes for various enzymes activities. Liver and kidney were the organs where Se typical distributed. Numbers of studies on the adverse effect of As on Se has been carried out. The interaction between Se and As has been shown in many experimental studies (Zeng et al. 2005). Moreover, Zeng et al. reported that at subacute doses, excretion of Se in the gastrointestinal tract was markedly increased by As and Se co-injected (Zeng et al. 2005). However, we found that the level of Mn was not affected by As exposure. Cui and Okayasu also demonstrated that Mn concentration in mouse liver and kidney remained not influenced by the treatment of sodium arsenate (Cui and Okayasu 2008), being accordant with our results. These results suggest that As exposure may affect trace element in liver and kidney such as the down-regulation of Se and Fe levels, or up-regulation of Cu level.

Many studies and our previous study reported that the As-induced oxidative stress might be related to pathological changes in mouse liver and kidney exposed to As. It was known that anti-oxidant therapy had been widely used in the treatment of heavy metal toxicity. Taurine was important in bile secretion and lipid digestion, by conjugation with bile acids in liver (Takatani et al. 2004). Taurine supplement could accelerate the hepatotoxicity and renotoxicity induced by As_2O_3 (Li et al. 2009; Bai et al. 2016). The obvious up-regulated or down-regulated levels of Se, Cu, Fe induced by As, which were rescued by taurine in present study. It a word, taurine can alleviate the adverse effect of As caused in liver and kidney, this symptom might related to the antioxidant function of taurine. So, the exact mechanisms of taurine benefits in trace elements need to be further explored.

5 Conclusion

In conclusion, exposure to As could induced the level of Cu significantly higher in liver and kidney, on the contrary, the levels of Se and Fe in liver and kidney were lower after As exposure. Moreover, the antioxidant taurine normalized the interference levels of As and these trace elements. Possible potential mechanisms of taurine function need to be clarified.

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Taurine Protects Mouse Liver Against Arsenic-Induced Apoptosis Through JNK Pathway

Shuangxing Li[$], Lijun Yang[$], Guangtao Dong, and Xiujie Wang

Abstract A great number of evidences demonstrated that the increased apoptosis is related to arsenic (As)-induced liver injury. The object of the present study was to explore the protection of taurine (Tau) against As-induced impairment in liver and the related mechanism. Adult mice were divided into control group, As exposure group and Tau protection group. The results of RT-PCR and WB showed that Tau treatment significantly reversed the disturbance of Bax and Bcl-2 expression. The release of cytochrome c and caspase-3 activation in liver both were prohibited by Tau in As-intoxicated mice. Furthermore, Tau markedly attenuated As-induced decrease of p-JNK level in mouse liver. These results indicated that Tau attenuated As-induced hepatic injury via JNK pathway.

Keywords Taurine • Arsenic • Apoptosis • JNK signaling • Liver

Abbreviations

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
As	Arsenic
Cyt C	Cytochrome c
LDH	Lactate dehydrogenase
Tau	Taurine

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1 Introduction

Arsenic (As) is an omnipresent naturally toxicant that found in food, groundwater, ambient air and dust. The toxin could enter the body system though diet, inhalation or dermal contact, then influence the morphology and function of targeted organs (Das et al. 2010; Meliker et al. 2007; Rodriguez-Lado et al. 2013). Inorganic As, the more toxicant than its organic compound, which were converted to intermediated toxin, dimethyl arsenic acid and monomethyl arsenic acid, mainly in liver tissue. Ayala-Fierro et al. treated primary rat hepatocytes with arsenical compounds, as early as 1999, and found that As exposure increased the viability of hepatocytes in a time-dependent manner (Ayala-Fierro et al. 1999). It was reported that As treatment induced the decrease of body and liver weight of animal model, the markers of liver damage, and the increase of indicators of liver function, including the alkaline phosphatase (ALP) and alanine aminotransferase (ALT) in serum (Das et al. 2010). These results all showed that As has toxic effects on liver tissue, which has get an increasing number of attention.

Taurine, a natural substance in many kinds of foods, exists in the liver with a high concentration (Batista et al. 2013). Numerous results indicated that Tau has liver protective effects (Heidari et al. 2013, 2016; Nagai et al. 2016; Zhang et al. 2014). Several groups have reported that Tau treatment attenuated cisplatin or iron-overload induced oxidative stress in liver (Liao et al. 2008; Zhang et al. 2014). Tau also has protective potentials in hepatocytes being an osmoregulator, antioxidant or intracellular calcium flux regulator (Das et al. 2010, 2012; Heidari et al. 2014). Moreover, the absence of Tau could initiate apoptosis and disease in liver tissue which had been proven in mice (Warskulat et al. 2006, 2007). Tau can be used as a potential effecter to intervene As-induced damage in liver.

In order to clarify the positive role of Tau in As-induced hepatocyte injury and related mechanism, real time RT-PCR was used to examine the gene level of Bax and Bcl-2. The level of cytochrome c protein in mitochondria and cytosolic were assessed by Western Blotting (WB). Commercial kits were used to evaluate caspase-3 activity. The protein levels of JNK and p-JNK in mouse liver, the related mechanism pathway, were measured with WB.

2 Methods

2.1 Animals

19.2–24.7 g male mice were provided by Experimental Animal Center, Harbin Medical University. During experiments, all mice were raised under 20–24 °C temperature, 55% humidity, 12 h dark-light cycle environment with an ad libitum diet and water. The 30 mice were divided into control group, As exposure group and Tau protection group randomly. As exposure group exposed to 4 mg/L As_2O_3 in double-distilled water orally; tau protection group received 4 mg/L As_2O_3 in double-distilled water orally and 150 mg/kg Tau once daily by gavage; control group only received

double-distilled water. After 60-day treatment, all model mice were sacrificed and samples were collected carefully. The animal experiments were carried out according to the guidelines of the committee of Harbin Medical University.

2.2 Real Time RT-PCR

Trizol® reagent (Takara, China) was used to extract RNA sample according to the instructions. Transcriptor First Strand cDNA Synthesis Kit (Roche, \USA) was used to perform RT reactions. TP800 System and SYBR Green PCR kit (Takara, Japan) were used to carry out Real time RT-PCR. 95 °C 5 min, followed by 95 °C for 30 s, 40 cycles, then 55 °C 30 s, 72 °C 30 s were used as reaction conditions. The followed primers were used: Bcl-2, GACTGAGTACCTGAACCGGCATC, CTGAGCAGCGT CTTCAGAGACA; Bax, CGAATTGGCGATGAACTGGA, CAAACATGTCAGCTGCCACAC; β-actin, GGAGATTACTGCCCTGGCTCCTA, GACTCATCG TACTCCTGCTTGCTG.

2.3 Western Blot

Total proteins were extracted from liver tissue with lysis buffer. BCA method was used to qualify protein concentration. SDS-polyacrylamide gel electrophoresis was carried out with same gram of loading sample protein, and the protein samples were transferred to a PVDF membrane. After blocking with 10% milk for 30 min, the blots were incubated with Cyt C, JNK, p-JNK and β-actin (1:500, R&D, USA) primary antibodies, respectively. The blots were treated with HRP-conjugated secondary antibodies, and then detected by Bio-Rad ChemiDoc™ MP imaging system (Bio-Rad, USA), and then qualified with the Gel-Pro software.

2.4 Caspase-3 Activity

Caspase-3 Colorimetric Assay Kit (Beyotime, China) was used to detect caspase-3 activity of liver according to the manufacturer's manipulations. The liver lyses were incubated in ice-cold lysis buffer for 20 min, then centrifuged at 10,000 × *g* 2 min. The related results were showed as a ratio to control.

2.5 Statistical Analysis

Statistical analysis was performed with SPSS 17.0 statistical software. Data were analyzed using one-way ANOVA and expressed as means ± SD in triplicate.

3 Results

3.1 Tau Attenuated As-Induced Caspase-3 Activation in Liver

To determine whether apoptosis inhibition was responsible for the potential of Tau against As-induced hepatoxicity in mice, caspase-3 activity was assessed. As Fig. 1 shown, compared with control group, As exposure increased the activity of caspase-3 of liver for about threefold. However, Tau treatment markedly prohibited the increased activity of caspase-3 induced by As exposure nearly a half (Fig. 1).

3.2 Tau Inhibited As-Induced Disturbance of Bax and Bcl-2 Expression

Bax and Bcl-2 are the predominant members of Bcl-2 family, and cast an unique role in apoptosis regulation (Das et al. 2010). Real time RT-PCR was used to analysis the gene expression of Bax and Bcl-2. As shown in Fig. 2a, Bax level was markedly increased in the liver of As-intoxicated mice. However, compared with As group, Tau treatment significantly blocked the increase of Bax expression in liver. On the contrary, As exposure induced a decrease of Bcl-2 level, which was significantly inhibited by Tau treatment (Fig. 2b).

3.3 Tau Blocked As-Induced Cyt C Release of Mouse Liver

The levels of mitochondrial and cytosol Cyt C were examined by WB. Compared with control group, As exposure significantly increased Cyt C level in cytosol, which was markedly blocked with Tau treatment. The level of mitochondrial Cyt C

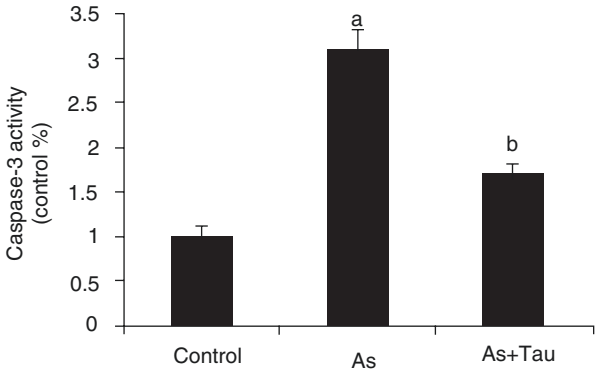

Fig. 1 Potential of Tau on caspase-3 activity in As-exposed mouse liver. [a]$p < 0.05$, vs. control group; [b]$p < 0.05$, vs. As group

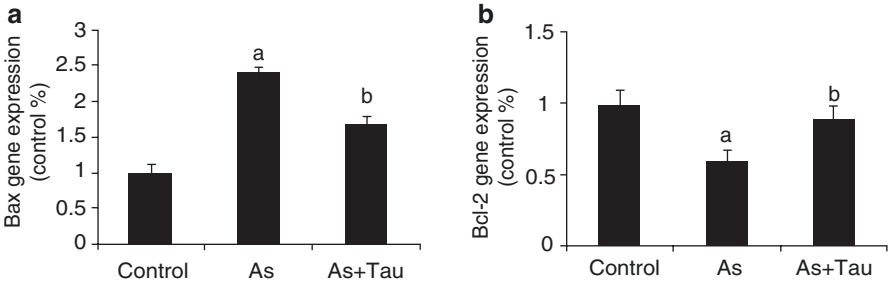

Fig. 2 Effect of Tau on Bax (**a**) and Bcl-2 (**b**) gene expression in As-exposed mouse liver. Data were presented as mean ± SD. $^{a}p < 0.05$, vs. control group; $^{b}p < 0.05$, vs. As group

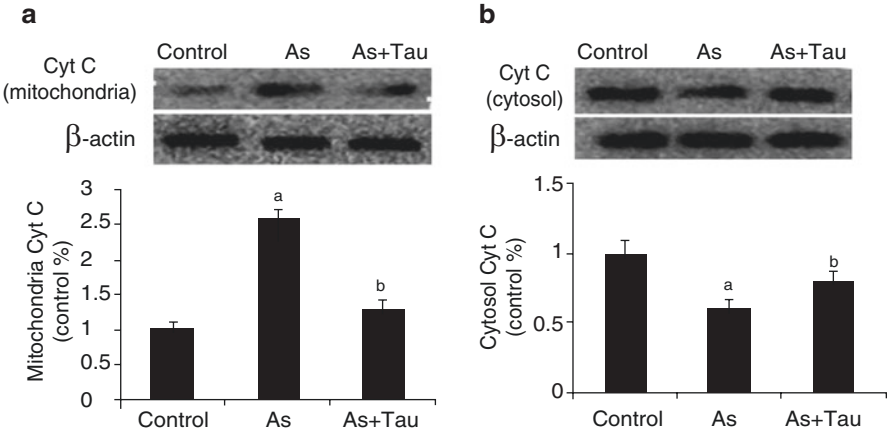

Fig. 3 Effect of Tau on the release of Cyt C in As-exposed mouse liver. Cytochrome c in mitochondrial fraction (**a**) and cytosol fraction (**b**) was determined by WB. Data were presented as mean ± SD. $^{a}p < 0.05$, vs. control group; $^{b}p < 0.05$, vs. As group

markedly reduced in As-intoxicated liver, which were blocked with Tau treatment (Fig. 3), suggesting that Tau blocked As-induced Cyt C release in liver of mice.

3.4 Tau Reversed As-Induced Decrease of p-JNK Level in Liver

To determine the pathway mechanism of the Tau protection against As-induced apoptosis in liver, we examined the protein expression of JNK and p-JNK by WB. As Fig. 4 showed, both As and Tau treatment didn't affect the expression of JNK in liver of mice. However, the level of p-JNK was significantly decreased after As treatment. With Tau treatment, p-JNK protein level was markedly increased, suggesting the activation of JNK pathway may involved in the protection of Tau against As-induced injury in liver.

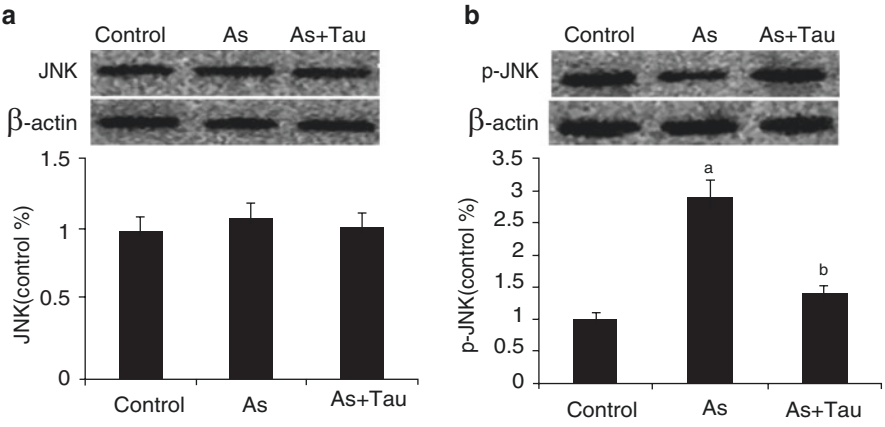

Fig. 4 Effect of Tau on JNK (**a**) and p-JNK (**b**) level in As-exposed mouse liver. Data were presented as mean ± SD. [a]$p < 0.05$, vs. control group; [b]$p < 0.05$, vs. As group

4 Discussion

Our study demonstrated the protective capacity and its mechanism of Tau on apoptosis in As-intoxicated liver. Apoptosis is a basic process under both physiological and pathological condition, which regulates cell death in a controlled according to surrounding environmental (Espe and Holen 2013; Sinha et al. 2009). The process is regulated by several protein families, and one of the most important families is Bcl-2 family. Bcl-2 protein family consists of two type regulators. One is pro-apoptotic protein regulator, such as Bax; the other is anti-apoptotic protein regulator, such as Bcl-2. These two types of protein have opposite effects: Bax promotes apoptotic process, while Bcl-2 inhibits the program of cell death. The elevation of Bax promotes Bax homodimers formation, then triggered mitochondrial Cyt C release, causing, which subsequently induced the activation of caspase and apoptosis (Gross et al. 1998; Leon et al. 2009). Tau exposure has been proved to attenuate the disturbance of Bcl-2 family and rescue cells from apoptosis (Taranukhin et al. 2012; Wu et al. 2009). Our results showed that Tau exposure significantly blocked the disturbance of Bax and Bcl-2, the Cyt C release and the activation of caspase-3 in As-treated liver. It is indicated that Tau rescues mouse liver from apoptosis through Bax/Bcl-2 related pathway.

JNK, one of the most important member of phosphorylating proteins for serine/threonine, involved in several vital signals for cell survival (Das et al. 2010). Taranukhin et al. reported that ischemia-induced cleavage of caspases-3 and 9 was prohibited via Tau treatment (Taranukhin et al. 2008). Das et al. reported that JNK pathway inhibited the release of mitochondria Cyt C and mediated mitochondrial apoptotic signaling (Das et al. 2010). In order to clarify if JNK pathway took part in the anti-apoptotic potential of Tau in As-exposed liver, the levels of JNK and p-JNK were examined with WB. Interestingly, a significantly increase of p-JNK level was

found in liver of Tau-treated mice. Das et al. also reported that JNK inhibitor treatment, SP600125, markedly suppressed the As-induced caspase-3 activation and cellular damage of cultured hepatocytes (Das et al. 2010). Takatani et al. also report that Tau cardioprotection was regulated by JNK activity in ischemia model (Takatani et al. 2004). It suggests that Tau would trigger the activation of JNK, which may be relate to the protection of Tau against As-induced hepatoxicity.

5 Conclusion

Our results indicated Tau treatment markedly attenuated As-induced apoptosis in mouse liver, which may be related with the activation of JNK pathway. It provides a new approach to moderate As-related hepatotoxicity via Tau supplement.

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Taurine Attenuates As_2O_3-Induced Autophagy in Cerebrum of Mouse Through Nrf2 Pathway

Fengyuan Piao, Yan Zhang, Lijun Yang, Cong Zhang, Jing Shao, Xiaohui Liu, Yachen Li, and Shuangyue Li

Abstract We previously reported that the impairment of cerebrum may relate with neurotoxicity induced by arsenic (As) exposure. In the present study, we investigated whether autophagy of the cerebrum neurons were responsible for As-induced neurotoxicity and the protective role of taurine (Tau). Forty mice were randomly divided into control group, Tau control group, As exposure group and Tau protection group. The results showed that LC3 II expression was elevated and P62 expression was lower after As exposure, whereas the effects were obviously attenuated by Tau treatment. More important, As induced increase of MDA level and decrease of Nrf2 expression were significantly inversed in protective group. In sum, autophagy inhibition might play a strong role in the neuroprotection of Tau in As-induced toxicity via Nrf2 pathway.

Keywords Taurine • Arsenic • Autophagy • Nrf2 pathway • Cerebrum

Abbreviations

As	Arsenic
SD	Standard deviation
Tau	Taurine

Fengyuan Piao and Yan Zhang contributed equally to this work.

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1 Introduction

Arsenic (As) is a kind of carcinogen and toxicant which is widely spread in nature. As was found in food, groundwater, ambient air and dust and could enter the body system though diet, inhalation or dermal contact, then influence the morphology and function of targeted organs (Hata et al. 2012). It was showed that exposure to As was responsible for a number of deficits in nerve system and resulted in permanent neuronal damage (Chen et al. 2015; Liu et al. 2012; Yorifuji et al. 2016). Recently, increasing evidences showed that a self-degradative process, autophagy, takes part in the origin and development of As-induced neurotoxicity (Cholanians et al. 2016; Escudero-Lourdes 2016).

Autophagy is necessary cellular process which sequester cytoplasmic materials into autophagosomes, literally double-membrane vesicles, and then transfer to lysosomes to degrade or recycle (Yang et al. 2015). The damaged organelles which were facilitated to digestive degrade including endoplasmic reticulum, mitochondria and Golgi complex (Donadelli et al. 2011). The high conserved process can be triggered and prolonged in answer to extracellular or intracellular stress and then results in selective self-digestion to maintain cellular homeostasis. However, over-unregulated autophagy would result in cells damage and death, defining as autophagic cell death (Li et al. 2012a; Park et al. 2013). It is reported that As could cause cell death via ROS pathway due to prolonged autophage (Zhu et al. 2014). Several evidences had demonstrated that ROS involved in the origination and development of As-induced neurotoxicity (Hong et al. 2009; Lu et al. 2014; Yu and Liao 2014).

Taurine (Tau), a natural substance in many kinds of foods, particularly in seafood and meat, exists in the brain with a high concentration. Numerous evidences demonstrated that Tau belongs to a powerful endogenous antioxidant (Chang et al. 2014). Tau could decrease the yield of oxidative stress and increase the activations of antioxidative enzymes in kinds of models (Li et al. 2012b; Rodriguez-Martinez et al. 2004; Xu et al. 2015). An increasing number of evidences also indicate that Tau possesses cytoprotective properties, which can prevent the damage from autophagy induced by various toxicants (Bai et al. 2016; Li et al. 2012b). However, very limited studies have suggested that the neuroprotection of Tau against As neurotoxicity is regulated by mediating autophagy. Thus, the present study was undertaken to examine the beneficial action of Tau against neuronal damage induced by As exposure in mice. The goals of this study were to clarify whether autophagy involved in neuroprotection of Tau in As-exposed mice and its related mechanisms.

2 Methods

2.1 Animal and Treatment

19.2~24.7 g male mice were provided by Animal Center, Dalian Medical University. During experiments, all mice were raised under 20~24 °C temperature, 55% humidity, 12 h dark-light cycle environment with an ad libitum diet and water. The 30 mice were divided into control group, As exposure group and Tau protection group

randomly. As exposure group exposed to 4 mg/L As_2O_3 in double-distilled water orally; tau protection group received 4 mg/L As_2O_3 in double-distilled water orally and 150 mg/kg Tau once daily by gavage; control group only received double-distilled water. After 60-day treatment, all model mice were sacrificed and samples were collected carefully. The animal experiments were carried out according to the guidelines of the committee of Dalian Medical University.

2.2 Western Blot

Total proteins were extracted from cerebrum tissues with lysis buffer. BCA method was used to qualify protein concentration. SDS-polyacrylamide gel electrophoresis was carried out with same gram of loading sample protein, and the protein samples were transferred to a nitrocellulose membrane. After blocking with 10% milk for 30 min, the blots were incubated with LC3 II, p62, Nrf2 and β-actin (1:500, Abcam, USA) primary antibodies, respectively. The blots were treated with horseradish peroxidase-conjugated secondary antibodies, and then detected by Bio-Rad ChemiDoc™ MP imaging system (Bio-Rad, CA, USA), and then qualified with the Gel-Pro software.

2.3 Malonic Dialdehyde (MDA) Assay

MDA assay were used to examine the status of oxidative stress. Cerebrum tissue were homogenized with phosphate buffer and then centrifuged. The supernatants were collected and used for MDA analysis with MDA test kit (Keygen Biotech) at the absorption at 532 nm.

2.4 Statistic Analysis

Statistical analysis was performed with SPSS 11.0 statistical software. Data were analyzed with one-way ANOVA and expressed as means ± SD in triplicate.

3 Results

3.1 Tau Inhibited As-Induced Elevation of LC3 Expression in Cerebrum

To evaluate whether autophagy involves in the neurotoxicity of As intoxication in mice, autophagy biomarker protein LC3-I/II expression of all groups were assessed by WB. Figure 1 showed that, in comparison with control, As intoxication sharply increased the expression of LC3-II. Interestingly, Tau markedly attenuated the elevation of LC3-II induced by As intoxication.

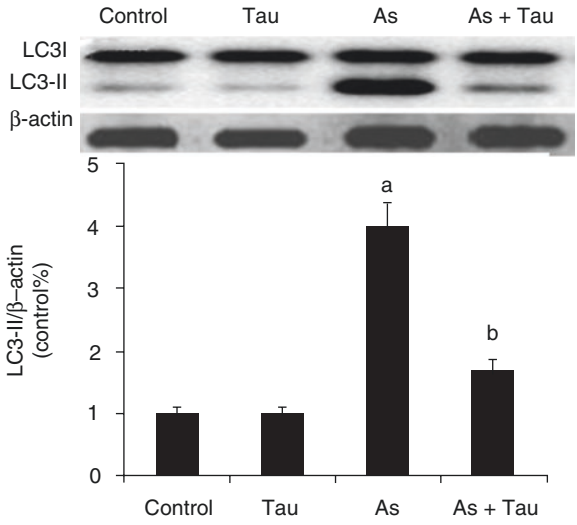

Fig. 1 Protect potential of Tau on LC-3 expression in cerebrum of As-intoxicated mice. LC-3 expression was determined by WB. [a]$p < 0.05$, compared with control group; [b]$p < 0.05$, compared with As group

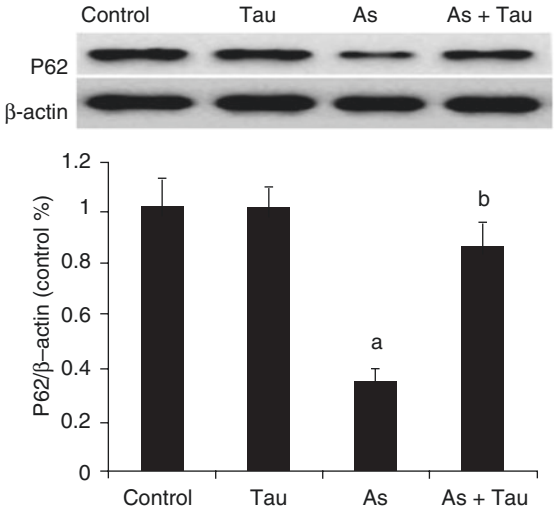

Fig. 2 Protect potential of Tau of Tau on P62 expression in cerebrum of As-intoxicated mice. P62 expression was determined by WB. [a]$p < 0.05$, compared with control group; [b]$p < 0.05$, compared with As group

3.2 Tau Blocked As-Induced Decrease of P62 Expression in Cerebrum

We also studied the protect potential of Tau on autophagy induced by As exposure in cerebrum by determining P62 protein level. As shown in Fig. 2, the level of P62 protein was increased dramatically in the cerebrum of As-intoxicated mice

compared with control. Once treated with Tau, the increase of P62 expression was markedly blocked in cerebrum. These results indicate that Tau protect cerebrum from As-induced cerebrum injury through autophagy pathway.

3.3 *Tau Suppressed As-Induced Decrease of Nrf2 Expression in Cerebrum*

The expression of Nrf2 was examined by WB assay. As seen in Fig. 3, in comparison with control, As intoxication significantly increased the expression of Nrf2, which was markedly suppressed once As-intoxicated mice were treated with Tau. These results suggest that Nrf2 pathway involved in the protect capacity of Tau against As-induced cerebrum injury in mice.

3.4 *Tau Prohibited As-Induced Increase of MDA Level in Cerebrum*

As could induce autophagy through ROS generation in vitro. To investigate the level of oxidative stress, we used MDA test kit to measure the level of MDA. We found that the level of MDA was increased significantly after treatment with As. After pretreatment with Tau, the level of MDA was decreased in the cerebrum of As-treated mice (Fig. 4). It suggested that treatment with As could increase the generation of ROS, and pretreatment with Tau could withstand the oxidative stress induced by As intoxication.

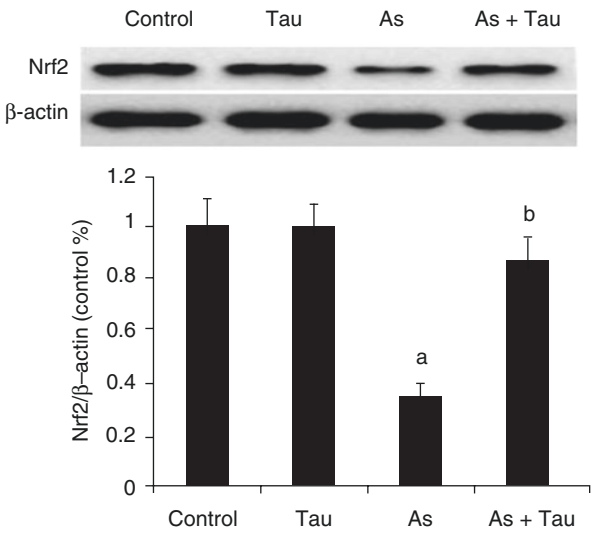

Fig. 3 Protect potential of Tau on Nrf2 expression in cerebrum of As-intoxicated mice. [a]$p < 0.05$, compared with control group; [b]$p < 0.05$, compared with As group

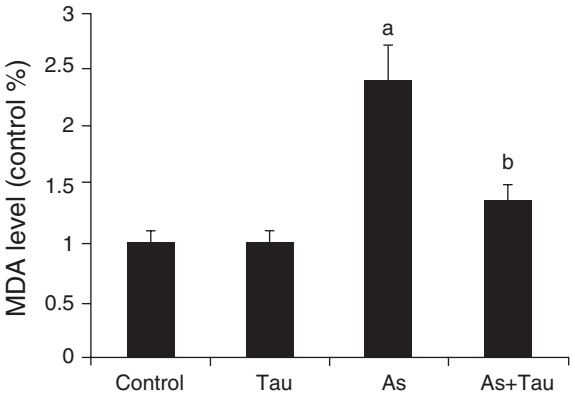

Fig. 4 Protect potential of Tau on MDA level in cerebrum of As-intoxicated mice. [a]$p < 0.05$, compared with control group; [b]$p < 0.05$, compared with As group

4 Discussion

Autophagy is a vital degraded process that services as a character controller that shields neurons from toxic protein accumulation and nonadaptive organelles by causing fast clearance (Li et al. 2012b). On contrast, prolonged or over-activated autophagy process do harm to cells and tissues and takes part in the development of pathological conditions (Zhang et al. 2013). It was reported that the over-activation of autophagy was responsible for cell death in the spine (Sekiguchi et al. 2012; Walker et al. 2012). To explore the effect of Tau on autophagy in cerebrum of As-intoxicated mice, LC3 was employed to detect autophagic vacuoles. Cytosolic LC3-I and enzymatic LC3-II are the two different forms of the autophagy protein marker, LC3 (Kabeya et al. 2000). When autophagy is activated, LC3-I converts to LC3-II, thus, LC3-II level is a standard marker for the detection of autophagy. Li et al. reported that METH treatment induced an increased expression of LC3-II, whereas such elevation was markedly alleviated by Tau (Li et al. 2012b). It was showed that As intoxication induced LC3-II level decrease dose-dependently in pancreas of rats. However, after Tau pretreatment, the level of LC3-II was decreased dramatically (Bai et al. 2016). In the present study, the amount of LC3-II in cerebrum increased after As exposure, which was markedly inhibited after Tau treatment, suggesting the activation of autophagy triggered by As in cerebrum was blocked by Tau.

P62 is a characterized shuttling factor that induces targeted proteins to degrade by either proteasome or autophagy pathways (Zhang et al. 2013). P62 protein, via binding to LC3-II, is related to autophagosomes, and mark the beginning of late stage autophagosome. We also assess protein expression of p62 via Western blotting. The results showed that p62 expression decreased after As exposure, while, which expression was enhanced after Tau treatment, indicating that the neuroprotection of Tau on As-induced autophagy may also correlated with p62 expression.

The formation of ROS has been illustrated to play an important role in the process of autophagy (Lee et al. 2012). It was showed that As evoked autophagic flux

and, therefore, induced autophagic cell death via ROS signaling in cultured INS-1 cells (Zhu et al. 2014). During the oxidative stress reaction, Nrf2, being a vital regulator, played a decisive role in protecting against oxidative stress (Saw et al. 2014). In our study, we found that Tau treatment prohibit the decrease of Nrf2 in cerebrum of As-exposed mice, which correlates with the reduction in As-induced the elevation of LC3-II and P62 expression.

5 Conclusion

In conclusion, we found that taurin could inhibit the activation of autophagy in cerebrum of As-intoxicated mice and that the decrease of Nrf2 expression and MDA level may take part in this process.

Acknowledgements This work was supported by National Natural Science Foundation of China (grant numbers 81273038 and 81102160) and China Postdoctoral Science Foundation Funded Project (No. 2015M581338).

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Effects of Taurine on ACE, ACE2 and HSP70 Expression of Hypothalamic-Pituitary-Adrenal Axis in Stress-Induced Hypertensive Rats

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Abstract The experiment was to elucidate protective mechanism of taurine against stress-induced hypertension. Thirty two male Wistar rats were randomly divided into four groups. Normal control group and stress control group were intragastrically administered saline; β-alanine stress group were fed with β-alanine (200 mg/kg/day) and taurine stress group with taurine (200 mg/kg/day). The hypertensive model was established by giving rats stress for 3 weeks.

Results showed that significant expression levels of angiotensin converting enzyme (ACE) in the hypothalamus, pituitary and adrenal were observed in β-alanine stress group and stress control group ($P < 0.05$), but significant mRNA expression levels of angiotensin-converting enzyme 2 (ACE2) in taurine stress group and normal control group ($P < 0.05$). All the groups showed no significant differences in HSP70 mRNA expression levels in hypothalamus ($P > 0.05$), while taurine stress group exhibited the highest HSP70 mRNA expression levels both in pituitary and in adrenal ($P < 0.05$). It was also found that β-alanine stress group and stress control group had significantly higher protein expression levels of ACE in hypothalamus, pituitary and adrenal ($P < 0.05$), but significantly lower protein expression of ACE2 compared to taurine stress group and control groups ($P < 0.05$). The results indicated that taurine regulated the hypothalamus pituitary adrenal (HPA) axis of the renin-angiotensin-aldosterone system (RAAS) by inhibiting ACE gene and protein expressions and promoting ACE2 and HSP70 protein expressions, thereby contributing to the prevention of stress-induced hypertension.

Keywords Stress • Hypertension • Prevention • Rat

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Abbreviations

ACE	Angiotensin converting enzyme
ACE2	Angiotensin-converting enzyme 2
HPA	Hypothalamus pituitary adrenal
RAAS	The renin angiotensin aldosterone system

1 Introduction

Hypertension, a common and sometimes fatal condition, ranks among major threats to human health. Stressful situations can cause stress-induced hypertension temporarily, and may also cause long-term high blood pressure when under stress for too long. Many recent studies have found that there exists an important hypothalamus pituitary adrenal (HPA) stress-induced system in living organisms, and that renin-angiotensin-aldosterone system (RAAS) is also a stress-induced one that cannot be ignored (Wan et al. 1996). ACE and ACE2 are key enzymes in RAAS. AngI passes lungs and renal circulation through ACE to form vasoexcitor material AngII, which removes D-Aspartic acid through ACE2 to be converted to vasodilators AngIII. Stressful situations lead to higher concentrations of AngII, which may cause strong contraction around peripheral artery and fast beating heart, leading to hypertension (Donoghue et al. 2000; Tipnis et al. 2000; Ferrario 2003; Danilczyk and Penninger 2006). Therefore, RAAS plays a critical role in the mechanism of stress-induced hypertension. The current investigation may reflect the changing mechanism of hypertension by observing gene and protein expressions of ACE and ACE2 in the HPA axis.

HSP70 is a heat shock protein and has a molecular weight of about 70 kDa. It is an important part of the family of heat shock proteins, and is known as major heat shock protein, serving essentially as protein folding, stretching, transport, and oligomeric formation and depolymerization, and helping to protect cells from stress.

2 Methods

2.1 Materials

RNAiso Plus (total RNA Extraction) kits and TaKaRa RNA-PCR Kit Ver.3.0 kits were from Japan Takara Biotechnology (Dalian) Co. Ltd.; Agarose gelpurification kits from Beijing Bomaide Technology Development Co. Ltd.; Trans1-T1

competent cells and the pEASY-T1 cloning vector from Beijing TransGen Biotech Co. Ltd.; Rat polyclonal antibody of ACE and ACE2 and SABC immunohistochemistry kits from Wuhan Boster Biological Engineering Company; and DAB kits from Beijing Dingguo Biotech Co. Ltd.

2.2 Study Design and Grouping

Thirty two clean male Wistar rats were randomly and evenly distributed into four groups, including normal control group, β-alanine stress group, stress control group, and taurine stress group. At 8 a.m. during the experiment, normal saline was administered to normal control group and stress control group, β-alanine (200 mg/kg/day) to β-alanine stress group in order to exhaust endogenous taurine for comparison, and taurine (200 mg/kg/day) to taurine stress group. β-Alanine stress group and taurine stress group were subjected to shock and noise stimulus as the stress model.

Electric shocks and noise were given as multiple stress on a daily basis, and discontinuous irregular foot shock and noise as stressors. An output voltage of 150 V discharged every 5~10 s, combined with a 100 dB industrial noise stimulus, lasting 2 h every day in 20 consecutive days. The systolic arterial pressure was measured with a blood pressure monitor for small animals between 8:30 and 10:00 a.m. of D 1, 6, 11, 16 and 21. On the 22nd day, all rats were killed; their hypothalamus, pituitary and adrenal were collected and washed with 4 °C pre-cooled normal saline before frozen in freezing tubes at −80 °C for future use.

2.3 Determination of Expressions of ACE, ACE2 and HSP70 mRNA in Rat Hypothalamus, Pituitary, and Adrenal

Design of primer was based on the relevant rules and partial sequences of ACE gene, ACE2 gene and HSP70 gene sequences in rat muscle tissues previously reported. The amplified primer sequences of ACE, ACE2, HSP70 genes and β-actin endogenous gene were synthesized by Shenggong Biological Engineering (Shanghai) Co. Ltd. (Table 1).

PCR amplification is as follows: After initial denaturation of 3 min at 94 °C, 34 cycles of denaturation at 94 °C for 30 s, annealing at 51.2 °C for 30 s, extension at 72 °C for 1 min, and termination at 72 °C for 5 min for PCR amplification of ACE gene fragments; 32 cycles of denaturation at 94 °C for 30 s, annealing at 53.5 °C for 30 s, 72 °C extension for 1min, and termination at 72 °C for 5 min for PCR amplification of ACE2 gene fragments; and 34 cycles of denaturation at 94 °C for 30 s, annealing at 51.7 °C for 30 s, 72 °C extension for 1min, and termination at 72 °C for

Table 1 Design of primer

Gene		Primer	Length of amplified fragments (bp)
ACE	Upstream	CCTATTCCCGCTCATCT	510
	Downstream	ATTGGCAACACGGTCTA	
ACE2	Upstream	ATGAATGGACCGACAA	374
	Downstream	AATGCCAACCACTACC	
HSP70	Upstream	TAGAGTGAGCCTTAGCGT	530
	Downstream	GTGATTACAAAGACCCAGA	
β-Actin	Upstream	TTGTTACAAACTGGGACG	764
	Downstream	GATATTGATCTTCATGGTG	

5 min for PCR amplification of HSP70 gene fragments. PCR products using agarose gel electrophoresis were scanned and analyzed in the gel imaging system, thereby calculating the relative expressions of ACE, ACE2 and HSP70 mRNA to β-actin gene expression (%), as shown in the following formula,

$$\text{relative expression of a target gene} = \text{mRNA optical density of a target gene} / \beta\text{-actin mRNA optical density value} * 100\%$$

2.4 Determination of Protein Expressions of ACE, ACE2 and HSP70 in Rat Hypothalamus, Pituitary, and Adrenal Tissues

The slices were incubated for 10 min at room temperature with 0.3% methanol solution of hydrogen peroxide, for another 15 min at room temperature with 6% BSA non-immune animal serum, then excess serum was dried with filter paper. The slices were subsequently incubated with primary antibody (1:100) overnight at 4 °C, rewarmed at 37 °C for 45 min; with biotinylated secondary antibody at 37 °C for 30 min, with SABC-HRP at 37 °C for 30 min; DAB and hematoxylin counterstained; dehydrated and cleared; mounted in neutral balsam and dried at room temperature. The SABC slices stained by Immunohistochemistry were magnified 100 times under light microscope, and each slice selected 10 fields randomly by image analyzer. The gray level unit was converted into optical density unit and corrected using Image Pro Plus 6.0. The yellow image area (protein staining of ACE and ACE2) was used as AOI (area of interest) for determination of optical density. The area, density (mean) and integrated optical density (IOD) were measured selectively.

2.5 Statistical Analysis

Statistical analysis was performed with SPSS17.0 statistical software, and all the data were expressed as (mean ± standard deviation).

3 Results

3.1 ACE, ACE2 and HSP70 mRNA Expressions in Rat Hypothalamus, Pituitary and Adrenal

3.1.1 The Result of ACE Gene mRNA Expression

As shown in Table 2, Figs. 1, 2, and 3, electrophoresis analysis was used for RT-PCR products of mRNA in hypothalamus, pituitary and adrenal. Specific bands of ACE were shown in all trial groups, indicating that ACE gene transcription existed in rat hypothalamus, pituitary and adrenal. There were no significant differences in ACE mRNA expression of hypothalamus between taurine stress group and normal control group ($P > 0.05$), whereas significant differences were observed in that of pituitary and adrenal ($P < 0.05$). There were differences in tissues between β-alanine stress group and normal control group ($P < 0.05$), between stress control group and normal control group ($P < 0.05$), between β-alanine stress group and taurine stress group ($P < 0.05$), and between stress control group and taurine stress group ($P < 0.05$). Stress control group and β-alanine stress group observed significant differences in pituitary and adrenal ($P < 0.05$), but no significant differences in hypothalamus ($P > 0.05$).

Table 2 Taurine stress rat hypothalamus, pituitary and adrenal ACE gene expression

Group	Hypothalamus	Pituitary	Adrenal
Control group	22.57 ± 3.29^{a}	12.59 ± 2.49^{a}	15.32 ± 2.61^{a}
β-Alanine stress group	35.67 ± 4.57^{b}	31.92 ± 3.68^{c}	47.23 ± 3.12^{c}
Stress group	37.36 ± 2.34^{b}	18.83 ± 2.13^{b}	24.36 ± 3.19^{b}
Taurine stress group	24.38 ± 1.98^{a}	14.02 ± 1.76^{a}	15.69 ± 3.87^{a}

Note: Same letters indicate insignificant difference between two groups ($P > 0.05$). Different letters indicate significant difference between two groups ($P < 0.05$). The data in the table are mean ± standard deviation

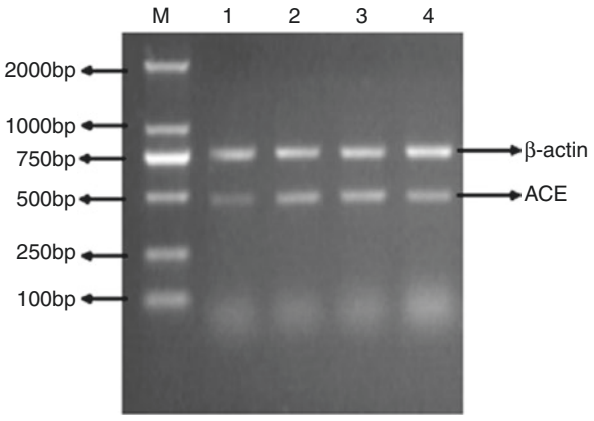

Fig. 1 The ACE gene mRNA expression in rat hypothalamus. Note: M. DL2, 000 DNA Marker, 1. Normal control group; 2. β-Alanine stress group; 3. Stress group; 4. Taurine stress group

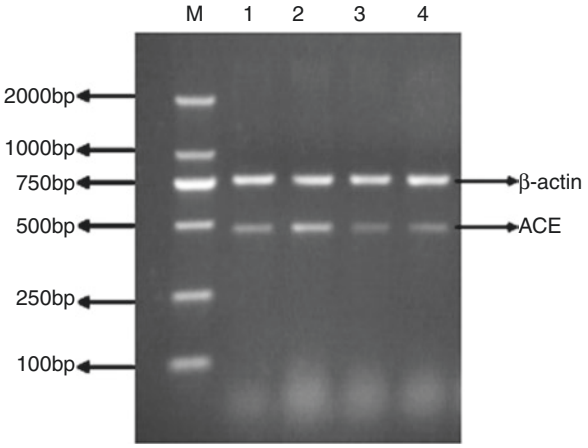

Fig. 2 The ACE gene mRNA expression in rat pituitary. Note: M. DL2, 000 DNA Marker, 1. Normal control group; 2. β-Alanine stress group; 3. Stress group; 4. Taurine stress group

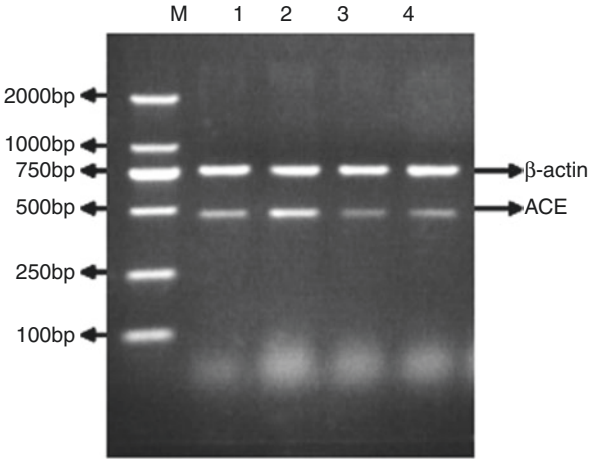

Fig. 3 The ACE gene mRNA expression in rat adrenal. Note: M. DL2, 000 DNA Marker, 1. Normal control group; 2. β-Alanine stress group; 3. Stress group; 4. Taurine stress group

3.1.2 The Result of ACE2 mRNA Expression

While there were differences in ACE2 gene mRNA expression in rat hypothalamus and pituitary among all groups, no significant differences were found ($P > 0.05$), as shown in Table 3, Figs. 4, 5, and 6. As for the expression of ACE2mRNA of rat adrenal, taurine stress group demonstrated the highest expression of ACE2 gene, higher than stress control group and β-alanine stress group, and normal control group was the lowest. There were significant differences among different groups ($P < 0.05$).

Table 3 Taurine stress rat hypothalamus, pituitary and adrenal ACE2 gene expression

Group	Hypothalamus	Pituitary	Adrenal
Control group	12.37 ± 1.89[a]	72.59 ± 2.65[a]	9.32 ± 0.91[a]
β-Alanine stress group	13.81 ± 3.12[a]	71.27 ± 3.73[a]	11.71 ± 1.01[b]
Stress group	13.36 ± 2.21[a]	72.98 ± 3.06[a]	14.36 ± 0.78[c]
Taurine stress group	13.01 ± 1.56[a]	73.43 ± 4.38[a]	18.89 ± 1.02[d]

Note: Same letters indicate insignificant difference between two groups ($P > 0.05$). Different letters indicate significant difference between two groups ($P < 0.05$). The data in the table are mean ± standard deviation

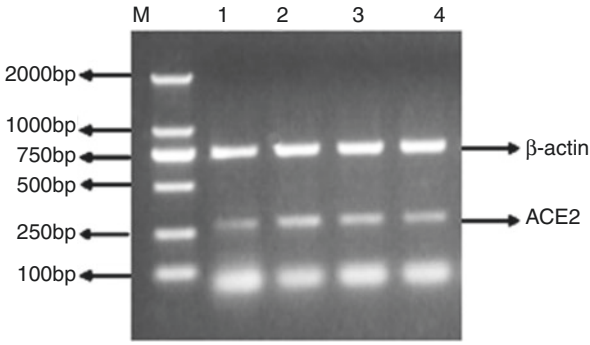

Fig. 4 The ACE2 gene mRNA expression in rat hypothalamus. Note: M. DL2, 000 DNA Marker, 1. Normal control group; 2. β-Alanine stress group; 3. Stress group; 4.Taurine stress group

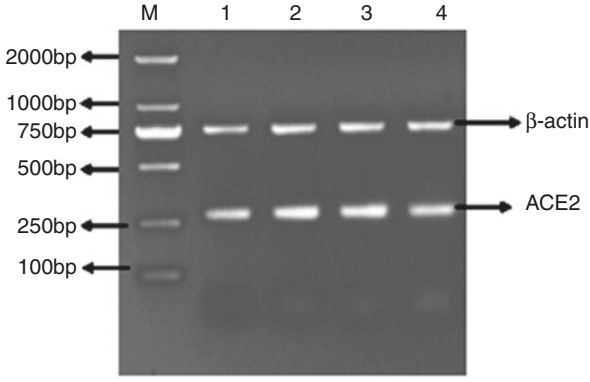

Fig. 5 The ACE2 mRNA expression in rat pituitary. Note: M. DL2, 000 DNA Marker, 1. Normal control group; 2. β-Alanine stress group; 3. Stress group; 4. Taurine stress group

3.1.3 The Result of HSP70 mRNA Expression

As shown in Table 4, Figs. 7, 8, and 9, electrophoresis analysis was used for RT-PCR products of mRNA in rat hypothalamus, pituitary and adrenal, and specific bands of HSP70 were shown in all trial groups, indicating that HSP70 gene transcription existed in rat hypothalamus, pituitary and adrenal. No significant differences were found in

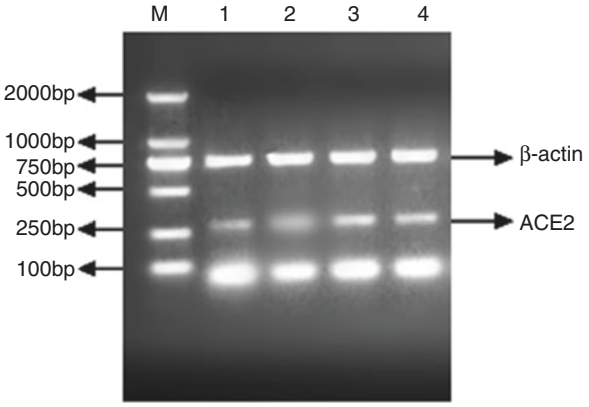

Fig. 6 The ACE2 mRNA expression in rat adrenal. Note: M. DL2, 000 DNA Marker, 1. Normal control group; 2. β-Alanine stress group; 3. Stress group; 4. Taurine stress group

Table 4 Taurine stress rat hypothalamus, pituitary and adrenal HSP70 gene expression

Group	Hypothalamus	Pituitary	Adrenal
Control group	10.28 ± 1.26^{a}	12.31 ± 2.10^{a}	12.13 ± 1.02^{a}
β-Alanine stress group	11.21 ± 1.63^{a}	17.53 ± 1.68^{b}	23.38 ± 1.09^{b}
Stress group	11.59 ± 1.82^{a}	22.29 ± 2.64^{c}	26.43 ± 1.78^{c}
Taurine stress group	11.72 ± 1.56^{a}	27.57 ± 1.16^{d}	32.36 ± 2.67^{d}

Note: Same letters indicate insignificant difference between two groups ($P > 0.05$). Different letters indicate significant difference between two groups ($P < 0.05$). The data in the table are mean ± standard deviation

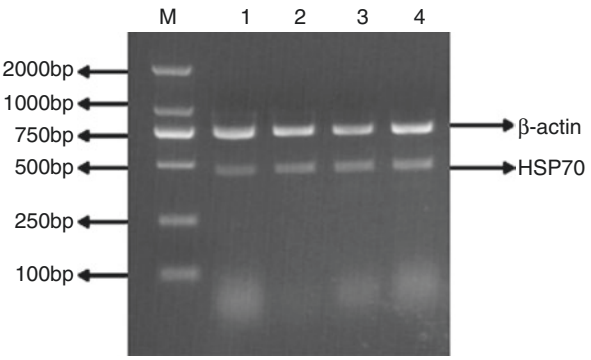

Fig. 7 The HSP70 gene mRNA expression in rat hypothalamus. Note: M. DL2, 000 DNA Marker, 1. Normal control group; 2. β-Alanine stress group; 3. Stress group; 4. Taurine stress group

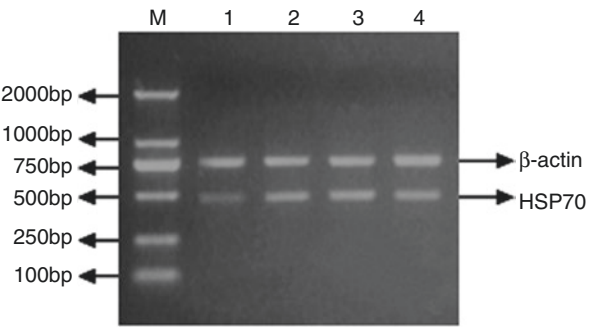

Fig. 8 The HSP70 gene mRNA expression in rat pituitary. Note: M. DL2, 000 DNA Marker, 1. Normal control group; 2. β-Alanine stress group; 3. Stress group; 4. Taurine stress group

HSP70mRNA expression of rat hypothalamus among all groups ($P > 0.05$). The highest HSP70mRNA expression of rat pituitary was observed in taurine stress group, higher than stress control group and β-alanine stress group, the lowest was in normal control group, and there were significant differences among the groups ($P < 0.05$). Similarly, taurine stress group showed the highest HSP70 expression, higher than stress control group and β-alanine stress group, and normal control group did the lowest. There were significant differences among the groups ($P < 0.05$).

3.2 Results of ACE, ACE2 and HSP70 Protein Expressions in Rat Hypothalamus, Pituitary and Adrenal

3.2.1 The Result of ACE Protein Expression

As shown in Table 5, Figs. 10, 11, and 12, the result of immunohistochemical staining suggested that ACE expression was scattered in hypothalamus tissues of normal control group and taurine stress group, that cell shading was lighter, and that grey value showed no significant differences ($P > 0.05$). However, stress control group displayed strongly positive ACE expression in hypothalamus tissues and cytoplasm

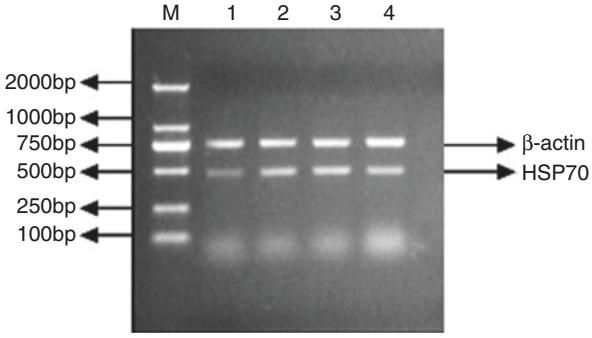

Fig. 9 The HSP70 gene mRNA expression in rat adrenal. Note: M. DL2, 000 DNA Marker, 1. Normal control group; 2. β-Alanine stress group; 3. Stress group; 4. Taurine stress group

Table 5 ACE protein immunohistochemical analysis results in rats

Group	IOD		
	Hypothalamus	Pituitary	Adrenal
Control group	1038.26 ± 186.09[a]	1198.63 ± 152.48[a]	1462.53 ± 172.31[a]
β-Alanine stress group	2937.31 ± 147.74[b]	3136.94 ± 201.34[b]	4259.65 ± 249.37[b]
Stress group	2676.29 ± 142.37[c]	2691.65 ± 199.27[c]	3159.16 ± 257.31[c]
Taurine stress group	1105.24 ± 98.78[a]	1302.64 ± 129.64[a]	1632.83 ± 182.06[a]

Note: Same letters indicate insignificant difference between two groups ($P > 0.05$). Different letters indicate significant difference between two groups ($P < 0.05$). The data in the table are mean ± standard deviation

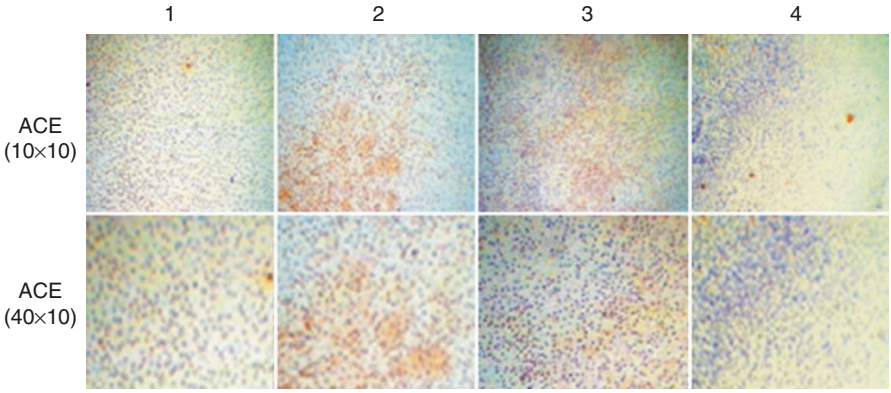

Fig. 10 The rat hypothalamus ACE protein immunohistochemical staining. Note: 1. Normal control group; 2. β-Alanine stress group; 3. Stress group; 4. Taurine stress group

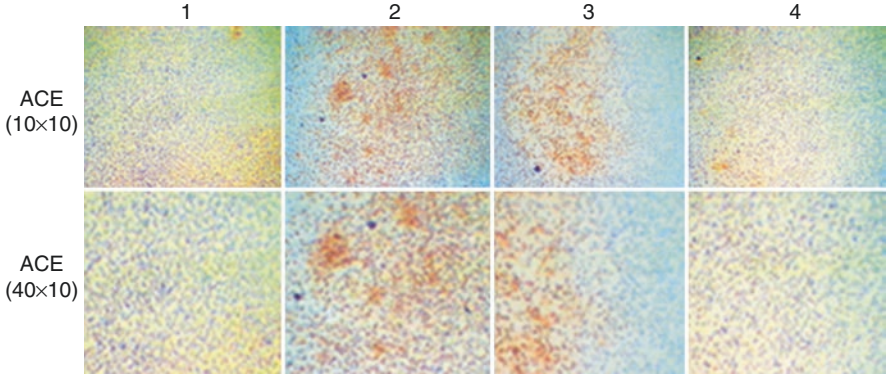

Fig. 11 The rat pituitary ACE protein immunohistochemical staining. Note: 1. Normal control group; 2. β-Alanine stress group; 3. Stress group; 4. Taurine stress group

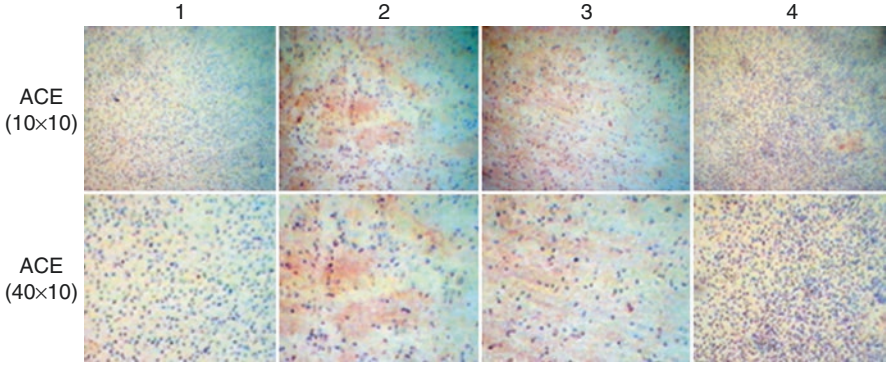

Fig. 12 The rat adrenal ACE protein immunohistochemical staining. Note: 1. Normal control group; 2. β-Alanine stress group; 3. Stress group; 4. Taurine stress group

was tan, and the positive cells increased more dramatically compared to taurine stress group ($P < 0.05$); the expression of ACE in β-alanine stress group was positive, and cytoplasm was claybank and significantly increased compared to other groups ($P < 0.05$). As for ACE protein expression of rat pituitary and adrenal tissues, β-alanine stress group performed the highest, significantly higher than other groups ($P < 0.05$). Significant differences were also observed in stress control group, normal control group and taurine stress group ($P < 0.05$), while there were no significant differences between taurine stress group and normal control group ($P > 0.05$).

3.2.2 The Result of ACE2 Protein Expression

The result of Immunohistochemical staining (Table 6, Figs. 13, 14, and 15) showed that ACE2 protein expression in rat hypothalamus decreased progressively in taurine stress group, stress control group, β-alanine stress group and normal control group, and exhibited either significant differences ($P < 0.05$) or no significant differences ($P > 0.05$) between different groups.

Table 6 ACE2 protein immunohistochemical analysis results in rats

	IOD		
Group	Hypothalamus	Pituitary	Adrenal
Control group	614.55 ± 76.74[a]	762.34 ± 59.87[a]	986.31 ± 102.34[a]
β-Alanine stress group	643.19 ± 81.31[a]	873.65 ± 71.32[ab]	2096.68 ± 118.94[b]
Stress group	725.62 ± 70.26[a]	939.64 ± 84.32[b]	2451.58 ± 149.63[c]
Taurine stress group	894.68 ± 56.23[b]	1081.56 ± 94.25[bc]	3623.06 ± 314.56[d]

Note: Same letters indicate insignificant difference between two groups ($P > 0.05$). Different letters indicate significant difference between two groups ($P < 0.05$). The data in the table are mean ± standard deviation

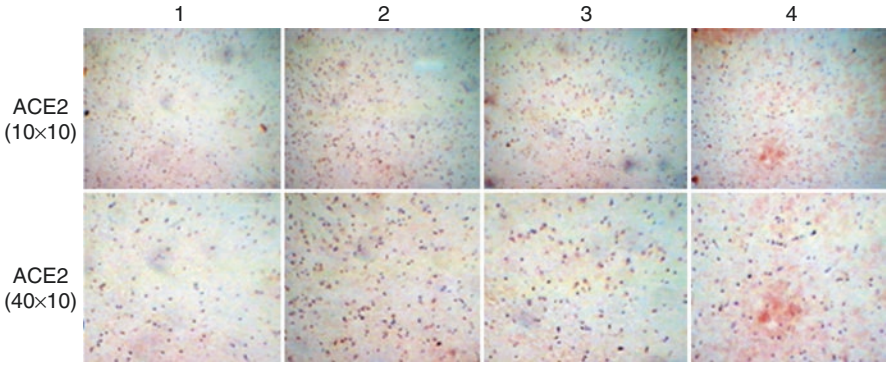

Fig. 13 The rat hypothalamus ACE2 protein immunohistochemical staining. Note: 1. Normal control group; 2. β-Alanine stress group; 3. Stress group; 4. Taurine stress group

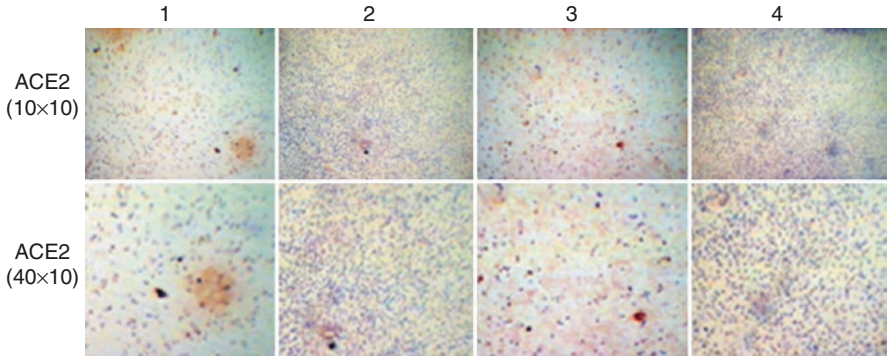

Fig. 14 The rat pituitary ACE2 protein immunohistochemical staining. Note: 1. Normal control group; 2. β-Alanine stress group; 3. Stress group; 4. Taurine stress group

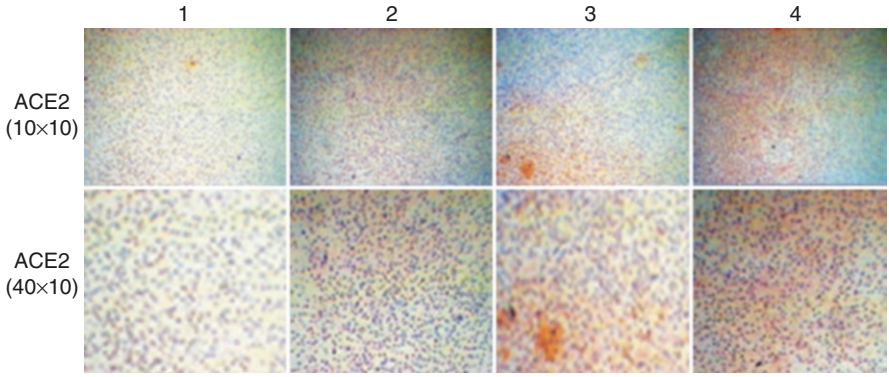

Fig. 15 The rat adrenal ACE2 protein immunohistochemical staining. Note: 1. Normal control group; 2. β-Alanine stress group; 3. Stress group; 4. Taurine stress group

3.2.3 The Result of HSP70 Protein Expression

As shown in Table 7, Figs. 16, 17, and 18, the result of immunohistochemical staining indicated that there were some differences in rat hypothalamus HSP70 protein expression, but overall no significant differences were observed ($P > 0.05$). Taurine stress group showed the highest HSP70 expression in rat pituitary, higher than stress control group, β-alanine stress group, and normal control group, while there were no significant differences between β-alanine stress group and stress control group ($P > 0.05$), likewise between stress control group and taurine stress group ($P > 0.05$). Significant differences were found among other groups ($P < 0.05$). Taurine stress group also observed the highest HSP70 expression in rat adrenal, higher than stress control group, β-alanine stress group, and normal control group, while there were no significant differences between β-alanine stress group and stress control group ($P > 0.05$). Significant differences were found among other groups ($P < 0.05$).

Table 7 HSP70 protein immunohistochemical analysis results in rats

Group	IOD		
	Hypothalamus	Pituitary	Adrenal
Control group	1236.9 ± 172.14[a]	1598.65 ± 148.41[a]	2475.68 ± 412.02[a]
β-Alanine stress group	1393.17 ± 129.97[a]	1872.47 ± 102.15[b]	3351.61 ± 169.32[b]
Stress group	1332.36 ± 159.56[a]	2024.04 ± 149.32[bc]	3749.74 ± 309.52[b]
Taurine stress group	1413.63 ± 144.68[a]	2278.55 ± 127.86[c]	4856.24 ± 314.52[c]

Note: Same letters indicate insignificant difference between two groups ($P > 0.05$). Different letters indicate significant difference between two groups ($P < 0.05$). The data in the table are mean ± standard deviation

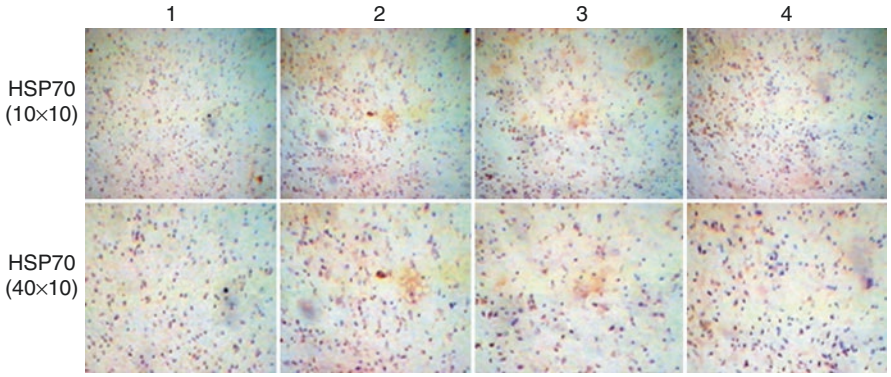

Fig. 16 The rat hypothalamus HSP70 protein immunohistochemical staining. Note: 1. Normal control group; 2. β-Alanine stress group; 3. Stress group; 4. Taurine stress group

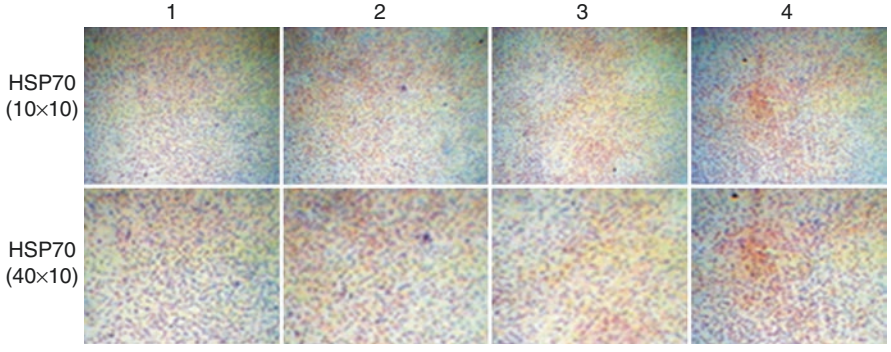

Fig. 17 The rat pituitary HSP70 protein immunohistochemical staining. Note: 1. Normal control group; 2. β-Alanine stress group; 3. Stress group; 4. Taurine stress group

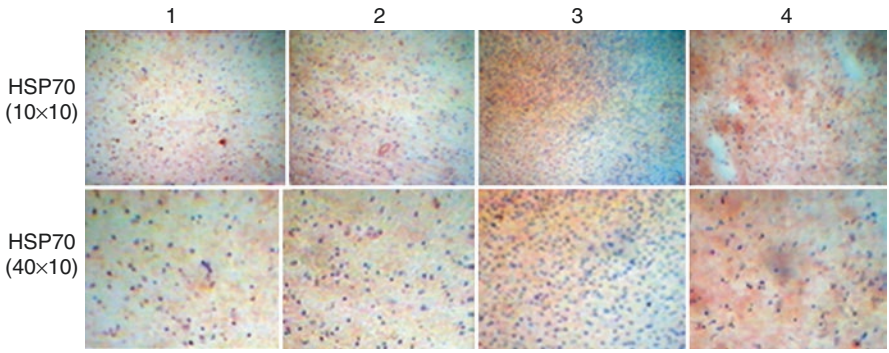

Fig. 18 The rat adrenal HSP70 protein *immunohistochemical staining*. Note: 1. Normal control group; 2. β-Alanine stress group; 3. Stress group; 4. Taurine stress group

4 Discussion

Hypertension is highly heterogeneous, most of which may result from genes and other multiple factors. It was reported by Maier early in the 1990s that animals were subjected to long-term high blood pressure in continuous stressful situations, resulting in dysfunction of central cardiovascular regulation. This type of hypertension was attributed to long-term stressful environment and therefore was known as stress-induced hypertension. It was currently believed that many genes in human beings and animals were involved in regulating and controlling body's blood pressure and electrolyte. It was possible that many means were employed to investigate the role of genes in hypertension incidence and development. RAAS, an important system for regulating and controlling blood pressure, was directly involved in vasoconstriction, metabolism and sympathetic regulation.

The early stage of the current experiment found that rats in stress control group significantly increased blood pressure when given discontinuous noise and foot shock, indicating that RAAS had been activated, and that taurine stress group could significantly inhibit high blood pressure (Lv et al. 2015). Our current study focused on the rat hypothalamus, pituitary and adrenal axis in RAAS as well as expressions of ACE and ACE2 genes and protein, showing that there were expressions of ACE and ACE2 genes in hypothalamus, pituitary and adrenal, and the expressions gradually increased from top to bottom. Taurine reduced ACE gene and protein expressions in rat hypothalamus, pituitary and adrenal, showed no significant differences in ACE2 gene expression in hypothalamus and pituitary, but significantly increased ACE2 gene expression in adrenal. Stress control group and β-alanine stress group reduced ACE2 expression more or less in rat hypothalamus, pituitary and adrenal, compared to normal control group, but increased ACE expression, compared to control groups. β-Alanine stress group inhibiting taurine performed the best; there were no significant differences between taurine stress group and normal control group. Moreover, the current study found that ACE2 expression in stress control group and β-alanine stress group may not be enough against over-activated ACE activity, thus causing rise in

blood pressure, whereas taurine stress group was administered taurine, which prompted ACE2 gene expression and inhibited ACE gene expression, thus contracting blood vessels, reducing the total peripheral vascular resistance, and inhibiting hypertension. Our study results conformed to the previous reports (Gallagher et al. 2003; Yamamoto et al. 2006; Diez-Freire et al. 2006), suggesting that taurine played a role in the prevention of stress-induced hypertension by affecting the HPA axis in RAAS.

Heat shock proteins (HSP) are a group of proteins that cells give priority to synthesize under stress, known as Stress Proteins, a family of conserved ubiquitously expressed heat shock proteins that exist in virtually all living organisms. Inducible expressions may occur at high temperatures, trauma, infection, hypoxia, heavy metal poisoning, oxygen free radicals, cancer and other stress (Burdon 1986). Many studies have shown that HSP70 generated in various stressful situations may enhance the tolerance of next damage, maintain normal cellular metabolism, and increase the survival rate of cells. Furthermore, HSP induced by heat shock could improve the ability to tolerate not only the current stimulus, but also other sources of stress, which was so-called "cross-protection" of HSP70 (Gabai et al. 1997). It was also reported that HSP70 may delay protecting cells, and that they did not protect cells until reaching a certain value and within limitation. Our study found that taurine showed no significant differences in HSP70 expression of rat hypothalamus, but promoted that of pituitary and adrenal, thereby playing a role in fighting against stress to somedegree.

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Taurine Protects Glutamate Neurotoxicity in Motor Neuron Cells

Na-Young Lee and Young-Sook Kang

Abstract Amyotrophic lateral sclerosis (ALS) is a fetal neurodegenerative disease that results in motor dysfunction and death. However, there is no cure or effective therapy for ALS. In our previous results, taurine protects motor neurons by repairing for constitutive oxidative stress in an ALS model. ALS is caused by multiple factors including inflammation, oxidative stress, mitochondrial dysfunction, apoptosis, glutamate excitotoxicity and proteasomal dysfunction. Especially, glutamate excitotoxicity has been well known as a mediator in the disease process, and may occur from changes in the excitability of the neurons being stimulated. D-serine is known to a key factor of determination on glutamate toxicity in ALS. Therefore, in the present study, we investigated neuroprotective effects of taurine from glutamate excitotoxicity using motor neuron cells, mtSOD1 (G93A) transgenic cell line model of ALS (NSC-34/hSOD1G93A cells). We evidenced that taurine protects cultured motor neurons from neurotoxic injury. Our findings indicated that taurine has neuroprotective properties and may be a good candidate for therapeutic trials in ALS.

Keywords Taurine • Serine • Amyotrophic lateral sclerosis • Motor neuron-like cell line • Glutamate toxicity

Abbreviations

ALS	Amyotrophic lateral sclerosis
MTT	3-(4,5-Dimethyldiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NMDA	N-methyl-D-aspartate
NSC34	Motor neuron-like cell line
TAUT	Taurine transporter

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1 Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the death of motor neurons in the nervous system (Jung et al. 2013). The pathogenesis of selective motor neuron degeneration in ALS remains unclear. So far, glutamate-induced excitotoxicity is a major contributor to motor neuron degeneration in the pathogenesis of ALS. It was caused by mitochondrial dysfunction, oxidative stress, and protein aggregation (Blasco et al. 2014). Moreover, riluzole only has proven neuroprotective effect as an anti-excitotoxic agent for ALS (Blasco et al. 2014). D-serine is an agonist at the N-methyl-D-aspartate (NMDA) receptors mediating both physiological actions of glutamate and also pathological effects mediated by excitotoxicity (Mothet et al. 2000). Recently, it was found that D-serine levels are elevated in sporadic ALS and G93A SOD1 mouse model of ALS (Sasabe et al. 2007, 2012).

Taurine, a free sulfonic acid, plays several roles in the CNS including acting as a neuromodulator, a neuroprotector, and an antioxidant (Foos and Wu 2002; Pan et al. 2010; Sun et al. 2011). Recently, levels of taurine were found to be increased in the motor cortex and the spinal cord of ALS patients, suggesting that it may be affected the disease progresses in ALS patients (Yoshino et al. 1979; Iłzecka et al. 2003; Jung et al. 2013).

Intracellular levels of taurine in mammalian tissues are very high compared to in plasma levels (Mozaffari et al. 2006). Taurine supplies to the tissues are directly mediated by Na^+, Cl^--dependent taurine transporter (TAUT) (Kang et al. 2002). This TAUT is involved in the maintenance of the high levels of taurine in many tissues. Therefore, in present study, we first investigated whether taurine protects motor neuron by suppressing glutamate-induced neurotoxicity with or without D-serine using MTT assay in motor neuron like cell line, NSC34. We further examined whether TAUT expression and activity was regulated by glutamate-induced neurotoxicity or/and D-serine in NSC34 cells.

2 Methods

2.1 Cell Culture

Motor neuron-like cell line (NSC34) was culture according to the previous report (Jung et al. 2013).

2.2 Glutamate Cytotoxicity and Determination of Motor Neuron Survival

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltertazolium bromide (MTT) assay was performed for determination of cell viability. NSC34 cells were pretreated with

glutamate (100 μM) and/or D-serine (10 μM) with/without taurine (10 mM). The absorption values were read using a microplate spectrophotometer at 550 and 630 nm.

2.3 Real-Time Reverse Transcription Polymerase Chain Reaction

According to the manufacturer's instructions, from cultured NSC34 cells isolated total RNA using the RNeasy kit from Quiagen (Quiagen, Valencia, CA) and total RNA was reverse-transcribed by RT kit (Applied Biosystems, Foster City, CA). TAUT mRNA was quantified using an ABI 7500 Sequence Detector System (Applied Biosystems, Foster City, CA). Five microliters of complementary DNA (cDNA) were used for real-time PCR.

2.4 [^{3}H]Taurine Uptake Study in NSC34 Cells

Uptake of [^{3}H]taurine was done according to the previous report (Lee et al. 2012). [^{3}H]Taurine uptake was performed by addition of ECF buffer containing [^{3}H] taurine (28 nM) at 37 °C. Incubation time was 5 min. After uptake experiment, radioactivity was counted by liquid scintillation counter (LS6500; Beckman, Fullerton, CA).

2.5 Statistic Analysis

One-way ANOVA with Dunnett's post-hoc test were used for statistical significance. The value was expressed as the mean ± SEM.

3 Results

3.1 Taurine Prevents Glutamate-Induced Motor Neuron Death

Whether taurine protects the glutamate-induced neurotoxic effect was investigated in motor neuron like cell line, NSC34 cells. The treatment of 100 μM glutamate for 24 h significantly generated the cell death (~60% of cell viability compared to that of control) (Fig. 1). However, co-administration with 10 mM taurine completely recovered glutamate-induced cell death (Fig. 1).

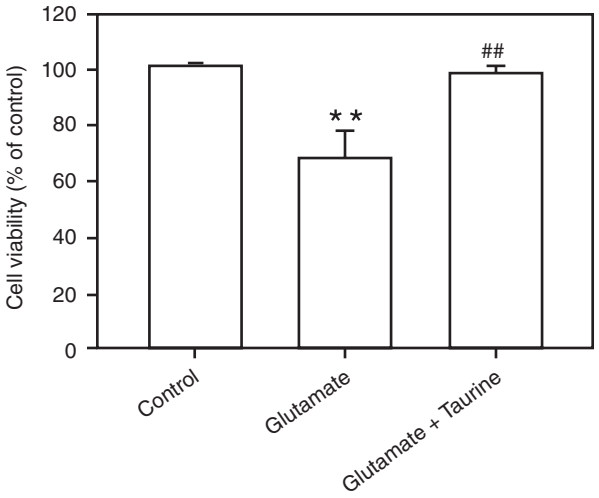

Fig. 1 Taurine eliminated glutamate-induced neurotoxicity in NSC34 cells. The cells were incubated with 100 μM glutamate with/without 10 mM taurine for 24 h, and viability was then measured by MTT assay. The value represents the mean ± SEM (n = 3). $^{**}p < 0.01$, versus control; $^{\#\#}p < 0.01$, versus glutamate treatment

3.2 Taurine Prevents D-serine-Induced Motor Neuron Death

Next, we examined the neuroprotective effect of taurine on D-serine-induced cytotoxicity in NSC34 cells. As shown in Fig. 2, pre-treatment of 10 μM D-serine for 24 h very highly caused the cell death (~70% of cell viability compared to that of control). Pre-incubation with taurine inhibited motor neuronal cell death formed by D-serine-induced cytotoxicity (Fig. 2).

3.3 Taurine Prevents Glutamate and D-serine-Induced Motor Neuron Death

When the cells were pre-treated for 24 h with both glutamate and D-serine together, cytotoxic effect was significantly induced compared to that of alone in NSC34 cells (Fig. 3). However, the synergic effect by both substance was not big. Pre-incubation with taurine also restored motor neuronal cell death caused by both glutamate and D-serine (Fig. 3).

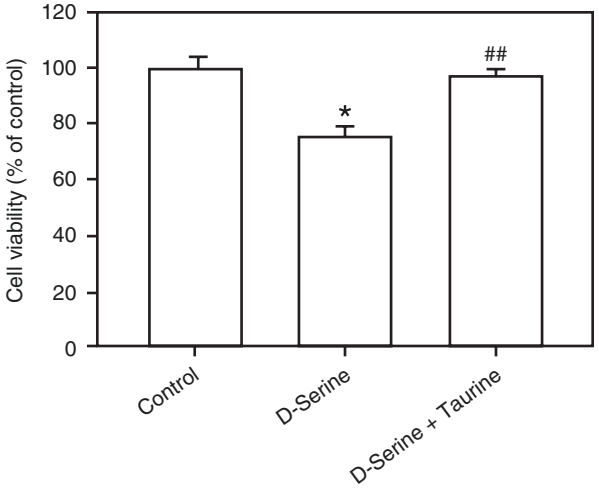

Fig. 2 Taurine restored cell viability against by D-serine treatment in NSC34 cells. The cells were incubated with 10 μM D-serine only or with 10 mM taurine for 24 h, and cell viability was measured by MTT assay. The value represents the mean ± SEM (n = 3). *p < 0.05, versus control; ##p < 0.01, versus glutamate treatment

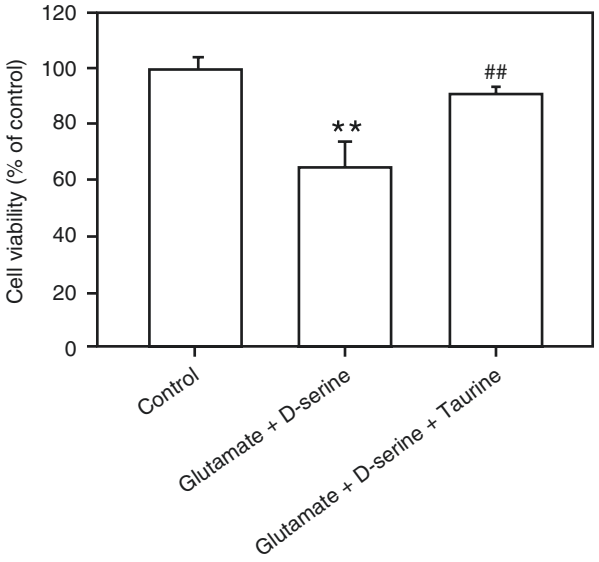

Fig. 3 Taurine removed glutamate and D-serine-induced neurotoxicity in NSC34 cells. The cells were incubated with 100 μM glutamate and 10 μM D-serine or adding 10 mM taurine for 24 h, and viability was measured by MTT assay. The value represents the mean ± SEM (n = 3). **p < 0.01, versus control; ##p < 0.01 versus glutamate treatment

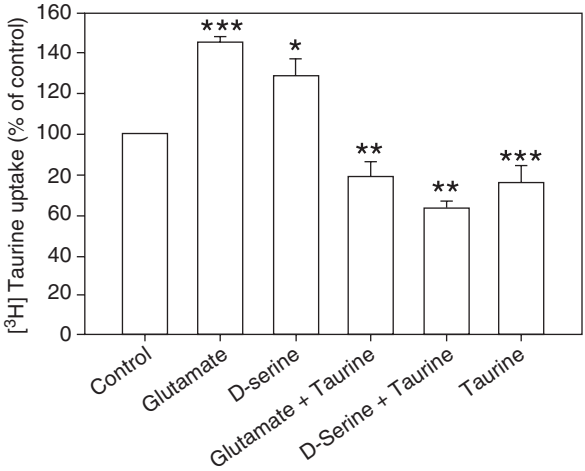

Fig. 4 Effect of glutamate or/and D-serine on [^{3}H]taurine uptake in NSC34 cells. The cells were incubated with both 100 μM glutamate or 10 μM D-serine in the absence or presence of 10 mM taurine for 24 h. [^{3}H]Taurine uptake was executed with ECF buffer containing [^{3}H]taurine (28 nM) for 5 min at 37 °C. The value represents the mean ± SEM (n = 3). *p < 0.05, **p < 0.01, **p < 0.001, versus control

3.4 The Taurine Uptake Is Increased by Glutamate in NSC34 Cells

To further confirm the control of taurine transport by glutamate or/and D-serine, we carried out cellular uptake study using radiolabeled taurine ([^{3}H]taurine) in NSC34 cells. [^{3}H]Taurine uptake by NSC34 cells was significantly higher at pre-treatment of glutamate or/and D-serine than in control (Fig. 4). In contrast, after 10 mM taurine treatment for 24 h, [^{3}H]taurine uptake by NSC34 cells decreased as compared with that of control (Fig. 4). Also, co-treatment of taurine reduced the increment of [^{3}H]taurine uptake induced by glutamate or/and D-serine (Fig. 4). These phenomena were consistent with results of TAUT mRNA expression in NSC34 cells.

3.5 The Expression of TAUT Is Increased by Glutamate in NSC34 Cells

We performed whether glutamate or/and D-serine altered expression of taurine transporter, TAUT in NSC34 cells using RT-PCR. TAUT gene expression was significantly elevated at 100 μM glutamate, as compared with that of control (Fig. 5). Pre-treatment for 24 h with glutamate and D-serine increased levels of TAUT mRNA

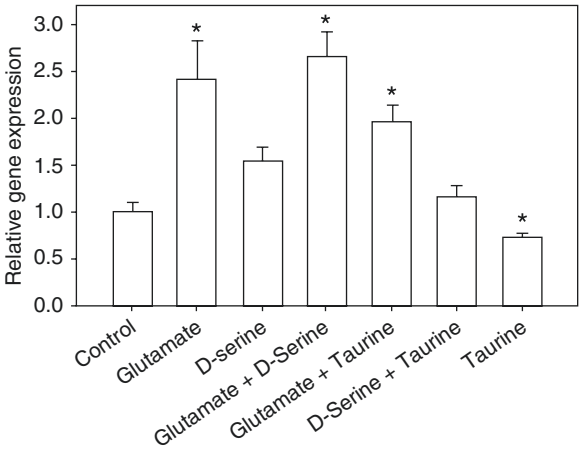

Fig. 5 Effects of glutamate or/and D-serine on the mRNA expression of TAUT in NSC34 cells, using RT-PCR analysis. The cells were incubated with 100 μM glutamate and 10 μM D-serine or adding 10 mM taurine for 24 h. The value represents the mean ± SEM (n = 3). *p < 0.05, versus control

in cells as compared with control (Fig. 5). In contrast, after 10 mM taurine treatment for 24 h, the TAUT mRNA level decreased as compared with control (Fig. 5). Also, co-treatment of taurine diminished the increment of TAUT expression caused by glutamate (Fig. 5).

4 Discussion

Motor neurons are particularly susceptible to excitotoxicity was compared with other neurons (Pandya et al. 2013). Glutamate-induced excitotoxicity leading to motor neuron death is a core pathogenic mechanism of ALS (Plaitakis et al. 1988; Rothstein et al. 1995). In addition, D-serine, which is an activator at the NMDA glutamate receptors, is elevated in ALS patients and also in the G93A SOD1 mouse model of ALS (Sasabe et al. 2007, 2012). It was found that these abnormal amounts of D-serine contribute to the progress of glutamate toxicity in ALS model mice (Sasabe et al. 2007). Furthermore, D-serine has been shown to enhance the binding affinity of glutamate to the NMDA receptors (Crow et al. 2012). Our study also showed the cytotoxic effect by glutamate and D-serine in motor neuron cell line (Figs. 1, 2, and 3). When the cells were pre-treated with both glutamate and D-serine together, cytotoxic effect was increased compared to that of alone (Fig. 3). Furthermore, we found that taurine protected motor neurons against glutamate or/and D-serine induced toxicity *in vitro* (Figs. 1, 2, and 3). Taurine has been investigated the therapeutic effects in experimental models of several neurodegenerative diseases such as stroke, Alzheimer's disease, and Huntington's disease. Taurine has been shown to have neuroprotective activity to H_2O_2 induced cell injury (Leon et al. 2009; Jung et al. 2013). Previously, it was also demonstrated that taurine protected against glutamate toxicity (Prentice et al. 2015). It was reported that taurine

inhibited glutamate induced calcium influx through L-, P/Q, and N-type voltage gated calcium channels and the NMDA receptor channel (Wu et al. 2005; Prentice et al. 2015). In our results, taurine protected motor neurons from glutamate or/and D-serine induced toxicity effectively, so taurine may be a good candidate for therapeutic trial in motor neuron diseases such as ALS. The investigation of mechanisms of taurine neuroprotection against D-serine induced toxicity is needed in further studies.

Intracellular taurine concentration is maintained by TAUT is known to be regulated by glucose, oxidative stress, and changes in osmolality (Kang et al. 2002; Lee and Kang 2013). In previous study, we demonstrated that levels of taurine were increased via induction of TAUT expression in the motor cortex and the spinal cord of ALS patients (Jung et al. 2013). In our study, we also found that expression and activity of taurine transport in NSC34 cells were significantly higher at pre-treatment of glutamate or/and D-serine than in control (Figs. 4 and 5). These results showed that altered expression and activity of TAUT can contribute to motor neuron survival under glutamate or/and D-serine toxicity. Consequently, elevated intracellular taurine levels reduced motor neuronal death in the context of cellular stress. It was also shown that chronic deficit in the NMDA receptor function produced by diminution in the extracellular D-serine levels could result in the reduction of the basal extracellular taurine release (Ishiwata et al. 2015). Therefore, the activation of the TAUT pathway in response to glutamate-induced excitotoxicity may be one of the protective responses that prevent motor neuron death.

5 Conclusion

In conclusion, our study indicates that taurine has potent neuroprotective effects against excitotoxicity induced by glutamate in motor neuronal cell lines. The intrinsic mechanism of these effects is unclear yet, however, our results suggest that taurine may be a good candidate for therapeutic trial in motor neuron diseases such as ALS.

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Role of Taurine in BDE 209-Induced Oxidative Stress in PC12 Cells

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Abstract Polybrominated diphenyl ethers (PBDEs) are globally dispersed throughout the environment, and the levels of some PBDEs in the environment may still be increasing. Previous studies showed that BDE 209 exerted neurodevelopmental and neurobehavioral effects in humans and animals. Oxidative stress is a common mechanism reported in PBDEs-induced neurotoxicity. Taurine, as an antioxidant, whether it is effective in alleviating BDE 209-induced neurotoxicity is still unknown. PC12 cells were exposed to various concentrations of BDE 209 (6.25, 12.5, 25, 50, and 100 μM). 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was used to assess the cell viability. 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) detector was used to explore the production of ROS. Acridine orange was used to reflect the permeation of lysosomal membrane. Rhodamine 123 was used to reflect the permeation of mitochondrial membrane. Lactate dehydrogenase and catalase in PC12 cells exposed to BDE 209 were examined by kits. The results showed that taurine could significantly reverse the decreased viability, the serious oxidative stress and abnormal autophagy in PC12 cells exposed to BDE 209. Collectively, our results indicated that taurine could protect PC12 cells from BDE 209-induced neurotoxicity by alleviating oxidative stress.

Keywords BDE 209 • Oxidative stress • Taurine • PC12

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Abbreviations

CAT	Catalase
LDH	Lactate dehydrogenase
PBDEs	Polybrominated diphenyl ethers
ROS	Reactive oxygen species

1 Introduction

Polybrominated diphenyl ethers (PBDEs) are flame retardants that were widely used in many consumer products such as electronic equipment, textiles, upholstered furniture and plastics (Rahman et al. 2001). PBDEs could be easily released into the environment, such as emissions from the manufacture of PBDE-containing products and from the products themselves. Consequently, these chemicals are detected in samples of house dust and air extensively. PBDEs revealed a potential exposure for human (Frederiksen et al. 2009). Of the 209 possible PBDE congeners, penta-BDE, octa-BDE and deca-BDE are typically detected in the environment (La Guardia et al. 2006). Though the lower brominated forms are highly toxic and are presently not in commercial use, the highest brominated (2,2′,3,3′,4,4′,5,5′,6,6′-decabromodiphenyl ether, BDE 209) remains in use and can debrominate to more toxic lower brominated congeners. As a congener of PBDEs, BDE 209 had been found in biological samples, and the level of which has been increasing in human breast milk and blood serum (Sarkar et al. 2015). It was reported that BDE 209 could induce the toxic effect on endocrine and central nervous system (CNS), and especially the neurobehavioral effects. Human epidemiological evidence and animal studies both showed that BDE 209 could induce neurodevelopmental and neurobehavioral effects (Costa and Giordano 2011). Hence, it is urgent to find substitutions or prevention to protect biological systems from toxicity induced by BDE 209.

Oxidative stress is a common mechanism reported in PBDEs-induced neurotoxicity both *in vivo* and *in vitro*. In particular, several PBDEs have been shown to cause oxidative stress in neurons, leading to apoptotic neuronal death (Tagliaferri et al. 2010). In addition to apoptosis, autophagy is another way of program cell death, which is important in eliminating toxic protein aggregates, defective organelles and pathogens from cells, as well as intracellular pathogens. It is reported that diverse stimuli can induce autophagy including nutritional depletion, endoplasmic reticulum (ER) stress, hypoxia, hyperoxia, mitochondrial damage, oxidative stress. Previous study in our lab showed that BDE 47 could induce autophagy related to oxidative stress in HepG2 cells (Liu et al. 2015). However, autophagy had not been reported in the neurotoxicity induced by BDE 209.

Taurine (2-aminoethanesulfonic acid) is an amino acid, and could be synthesized from cysteine, which is toxic in mammals. It was reported that taurine is important for brain by the facts that it not only protects neurons against pollutants-induced

cytotoxicity, and also benefits neuronal proliferation and synaptogenesis (Sayed et al. 2012). As an antioxidant, whether taurine is effective in alleviating BDE 209-induced neurotoxicity is still unknown.

In the present study, the role of taurine in BDE 209-induced oxidative stress in PC-12 cells was evaluated by the indexes of cell viability, ROS production, mitochondrial membrane potential, lysosomal membrane permeability, lactate dehydrogenase and catalase.

2 Methods

2.1 Chemicals

BDE 209 (99% purity) was supplied by Chem Service (PuertoArmuelles, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA), acridine orange, Rhodamine 123 and the kits for lactate dehydrogenase and catalase were purchased from Beyotime Institute of Biotechnology in China. All the other chemicals were analytical degrade.

2.2 Cell Culture

The rat pheochromocytoma cell line (PC12) was purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences. PC12 cells were cultured and grown in antibiotic-free Dulbecco's modified Eagle medium (DMEM) (Hyclone, USA) supplemented with 10% horse serum and 5% fetal bovine serum (FBS) (Hyclone, USA). Cells were trypsinized with 0.125% Trypsin-EDTA (Beyotime, Chine), subcultured, and maintained in a humid incubator (37 °C, 5% CO_2).

2.3 Determination of Cell Viability

The cell viability was determined by MTT (tetrazolium) assay (Tang et al. 2004). PC12 cells were seeded at the density of 1×10^4 cells per well in 96-well plates. After the cells were treated with PFOS, 100 μL (0.5 mg/mL) 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added to the cell for a further 2 h incubation at 37 °C, 5% CO_2. Then the medium was discarded and 100 μL dimethyl sulfoxide (DMSO) was added to dissolve the formazan blue formed by mitochondria reducing in the cell. The cell viability was determined by measuring the absorbency of the DMSO-dissolved solution at 570 nm with ELISA Reader

(DG-I, China). At least three independent experiments were performed to determine the percentage of viable cells.

2.4 Measurement of Reactive Oxygen Species (ROS) Production

The level of intracellular ROS was quantified by Reactive Oxygen Species Assay Kit. DCFH-DA, a fluorescent probe, is oxidized by reactive oxygen species in viable cells to 2′,7′-dichlorofluorescein (DCF). With designated treatment, the same density of PC12 cells were harvested by cell counting. The cells were then incubated with 100 μM DCFH-DA (dissolved in DMSO) for 30 min at 37 °C. After three times washes with PBS, the relative levels of fluorescence were quantified by a multi-detection microplate reader (485 nm excitation and 535 nm emission).

2.5 Mitochondrial Membrane Potential (MMP)

A fluorescent dye, Rhodamine 123, was used to determine the effect of BDE 209 on MMP in PC12 cells as described elsewhere (Shaki et al. 2012). Rhodamine 123 is a specific fluorescent cationic dye, and could be readily sequestered by active mitochondria with dependence of transmembrane potential, and therefore, the decrease in the fluorescence can directly reflect the decrease in MMP. Briefly, PC12 cells were cultured in a 6-well plate at a density of 2×10^5 mL^{-1} overnight. Then, the cells were treated with BDE 209. After being washed with PBS, the cells was resuspended in 2 mL of fresh medium containing1.5 μM Rhodamine 123, and incubated at 37 °C for 15 min. Then, the cells were washed with PBS to remove the unbound dye and measured at 490 ex/520 em in a fluorescence spectrophotometer.

2.6 Lysosomal Membrane Permeability (LMP)

The effect of BDE 209 on lysosomal membrane stability in PC12 cells was evaluated by acridine orange (AO). AO is a fluorescent dye used to reflect changes of pH by reversibly accumulating into acidified membrane-bound compartments, such as lysosomes. Briefly, PC12 cells were treated with BDE 209. Then the cells were washed twice with PBS and incubated with AO at a final concentration of 5 μM at 37 °C in the dark for 15 min. The cells were washed with PBS to remove the fluorescent dye and resuspended in PBS. The changes in lysosomal membrane stability were measured by a fluorescence spectrophotometer at 495 ex/530 em.

2.7 Lactate Dehydrogenase and Catalase

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme that could be rapidly released into the cell culture medium when the integrity of plasma membrane is damaged. In brief, the culture medium was collected after BDE 209 exposure for 24 h. And LDH reagent was added. The release of LDH into culture medium was measured at 490 nm, and was defined as 100%. CAT is an important antioxidant enzyme in alleviating oxidative stress. Hence, the activity of CAT was also evaluated in the present study. For CAT activity, cell extract samples were measured with hydrogen peroxide to generate N-4-antipyryl-3-chloro-5-sulfonate-p-benzoquinone monoimine, which absorbed maximally at 520 nm.

2.8 Statistical Analysis

All results were expressed as mean ± standard error, and statistical analysis was performed by one-way analysis of variance (ANOVA), followed by LSD and Dunnett's T3 test using SPSS 13.0 (SPSS Inc, Chicago, IL). Differences were considered statistically significant at $P < 0.05$.

3 Results

3.1 Effect of Drugs Treatment on Cytotoxicity in Neuronal Differentiated PC12 Cells

As shown in Fig. 1, BDE 209 at concentrations of 25 μM or over could significant decrease the viability of PC12 cells, compared with the control group ($P < 0.05$). For example, about 20% of PC12 cell population was decreased in 50 μM BDE 209 exposed groups. Taurine pretreatment could increase the viability of PC12 cells exposed to 50 μM BDE 209 significantly ($P < 0.05$).

3.2 Effect of Drugs Treatment on ROS Production in Neuronal Differentiated PC12 Cells

DCFH-DA dye was used in the present study to detect the production of ROS. And the result in Fig. 2 showed that 50 μM BDE 209 could increase the production of ROS significantly, compared with the control group ($P < 0.05$). And pretreatment with taurine could decrease the production of ROS induced by BDE 209 exposure alone.

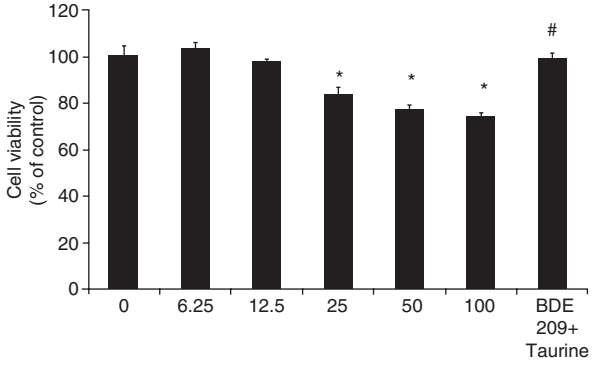

Fig. 1 Effect of taurine on BDE 209-induced cytotoxic effect in neuronal differentiated PC12 cells. *: compared with the control group. $p < 0.05$; #: compared with the BDE 209 exposure alone. $p < 0.05$

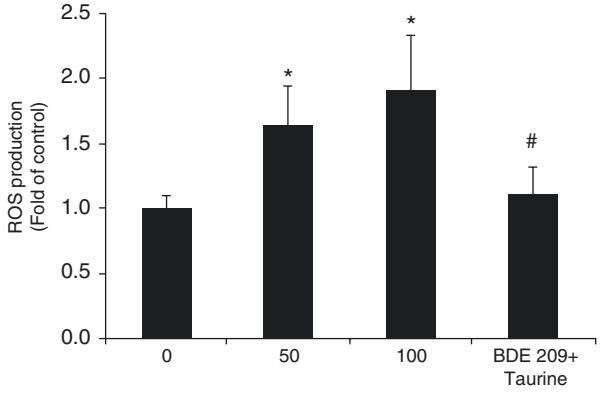

Fig. 2 Effect of taurine on BDE 209-induced ROS production in neuronal differentiated PC12 cells. *: compared with the control group. $p < 0.05$; #: compared with the BDE 209 exposure alone. $p < 0.05$

3.3 Effect of Drugs Treatment on MMP in Neuronal Differentiated PC12 Cells

The results in Fig. 3 showed that 50 μM BDE 209 exposure could decrease MMP of PC12 cells significantly, compared with the control ($P < 0.05$). Pretreatment with taurine, MMP in PC12 cells exposed to BDE 209 alone was increased significantly ($P < 0.05$).

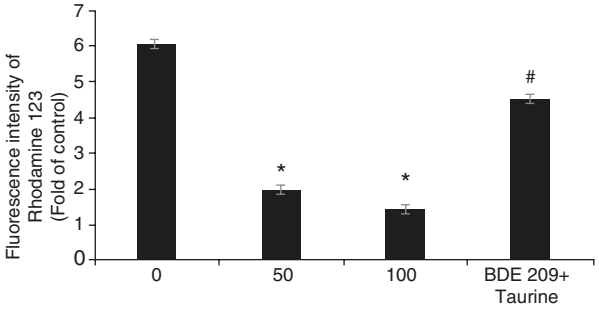

Fig. 3 Effect of taurine on BDE 209-induced MMP in neuronal differentiated PC12 cells. *: compared with the control group. $p < 0.05$; #: compared with the BDE 209 exposure alone. $p < 0.05$)

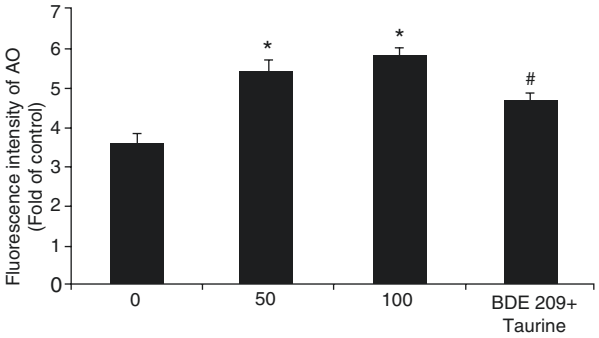

Fig. 4 Effect of taurine on BDE 209-induced LMP in neuronal differentiated PC12 cells. *: compared with the control group. $p < 0.05$; #: compared with the BDE 209 exposure alone. $p < 0.05$

3.4 Effect of Drugs Treatment on Autophagy in Neuronal Differentiated PC12 Cells

The dye acridine orange is a weak base that can enter autophagic vacuoles (and other acidic compartments) and become protonated thereby emitting fluorescence in the red range. In Fig. 4, the red fluorescence intensity was increased with the concentration of BDE 209 increased. However, the fluorescence intensity was decreased in PC12 cells pretreated with taurine.

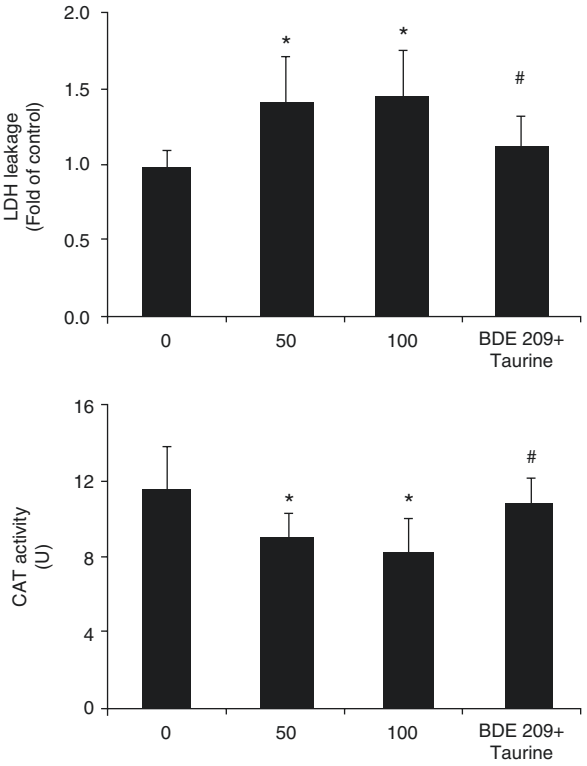

Fig. 5 Effect of taurine on BDE 209-induced LDH and CAT in neuronal differentiated PC12 cells. *: compared with the control group. $p < 0.05$; #: compared with the BDE 209 exposure alone. $p < 0.05$

3.5 Effect of Drugs Treatment on Lactate Dehydrogenase and Catalase in Neuronal Differentiated PC12 Cells

As shown in Fig. 5a, 50 or 100 μM BDE 209 increased the leakage of LDH significantly, compared with the control ($P < 0.05$). Then, PC12 cells pretreated with 80 μM taurine could decrease the leakage of LDH induced by BDE 209 exposure alone significantly ($P < 0.05$). In Fig. 5b, 50 or 100 μM BDE 209 decreased the activity of CAT significantly, compared with the control ($P < 0.05$). Then, PC12 cells pretreated with 80 μM taurine could increase the activity of CAT induced by BDE 209 exposure alone significantly ($P < 0.05$).

4 Discussion

As a group of synthetic organic flame retardants, polybrominated diphenyl ethers (PBDEs) have been widely used for several decades. Among 209 congeners, pentabromodiphenyl ethers (penta-BDEs), octabromodiphenyl ethers (octa-BDEs) and

decabromodiphenyl ethers (BDE 209) have been produced for commercial use. And it was reported that BDE 209 comprised 82% of the PBDEs present in electronic products, electrical appliances and industry products globally (Liang et al. 2010). PBDEs are lipophilic, persistently exist in the environment, and easily bio-accumulated in the food chain. Consequently, more attention has been focused on the potential toxicity of BDE 209, especially the neurotoxicity. The present studies showed that BDE 209 could decrease the viability and arouse oxidative stress in PC12 cells. Then, taurine pretreatment could alleviate the toxic effect induced by BDE 209.

ROS is mostly produced in mitochondria and a sensitive index in oxidative stress. ROS has been known to be a protector for bacterial invasion, while the stimulated production of ROS was originally called "the respiratory burst" because of the increased consumption of oxygen (Suzuki et al. 2011). Increased production of ROS could exhibit oxidative stress, inducing damage in DNA, lipids and proteins (Apel and Hirt 2004). CAT is one of the enzyme in defensing for these damage. In accordance with these mentioned mechanisms, the result showed that BDE 209 increased the production of ROS, caused the leakage of LDH, and decreased the activity of CAT in PC12 cells. And decreased mitochondria membrane potential in PC12 cells induced by BDE 209 was also companied, which further supported the destruction of oxidative stress. However, the toxic effects could be reversed in PC12 cells pretreated with taurine.

It was reported that oxidative stress leading to cell death is an acceptable mechanism for the neurotoxicity induced by BDE 209, though death-receptor pathway is also reported (Chen et al. 2016). Autophagy is another way of programmed cell death. Recently, autophagy is reported to be involved in pollutant-induced abnormal apoptosis caused by ROS production (Bodas et al. 2016). Autophagy is recognized as an evolutionary conserved pathway, keeping cellular homeostasis through removal and recycling of damaged macromolecules and organelles. However, increased autophagy is a way of leading to cell death. Our previous study showed that BDE 47 could induce autophagy in HepG2 cells depicted by increased lysosomal membrane permeability (Liu et al. 2015). In the present study, autophagy could also be observed in PC12 cells exposed to BDE 209. And taurine could alleviate the autophagy induced by BDE 209.

Considering the possible role of oxidative stress in apoptosis and autophagy, these aforementioned results indicated that oxidative stress exerted by BDE 209 could also be intervened by taurine, similar as N-acetylcysteine (Zhang et al. 2010).

5 Conclusion

In conclusion, the developmental neurotoxicity induced by BDE 209 has been received much concerns. Hence, finding the substitutions or prevention to protect biological systems from toxicity induced by BDE 209 is necessary. In the present study, the role of taurine in BDE 209-induced oxidative stress was investigated. And the results found that decreased cell viability, aggravated oxidative stress and autophagy could be alleviated in PC12 cells exposed to BDE 209 by taurine

pretreatment. The finding that taurine is an effective antioxidant in BDE 209-induced oxidative stress supply a clue in improving the toxicity induced by BDE 209.

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Protection of Taurine Against PFOS-Induced Neurotoxicity in PC12 Cells

Chunna Li, Xiaohui Liu, Qi Liu, Shuangyue Li, Yachen Li, Hong Hu, and Jing Shao

Abstract As a new member of persistent organic pollutants, the potent neurotoxicity of perfluorooctane sulfonates (PFOS) found in epidemiological studies and laboratory research has drawn increasing attention around the world. Previous studies showed that apoptosis driven by oxidative stress and autophagy were both observed in PFOS-induced toxicity. Taurine has been demonstrated to exert potent protections against oxidative stress as an efficient antioxidant. Whether taurine could protect against the PFOS neurotoxicity is not known. In the present study, PC12 cells were treated with several concentrations of PFOS (31.25, 250 μM) for 24 h. 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was applied to assess the cell viability. DCFH-DA detector was used to explore the production of ROS. Caspase 3 activity was used to reflect the possible apoptosis pathway. The lyso-tracker red dying was invited to evaluate the autophagy. Our data showed that taurine could significantly reverse the decreased viability and the increased ROS production in PC12 cells treated with PFOS. Moreover, the increased autophagy and apoptosis elicited by PFOS in PC12 cells could also be attenuated by taurine. Collectively, our results indicate that taurine may be an effective antioxidant in fighting against PFOS cytotoxicity and therefore could potentially serve as a preventative and therapeutic agent for environmental pollution-related toxicities.

Keywords Taurine • Perfluorooctane sulfonates (PFOS) • PC12 cells • Neurotoxicity • Antioxidant

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Abbreviations

MTT	4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
PFOS	Perfluorooctane sulfonates
ROS	Reactive oxygen species

1 Introduction

Perfluoroalkyl substances (PFASs) are a class of organic chemicals that most of them are synthetic chemicals (Conway et al. 2016). All the hydrogen atoms of the hydrocarbon backbone in PFASs are substituted by fluorine atoms. Due to the stability of C-F bond, PFASs were widely used in industrial and commercial applications, such as electrical wiring, clothing and food package (Zhou et al. 2016). Then, PFASs were widely found and detected in the environment, even in the biological specimens (tissues and serum) of animals and human life. Among these PFASs, perfluorooctane sulfonates (PFOS) is the representative of perfluorochemicals, which has a sulfonyl in the end of the structure. In 2000, PFOS had been phased out by 3M, the major manufacturer of PFOS in the USA. However, PFOS could still be detected because of the production of PFOS-related compounds around the world and the difficulty in the natural degradation of PFOS (Das et al. 2015).

PFOS could induce various toxic effects, such as hepatotoxicity, endocrine toxicity and carcinogenicity (Cerveny et al. 2016). Recent findings from epidemiological investigations showed that there are relationships between the concentrations of PFOS in cord blood and motor development in children (Papadopoulou et al. 2015). Animal experiments also found that PFOS could cross blood brain barrier and cause neurological deficits (Surma et al. 2015). Previous studies in vivo and in vitro both disclosed that PFOS could induce apoptosis mediated by oxidative stress in neurons, astrocytes and other kinds of cells in brain (Dong et al. 2015; Li et al. 2015; Wang et al. 2015). Hence, it was necessary to intervene the neurotoxicity induced by PFOS exposure.

As one of the major intracellular free amino acids, taurine could be found most in brain, then in liver and retina of mammalian tissues (Li et al. 2014). Taurine could be acquired from natural diet or bio-synthesized by human. Several processes have been found to be associated with taurine, such as brain development, neurotransmission and neuromodulation (Wenting et al. 2014). Taurine has been found to be neuroprotective against various pathological conditions including hypoxia, hypoglycemia and ischemia as a type of antioxidant (Kumari et al. 2013). Especially, taurine could interact with mitochondria, which is the major source of oxidative stress (Jong et al. 2012). Hence, the effect of taurine on PC12 cells-treated by PFOS was investigated.

2 Methods

2.1 Chemicals

PFOS (at purity over 98%) was supplied by Sigma, USA. 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA), and the detection kits for cell autophagy (Lyso-tracker red) and Caspase 3 activity reflective of apoptosis were bought from Beyotime Institute of Biotechnology, China. All the other chemicals were analytical grade.

2.2 Cell Culture

The PC12 cell line derived from the rat pheochromocytoma was proveded from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cells were grown in antibiotic-free Dulbecco's modified Eagle medium (DMEM) (Hyclone, USA) supplemented with 10% horse serum (Hyclone, USA) and 5% fetal bovine serum (FBS) (Hyclone, USA). For passaging, cells were trypsinized with 0.125% Trypsin-EDTA (Beyotime, China), and maintained in a humid incubator at 37 °C, 5% CO_2.

2.3 Cell Viability

The impact of PFOS on PC12 cell viability was measured with the MTT (tetrazolium) assay as described elsewhere (Mendonça-Dias et al. 1983). In brief, PC12 cells were seeded in 96-well plates at the density of 1×10^4 cells per well. Following exposure to PFOS or the vehicle control, 100 μL MTT at 0.5 mg/mL was applied to the culture, and incubated at 37 °C, 5% CO_2 for an additional 2 h period. The culture medium was then removed, and 100 μL dimethylsulfoxide (DMSO) per well was added to extract the formazan blue. The absorbance of the DMSO-dissolved solution, as an index of cell viability, was measured at 570 nm on the ELISA Reader (Thermo, USA). At least three replicate experiments with three technical repeats were conducted for the determination of the PFOS-induced cell viability.

2.4 Reactive Oxygen Species (ROS) Generation

The level of oxidative stress by PFOS in PC12 cells, reflected by ROS generation, was analyzed using the commercial ROS assay kit. According to on the manufacturer, DCFH-DA, supplied in the assay kit, is oxidized by ROS to

2′,7′-dichlorofluorescein (DCF) which serves as a fluorescent probe. Briefly, post exposure of PC12 cells with PFOS or the vehicle control, the same density of cells were harvested and subsequently incubated for 30 min at 37 °C with 100 μM DCFH-DA dissolved in DMSO for conversion of DCF from DCFH-DA. After three washes with PBS, the ROS generation was recorded as relative levels of fluorescence measured on a multi-detection microplate reader (485 nm ex/535 nm em).

2.5 Caspase 3 Activity

Caspase 3 activity can be reflected by the level of conversion of acetyl-Asp-Glu-Val-Asp p-nitro-aniline by caspase 3 to p-nitro-aniline (pNA), a yellow formazan product. In this study, PC12 cells at a density of 3×10^6/mL were exposed to PFOS or the vehicle control for 24 h, spinned at 600 g for 5 min, resolved in 200 μL lysis buffer (C1115-1, Beyotime, China) and kept on ice for 15 min, followed by centrifugation at 4 °C, 14,000 rpm for 15 min. The cell lysates were then mixed with the reaction buffer (Beyotime, China) and incubated overnight at 37 °C. The absorbance of the fluorescent pNA at 405 nm was recorded on the microplate reader (Multiscan Ascent, Thermo Fisher Scientific, and Waltham, MA, USA), and the chemical-mediated changes in caspase 3 activity, expressed as nmol pNA/mg protein, was calculated as the ratio of measurements at OD 405 nm of the treated cells to the control cells, according to Lu et al. (2015). The protein concentrations were determined with the Bradford Protein Assay Kit (Beyotime Institute of Biotechnology, China).

2.6 Cell Autophagy

The Lyso-Tracker Red dye was supplied by Beyotime Institute of Biotechnology in China. Briefly, PC12 cells were seeded at the density of 8×10^5 cells per well in 6-well plates. After treatment with PFOS, 50 nM of Lyso-Tracker Red was incubated with PC12 cells in the plates in dark for 45 min. Then the number of lysosomes was recorded by red fluorescence under the inverted fluorescence microscopy (Olympus, Japan).

2.7 Statistical Analysis

All experiments in this study were repeated three times and each experiment was conducted in three technical repeats. The data were typically expressed as mean ± standard error of mean (SEM). SPSS 13.0 (SPSS Inc, Chicago, IL) was implemented for data analysis of one-way analysis of variance (ANOVA), followed by LSD and Dunnett's T3 test. The statistical significance was determined as treatment-related at a p value <0.05.

3 Results

3.1 Effect of Taurine on Cytotoxicity in Neuronal Differentiated PC12 Cells Exposed to PFOS

Microscopic observations on PC12 cells showed that PFOS exposure decreased the number of cells (Fig. 1a). The morphology for PC12 cells in PFOS exposure group exhibited shrinkage, while the intact cells could be observed in control and taurine-pretreated PC12 cells (Fig. 1a). The cell viability was shown in Fig. 1b. Compared with the untreated group, 250 μM PFOS could significantly decrease the viability of PC12 cells ($p < 0.05$). For instance, about 40% loss of PC12 cell population was detected in the 250 μM PFOS exposed group. In contrast, the taurine pretreatment (80 mM, 30 min) could significantly increase the viability of PC12 cells treated with 250 μM PFOS ($p < 0.05$).

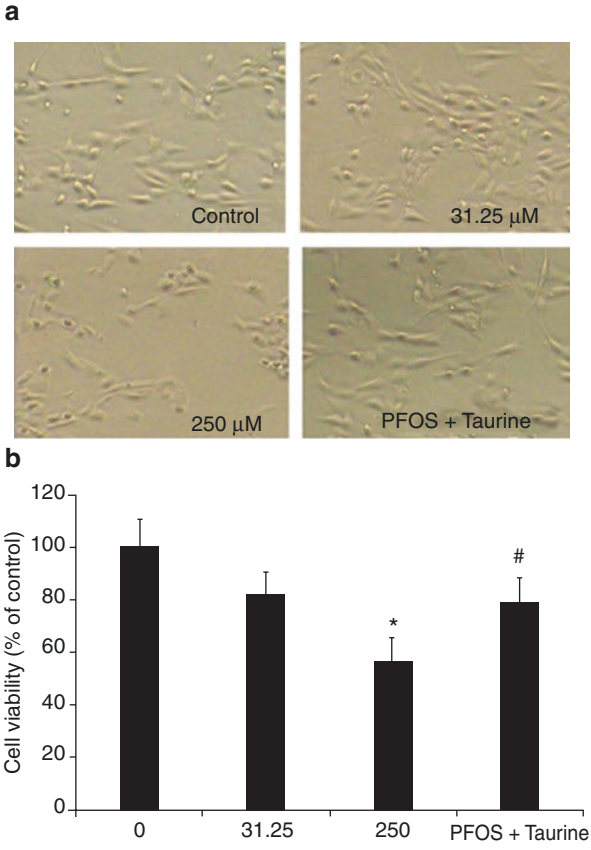

Fig. 1 Effect of taurine on PFOS-induced cytotoxicity in neuronal differentiated PC12 cells. * compared with the control group, $p < 0.05$; # compared with the PFOS exposure alone, $p < 0.05$

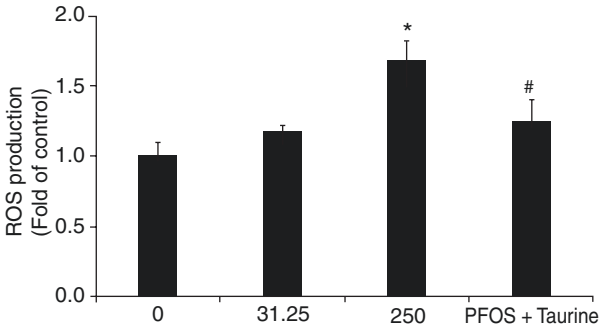

Fig. 2 Effect of taurine on PFOS-induced ROS production in neuronal differentiated PC12 cells. * compared with the control group, $p < 0.05$; # compared with the PFOS exposure alone, $p < 0.05$

3.2 Effect of Taurine on ROS Production in Neuronal Differentiated PC12 Cells Exposed to PFOS

The PFOS-induced ROS generation was evaluated using DCFH-DA dye in the present experiment. Figure 2 suggested that 250 μM PFOS could dramatically increase the generation of ROS, in comparison with the control group ($p < 0.05$). Similar to the cell viability, pretreatment with 80 mM taurine for 30 min showed a protective effect by reducing PFOS-mediated increase in ROS generation ($p < 0.05$).

3.3 Effect of Taurine on Caspase 3 Activity in Neuronal Differentiated PC12 Cells Exposed to PFOS

Caspase 3 is a key protease in early stage of apoptosis activation. Caspase 3 is inactive when cells were undergoing apoptosis by self-proteolysis and/or cleavage by another protease. However, the active caspase 3 could proteolytically cleave and activate other caspases, as well as relevant targets both in the cytoplasm and in the nucleus, leading to downstream events. Hence, the activity of caspase 3 was also evaluated with a caspase 3 activity kit. According to the instructions, the activity is according to the ability of caspase 3 to convert acetyl-Asp-Glu-Val-Asp p-nitro-aniline into p-nitro-aniline (pNA). As shown in Fig. 3, 250 μM PFOS could increase the activity of caspase 3 significantly, compared with the control ($p < 0.05$). By pretreating the cells with 80 mM taurine for 30 min, the caspase 3 activity in PC12 cells induced by PFOS was decreased significantly ($p < 0.05$).

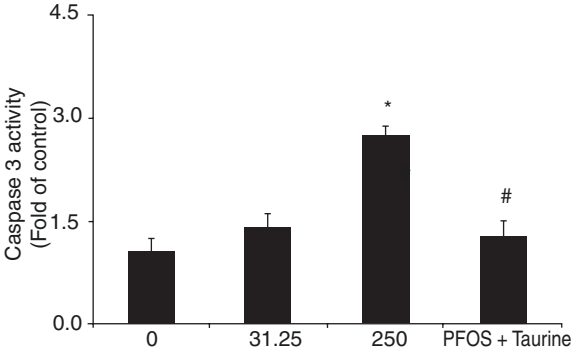

Fig. 3 Effect of taurine on PFOS-induced caspase 3 activity in neuronal differentiated PC12 cells. * compared with the control group. $p < 0.05$; # compared with the PFOS exposure alone. $p < 0.05$

3.4 Effect of Taurine on Autophagy in Neuronal Differentiated PC12 Cells Exposed to PFOS

The dye Lyso-Tracker Red is a specific probe for analyzing numbers of lysosomes. An intensity of the Lyso-Tracker Red staining reflects lysosomal acidification and the number of lysosomes, and therefore has been utilized to monitor activation of autophagy. As revealed in Fig. 4, PFOS could induce a dose-dependent increase in the red fluorescence intensity, indicating the activation of autophagy by PFOS; and this action could be alleviated by pretreating the cells with 80 mM taurine for 30 min (Fig. 4a). The statistical significance of the effect of taurine on autophagy in PC12 cells treated with PFOS was shown in Fig. 4b.

4 Discussion

PFOS was listed as a new kind of persistent organic pollutant in 2009, and its potential adverse effects on living species have continuously received much concerns. Some harmful effects in human beings have been reportedly linked with PFOS by epidemiological investigations, such as abnormal neurobehavioral effects (Lin et al. 2013). PFOS has been considered as a developmental neurotoxin in different animal species. It has been well accepted that exposure to environmental chemicals constitutes one of etiological factors in the progressions of neurodegenerative diseases (Grandjean and Landrigan 2014). Hence, intervention studies for PFOS-induced neurotoxicity are in urgent needs.

In the present study, the decreased viability of PC12 cells exposed to PFOS indicated that PFOS had a cytotoxic effect on the neuronal cells. These results were coincided with the findings in the cultured neurons and astrocytes (Dong et al. 2015; Lee et al. 2013). It has been reported that the neurotoxic effects induced by PFOS

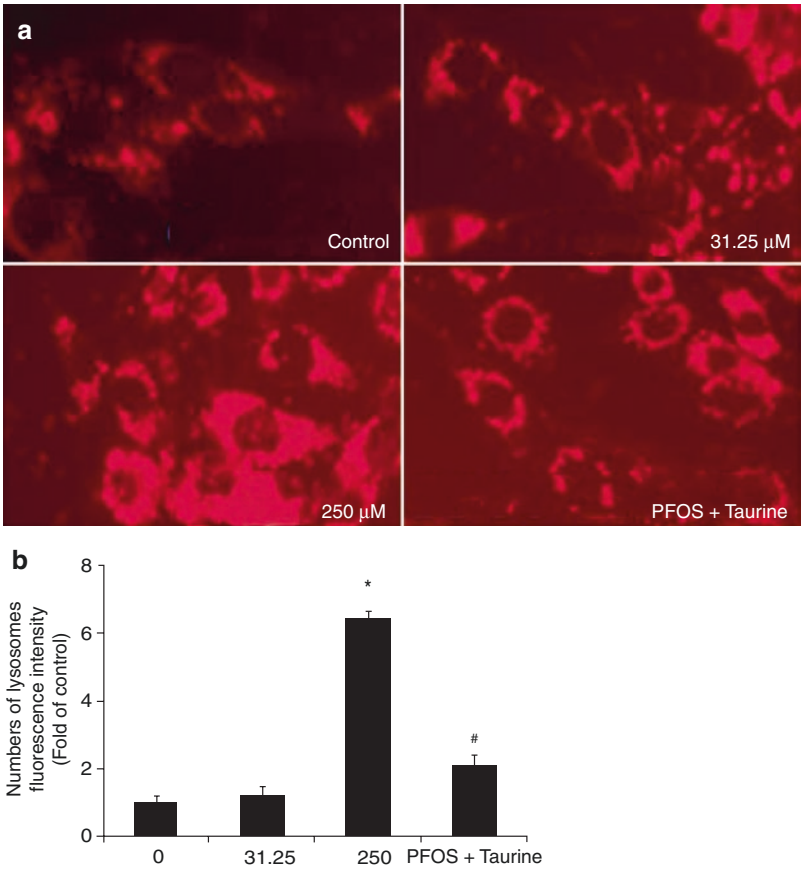

Fig. 4 Effect of taurine on PFOS-induced autophagy in neuronal differentiated PC12 cells. (**a**) As indicated by the Lyso-Tracker Red fluorescence intensity, PFOS could induce autophagy with a dose-dependency. However, PFOS-induced the increase in the fluorescence intensity was alleviated in PC12 cells pretreated with taurine. (**b**) Statistical analysis. *$p < 0.05$

were associated with oxidative stress. Oxidative stress has an important role in neurodegeneration induced by chemicals (Selvakumar et al. 2013). The impairment in redox-homeostasis would damage the critical biomolecules such as DNA, lipids and proteins. Because of its high oxygen content, low level of antioxidant activity and its abundant level of polyunsaturated fatty acids, the brain is very susceptible to oxidative damage (Butterfield et al. 2011; Moreira et al. 2005). Experimental evidence suggests that PFOS could induce oxidative stress, which is characterized by ROS production. Therefore, ROS production is an important index in the mechanism of PFOS-induced neurotoxicity. In the present study, the risk of PFOS-induced neurotoxicity was increased by the finding that PFOS arouse an increased production of ROS in PC12 cells. As an antioxidant, taurine could alleviate the risk of PFOS by decreasing the production of ROS.

Apoptosis is defined as a type I programmed cell death (PCD), and has been found to be initiated in Parkinson's disease (Yalcinkaya et al. 2016). Additionally, autophagy (type II PCD) has been recognized as another cytoprotective mechanism for cell survival during the stress, while the excessive autophagy will ultimately lead to death of cells in the disease conditions. Whether PFOS could induce both of these two events in PC12 cells was also examined in the present study. And our data derived from increases in caspase 3 activities and Lyso-Tracker Red fluorescence intensity indicate that both apoptosis and autophagy were involved in PFOS-induced neurotoxic effects in PC12 cells. It has been well understood that oxidative stress resulting from excessive ROS generation could induce multiple biological responses, such as DNA damage, cell cycle arrest, apoptosis and autophagy. In the result, the apoptosis induced by PFOS could be alleviated by taurine. Though the upstream role of ROS in mediating apoptosis induced by PFOS had been reported (Wang et al. 2013), the exact role of ROS in autophagy induced by PFOS was not well understood. As with other antioxidants, taurine was supplied to the PC12 cells as a first line of protection before PFOS treatment, and the results showed that autophagy was also relieved. Considering the antioxidant effect of taurine, our data disclosed that ROS might also be an upstream event of autophagy induced by PFOS.

5 Conclusion

In the present study, the role of antioxidant effect of taurine in PFOS-induced increased ROS production, apoptosis and autophagy could be observed. And the ROS is found to be an upstream molecule in PFOS-induced apoptosis or autophagy. As we know, mitochondria are the main sources of ROS generation. PFOS-mediated ROS production might be attributed to the mitochondrial malfunction exerted by PFOS. Thus, taurine might serve as a mediator of protein synthesis in mitochondria through enhancement in the electron transport chain activity and as a line of defense against excessive superoxide generation by PFOS. Nevertheless, the further studies in seeking for the role of taurine in PFOS toxicity are still needed.

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Protection of Taurine Against Arsenic-Induced DNA Damage of Mice Kidneys

Yinghua Zheng, Hongxin Qu, Dunjia Wang, Shuangyue Li, Cong Zhang, and Fengyuan Piao

Abstract The purpose of this study was to explore the protective capacity of taurine on arsenic (As)-induced neurotoxicity. Thirty mice were used and ten rats in each group. We treated the As exposure group with 4 ppm As_2O_3 for 60 days by drinking water and the protective group with 4 ppm As_2O_3 and 150 mg/kg taurine. Drinking water was only given in the control group. Pathologic changes and DNA damage in the mice kidney were examined by HE staining, immunohistochemistry and comet assay. Abnormal morphological changes were found in the kidney of As exposed mouse. Moreover, 8-hydroxy-2-deoxyguanosine (8-OHdG) expression and comet number, tail moment, and tail length of comet were markedly elevated in the As intoxication mice. However, histopathological changes and low expression of 8-OHdG were shown in the protective group. Our results indicate that supplementation of taurine protects against the histopathologic changes and DNA damage of mouse kidneys in As exposure group.

Keywords Taurine • Arsenic • DNA damage • Kidney • Protection

Yinghua Zheng and Hongxin Qu contributed equally to this work.

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Abbreviations

As	Arsenic
DAB	Diaminobenzidine
DMSO	Dimethyl sulfoxide
8-OHdG	8-Hydroxy-2-deoxyguanosine
SCG	Single cell gel

1 Introduction

Arsenic (As) is one of the common pollutants and its pollution is involved all over the world. Sources of human exposure to As mainly include well water, contaminated soil, occupational environment and seafood rich in methylated As (Carlin et al. 2016). Therefore, As exposure is a major public health problem that influences hundreds of millions of people around the world. Especially, the risk suffering from diseases due to As exposure is high in some countries including China, Bangladesh and India (Zheng et al. 2014). As exposure has been linked to cancer, cardiovascular disease, neurodevelopmental neurologic deficits, reproductive abnormalities and Kidney disease (Kuo et al. 2013; Navas-Acien et al. 2005; Peters et al. 2015). It has been demonstrated that As exposure results in renal injury and cancer (Ratnaike 2003), indicating that kidneys is a target organ of As-induced toxicity.

In vitro and in vivo studies indicated that toxicity of As is mediated via oxidative stress (Flora 2011). As is a well-known reactive oxygen species (ROS) inducer. The generation of these species has been shown to play a fundamental role in As-induced adverse health effects and diseases (Shi et al. 2004; Kumagai and Sumi 2007). Oxidative stress causes damage to lipids, proteins, and DNA. It was reported that Arsenite with low concentrations increased ROS levels and led to oxidative DNA damage of vascular smooth muscle cells. Arsenite (20–80 mmol/L) was shown to induce breaks of DNA strand in cultured mammalian cells. As exposure in rodent studies has resulted in increased ROS, reduced activities of antioxidant enzymes and oxidative damage in kidneys (Roy et al. 2009; Bera et al. 2011). Administration of antioxidants can mitigate or prevent the oxidative damage in As treated rats, suggesting that antioxidants may protect against As-induced nephrotoxicity. Therefore, treatment with antioxidants has been an efficient strategy to prevent from oxidative injury of kidneys by As.

2-Aminoethanesulfonic acid (taurine) is a free-amino acid and abundantly in kidney tissue (Lerdweeraphon et al. 2013). It obtained largely from in food, particularly from seafood and meat (Das et al. 2011). The studies have demonstrated that taurine can play a protective role by its antioxidant activity (Das et al. 2011). The unbalance between ROS production and antioxidant defenses induces oxidative stress, which may result in the pathogenesis of human diseases. Therefore, we are interested in whether treatment of taurine as an antioxidant protects against As-induced nephrotoxicity including oxidative DNA damage in mice.

Because attacking the 8-hydroxy-2-deoxyguanosine (8-OHdG) is one of the main mechanisms that ROS induces the damage of DNA, the 8-OHdG expression has been used as a biomarker for determining oxidative DNA damage (Ding et al. 2005). Moreover, the DNA damage induced by ROS can lead to breakage of DNA single- or double-strands. Comet assay is a rapid, sensitive and inexpensive method for detecting breaks of DNA strand (Lee and Steinert 2003). In the present study, the experimental animals were given with As alone or both As and taurine for 60 days. To assess As-induced DNA damage of kidneys and protection of taurine, the 8-OHdG expression, the DNA strand breaks and abnormal morphological changes of the kidneys were examined by immunohistochemistry, SCG assay and HE staining.

2 Methods

2.1 Chemicals

Arsenic trioxide (As_2O_3), taurine and anti-8-OHdG antibody were bought from Sigma (St. Louis, USA). Dimethyl sulfoxide (DMSO) and ethidium bromide (EtBr) came from Sigma (St. Louis, MO). UltrasensitiveTM S-P kitand Diaminobenzidine (DAB) color reagent kit and agarose were purchased from Gibco (Paisley, UK).

2.2 Animal and Treatment

Thirty KM mice were provided by Animal Center of Dalian Medical University. The experiment was divided into three groups. Ten mice were used in each group. We treated the As exposure group with 4 ppm As_2O_3 for 60 days by drinking water and the protective group with 4 ppm As_2O_3 and 150 mg/kg taurine. Drinking water was only given in the control group. Taurine was treated by gavage twice weekly. Mice were given standard diet and water ad libitum. Moreover, they were kept in circumstance of a 12 h dark-light cycle, temperature with 18–22 °C and humidity with 50%. The experiment of animal was carried out according to the guidance for care and the ethical protocols of standard experimental animals study.

2.3 Histopathological Examination

Following final administration, the kidney was taken out and put in fixative. The kidneys were embedded, cut into sections with 5 μm, fixed on slides and stained with hematoxylin and eosin in the light of conventional methods. Morphological abnormalities were examined under light microscopy.

2.4 Comet Assay

Following final administration, the isolation of kidney cells was performed according to the methods reported previously (Conner and Grisham 1996). The abdomen was opened, the aorta of the renal artery was ligated and a catheter was inserted into the abdominal aorta. The kidneys were rinsed for 1 min. After cutting superior vena cava, the collagenase buffer (HBSS) was perfused into the kidneys for 10 min. The tissues were then taken out and cut in 2~3 mm thickness. They were digested by the solution of trypsine (0.05%) and EDTA (0.02%) for 20 min at 37 °C. 10% fetal calf serum was added to stop the enzymatic reaction and the suspension of cells was moved into a 250 μm nylon filter-funnel on a sterilized tube and then centrifuged.

The comet assay was based on the method reported previously (Singh and Stephens 1997) and appropriate modifications were made. To avert artifacts due to apoptotic or necrotic cells, the cell viability and apoptosis were determined by trypan blue and Hoechst 33342. The cell suspension with cell viability greater than 90% and no apoptotic cells was used to measure migration of DNA on gels. The cell suspension was adjusted to 300 μL volume after rinsed by phosphate-buffered saline twice. The following procedures please refer to the literature (Singh and Stephens 1997). Finally, comets were observed by fluorescent microscopy were analyzed with Software. There were three parallel cultures at each experimental point and the tail DNA in 50 cells from each culture was detected.

2.5 Determination of 8-OHdG Expression by Immunohistochemistry

Three μm slices were deparaffinized, rehydrated, treated with 1% H_2O_2 and blocked. After incubation with -OHdG antibody (1:300) overnight, the samples were incubated with biotin-labeled rabbit anti-mouse IgG for 60 min and then with diaminobenzidine (DAB) solution. Five fields were examined per slide and the quantity value was determined via Image-Pro Plus 4.5 software (Media Cybernetics).

2.6 Data Analysis

All data were presented as means ± SD Student's t-test and One-way analysis of variance (SPSS v10.0 software) were used. $P < 0.05$ indicated significant and highly significant compared to controls.

3 Results

3.1 Effect of Taurine on As-Induced Morphological Changes in Kidneys

As shown in Fig. 1, Endochylema vacuolar degeneration, karyopyknosis, karyolysis and contracted lumens were seen in As-exposed mice's kidney tissues. Particularly, abnormal morphological changes in the proximal convoluted tubules (PCT) were more pronounced. Abnormal changes of morphology in glomerulus were largely seen in the region of Bowman's capsule (BC). The number of BC was reduced or vanished in treated mice. However, the morphological changes in the protective group were slight in the renal tissues. On the other hand, there were no abnormal changes of the renal morphology in the control mice.

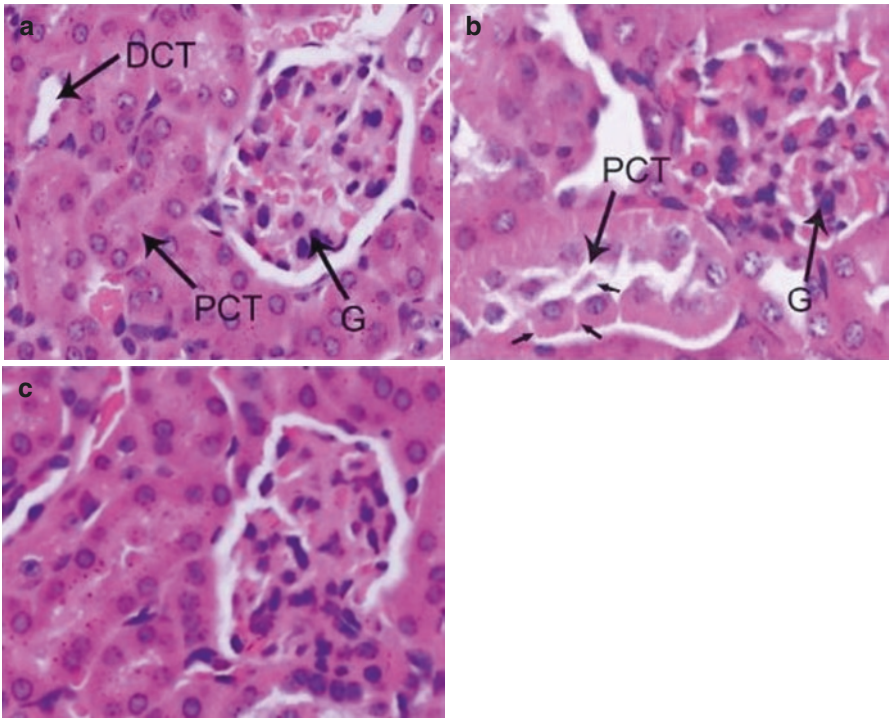

Fig. 1 Changes in renal morphology. (**a**) Control group; (**b**) As exposure group; (**c**) protective group. *G* glomerulus, *BC* Bowman's capsule, and *PCT* proximal convoluted tubule. Original magnification ×200. As-exposed mice showed cellular swelling, endochylema vacuolar degeneration, karyopyknosis, karyolysis, contracted lumens and diminished Bowman's capsule in renal tissues. However, the pathological changes in the protective group were slight in the renal tissues. These morphological changes were not shown in controls. *Short arrow* demonstrate epithelial cell of PCT

3.2 *Effect of Taurine on As-Induced Breaks of DNA Strand in Kidney Tissues*

DNA strand breaks in the kidneys of mice were assessed using Comet assay (Fig. 2 and Table 1). The renal cells in the control and protective groups were round shaped (Fig. 2a, c), while mice treated with As showed comet cells with longer tail (Fig. 2b). Moreover, there was significant increase in the quantity of comet cells, tail moment

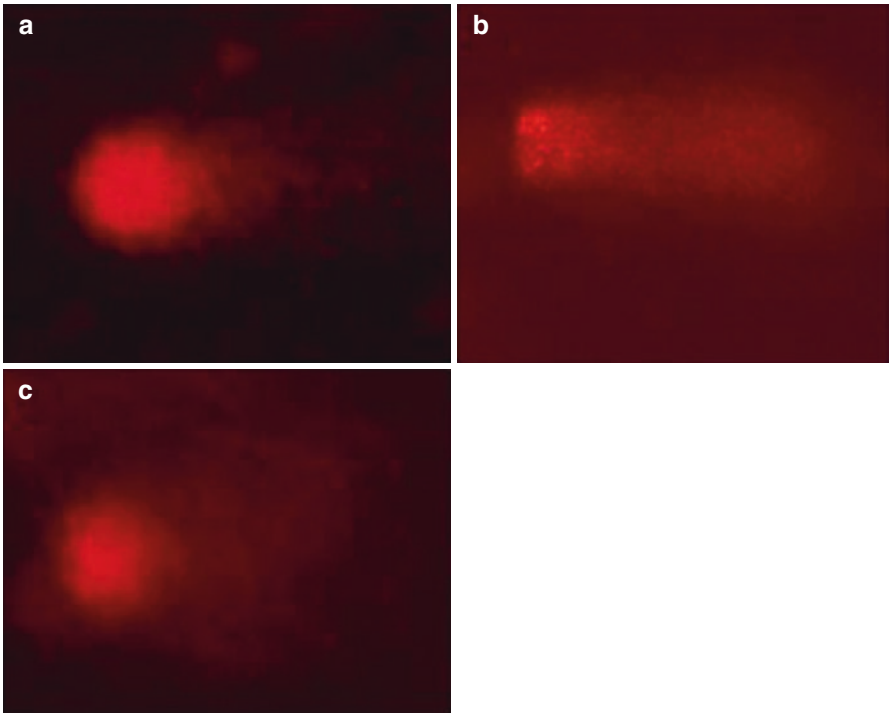

Fig. 2 Pictures of cell comet in renal tissues by SCGE. (**a**) Control group; (**b**) As exposure group; (**c**) protective group. DNA damage in the mice kidney tissues were examined by single cell gel electrophoresis (SCGE). The treated group showed longer tail and increased moment of comet cells in the renal tissues compared to controls

Table 1 Results of comet in renal tissues of mice in three groups by SCGE

Groups	No. of cells	No. of comet	Tail length (mean ± SD, μm)	Tail moment (mean ± SD, μm)
Group 1	50	5	6.80 ± 3.73	0.48 ± 0.32
Group 2	50	38	43.82 ± 4.78[a,b]	25.32 ± 5.72[a,b]
Group 3	50	8	11.40 ± 2.46	1.12 ± 0.49

[a]$P < 0.01$, compared with controls
[b]$P < 0.01$, compared with group 3

of comet and tail length in the group administrated with As compared to the controls (Table 1). However, there were no markedly differences in these parameters between the protective and control groups.

3.3 *Effect of Taurine on As-Induced 8-OHdG Expression in Kidneys*

As shown in Fig. 3, the intensive immunoreactivity of 8-OHdG was observed in the renal tissues of treated mice. The 8-OHdG expression most existed in the renal tubule area and glomerulus. Particularly, high 8-OhdG expression in the podocytes

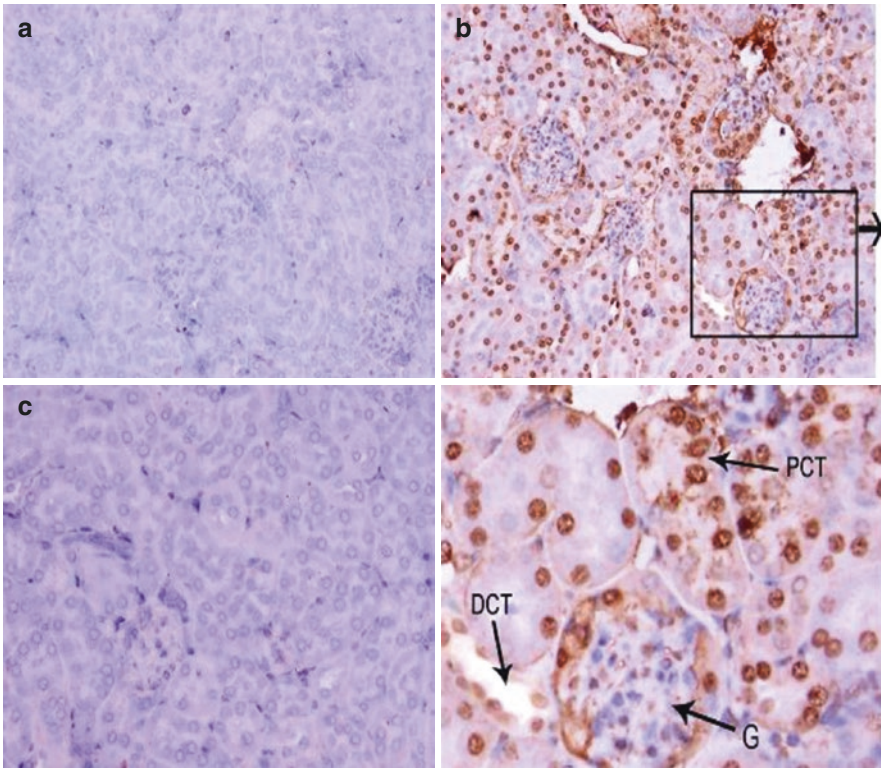

Fig. 3 The 8-OHdG immunoreactivity in the renal tissues. (**a**) Control group; (**b**) As exposure group; (**c**) protective group. *DCT* distal convoluted tubule, *G* glomerulu, and *PCT* proximal convoluted tubule. Positive cells of 8-OHdG appeared *brown* and their nuclei were stained. Original magnification ×200. The 8-OHdG immunoreactivity was increased in the renal tissues of treated mice. The 8-OHdG expression most existed in the glomerulus and renal tubule area. Particularly, the high 8-OhdG expression in the podocytes and PCT was found. However, the protective group showed weak 8-OHdG expression in the renal tissues. In control group, no immunoreactivity was found in the renal tissues. *Arrow*, the enlargement inside the frame shown in the last graph

Table 2 Optical density value of 8-OHdG in the renal tissues of mice

Groups	Chemicals	No. of animals	Optical density value (mean ± SD)
Group 1	Drinking water	10	4.941 ± 1.674
Group 2	4 mg/L As_2O_3	10	5522.710 ± 686.338[a,b]
Group 3	4 mg/L As_2O_3 + 150 mg/kg taurine	10	25.833 ± 5.901

[a]$P < 0.01$, compared with controls
[b]$P < 0.01$, compared with group 3

and PCT was found. On the other hand, a low 8-OHdG expression was shown in protective group. In control group, no immunoreactivity was found in the renal tissues. The 8-OHdG immunoreactivity distribution in the renal tissues of treated mice was consistent with the histopathological changes. Moreover, the 8-OHdG immunoreactivity was assessed by image analyzer. As shown in Table 2, the optical density value of 8-OHdG immunoreactivity in the treated group significantly increased compared to the protective or control group ($p < 0.01$).

4 Discussion

It was reported that accumulation of As was found in mice kidney tissues after oral administration of sodium arsenite ($NaAsO_2$) with 10 mg/kg for 2 days and abnormal morphological changes were shown in the epithelial cells of kidneys (Sinha et al. 2008). The study showed that level of lipid peroxidation significantly increased in the kidneys of mice exposed to 25 mg/L $NaAsO_2$ by drinking water for 3 month (Gupta and Flora 2005). In the present study, our results showed that abnormal morphological changes in the PCT and the area of BC in kidney tissues of As-treated mice, indicating that the kidneys are a major target organ of toxicity induced by As. On the other hand, the cells in the PCT showed slightly swollen in the protective group, but the boundary of cells was clear, implying taurine prevents from abnormal morphological changes in the renal tissue of treated mice. Das et al. reported that the seminiferous tubule atrophy and spermatogenic cell layer loss in As intoxicated rats (Das et al. 1995). However, pretreatment with taurine prevented As toxicity so as to testicular structure maintained in the normal state. It is indicate that taurine supplementation protects against As-induced nephrotoxicity in the kidneys of mice.

It was shown that As-induced reactive oxygen species (ROS) leads to the damage of DNA. In this study, to assess As-induced DNA damage in kidneys, we examined the 8-OHdG expression and the breaks of DNA strand in the renal tissue by SCG assay and immunohistochemistry. Our results showed that comet cells with longer tail, the number of comet cells and tail length of comet significantly raised in the As exposure group compared to the controls. Moreover, the increased 8-OHdG immunoreactivity in the renal tissue was found in the treated mice. The 8-OHdG expression most existed in the glomerulus and renal tubule area. Particularly, the high

8-OhdG expression in the podocytes and PCT was found. Furthermore, the distribution of 8-OHdG immunoreactivity was consistent with the histopathological changes. These results suggest that there may be a link between the As-induced DNA damage and abnormal morphological changes in the renal tissue of treated mice. In the present study, protection of taurine against the DNA damage of As to kidneys of mice also examined. The results showed no significant the DNA strand breaks such as increased tail length and elevated tail moment of comet in the renal tissue of mice with taurine compared with controls. Moreover, weak 8-OHdG expression was shown in the protective group. The 8-OHdG immunoreactivity significantly decreased in taurine group compared to the treated group. Pretreatment with taurine significantly reduced the DNA damage and DNA-protein cross-linking in the rat intestine induced by potassium bromate (Ahmad et al. 2015). It was also shown that lipid peroxidation and DNA damage were significantly decreased in testes of diabetic rats after administration of taurine (Abd El-Twab et al. 2016), being accordance with our results. Our results indicate that administration of taurine protects against the As-induced DNA damage to kidney of mice.

Taurine is known as a conditionally essential amino acid that plays an important role in antioxidation, membrane stabilization, osmoregulation and the modulation of calcium signaling (Bouckenooghe et al. 2006; Marcinkiewicz and Kontny 2014). Taurine was also found to protect against atherosclerosis (Balkan et al. 2002), diabetic complications (Franconi et al. 2004) and gastrointestinal damage (Cetiner et al. 2005) induced by oxidative stress. These beneficial effects of taurine on some diseases or injuries may be attributed to its antioxidant activities. Some mechanisms may be involved in the regulation of taurine to reduce oxidative stress (Giris et al. 2008). It has been indicated that the antioxidant mechanism of taurine may include the following: (1) upregulating the antioxidant defences; (2) forming chloramines with HOCl; (3) binding free metal ions such as Fe^{2+} by its sulphonic acid group.

5 Conclusion

Taken together, the present study showed that exposure to As resulted in histopathological changes in the Bowman's capsules and the PCT of mice, the DNA strand breaks and an increase in 8-OHdG expression. However, taurine supplementation significantly reversed these abnormal changes in the renal tissues of treated mice. It is suggested that taurine supplementation protects against the histopathologic changes and oxidative damage of DNA in kidney tissue of As-exposed mice. Meanwhile, it is also suggested that taurine may be potentially used as a therapeutic/protective agent against As-induced nephrotoxicity including DNA damage. In the future, it is necessary to explore further exact mechanism that taurine protects against As-induced DNA damage in the kidney tissues.

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Part VII
Effects of Taurine on Immunity

Mussel (*Mytilus coruscus*) Water Extract Containing Taurine Prevents LPS-Induced Inflammatory Responses in Zebrafish Model

Sun Hee Cheong, Seung-Hong Lee, You-Jin Jeon, and Dong-Sung Lee

Abstract Mussel (*Mytilus coruscus*) water extract had strong anti-inflammatory activities, but the effects and its mechanisms of mussel on anti-inflammatory properties *in vivo* remain to be determined. This study, therefore, was designed to investigate anti-inflammatory activities of mussel water extract containing a large amounts of taurine (151.96 nmol/mg) using the lipopolysaccharide (LPS)-induced inflammatory zebrafish model. In this study, mussel water extract containing taurine shows potent protective effects against the cell death stimulated by LPS exposure in zebrafish embryos. In addition, zebrafish subjected to LPS treatment exhibited significantly increased reactive oxygen species (ROS) and nitric oxide (NO) levels. However, mussel water extract markedly suppressed LPS-induced ROS and NO production. Our results indicate that mussel water extract attenuated inflammation by inhibiting the LPS-induced intracellular ROS and NO production in zebrafish embryos. These findings could demonstrate the anti-inflammatory activity of mussel water extract containing taurine, which might have a protective effects on inflammatory diseases.

Keywords Mussel (*Mytilus coruscus*) • Taurine • Zebrafish • LPS treatment • Anti-inflammatory

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Abbreviations

AIA	Adjuvant-induced arthritis
CIA	Collagen-induced arthritis
Cys	Cysteine
DAF-FM-DA	Diaminofluorophore 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate
DCF	Dichlorofluorescein
DCF-DA	2′,7′-Dichlorodihydrofluorescein diacetate
LPS	Lipopolysaccharide
Met	Methionine
NO	Nitric oxide
NOS	Nitric oxide synthase
iNOS	Inducible NOS
ROS	Reactive oxygen species

1 Introduction

Taurine (2-aminoethanesulfonic acid) is one of the sulfur-containing β-amino acid and it exists at high levels in most animal cells (Sturmann 1993). In the immune system, taurine acts beneficial roles as natural antioxidant to prevent the oxidative stress (Schaffer et al. 2009; Wang et al. 2009). Oxidative stress is well known as a major factor for tissue injuries in several conditions including cancer, infection, aging as well as inflammation. In particular, oxidative stress can be induced by reactive oxygen species (ROS) produced from activated leukocytes at the inflammation sites (Smith 1994). ROS has several beneficial effects in host defense system, whereas they are also responsible for several cellular damages (Weiss 1988; Smith 1994). Numerous reports have indicated that taurine is an effective antioxidant, but the mechanisms underlying its antioxidant and anti-inflammatory activities remain unclear.

Mussel (*Mytilus coruscus*) is one of the major marine shellfish in Korea. For thousands of years, it has been used as a source of diet and traditional medicine. It has been known that mussel has various beneficial effects including regulator of the organ function and immune system, treatment of chronic arthritis, treatment of female menoxenia and male impotence (de Lorgeril et al. 1997; Brien et al. 2008). Although several previous studies have investigated the cultivation conditions and biological activity of bioactive substances—such as peptides, polysaccharide and lipids—extracted from mussel (Miller et al. 1993; Kim et al. 2013; Fu et al. 2015), little research has addressed mussel water extract possessing taurine and its anti-inflammatory activity in a zebrafish model.

The zebrafish, *Danio rerio*, is a vertebrate model of development that is increasingly used in biomedical science (Phillips and Westerfield 2014). In particular, zebrafish obtained well-developed immune systems, which are very similar with the

mammals (Trede et al. 2001). Because of these characteristics, zebrafish are an alternative animal model for several studies for inflammation (Liao et al. 2011; Park and Cho 2011). Therefore, this study assessed the anti-inflammation activities of mussel water extract containing taurine using the LPS-stimulated zebrafish model.

2 Methods

2.1 Analysis of Chemical and Amino Acid Composition

Crude protein, crude fat, and ash contents of the mussel sample were analyzed for according to the methods of the Association of Official Analytical Chemists (AOAC 1990). To analyze the amino acid composition, 80 mg of mussel sample was mixed with 6 N HCl (10 mL) and then hydrolyzed at 110 °C for 24 h after purging with N_2 gas in a test tube. Each sample was evaporated and added a sodium-distilled buffer (pH 2.2). After filtering using a syringe filter (0.45 μm), amino acid composition was analyzed by an amino acid autoanalyzer (Pharmacia Biotech Biochrom 20) by absorbance at 440 and 570 nm, respectively.

2.2 Intracellular LPS Scavenging Assay in Vero Cells

Vero cells were incubated at 37 °C under a humidified atmosphere containing 5% CO_2. The cells were maintained in DMEM containing 100 μg/mL of streptomycin, 110 mg/L of sodium pyruvate, 100 units/mL of penicillin, and 10% heat-inactivated FBS. The cells (1.0×10^5 cells/mL) were maintained in 96-well plates for detection of intracellular LPS (10 μg/mL). After 16 h, the cells were added with 10 μL of each sample and incubated at 37 °C under a humidified atmosphere and then LPS (10 μg/mL) was added after 30 min. The cells were incubated for a further 30 min at 37 °C under a humidified atmosphere. Finally, 5 μg/mL of DCF-DA was added, and then the fluorescence of 2′,7′-dichlorodihydrofluorescein was observed at an emission wavelength of 535 nm and an excitation wavelength of 485 nm, using a Perkin-Elmer LS-5B spectrofluorometer (Waltham, MA, USA).

2.3 Inflammation-Induced by LPS in Zebrafish Model

Adult zebrafish (n = 10) were purchased from a commercial company (Seoul aquarium, Seoul, Korea). They were maintained in acrylic tank (3 L) at 28.5 °C with a 14:10 h light: dark cycle. The zebrafish were fed tetramin flake foods (*Artemia salina*; Sewhapet food Co., Seoul, Korea) three times per day for 6 days per a week.

Synchronized zebrafish embryos (10–15 embryos/well) were collected by pipette in 12-well plates supplemented with embryo medium (2 mL) for 7–9 hpf. Zebrafish embryos were then incubated with or without the treatment of samples for 1 h. For inflammation induction, LPS (10 μg/mL) was treated to the embryo medium at 28.5 °C for 15–17 hpf, then the zebrafish embryos were transferred to fresh embryo medium.

2.4 *Effect of Mussel Water Extract on Inflammation-Induced Cell Death*

Inflammation-induced cell death was assessed by the method described by Cheong et al. (2015). Briefly, zebrafish larvae were moved to 96-well plates at 3 dpf, and then added 7 g/mL of acridine orange solution in each well and incubated for 30 min under the dark at 28.5 °C. The zebrafish larvae were washed with fresh embryo medium and then anesthetized using 2-phenoxy ethanol (1/500 dilution; Sigma) and observed by a CoolSNAP-Pro color digital camera (Olympus, Japan). The fluorescence intensities were quantified using the Image-J software.

2.5 *Intracellular ROS Production in the Inflammatory Zebrafish Model*

Intracellular ROS and NO generation were measured using the method described by Lee et al. (2013). In the inflammatory zebrafish model induced by LPS, ROS production was determined using an 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA). In the condition of the presence of cellular peroxides, DCF-DA can be easily deacetylated by nonspecific esterases and then oxidized to dichlorofluorescein (DCF) (Rosenkranz et al. 1992). The zebrafish embryos and larvae were moved into 96-well plates after LPS treatment, 20 μg/mL of DCF-DA solution was treated in each well of 96-well plates. After the incubation for 1 h in the dark at 28.5 °C, the zebrafish embryos and larvae were washed with fresh embryo medium and then anesthetized using tricaine methanesulfonate. The fluorescence intensities of zebrafish embryo and larvae were quantified by a spectrofluorometer (Perkin-Elmer LS-5B, Norwalk, CT, USA). After staining of the embryos the larvae, they were photographed by a fluorescence microscope equipped with a Moticam color digital camera (Motix, Xiamen, China).

2.6 *Intracellular NO Production in the Inflammatory Zebrafish Model*

In the inflammatory zebrafish model induced by LPS, NO production was determined using the diaminofluorophore 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-DA), which is well known as fluorescent probe dye.

The DAF-FM-DA produced highly fluorescent triazole derivatives by NO in the condition of the presence of dioxygen (Itoh et al. 2000). The zebrafish embryos and larvae were moved into 96-well plates after LPS treatment, 5 μM of DAF-FM-DA solution was treated in each well. After the incubation for 1 h, the zebrafish embryos and larvae were washed with fresh embryo medium and then anesthetized using tricaine methanesulfonate. The fluorescence intensities of zebrafish embryos and larvae were quantified by a spectrofluorometer (Perkin-Elmer LS-5B, Norwalk, CT, USA). After staining of the embryos the larvae, they were photographed by a fluorescence microscope equipped with a Moticam color digital camera (Motix, Xiamen, China).

2.7 Statistical Analysis

All data are expressed as means ± SEM. The differences between means were assessed by one-way ANOVA followed by Tukey-Kramer multiple comparison test, and statistical significance was defined at $P < 0.05$.

3 Results

*3.1 Amino Acid Content and Composition of Mussel (***Mytilus coruscus***)*

Dry matter, crude protein, crude lipids and ash contents were 97.12%, 55.71%, 9.63% and 7.21%, respectively, of the mass of mussel (data not shown). Total amino acid content of the mussel water extract was higher than that of the lysis buffer. As shown in Table 1, the predominant amino acids of mussel water extract was taurine followed by glycine. In particular, the taurine content of mussel water extract was 151.96 nmol/mg, which was 4.23-fold increased compared to that of the lysis buffer extract. Therefore, we used mussel water extract to evaluate the anti-inflammatory effects in the LPS-induced inflammatory zebrafish model for the next experiments.

3.2 Inhibitory Effect of Mussel Water Extract Against Cell Death Induced by LPS Treatment

Figure 1a shows the viabilities of cells treated with LPS or co-treated with mussel water extract in Vero cells. The viability of control cells was assigned as 100%, whereas was lower in the LPS-treated cells. However, the treatment of mussel water extract showed a tendency of reduction of cell viability compared to the LPS non-treated or LPS-treated cells. These results demonstrate that mussel water extract had

Table 1 Amino acid content and composition of mussel (*Mytilus coruscus*)

Amino acid contents (nmol/mg)	Water extract	Lysis buffer extract
Aspartic acid	25.13	9.29
Glutamic acid	39.52	11.13
Asparagine	7.09	1.07
Serine	22.63	6.01
Glutamine	17.56	3.97
Glycine	169.58	38.46
Histidine	5.12	2.65
Arginine	8.03	5.96
Taurine	151.96	35.91
Threonine	16.22	3.13
Alanine	80.39	21.62
Proline	20.13	5.01
Tyrosine	6.85	3.12
Valine	7.22	2.69
Methionine	3.09	0.63
Cysteine	1.52	0.12
Isoleucine	6.54	1.98
Leucine	8.69	2.81
Phenylalanine	3.11	1.52
Tryptophan	3.78	1.19
Lysine	4.97	2.91
Total	609.13	161.17

no inhibitory effect on the cell viabilities induced by LPS *in vitro* in Vero cells. On the other hand, the cell death induced by LPS exposure in zebrafish was significantly increased compared to that in the control group (Fig. 1b). However, cell death was significantly decreased by mussel water extract treatment to LPS-stimulated zebrafish.

3.3 Effect of Mussel Water Extract Against ROS Production Induced by LPS Treatment

In the present study, we investigated the inhibitory effect of mussel water extract against ROS production using the LPS-stimulated inflammatory zebrafish model. Figure 2a shows a typical fluorescence micrograph images, and the negative control, which was not treated mussel water extract or LPS co-treatment with mussel water extract generated a clear image. However, only LPS treatment group as the positive control showed a fluorescence image. These results suggest that ROS generated by LPS in the zebrafish larvae. In contrast, mussel water extract significantly and

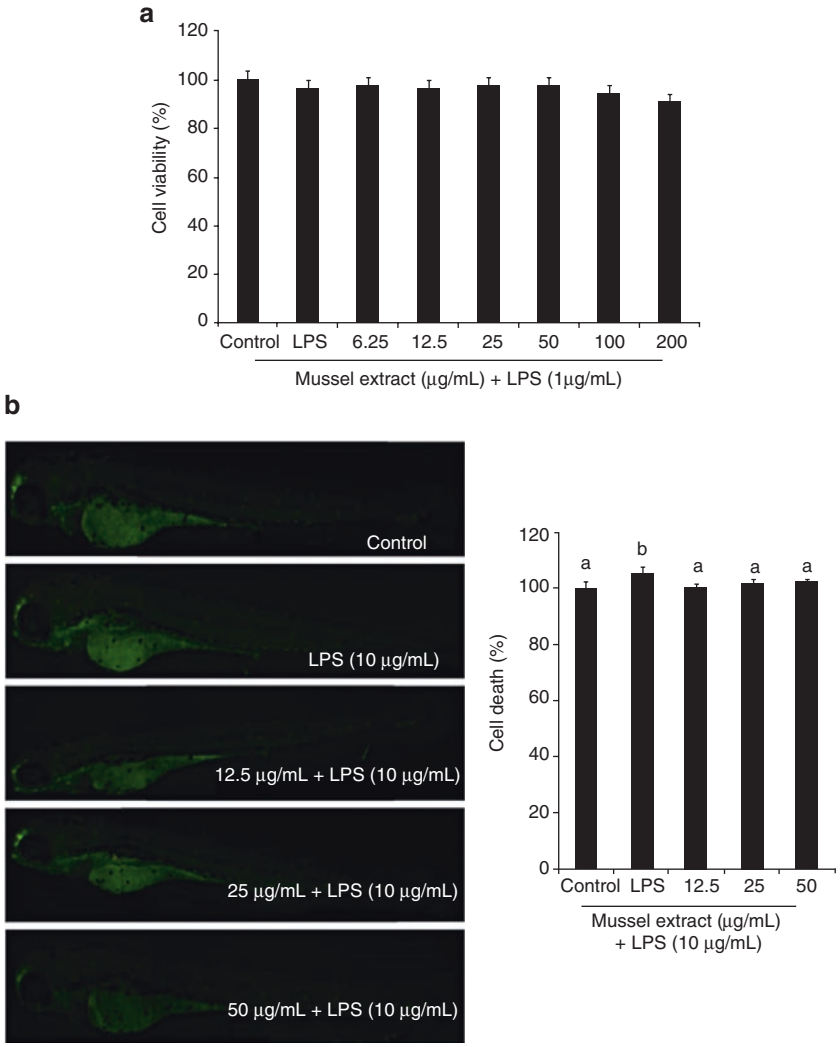

Fig. 1 Inhibitory effects of mussel water extract containing taurine on the cell death (%) induced by LPS in Vero cells (**a**). Effect of co-treatment of LPS with mussel extract on cell death in zebrafish (**b**). Experiments were conducted in triplicate and values are expressed as means ± SEM. Values with different letters are significantly different by Tukey-Kramer multiple comparison test at $P < 0.05$

dose-dependently reduced the elevated ROS production treated by LPS in the zebrafish larvae (Fig. 2b). These results indicate that LPS treatment induced the ROS generation in zebrafish, whereas mussel water extract possessing a large amounts of taurine effectively suppressed the LPS-induced ROS generation.

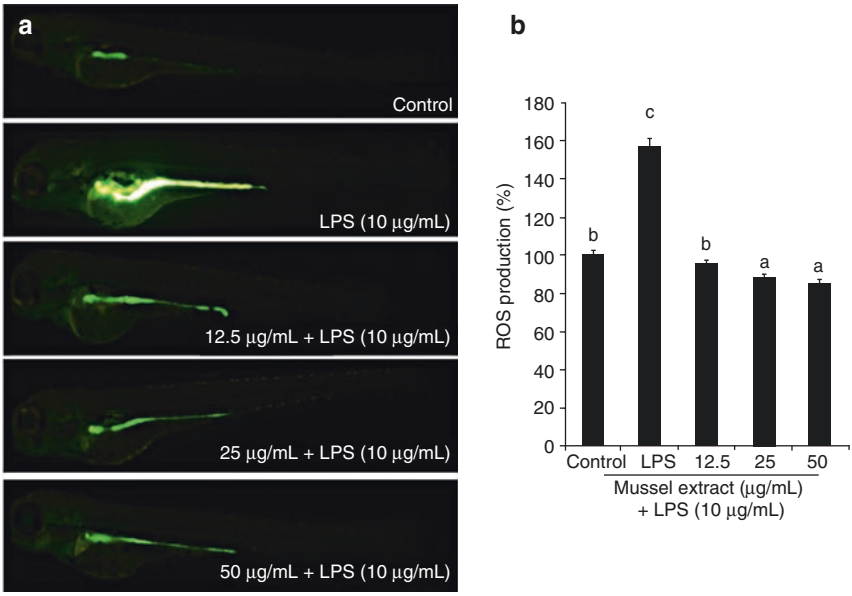

Fig. 2 Effect of mussel water extract against ROS production induced by LPS in zebrafish larvae. ROS production was measured using DCF-DA staining method. (**a**) Fluorescence micrographs of ROS production induced by LPS in zebrafish larvae. (**b**) Quantitative analysis of ROS production using a fluorescence spectrophotometer. Experiments were conducted in triplicate and values are expressed as means ± SEM. Values with different letters are significantly different by Tukey-Kramer multiple comparison test at $P < 0.05$

3.4 Effect of Mussel Water Extract Against NO Production Induced by LPS Treatment

Figure 3 shows the protective effect of mussel water extract against NO production induced by LPS treatment in zebrafish. The NO level was markedly increased by LPS compared to that of the non-LPS-treated zebrafish. In contrast, the level of NO was markedly and dose-dependently reduced in the range 6.25–200 μg/mL by addition of the mussel water extract. These results demonstrated that mussel water extract markedly inhibited the NO production in the inflammatory zebrafish model induced by LPS.

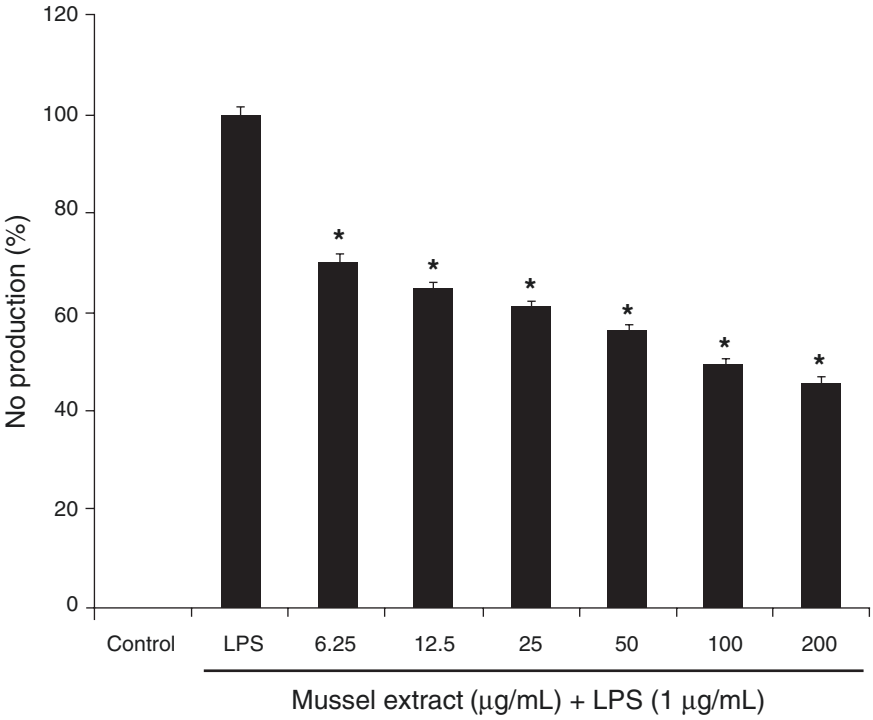

Fig. 3 Effect of mussel water extract against NO production induced by LPS in zebrafish larvae. The NO production was measured using DAF-FM-DA staining method. For quantitative analysis of NO production, a fluorescence spectrophotometer was used. Experiments were conducted in triplicate and values are expressed as means ± SEM. Values with different letters are significantly different by Tukey-Kramer multiple comparison test at $P < 0.05$

4 Discussion

Taurine is a semi-essential sulfur amino acid synthesized from cysteine (Cys) or through transformation from methionine (Met) within the body, but also present in the diet including fish, shellfish and seafoods. In the present study, we showed that taurine was the major amino acid in mussel water extract. In general, taurine acts as a trophic factor during central nerve system development, structural integrity of cell membranes, regulating calcium homeostasis and is an antioxidant, an osmolyte, a neuroprotective and a neuromodulatory agent (Hultman et al. 2007). Taurine also protects cells against oxidative stress (Pan et al. 2010), and decreases apoptosis and

necrosis (Yalcinkaya et al. 2009). These findings led to the hypothesis that water extracts from shellfish (such as mussel) containing high levels of taurine may have strong anti-inflammatory effects against oxidative stress mediated by LPS in zebrafish embryos.

LPS is generally present in the cell walls of Gram-negative bacteria and it is able to activate the innate immune response causes of ROS and nitrogen species as well as production of several proinflammatory cytokines (Raetz and Whitfield 2002; Bishop 2005). Oxidative stress is mediated by ROS produced by activated leukocytes during inflammation. ROS is advantageous in host defense system against several pathogens, whereas they are also responsible for several tissue damages (Smith 1994). In the present study, zebrafish embryos treated with LPS exhibited a significantly increased intracellular ROS level. In contrast, mussel water extract markedly inhibited LPS-stimulated intracellular ROS production with no toxicity in the zebrafish model. These findings show that mussel water extract possessing taurine alleviated inflammation by inhibition of the ROS production induced by LPS. Similarly, several investigators have demonstrated that taurine and taurine derivatives protect against inflammatory responses and oxidative tissue injury in animal models (Hwang et al. 2000; Balkan et al. 2002). Oliveira et al. (2010) reported that taurine was able to attach the ROS and reactive nitrogen species (RNS) at physiological concentrations.

Nitric oxide (NO) is one of the bioactive substances in the cells and functions as an intra- or extra-cellular mediator of several functions of cell (Gong et al. 2004). In general, it has been known that NO generated by the nitric oxide synthase (NOS) acts as a regulator of homeostasis, whereas NO produced by inducible NOS (iNOS) plays a major role in cytoprotection against oxidative stress and inflammation (Agnisola 2005; Pautz et al. 2010). In our study, we showed that NO generation was markedly increased by LPS in zebrafish. In contrast, the mussel water extract significantly reduced the rises in the NO level in the inflammatory zebrafish model stimulated by LPS. Recently, several materials obtained from marine sources have been reported as having numerous biological properties, with some reported to protect the pathogenesis of diseases (Rasmussen and Morrissey 2007). Several previous studies have shown that enzymatic hydrolysates derived from marine sources such as *Ruditapes philippinarum* and *Crassostrea gigas* possess NO-inhibitory activities (Hwang et al. 2012; Lee et al. 2012). However, the mechanisms by which taurine plays as an antioxidant as well as anti-inflammatory agent have not been determined. Another previous *in vivo* study showed that lipid extract of hard-shelled mussel possessed potent anti-inflammatory activities by reducing the arthritis index and paw swelling in both collagen-induced (CIA) and adjuvant-induced arthritis (AIA) rats. These anti-inflammatory activities may be relate to downregulation, proinflammatory cytokines—such as TNF-α, IL-6, IL-1β and IFN-γ and—and upregulation of several anti-inflammatory cytokines including IL-4, IL-10 in the blood and tissues of arthritic rats (Li et al. 2014). To our knowledge, this is one of the first studies to investigate the anti-inflammatory effect of mussel water extract containing a large amounts of taurine in zebrafish embryos. Moreover, our findings indicate that mussel water extract might have potent anti-inflammation effect against several

physicochemical damages in the zebrafish model, and these anti-inflammatory properties may be related to the amino acid compositions of mussel, especially taurine.

5 Conclusion

Our findings suggest that mussel water extract enriched in taurine exerted anti-inflammatory effects against the LPS-induced inflammation in zebrafish embryos by reducing intracellular ROS and NO generation. Further study of the clinical therapeutic effects and the underlying mechanism of mussel water extract on chronic inflammation is warranted.

Acknowledgement This study was financially supported by Chonnam National University, 2016.

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Anti-inflammatory Effects of Galactose-Taurine Sodium Salt in LPS-Activated RAW 264.7 Cells

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Abstract In this study, we synthesized Galactose-Taurine sodium salt (G-T) as a functional food ingredient to enhance biological activities of taurine. Also, anti-inflammatory effects of G-T were investigated in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. G-T found to reduce the generations of the LPS-

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stimulated nitric oxide (NO) and prostaglandin E2 (PGE_2) via down-regulating the expression levels of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2). Also, G-T reduced the secretion of inflammatory cytokines including interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF-α) in LPS-treated RAW 264.7 cells. Finally, we identified that G-T inhibits the activation of nuclear factor-κB (NF-κB) and the phosphorylation of inhibitor κB (IκB)-α. From these results, this study first suggests that G-T could be considered as an effective anti-inflammatory agent.

Keywords Galactose-taurine sodium salt • LPS • Anti-inflammatory

Abbreviations

COX-2	Cyclooxygenase-2
Gal-Tau	Galactose-Taurine sodium salt
IκB-α	Inhibitor κB-α
IL-1β	Interleukin-1β
LPS	Lipopolysaccharide
NF-κB	Nuclear factor-κB
NO	Nitric oxide
iNOS	Inducible NO synthase
PGE_2	Prostaglandin E2
TNF-α	Tumor necrosis factor-α

1 Introduction

Macrophages provide an important mediator against infection of bacterial pathogens in immune response. When macrophages are stimulated with lipopolysaccharide (LPS) which is an infectious material from Gram negative bacteria, mitogen-associated protein kinases (MAPKs) consisted of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinases (JNK), and p38 are activated to produce inflammatory mediators and proinflammatory cytokines. Cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) are the inflammatory mediators and produce prostaglandin E2 (PGE_2) and nitric oxide (NO), respectively. Also, proinflammatory cytokines include tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 (Ajizian et al. 1999; Uto et al. 2005). Inflammatory stimulation also causes phosphorylation of inhibitors κB (IκB) and detachment of nuclear factor-κB (NF-κB) and activates macrophages, partly with the increments of IL-6 and iNOS expression (Fang 2004; Miggin and O'Neill 2006). Among the various inflammatory mediators generated in the activated phagocytes such as microglia and macrophages, in particular, NO over-production has been considered as a critical mediator during inflammatory response (Jones et al. 2007). The increment of NO level followed by iNOS expression has been interested in various inflammatory diseases including asthma (Kharitinov and Barnes 2004), and rheumatoid arthritis

(Yasuda et al. 2004). Therefore, inhibiting roles of mediators involved in NF-κB activation may be a good therapeutic target against inflammation.

Taurine, a simple Sulphur-containing amino acid derivative, 2-aminoethane sulfonic acid, and is composed of most animal tissues. Although taurine is synthesized from other dietary sulfur-containing amino acids such as methionine and cysteine, endogenous production is generally inadequate, so dietary consumption of taurine is a necessity (Sturman 1993). Taurine acts as an important substance in several essential biological processes such as anti-oxidant (Stapleton and Bloomfield 1993), anti-apoptotic (Zhang et al. 2010), calcium modulation (Takahashi et al. 1992), membrane stabilization (Pasantes-Morales et al. 1985), development of the central nervous system, and immune function (Sturman 1993). Recent study has indicated that a new taurine analogue, ethane β-sultam showed anti-inflammatory action in macrophages isolated from rats as well as in vitro in an immortalized microglial cell line (Ward et al. 2011). However, it has also been reported taurine' disadvantages such as lower absorption and a rapid renal extraction rate. To solve them, development of several taurine derivatives and their biological capacities has been reported (Cho et al. 2014). Especially, considering the absorption through the carbohydrate transporter, taurine-carbohydrate derivatives were developed and their anti-oxidant and anti-adipogenic effects were verified (Kim et al. 2015; You et al. 2015).

Therefore, here, we revealed the anti-inflammatory effects of galactose-taurine sodium salt (G-T), a synthetic product derived from taurine, consists of taurine and galactose in a murine macrophage, RAW 264.7 cells.

2 Methods

2.1 Chemicals

RAW264.7 macrophage cell line was offered from Korean Cell Line Bank (KCLB; Seoul, Korea). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were bought from Invitrogen-Gibco (New York, USA). Trizol reagent was purchased from Molecular Research Center, Inc., Cincinnati, OH, USA. Antibodies including iNOS, COX-2, NF-κB p65, phosphorylated IκBα, β-actin were purchased from Cell signaling Technology (Beverly, MA, USA). NE-PER® Nuclear and Cytoplasmic Extraction Reagents, biotin 3'-end DNA labeling and LightShift™ chemiluminescent EMSA kit were of Pierce (Rockford, IL, USA). All used other reagents were offered from Sigma-Aldrich Chemical Co.

2.2 Cell Culture

RAW264.7 cells were incubated in DMEM with antibiotic (100 U/mL of penicillin/streptomycin) and 10% FBS. The cell line was incubated in an atmosphere of 5% CO_2 at 37 °C.

2.3 Determination of Nitric Oxide (NO) Production

RAW264.7 cells (1×10^5 cells/mL) were incubated with various concentrations of G-T for 2 h and stimulated by LPS (1 μg/mL). After 24 h, the generated NO levels were examined by using Griess reagent. The 100 μL of cultured medium was mixed with 100 μL of Griess reagent at RT for 10 min. The absorbance was measured using a microplate reader (SpectraMax® M2/M2^e, CA, USA) at 540 nm.

2.4 Determination of PGE_2 Production

RAW264.7 cells (1×10^5 cells/mL) were incubated with various concentrations of G-T for 2 h and stimulated by LPS (1 μg/mL). The produced PGE_2 levels in culture media were measured by the EIA kits.

2.5 Western Blot Analysis

The cells (1×10^6 cells/mL) were incubated with or without LPS (1 μg/mL) in the absence or presence of various concentrations of G-T for 20 min or 24 h. Cytosolic and nucleic proteins were obtained from the cells with the NE-PER® Nuclear and Cytoplasmic extraction kit (Thermo scientific, Rockford, USA). The protein concentrations of both cell lysates were measured using BCA™ protein assay kit. Both proteins (40 μg of each protein) were electrophoresed in 10% SDS-PAGE and transferred onto a nitrocellulose membrane with a glycine transfer buffer including 192 mM glycine, 25 mM Tris-HCl (pH 8.8), and 20% methanol (v/v). After blocking with 5% non-fat milk for 1 h, the blots were separately reacted with the following primary antibodies such as iNOS, COX-2, NFκB p65 and β-actin for 1 h. Then, the membranes were incubated for an additional 45 min with each HRP-conjugated secondary antibody (1:2000, Vector Lab. CA, USA) at RT and dark space. The targeted proteins were visualized by using enhanced chemiluminescence (ECL) reagents (Amersham, Arlington Heights, IL, USA).

2.6 RNA Preparation and RT-PCR

The cells (1×10^6 cells/mL) were incubated with or without LPS (1 μg/mL) in the absence or presence of various concentrations of G-T for 20 min or 24 h. Total cellular RNA (1 μg) was isolated from the cells using Trizol reagent and then was used

for the synthesis of the cDNA by using a Promega A3500 kit. PCR of this cDNA was performed with the primers by slightly modified method indicated by Lee et al. (2011) using the TaKaRa PCR machine (Takara Bio Inc., Otsu, Japan). PCR products were road 1.0% agarose gel with EtBr and visualized by UV transillumination.

2.7 Statistical Analysis

All data are expressed as means ± SE (n = 3), and all statistical analysis were performed by one-way analysis of variance followed by Duncan's test using PASW Statistics 21.0 software (SPSS, Chicago, IL, USA). A p-value <0.05 was considered to be statistically significant.

3 Results

3.1 Protective Effect of G-T Against LPS-Induced Cell Death and Production of NO and PGE_2 in RAW 264.7 Cells

The protective effects of G-T were assessed by MTT assays (Fig. 1a). G-T has no cytotoxic effect on RAW 264.7 cells at 25, 50, 100, or 200 μg/mL in comparison to control cells. To identify the inhibitory effect of G-T on LPS-stimulated NO and

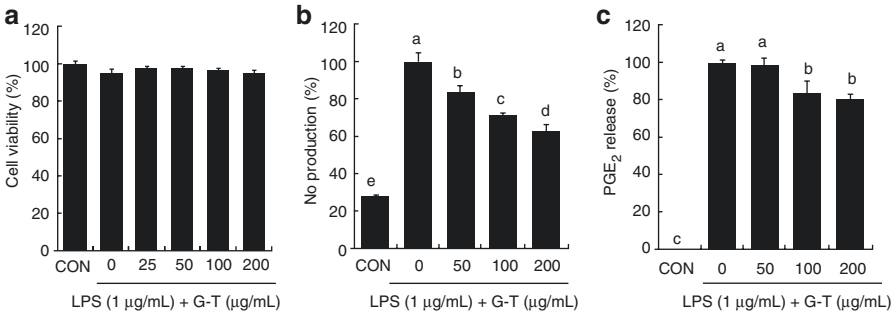

Fig. 1 The effect of G-T on cell viability (**a**), NO production (**b**), and PGE_2 release (**c**) in RAW 264.7 cells. RAW 264.7 cells were treated with LPS (1 μg/mL) and various concentrations of G-T (50, 100, and 200 μg/mL). After an incubation of 24 h, the viability of cells was determined by MTT and the production of NO and PGE_2 were analyzed. Each value indicates that the mean ± SE from three independent experiments. Values not sharing a common letter are significantly different at $P < 0.05$

PGE_2 release in RAW 264.7 cells, we used the Griess reaction and EIA kit. LPS stimulation significantly increased the NO and PGE_2 release (Fig. 1b, c). On the other hand, these increases were abolished by G-T treatment with an increment of concentrations.

3.2 *Effect of G-T on LPS-Induced iNOS and COX-2 Protein Expression*

In the present study, we also investigated the expression of iNOS and COX-2 protein to confirm the effects of G-T on inflammatory mediator production. The protein expressions of iNOS and COX-2 were markedly increased after LPS treatment, whereas they were inhibited by G-T (Fig. 2a–c).

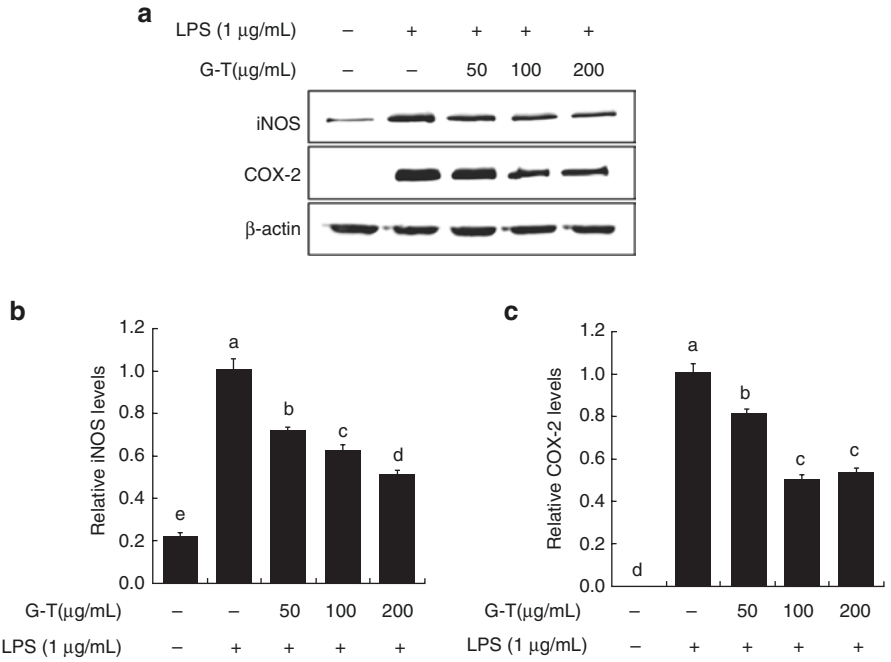

Fig. 2 Effect of G-T on the iNOS and COX-2 protein expressions in LPS-stimulated RAW 264.7 cells. (**a**) The protein expression levels of iNOS and COX-2 were determined by western blot analysis. (**b**, **c**) The intensity of the bands was measured by ImageJ software. Different alphabet letter means that the values significantly differ with the others at $P < 0.05$

3.3 *Effects of G-T on the mRNA Expression of IL-1β, IL-6 and TNF-α*

To examine effect of G-T on the mRNA expressions of inflammatory cytokines, RT-PCR was performed. Figure 3 showed that the stimulation of LPS increased the mRNA expression levels of IL-1β, IL-6 and TNF-α, whereas they were reduced by the application of G-T. It was dose-dependent.

3.4 *Effects of G-T on the NF-κB Activation*

To investigate whether the anti-inflammatory effect of G-T is associated to NF-κB signaling pathway, western blotting was examined. As shown in Fig. 4, it showed that G-T significantly suppressed the phosphorylation of IκB-α and the translocation of cytosol p65 into nucleus caused by the LPS stimulation. These results indicate G-T can inhibit the inflammatory response by the blockage of NF-κB signaling.

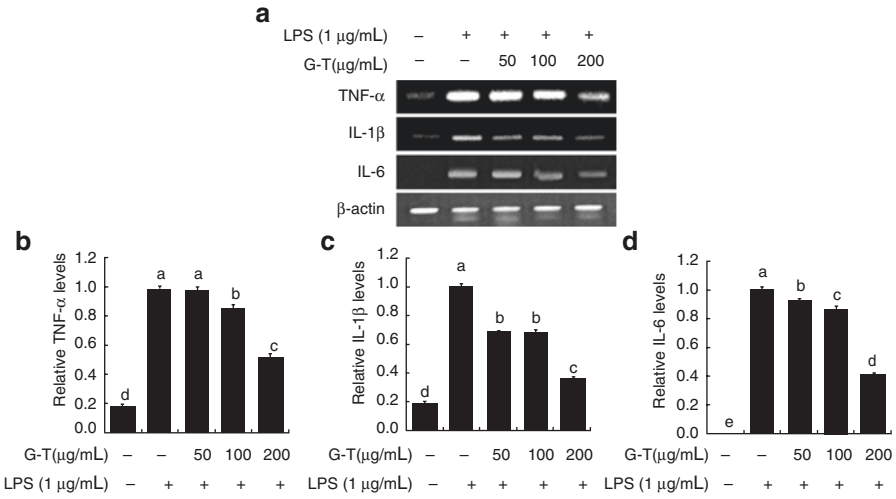

Fig. 3 Effect of G-T on TNF-α, IL-1β, and IL-6 expression in LPS-stimulated RAW 264.7 cells. (**a**) The mRNA expression levels of TNF-α, IL-1β, and IL-6 were determined by RT-PCR. (**b**–**d**) The intensity of the bands was measured by ImageJ software. Different alphabet letter means that the values significantly differ with the others at $P < 0.05$

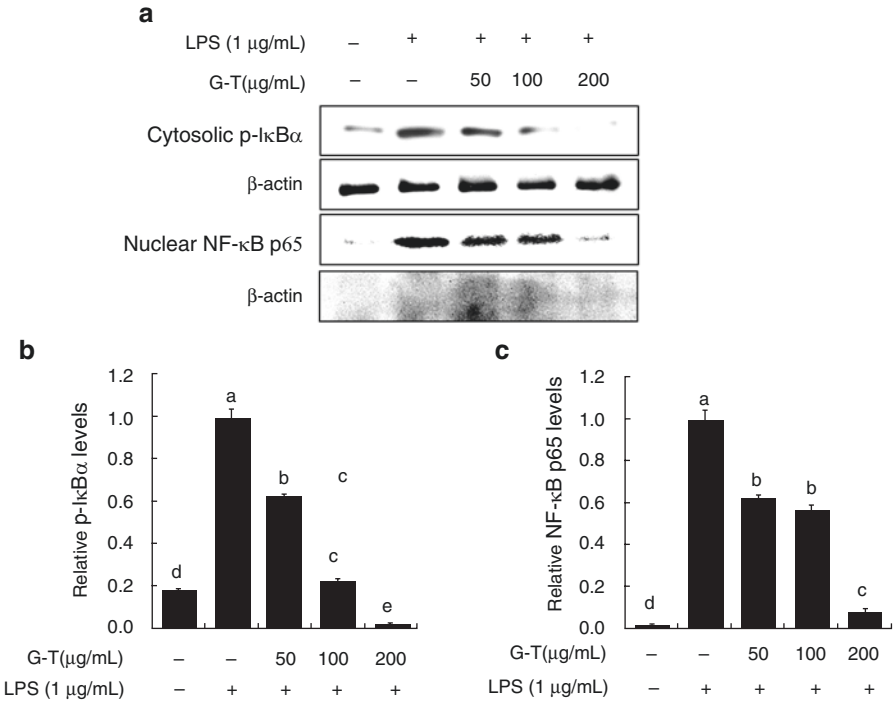

Fig. 4 Effect of G-T on the activation of NF-κB in LPS-stimulated RAW 264.7 cells. (**a**) The protein expression levels of cytosolic IκB-α NOS and nuclear NF-κB were determined by western blot analysis. (**b**, **c**) The intensity of the bands was measured by ImageJ software. Different alphabet letter means that the values significantly differ with the others at $P < 0.05$

4 Discussion

We first revealed the anti-inflammatory effects of G-T in LPS-stimulated RAW 264.7 cells. Stimuli of LPS promote several inflammatory mediators such as iNOS and COX-2, which synthesize NO and PGE_2 during inflammation in macrophages (Posadas et al. 2000). In general, NO plays a positive role as a neurotransmitter vasodilator, and in the immunological system as a defense against parasites, bacteria, and tumor cells (Nakagawa and Yokozawa 2002). In contrast, the abnormal NO production can cause cellular damages, cytotoxicity and tissue injury under pathological condition (Kim et al. 1999). So, the inhibition of NO is a critical key for the improvement of inflammatory diseases. Here, we identified that the NO and PGE_2 generations decreased in RAW 264.7 cells with the treatment of G-T as well as the down-regulated iNOS and COX-2 expression during the inflammatory response.

On the other hand, LPS known as a strong immune activator is known to elicit secretion of cytokines such as IL-1β, IL-6, IL-8, and TNF-α (Higashimoto et al. 2006; Yoon et al. 2010). Also, it increases the expression of adhesion molecules; induce the production of reactive oxygen species (ROS) (Higashimoto et al. 2006;

Yoon et al. 2010). It has been noted that over production of ROS are associated with tissue damage (Higashimoto et al. 2006). Interestingly, we also confirmed the reduced expression of IL-6, IL-1β, and TNF-α by G-T in LPS-stimulated RAW 264.7 cells. Similarly, several researchers demonstrated that taurine and its derivatives remarkably inhibited the generation NO, IL-1β and TNF-α and reduced the expression of IL-1β, IL-6 and TNF-α in primary cultured mouse mammary epithelial cells as well as LPS-stimulated mouse macrophages (Marcinkiewicz et al. 2006; Miao et al. 2012). With these results, our data suggests that G-T, a taurine derivate has the potential anti-inflammatory effect by virtue of its inhibition of inflammatory mediators as well as inflammatory cytokines.

NF-κB, an important transcription factor controls roles of genes involved in immune system including inflammation and immunity. Normally, NF-κB is kept as an inactive state bound to the inhibitory protein such as IκB in the cytoplasm (Caramori et al. 2004). Stimulators such as TNF-α and LPS can induce the initiation of intracellular signaling cascades and then result in the degradation and phosphorylation of IκB. And then, the released NF-κB is translocated into the nucleus, resulting in the activation of inflammatory mediators and cytokines that are pivotal mediators of the inflammatory reaction (Bae et al. 2005; Jones et al. 2007). With these points, intervention of NF-κB signaling may be advantageous in preventing or improving the progression of various inflammatory diseases. In the present study, we have shown that the phosphorylated IκB-α and p65 expression was down-regulated by G-T treatment and it can be linked to the reduced NO generation.

5 Conclusion

Taken together, this is the first study about the anti-inflammatory effect of G-T synthesized from taurine indicating the reduced NO and PGE_2 generations by the inhibition of inflammatory mediators as well as the decreased inflammatory cytokines via the regulating of NF-κB signaling in LPS-stimulated RAW 264.7 cells. Therefore, our findings indicate that the anti-inflammatory capacity of G-T might affect to the improvement of inflammations.

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Radio-Protective Effects of *Octopus ocellatus* Meat Consisted of a Plentiful Taurine Against Damages Caused by Gamma Ray Irradiation

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Abstract Gamma ray irradiation causes immune suppressive responses by inducing oxidative stress such as reduction of cell viability and damages in immune cells. In this present study, we investigated whether *Octopus ocellatus* meet (OM) consisted of a plentiful taurine has protective effects against damages caused by oxidative stress in murine splenocytes. First of all, we prepared the aqueous extract from OM (OMA) and identified it contained a plentiful taurine content. The result also showed that OMA exhibited the antioxidant activity by scavenging DPPH and $ABTS^+$ radicals and hydrogen peroxide. In addition, OMA improved the cell viability without cytotoxicity in gamma ray-irradiated murine splenocytes. Moreover, OMA significantly reduced the production of reactive oxygen species (ROS) in

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gamma ray-irradiated splenocytes. In further study, we identified that OMA protected zebrafish embryo via improving the reduced survival rate and decreasing the formation of deformity caused by the exposure of gamma ray irradiation. Also, OMA decreased the production of NO and ROS in gamma ray-irradiated zebrafish embryos as well as the induction of cell death. In these results, this study suggests that the consumption of taurine-rich foods, such as *O. ocellatus*, may be useful for the useful material for the protection against oxidative stress.

Keywords Radio-protective effect • Gamma ray irradiation • *Octopus ocellatus* meet

Abbreviations

OM Octopus ocellatus meat
ROS Reactive oxygen species

1 Introduction

The major detrimental effect of gamma ray irradiation leads to the reduction of viability and proliferation of cells via the induction of cellular damages followed by the generation of reactive oxygen species (ROS) and nitric oxide (NO) which induces oxidative stress (Bing et al. 2010; Lee et al. 2013; Halliwell and Aruoma 1991; Shih et al. 2002; Styskal et al. 2012; Ozben 2007). Oxidative stress caused by gamma ray irradiation can destruct the lymphoid and hemopoietic systems by occurring apoptosis of proliferating stem cells and/or splenocytes known as peripheral immune cells and finally induces serious diseases and/or death (Bing et al. 2010). With these points, it seems worthwhile to study possible protectors that basically counteract oxidative stress such as the production of ROS and NO and the induction of cell death at early stage. Indeed, major radio-protectors have been designed to reduce the various oxidative stresses, averting initial cascades of radiochemical events in the cells after gamma ray irradiation (Park et al. 2008a, b). Among them, beneficial capacities of various natural products that can protect cells and tissues against oxidative stress by gamma ray irradiation without obvious side-effects have been studied for recent years (Lee et al. 2013; Park et al. 2008a, b; Kang et al. 2006).

Particularly, many kinds of natural products with antioxidant and cytoprotective properties in peripheral immune cells such as splenocytes have received attention (Park et al. 2008a, b; Bing et al. 2010; Byon et al. 2008; Checker et al. 2008; Lee et al. 2013). Furthermore, zebrafish has been recently regarded as a favorite model system for testing radiation modifiers that can potentially be used for radio-protection or -therapeutic effects in humans (Hwang et al. 2007; Curran 1998; McAleer et al.

2005; Bladen et al. 2005; Geiger et al. 2006; Daroczi et al. 2006; Dimri et al. 2015). Various studies have also indicated that the zebrafish model exposed to gamma ray irradiation yields shortened and curved bodies, defects in the head and eyes, pericardial edema as well as cellular damages such as the production of ROS and NO and the induction of cell death (Hwang et al. 2007; Curran 1998; McAleer et al. 2005; Bladen et al. 2005). Recent our studies have evaluated radio-protective effects of natural products in gamma ray-irradiated zebrafish embryo models (Lee et al. 2016).

The mollusc *Octopus ocellatus (O. ocellatus)*, which belongs to Octopodidae under the phylum Mollusca, provides an important resource of natural proteins for human beings (Wei et al. 2015). *O. ocellatus*, begin nekto-benthic or benthic life as miniatures of the adults (Boletzky 1977). Previous studies have reported the potential involvement of molluscan Serpin-SPI in antibacterial immune response and the post-hatching development of the brain in *O. ocellatus* as experimental animals (Yamazaki et al. 2002). There are no scientific reports about the composition, biological activity and mechanism of *O. ocellatus* until now; although it became known that it contains plentiful taurine contents for recovery of fatigue.

Therefore, the present study first reveals the composition of *O. ocellatus* and its radio-protective effects in splenocytes and zebrafish embryo model exposed to oxidative stress caused by gamma ray irradiation.

2 Methods

2.1 Chemicals and Materials

ABTS, DPPH, fluorescein sodium salt, 2,2-azobis(2-amidino-propane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 3-(4-5-dimethyl-2yl)-2-5-diphynyltetrasolium bromide (MTT), 2′,7′-dichlorodihydroflurescin diacetate (DCFH-DA), diphenyl-1-pyrenylphosphine (DPPP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's phosphate-buffered saline (DPBS), Dulbeco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute medium (RPMI medium), 10% fetal bovine serum (FBS), penicillin/streptomycin, and other materials for culturing the cells were obtained from Gibco BRL (Life Technologies, New York, USA).

2.2 Preparation of an Aqueous Extract from O. ocellatus Meat *(OMA) and Its Composition*

Korea *O. ocellatus* was purchased from Sorae Port market of South Korea during spring season, washed and stored at −60°C until required for analysis. To prepare the aqueous extract from *O. ocellatus* meat (OM), OM (100 g) was mixed with 5 L

of distilled water for 24 h at 25°C. Then, the mixture was centrifuged at 3000 rpm and filtered through a Whatman No. 4 filter paper. The obtained filtrate (OMA) was freeze-dried and stored at −20°C until further use.

2.3 Determination of Amino Acid Compositions

Free amino acid composition of OMA was analyzed by an amino acid analyzer (S433-H, Sykam GmbH, Germany). A 50 mg of OMA was hydrolyzed using 2 mL of 6.0 M HCl in a sealed-vacuum ampoule at 110°C for 24 h. Hydrochloric acid was removed by rotary evaporator and brought to a final volume of 10 mL with 0.2 M sodium citrate buffer (pH 2.2). Amino acids were determined using a cation separation column (LCA K06/Na, 4.6 × 150 mm) with flow rates of 0.45 mL/min (buffer) and 0.25 mL/min (reagent) at wavelengths of 440 and 570 nm. For determination of free amino acids, 2.0 g of OMA was homogenized at 12,000 rpm twice for 2 min with 75% ethanol, followed by centrifuging at 2000 × *g* for 30 min. The supernatant was then removed by rotary evaporator, and it was then added 8 mL of distilled water with 5′-sulfosalicylic acid (0.2 g) at 4°C for 1 h. The mixture was centrifuged at 2000 × *g* for 30 min, and the 2 mL supernatant was transferred into a tube containing 1 mL of 0.2 M lithium citrate buffer (pH 2.2) added.

2.4 Determination of Antioxidant Activities

2.4.1 DPPH Radical Scavenging Activity

The DPPH radical scavenging activity was measured according to the method of Blois (Blois MS, 1958) with slight modifications. Briefly, a 100 μL of OMA was added to 100 μL of 150 μM DPPH methanolic solution. The mixture was at room temperature for 30 min in dark, and the absorbance was read at 517 nm (Spectra Max Plus^{384}, USA). The absorbance indicated higher radical scavenging activity. The DPPH radical scavenging activity was calculated using the following equation:

$$\text{Scavenging activity } (\%) = ((A_{517} \text{ of control} - A_{517} \text{ of sample})/A_{517} \text{ of control}) \times 100$$

The IC_{50} value was defined as the concentration required for scavenging 50% of DPPH.

2.4.2 ABTS$^+$ Radical Scavenging Activity

The ABTS$^+$ radical scavenging activity was measured according to the method of Leong (Leong and Shui 2002). A stock solution (7 mM ABTS and 2.4 mM potassium persulfate) was prepared for 16 h in the dark. Prior to each measurement,

the distilled water was mixed with the stock solution until it displayed an absorbance of 1.50 ± 0.05 at 414 nm. A 50 μL of OMA and 150 μL of the ABTS solution were mixed, reacted to stand for 10 min at room temperature, and the absorbance was measured at 414 nm. The results were expressed as μM TE/mg sample.

2.4.3 Hydrogen Peroxide Scavenging Activity

The H_2O_2 scavenging activity was determined according to the method of Mueller (Mueller and Arnhold 1995). In Brief, 100 μL of OMA (0.125, 0.25, 0.5 and 1 mg/mL) was mixed with sodium phosphate buffer (100 μL, 0.1 M, pH 5.0) in a 96-well plate followed by addition of H_2O_2 (20 μL, 20 mM), and then the mixture was incubated at 37°C for 5 min. At later dates, ABTS (30 μL, 1.25 mM) and peroxidase (30 μL, 1 U/mL) were added to the mixture followed by incubation at 37°C for 10 min. The absorbance values were measured at 405 nm. The IC_{50} value was defined as the concentration required for scavenging 50% of H_2O_2.

2.5 Isolation of Splenocytes from Mice

To isolate splenocytes from mice, spleens were aseptically removed from C57/BL6 mice (ages 6–9 weeks) (SLC, Inc., Shizuoka, Japan). And then, blood cells were lysed by immersion in ACK lysis buffer at room temperature for 5 min in darkness. The purified splenocytes were washed with DPBS and suspended in RPMI (Roswell Park Memorial Institute) medium (Gibco BRL) including 10% FBS (Fetal bovine serum), and 1% antibiotic (100 U/mL penicillin and 100 mg/mL streptomycin).

2.6 The Origin and Maintenance of Parental Zebrafish and Experimental Design

Adult zebrafish purchased from a commercial dealer (Seoul Aquarium, Seoul, Korea) were kept in a 3.5 L acrylic tank at 28.5 ± 1°C. The zebrafish were fed twice a day (Tetra GmgH D-49304 Melle) in a 14/10 h light/dark cycle. The zebrafish were mated, and spawning was stimulated by the onset of light. Then, zebrafish embryos were obtained within 30 min of natural spawning and moved to petri dishes containing the media.

The zebrafish embryos (n = 30) were transferred to individual e-tube containing 950 μL of the embryo medium. At 1 day postfertilization (dpf), OMA (31.25, 62.5, and 125 μg/mL) was added to the tubes before irradiation of gamma ray.

2.7 Irradiation with ^{60}Co Gamma Rays

The isolated splenocytes and zebrafish embryos were exposed to gamma ray irradiation using a ^{60}COTheratron® (Best Theratronics Ltd, Ottawa, Ontaria, Canada) in the teletherapy unit of the applied radiological science research Institute, Jeju National University, Korea. The cells and zebrafish embryos were respectively exposed to 2 and 30 Gy at a dose rate of 1.5 Gy/min and source-surface distance of 150 cm, as previously reported (Park et al. 2010).

2.8 MTT Assay

The cell viability was determined using the MTT assay. The non-irradiated splenocytes (1.0×10^5 cells/100 μL/well) were seeded in a 96-well plates and treated with various concentrations of OMA (62.5, 125 and 250 μg/mL) at 37°C for 24 h. Also, before the exposure of gamma ray irradiation, the splenocytes were treated by OMA for 1 h. After 24 h of the irradiation, 15 μL of MTT solution (5 mg/mL) was added to each well, followed by incubation for 4 h in an atmosphere of 5% CO_2 at 37°C. The formazan crystals in viable cells were dissolved in 100 μL DMSO solution (10% SDS, and 50% DMSO in pH 7.4). The optical density of each well was measured at 540 nm using a microplate reader (SpectraMax® M2/M2^e, CA, USA).

2.9 DCF-DA Assay

To identify effect of OMA on the production level of intracellular ROS, DCF-DA assay was performed. First, splenocytes were pre-incubated by OMA at various concentrations (62.5, 125 and 250 μg/mL) at 37°C for 24 h. After the irradiation of 2 Gy, the cells were directly seeded in a 96 well culture plate at 1×10^5 cells/well and additionally incubated for 1 h. After the incubation, DCF-DA solution (25 μM) was added to each well for 10 min at 37°C. The intensity of DCF-DA was measured at 585 and 620 nm using a microplate reader (SpectraMax® M2/M2^e).

The intracellular ROS scavenging activity (%) was calculated as 100 × [(optical density of irradiated group) − (optical density of irradiated group with OMA treatment)]/(optical density of irradiated group).

2.10 Measurement of the Survival and Deformity Rates

To examine the effects of OMA on the survival rates, zebrafish embryos (n = 30) were treated with OMA (62.5, 125 and 250 μg/mL) at 1 dpf and the number of surviving zebrafish embryos was monitored daily until 7 dpf. For the next experiments, we used OMA at the nontoxic concentrations (62.5, 125 and 250 μg/mL).

To test the effects of OMA on the survival rates in gamma ray-irradiated zebrafish embryos, the latter (n = 30) were treated with OMA (62.5, 125 and 250 μg/mL) at 1 dpf for 2 h and irradiated with 30 Gy. The number of surviving zebrafish within each group was monitored daily until 7 dpf.

At 2 days after the exposure to gamma ray, the deformity rates were examined by monitoring each point during the formation of cup phenotypes in the zebrafish. The standard is the following: the failure of formation of the cup phenotypes, the mild formation of the cup phenotypes, the moderate formation of the cup phenotypes, and the severe formation of the cup phenotypes.

2.11 Measurement of NO and ROS Production and Cell Death

Protective effects of OMA on gamma ray-irradiation were evaluated by measuring the productions of ROS and NO, and cell death in gamma ray-irradiated zebrafish embryos. The test sample was treated to zebrafish at 3 dpf with the concentrations of 62.5, 125 and 250 μg/mL for 2 h. Then, the zebrafish embryos were irradiated with gamma ray (30 Gy) except Non-IR group. The production of NO and ROS were analyzed by the methods indicated in previous our study (Lee et al. 2016). After incubation for each experiments, the zebrafish embryos groups were rinsed in the fresh medium and anesthetized with 0.001% MS222 (ethyl 3-aminobenzoate methanesulfonate) before the observation. At 1 h after the irradiation, ROS production levels were checked by using an oxidation-sensitive fluorescent probe dye, DCF-DA solution (20 μg/mL). NO levels were evaluated at the 12 h after the irradiation by using a fluorescent probe dye, DAF-FM DA (10 μM). Cell death levels were measured at the 24 h after the irradiation using a nucleic acid-selective fluorescent cationic dye, acridine orange (7 μg/mL). These dyes were treated according to the same method indicated in previous our study (Lee et al. 2016). The images of the stained zebrafish embryos were observed by a fluorescent microscope equipped with Olympus IX2-SLP (Olympus Japan) camera. The fluorescence intensity of individual zebrafish embryos was quantified using image J computer software.

2.12 Statistical Analysis

All results were expressed as means ± SE, and all statistical comparisons were made by means of one-way analysis of variance followed by Duncan's test using PASW Statistics 21.0 software (SPSS, Chicago, IL, USA). A P-value <0.05 was considered to be statistically significant.

3 Results

3.1 The Aqueous Extract of OM (OMA) Contained the Plentiful Taurine Component

The aqueous extract of OM (OMA) was prepared and its extraction yield and free amino acid composition were measured. The extraction yield of OMA was 56.87 ± 0.07% and contained the large amounts of protein contents (61.33 ± 0.40%), compared to the other components (carbohydrate and phenol contents). Especially, OMA contained the higher amount of taurine contents (29.67%) than the other free amino acid components in indicated in Table 1.

Table 1 Free amino acid composition (%) of OMA

Amino acid	% Amino acid
Phosphoserine	0.23
Taurine	29.66
Phosphoethanolamine	0.45
Aspartic acid	1.64
Threonine	0.79
Serine	0.87
Glutamic acid	4.10
Proline	1.40
Glycine	2.34
Alanine	4.41
Citrulline	2.85
α-Aminobutyric acid	0.12
Valine	4.70
Methionine	5.31
Isoleucine	4.45
Leucine	9.90
Tyrosine	4.99
Phenylalanine	5.50
β-Alanine	1.17
Histidine	0.33
Anserine	0.11
Tryptopan	0.00
Hydroxylysine	0.27
Ornitine	2.85
Lysine	9.01
Ethanolamine	0.04
Arginine	2.50
Total	100.00

3.2 *Anti-oxidant Activity of OMA*

Antioxidant activity of OMA was determined by measuring the $ABTS^+$ and DPPH radicals, and hydrogen peroxide scavenging activities. As shown in Fig. 1a, OMA led to the high $ABTS^+$ radical scavenging activity in the used all concentrations. Also, Fig. 1b exhibited OMA scavenged the DPPH radicals and it was a dose-dependent manner. The effect of OMA against hydrogen peroxide was shown in Fig. 1c. OMA exhibited the hydrogen peroxide scavenging activity in a dose-dependent manner. The result indicated that OMA had the potent antioxidant activity.

3.3 *OMA Improved the Survival Rate of Gamma Ray-Irradiated Splenocytes*

Next, to investigate effect of OMA on the viability of splenocytes, we utilized MTT assay. Fig. 2a indicated that OMA did not show the cytotoxicity at the concentrations of 31.25, 62.5, and 125 μg/mL compared to non-treated control cells. So, non-toxic concentrations were used for further experiments. As shown in Fig. 2b, the exposure of

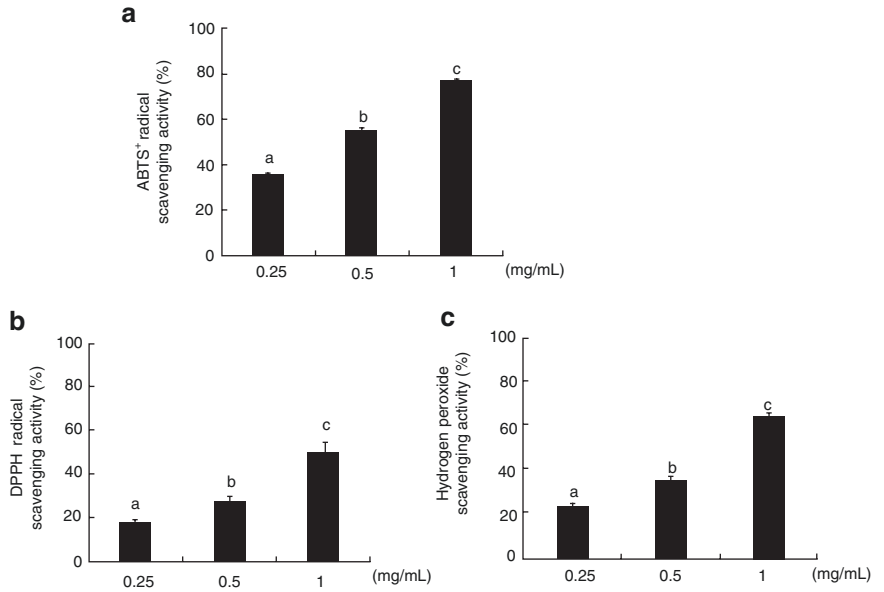

Fig. 1 Antioxidant activities of OMA. (**a**) $ABTS^+$ radical scavenging activity, (**b**) DPPH radical scavenging activity, and (**c**) hydrogen peroxide scavenging activity. All statistical analyses were performed with three independent experiments and data represented as means ± SE. $^{a–c}$The values with different subscripts indicate significant difference within the same concentration ($p < 0.05$)

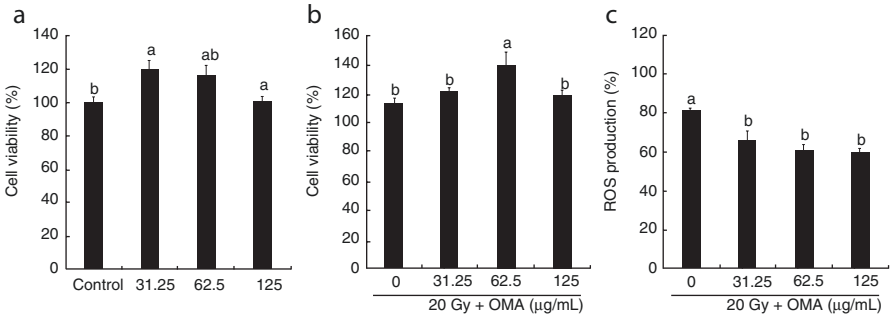

Fig. 2 Effect of OMA on the splenocyte's viability (**a**), the survival rate (**b**) and the production of ROS (**c**) in gamma ray-irradiated splenocytes. (**a**) Splenocytes were incubated with OMA for 24 h and the cell viability was determined by MTT assay. (**b**) Splenocytes were pretreated with OMA for 1 h and then exposed to 20 Gy irradiation for an additional 24 h. The cytoprotective effect was measured by MTT assay. (**c**) Splenocytes were pretreated with OMA for 1 h and then exposed to 20 Gy irradiation for an additional 1 h. Intracellular ROS generation was assessed by using the oxidation sensitive dye, DCFH-DA. [a, b]The bars with different letters represent significant differences ($p < 0.05$). Values are expressed as mean ± SE (n = 3)

gamma ray irradiation into the cells significantly reduced the viability of splenocytes ($p < 0.05$), compared to that of non-treatment group. However, the pretreatment of OMA dose-dependently improved the reduced cell viability, compared to that of only gamma ray-irradiated splenocytes (Fig. 2b). This result clearly indicates that the exposure of splenocytes to OMA confers to significant cytoprotective effect against gamma ray irradiation.

3.4 OMA Decreased the ROS Production in Gamma Ray-Irradiated Splenocytes

To evaluate the effects of OMA on intracellular ROS generation, we performed the well-characterized DCFH-DA fluorescence assay. As shown in Fig. 2c, gamma ray irradiation caused the increased ROS production in splenocytes, compared to that of the non-treated cells. However, OMA significantly decreased the intracellular ROS levels caused by the exposure to gamma ray-irradiation ($p < 0.05$) compared to the non-treated control. In addition, it was a dose-dependent manner. Therefore, this result suggests that OMA plays a role in a potential protector against oxidative stress.

3.5 OMA Improved the Survival Rate in Gamma Ray-Irradiated Zebrafish Embryos

Next, we utilized the zebrafish model for investigating the anti-oxidative effect of OMA in *in vivo* model. First of all, to determine effect of OMA on the toxicity of zebrafish embryos, we examined the survival rate. The result exhibited that the

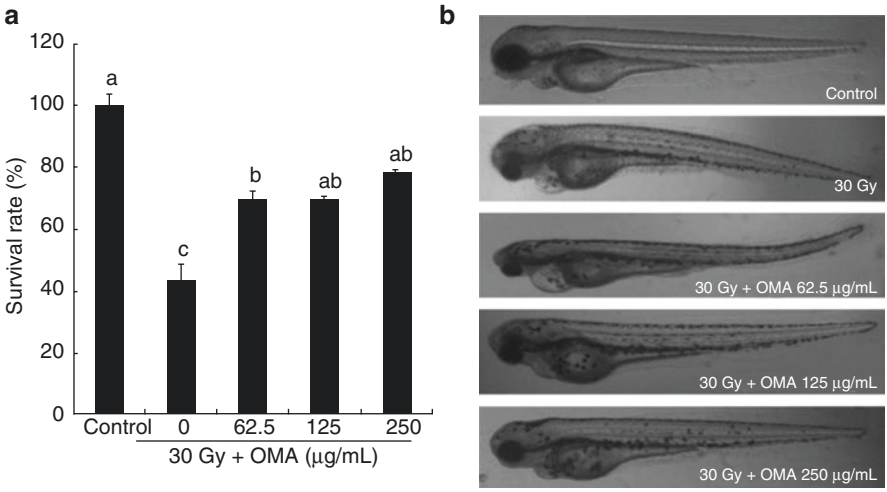

Fig. 3 Effect of OMA on the survival rate (**a**) and the formation of deformity (**b**) in gamma ray-irradiated zebraifh embryos. Zebrafish embyos were pretreated with OMA for 1 h and then exposed to 30 Gy irradiation. [a,b]The bars with different letters represent significant differences ($p < 0.05$). Values are expressed as mean ± SE (n = 30)

pre-treatment of OMA did not affect to the survival of zebrafish embryos at the tested all concentrations, compared to the non-treated zebrafish embryos (data not shown). We additionally checked the effect of OMA on survival rate in gamma ray-irradiated zebrafish embryos. In Fig. 3a, we identified that the survival rate was markedly reduced by the exposure of gamma ray irradiation in zebrafish embryos. Interestingly, it was significantly improved by the pre-treatment of OMA in a dose-dependent manner, comparing to the only gamma ray-irradiated zebrafish embryos (Fig. 3a). This result indicates that OMA protects zebrafish embryos against death caused by gamma ray irradiation.

3.6 OMA Inhibited the Formation of Deformity in Gamma Ray-Irradiated Zebrafish Embryos

We examined the effect of OMA on the formation of deformity caused in gamma ray-irradiated zebrafish embryos. When zebrafish embryos were exposed to gamma ray irradiation, the tail bending were markedly increased in zebrafish embryos comparing with the non-treated zebrafish embryos (Fig. 3b). However, the increased tail bending was recovered by the pre-treatment of OMA at all used concentrations, compared to the only gamma ray-irradiated zebrafish embryos. Form the result, we found out the protective effect of OMA against the morphological change in zebrafish embryos via inhibiting the formation of deformity.

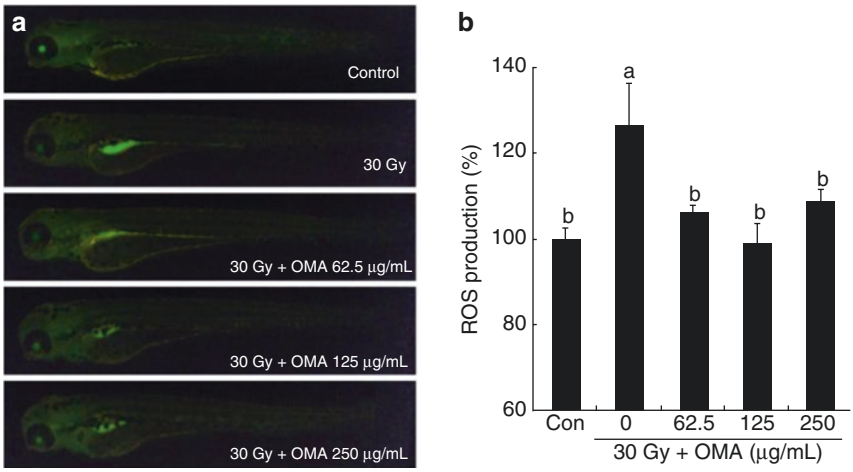

Fig. 4 Effect of OMA on the ROS production (**a**) and the image analysis (**b**) in gamma ray-irradiated zebraifh embryos. Zebrafish embryos were pretreated with OMA for 1 h and then exposed to 30 Gy irradiation for an additional 1 h. [a,b]The bars with different letters represent significant differences ($p < 0.05$). Values are expressed as mean ± SE (n = 30)

3.7 OMA Reduced the Production of ROS in Gamma Ray-Irradiated Zebrafish Embryos

To measure the effects of OMA on intracellular ROS generation, we carried out the DCFH-DA fluorescence staining assay. As shown in Fig. 4a, b, the gamma ray irradiation markedly caused the increased ROS production in zebrafish embryos, compared to that of the non-treated zebrafish embryos. In contrast, OMA significantly decreased the intracellular ROS levels produced by the exposure to gamma ray-irradiation ($p < 0.05$), compared to the only gamma ray-irradiated zebrafish embryos. This result indicates that OMA can inhibit the ROS productions in zebrafish embryo model as well as cells.

3.8 OMA Reduced the Production of NO in Gamma Ray-Irradiated Zebrafish Embryos

To elucidate the effect of OMA on the production level of NO of gamma ray-irradiated zebrafish embryos, the staining assay using a fluorescent probe dye, DAF-FM DA were performed. In zebrafish embryos, the exposure to gamma ray irradiation highly increased the fluorescence intensity based on the NO production level up to

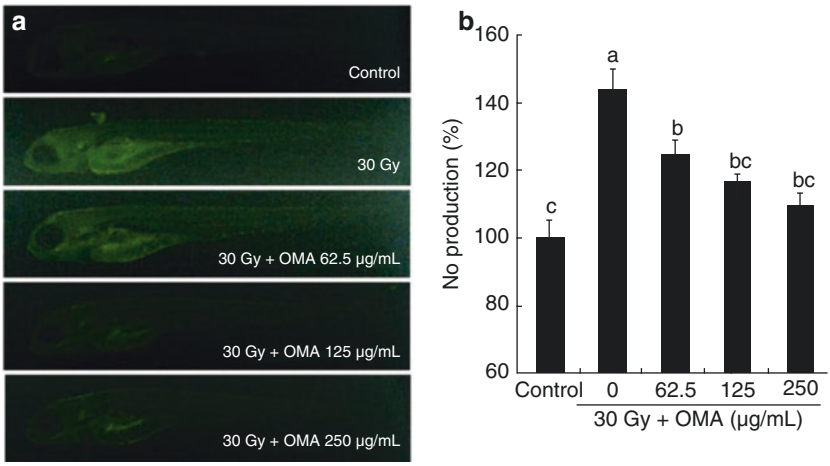

Fig. 5 Effect of OMA on the NO production (**a**) and the image analysis (**b**) in gamma ray-irradiated zebraifh embryos. Zebrafish embyos were pretreated with OMA for 1 h and then exposed to 30 Gy irradiation for an additional 12 h. [a,b]The bars with different letters represent significant differences ($p < 0.05$). Values are expressed as mean ± SE (n = 30)

approximately 145% (Fig. 5a, b). But, the treatments of OMA exhibited significant reductions of the NO production in a dose-dependently compared to the only-gamma ray-irradiated zebrafish embryos. This result suggests that OMA markedly reduced the abnormal NO production.

3.9 OMA Protected Zebrafish Embryos Against Cell Death Caused by Gamma Ray-Irradiation

The protective effect of OMA on cell death was examined by the acridine orange staining assay in gamma ray-irradiated zebrafish embryos. After the gamma ray irradiation, the fluorescence intensity of cell death was increased up to approximately 122% at 4 dpf of zebrafish embryos, compared to the non-treated zebrafish embryos (Fig. 6a, b). In contrast, the increased cell death induction was dose-dependently decreased by the pre-treatment of OMA, compared to the only-gamma ray-irradiated zebrafish embryos. Interestingly, it was similar to the pattern with the results on the productions of ROS and NO. This result indicates that OMA protects zebrafish embryos against the induction of cell death as well as the productions of ROS and NO caused by gamma ray-irradiation.

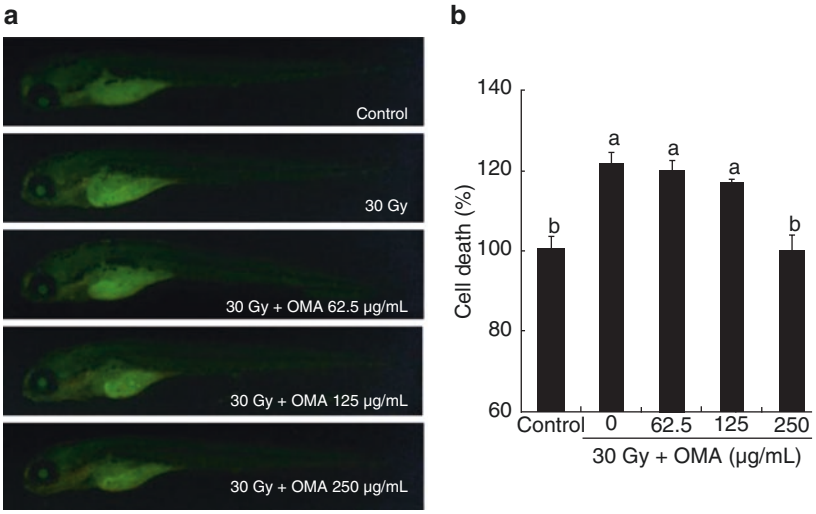

Fig. 6 Effect of OMA on the cell death (**a**) and the image analysis (**b**) in gamma ray-irradiated zebrafish embryos. Zebrafish embyos were pretreated with OMA for 1 h and then exposed to 30 Gy irradiation for an additional 24 h. [a,b]The bars with different letters represent significant differences ($p < 0.05$). Values are expressed as mean ± SE (n = 30)

4 Discussion

In the present study, we first indicated the direct evidence of the plentiful taurine composition of *O. ocellatus* meat and revealed its radio-protective effects in cells and *in vivo* model after the irradiation of gamma ray.

O. ocellatus, a kind of cephalopod plentifully produced in Korea, Japan and China is a popular taurine-rich seafood ingredient. Our result first showed the proximate compositions of *O. ocellatus* meat (OM) consisted of the plentiful taurine contents, compared to the others. Normally, taurine (2-aminoethane sulfonic acid) is one of the major intracellular and the most abundant free amino acid in most mammalian tissues. In our study, the aqueous extract of OM (OMA) was prepared and indicated its plentiful protein contents containing abundant taurine, compared to the other components. The pharmacological and physiological effects of taurine have been recently reported (Huxtable 1992; Zhou et al. 2011; Rashid et al. 2013; Islambulchilar et al. 2015). However, although a number of studies concerning the effect of taurine on oxidative stress have been reported, the cytoprotective effect of taurine-rich OMA, on oxidative stress has not been validated. Our data first exhibited the marked antioxidative activities on $ABTS^+$ and DPPH radical as well as hydrogen peroxide. Recent studies have reported oxidative stress represents an imbalance between ROS production and cellular antioxidant defense system (Rigoulet et al. 2011). The excessive produc-

tion of ROS causes damages of essential macromolecules of the cell, leading to the development of a number of human diseases, such as atherosclerosis, cancer, inflammation, rheumatoid arthritis and neurodegenerative diseases (Okezie 1998). So, the removal of ROS is an important key in various antioxidants (Kumar et al. 2012). Moreover, previous study have indicated the considerable evidence that taurine contents can play a role as a direct antioxidant by scavenging ROS (Niittynen et al. 1999) or as an indirect antioxidant by preventing changes in membrane permeability due to oxidative stress. Nevertheless, its biochemical and physiological functions are still undefined. With these reports, our data indicated that the taurine rich-OMA can be a beneficial component for its antioxidant capacities. Thus, exposure of gamma ray irradiation suppresses survival and proliferation of cells by causing DNA damage followed by ROS production which induces oxidative stress (Bing et al. 2010; Park et al. 2010). Present study has shown that OMA protected splenocytes, a peripheral immune cell against oxidative stress induced by the gamma ray-irradiation via the improving cell viability and the production of ROS. We also confirmed that the cytoprotective capacities of OMA contributed to the improved survival rate and deformity of zebrafish embryo model by reducing the productions of ROS as well as the cell death caused by gamma ray irradiation. The increased NO production subsequently brings about cytotoxicity, damages and tissue injury under pathological condition (Kim et al. 1999). Recent reports demonstrated that taurine and its derivatives remarkably inhibited the generation of NO in primary cultured mouse mammary epithelial cells as well as LPS-stimulated mouse macrophages (Marcinkiewicz et al. 2006; Miao et al. 2012). These results suggest that rich taurine of OMA affected to the decrease of NO and cell damage, and has been identified as a potent component in improved survival rate and deformity. Also, we believe that the consumption of taurine-rich foods, such as *O. ocellatus*, may be useful as the functional material for the protection against oxidative stress.

5 Conclusion

In conclusion, the findings of this study demonstrate that OMA can protect peripheral immune cells and zebrafish embryos against oxidative stress caused by gamma ray irradiation via the improvement of their survival rates by inhibiting the productions of ROS and NO and the induction of cellular damages. These results provide a scientific basis for the radio-protective effects of OMA consisted of plentiful taurine contents and suggest that it may be of potential value in the treatment of various diseases associated with oxidative stress.

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Part VIII
Taurine in Nutrition

The Effect on Blood Biochemical Factors of a ICR-Mice in a High-Fat Diet with Taurine 20%

Kyung-Ok Shin, Jin A. Yoon, and Kyung-Soon Choi

Abstract The purpose of this study was to examine the effects of taurine on lipid levels and liver function and the actions of insulin and leptin by biochemically analyzing the blood of albino mice fed a diet containing 20% taurine. The group fed a high-fat diet (HF) containing 20% taurine (HF + taurine 20%) showed higher blood HDL cholesterol levels as well as significantly lower total cholesterol and triglyceride levels ($p < 0.05$) than the group fed HF. No significant difference was observed among indicators of liver function, such as alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase activities. However, the HF + taurine 20% group showed significantly lower insulin and leptin levels than the HF group ($p < 0.05$). These findings show that 20% taurine had a significant effect on blood lipid levels and blood sugar maintenance in mice fed an HF.

Keywords Taurin • Organ weight • Lipid level • Liver function • Insulin • Leptin

Abbreviations

cAMP	Cyclic AMP
HF	High fat diet
HDL-cholesterol	High density lipoprotein-cholesterol
LDL-cholesterol	Low density lipoprotein-cholesterol

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1 Introduction

Modern people tend to suffer from more diseases or preexisting conditions with a lower frequency due to stress, a lack of exercise, and poor eating habits (Shin et al. 2007). Most of these diseases are caused by poor eating habits, such as diets containing excessive amounts of a particular nutrient. Therefore, a number of dietary therapies and nutrient-intake methods have been proposed. One such nutrient that has recently been attracting attention is taurine (Yoon et al. 2015). The amount of taurine synthesized within the human body is very low (50–125 mg/day), and synthesis is decreased in response to injuries or severe disease, such as sepsis (Redmond et al. 1998). The main source of taurine in the human diet is animal protein (Rana and Sanders 1986; Huxtable 1992; Yoon et al. 2015), and approximately 40–400 mg can be taken in per day through food consumption (Rana and Sanders 1986). The safety of taurine has been validated through experiments on animals (Olney and Hopas 1970; Takahashi et al. 1972; Park et al. 1998) and in humans (Lubec et al. 1997; Cha et al. 1999), and no side effects were noted. Taurine has various physiological activities, including metabolic and non-metabolic actions. The metabolic actions include antioxidant effects (You and Chang 1998), sulfur amino acid detoxification (Park et al. 1998, 2001a, b), xenobiotic conjugation, and bile acid conjugation. The non-metabolic actions include maintenance of cell membrane stability (Pasantes-Morales et al. 1985), electrolyte transport, osmoregulation (Kasuya et al. 1992), cell membrane phospholipids, and ion channel interactions, and are attributable to its zwitterionic properties (Yoon et al. 2015). In the human body, taurine promotes the synthesis of bile acid, discharges lipids (Huxtable 1992; Wasserhess et al. 1993; McKee 1999; Park 2002), promotes the growth and differentiation of neurons (Gaull 1986; Kang 2002), exerts effects on the retina and vision (Sturman et al. 1975), shows antioxidant activity (Green et al. 1991; Ogasawara et al. 1993; You and Chang 1998), promotes osmoregulation (Kasuya et al. 1992), prevents dementia (Huxtable 1989), promotes collagen synthesis within bones (Park et al. 2001a), protects the heart and liver (Nakashima et al. 1982, 1990), accelerates glycometabolism (Yim et al. 2010), and limits the production of melanin pigment (Joung 2007). In addition, experiments on cancer cells showed that taurine has anticancer effects. Taurine is mainly distributed in white blood cells, the heart, retina, and musculoskeletal regions (Sturman et al. 1975; Pierno et al. 1998; Kendler 1989; Gordon and Heller 1992; Son 2000; Matsuzaki et al. 2002; Yatabe et al. 2003; Grimble 2006), and accounts for approximately 0.1% of body weight because it is a component largely contained in the human body. More effects and diverse functions will be likely discovered in the future. Diverse studies on taurine as a biologically active substance have recently been reported. Not only in Korea but also in advanced nations, such as Japan, European countries, and the US, the use of taurine as a health drink and therapeutic agent is increasing (Park et al. 2001b). In a previous study, we found through experiments in which the blood of mice was biochemically analyzed, that adding 20% taurine to a high-fat diet (HF) is more effective than 10% taurine. Accordingly, in the present study, 20% taurine

was added to the HF of mice. Then, blood samples were biochemically analyzed to examine the effects of taurine on lipid levels and liver function and the actions of insulin and leptin.

2 Methods

2.1 Animal Experiments

All experimental animals were obtained from Orient Bio Co., Ltd. Eight-week-old ICR male mice were used, and five mice assigned to each experimental group. Mice were adapted to solid food (PicoLab® Rodent Diet) for 1 week, randomly grouped (depending on weight), and then housed with *ad libitum* access to water and feed for 60 days. The housing conditions were as follows: room temperature, 20 ± 2 °C; humidity, 40–60%; and an 11 ± 1-h light/dark cycle. The experimental period was 7 weeks. The animal procedures were performed in accordance with the guidelines of the Adventist University Animal Experiment Ethics Committee (approval number: SYUIACUC 2015-006).

2.2 Composition of Animal Feed

Taurine powder was purchased from Dong A Pharmaceutical Co., Ltd. The animal feed used for the control group was produced by converting solid feed to a powder form. The composition of the feeds was determined based on the weight ratio. The feed for the control groups was composed of 60% carbohydrate (starch + sucrose + glucose + fructose + lactose), 21% protein, 13% lipid (beef tallow), 1% vitamin, 3% mineral, and 2% fiber. The feed for the HF group was composed of 53% carbohydrate (starch + sucrose + glucose + fructose + lactose), 21% protein, 20% lipid (beef tallow), 1% vitamin, 3% mineral, and 2% fiber. The feed for the HF + taurine 20% group was composed of 33% carbohydrate (starch + sucrose + glucose + fructose + lactose), 21% protein, 20% lipid (beef tallow), 20% taurine, 1% vitamin, 3% mineral, and 2% fiber (Choi et al. 2013).

2.3 Average Body and Organ Weights

The body weight of the mice was measured once per week. Each group was fasted for 12 h before sacrifice. After administering ethyl ether as anesthesia, an incision was made on the abdomen and the organs were extracted. Then, each extracted organ was washed with saline solution, the connective tissue was removed, and the weight was measured.

2.4 Blood Collection

The mice were fasted for 12 h prior to sacrifice. After using ethyl ether for anesthesia, an incision was made on the abdomen and a syringe was used to collect blood from the heart. Collected blood samples were incubated at 4 °C for approximately 1 h. Then, the serum was separated using a centrifugal separator at 5 °C (3000 rpm/15 min). Aliquots of serum (100 μL each) were placed into microtubes and stored at −70 °C until use.

2.5 Blood Lipid Analysis

Serum cholesterol content was measured with o-phthaldehyde according to the methods of Cho and Choi (2007) and Rudel and Morris (1973). The sample was split into 0.1-mL aliquots, and 0.3 mL of a 33% KOH solution and 3.0 mL of 95% ethanol were added to each aliquot and mixed. Then, the serum was heated in a water bath at 60 °C for 15 min and then cooled. Nucleic acid (5.0 mL) was added, and the sample was mixed. Then, 3.0 mL of distilled water was added and mixed for 1 min. After separating the layers, 1.0 mL of nucleic acid layer was removed. Then, the nucleic acid layer was concentrated and dehydrated under nitrogen. Then, 2.0 mL of o-phthaldehyde reagent was added and mixed. After 10 min, 1.0 mL of thick sulfuric acid was added, and the sample was mixed to create the color reagent. Within 10–90 min after adding the sulfuric acid, the absorbance at 550 nm was measured with a spectrophotometer (Human corporation, Korea), and cholesterol content was quantified according to a standard calibration curve. HDL-cholesterol, LDL-cholesterol, and triglyceride contents were measured according to the method of Cho and Choi (2007) using HDL-cholesterol (HDL-C 555; Eiken Co., Japan) and LDL-cholesterol (BLF; Eiken Co.) Kits. To measure HDL-cholesterol, 0.3 mL of serum was placed in a test tube with 0.3 mL of precipitation reagent and mixed. After incubation at room temperature for 10 min, the sample was centrifuged for 10 min at 700 × *g*. Then, 50 μL of supernatant or 50 μL of standard solution (100 mg/dL) was mixed with 3.0 mL of HDL color reagent. Distilled water (50 μL) was used as the blank. The tubes were heated for 5 min in a water bath at 37 °C. The blank was used as a control for measuring the absorbance at 555 nm and quantifying HDL-cholesterol content. To measure LDL-cholesterol, 0.1 mL of a serum sample or 0.1 mL of standard serum was placed in a test tube, and 4.0 mL of BLF kit reagents I and II were added and mixed for 5 s. After incubation at room temperature (25 ± 3 °C) for 25 min, the absorbance at 650 nm was measured with a spectrophotometer, using distilled water as a blank, and then LDL-cholesterol content was quantified. Triglyceride content was analyzed with the TG kit (Sigma, USA) reagent. Briefly, 1.0 mL of TG kit reagent was mixed with

10 μL of serum, 10 μL of standard solution (300 mL/dL), or 10 μL of deionized water (the blank/control), and the tubes were incubated in a water bath at 37 °C for 5 min. The absorbance was measured at 540 nm with a spectrophotometer, and TG content was quantified.

2.6 Testing Liver Function and Analyzing Blood Insulin/ Leptin Levels

Phospholipid, alkaline phosphatase (ALP), aspartate transaminase (AST), and alanine transaminase (ALT) content in the blood samples was measured using a UV/VIS spectrophotometer (Specord 200; Analytik-Jena, Jena, Germany) according to the method of Reitaman and Frankel (1957). Serum lactate dehydrogenase (LDH) activity was measured with a UV/VIS spectrophotometer (Specord 200) according to the method of Martinek (1972). Blood insulin levels were measured using an enzyme-linked immunosorbent assay (ELISA) (kit supplied by Linco, Washington, USA) performed on plasma separated on the final experimental day, and the molecular device (USA) was used to make a request for analysis to Green Cross Corporation. Blood leptin levels were determined by ELISA (kit by R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol.

2.7 Statistical Analysis

All data are calculated as mean and standard deviation using SPSS package (ver. 18.0). The means of the groups were compared by one-way ANOVA, and the significance of differences ($p < 0.05$) between the means of the groups was determined by Duncan's multiple range test.

3 Results

3.1 Average Body Weight and Organ Weight

The body and organ weight changes of the mice are shown in Fig. 1 and Table 1, respectively. The body weight changes in the HF + taurine 20% group were significantly lower than those in the control and HF groups ($p < 0.05$) (Fig. 1). The liver weight of the mice was lower in the HF + taurine 20% group; however, no significant difference was detected among the groups. The weights of the spleen and kidney were significantly lower in the HF + taurine 20% group than in the HF group ($p < 0.05$) (Table 1).

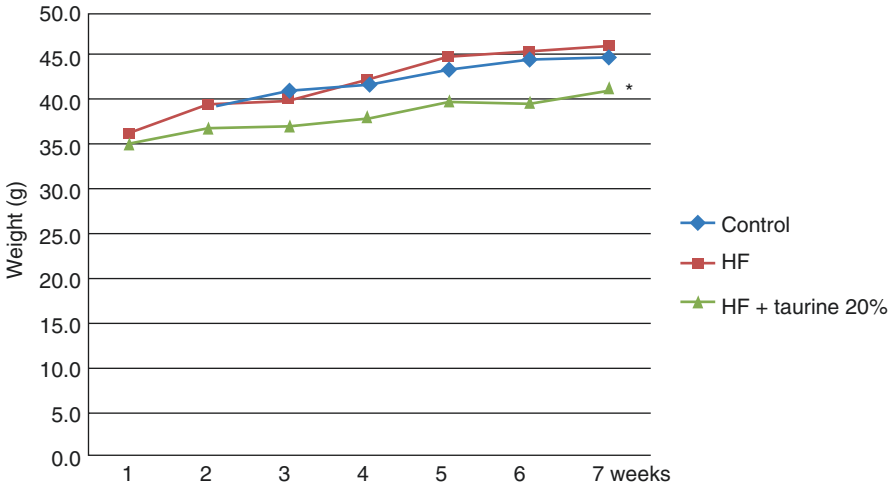

Fig. 1 Weight change of mice

Table 1 Organ weight in mice

	Total	Control	HF	HF + taurine 20%	Significance
Liver (g/100 g body wt.)	2.04 ± 0.31[A]	2.12 ± 0.31	2.14 ± 0.23	1.85 ± 0.33	NS[B]
Spleen (g/100 g body wt.)	0.16 ± 0.04	0.13 ± 0.02[a]	0.18 ± 0.05[b]	0.16 ± 0.02[ab]	0.05[C]
Kidney (g/100 g body wt.)	0.81 ± 0.09	0.84 ± 0.03[b]	0.84 ± 0.11[b]	0.74 ± 0.07[a]	0.05

[A]Mean ± SD
[B]NS: statistically no significant difference at $p < 0.05$ by ANOVA-test
[C]Significant at $p < 0.05$ by ANOVA-test

3.2 Blood Lipid Levels

The effect of taurine powder intake on blood lipid levels is shown in Table 2. Total blood cholesterol level was significantly lower in the HF + taurine 20% group (173.57 ± 28.24 mg/dL) than in the HF group (204.00 ± 23.29 mg/dL ($p < 0.05$). HDL-cholesterol levels were higher in the HF group (202.00 ± 21.68 mg/dL) and significantly lower in the HF + taurine 20% group (175.71 ± 29.07 mg/dL)

Table 2 Comparison of blood lipid in mice

	Total	Control	HF	HF + taurine 20%	Significance
Total cholesterol (mg/dL)	181.47 ± 29.14[A]	170.00 ± 27.39[a]	204.00 ± 23.29[b]	173.57 ± 28.24[ab]	0.05[B]
HDL-cholesterol (mg/dL)	179.12 ± 29.70	161.00 ± 26.55[a]	202.00 ± 21.68[b]	175.71 ± 29.07[ab]	0.05
LDL-cholesterol (mg/dL)	18.82 ± 4.16	17.00 ± 21.39	19.00 ± 4.18	20.00 ± 5.00	NS[C]
Triglyceride (mg/dL)	142.06 ± 43.91	192.00 ± 21.39[b]	133.00 ± 36.16[a]	112.86 ± 28.70[a]	0.05

[A]Mean ± SD
[B]Significant at $p < 0.05$ by ANOVA-test
[C]NS: statistically no significant difference at $p < 0.05$ by ANOVA-test
HDL-cholesterol high density lipoprotein-cholesterol, *LDL-cholesterol* low density lipoprotein-cholesterol

($p < 0.05$). In addition, triglyceride levels were significantly higher in the control group (192.00 ± 21.39 mg/dL) ($p < 0.05$) and were lower in the HF + taurine 20% group (112.86 ± 28.70 mg/dL) than the HF group (133.00 ± 36.16 mg/dL). However, there were no statistical differences between the HF and HF + taurine 20% groups.

3.3 Assessment of Liver Function and Analyzing Blood Phospholipid Levels

Blood phospholipid levels and liver enzyme activity are shown in Table 3. The activity of ALP, AST, ALT, and LDH, which are related to blood phospholipid levels and liver function, did not differ significantly among the experimental groups.

3.4 Analyzing Insulin/Leptin Levels

Blood insulin and leptin levels are shown in Table 4. Blood insulin levels were higher in the HF group (3.64 ± 3.21 ng/dL) and significantly lower in the HF + taurine 20% group (1.10 ± 0.92 ng/dL) ($p < 0.05$). Blood leptin levels were higher in the HF group (2.41 ± 1.05 ng/dL), and significantly lower in the HF + taurine 20% group (1.14 ± 0.49 ng/dL) ($p < 0.05$).

Table 3 Comparison of phospholipid and liver function test in mice

	Total	Control	HF	HF + taurine 20%	Significance
Phospholipid (mg/dL)	386.82 ± 121.71[A]	336.60 ± 138.12	442.20 ± 98.99	383.14 ± 124.80	NS[B]
Alkaline phosphatase (ALP) (U/L)	41.18 ± 12.06	40.00 ± 5.00	34.00 ± 9.62	47.14 ± 14.96	NS
Aspartate aminotransferase (AST) (U/L)	99.00 ± 57.48	117.50 ± 85.39	80.00 ± 20.3	102.50 ± 62.43	NS
Alanine aminotransferase (ALT) (U/L)	28.82 ± 12.31	34.00 ± 15.57	25.00 ± 3.54	27.86 ± 14.10	NS
Lactate dehydronase (LDH) (U/L)	821.33 ± 673.08	503.33 ± 136.05	591.00 ± 87.06	1122.14 ± 919.51	NS

[A]Mean ± SD
[B]*NS* statistically no significant difference at $p < 0.05$ by ANOVA-test

Table 4 Comparison of insulin and leptin level in mice

	Total	Control	HF	HF + taurine 20%	Significance
Insulin (ng/dl)	2.48 ± 2.24[A]	2.70 ± 1.29[ab]	3.64 ± 3.21[b]	1.10 ± 0.92[a]	0.05[B]
Leptin (ng/dl)	2.04 ± 1.09	2.62 ± 1.09[b]	2.41 ± 1.05[b]	1.14 ± 0.49[a]	0.05

[A]Mean ± SD
[B]Significant at $p < 0.05$ by ANOVA-test

4 Discussion

This study was conducted to assess the effects of adding taurine to an HF in mice by assessing various biomarkers in blood based on the results of previous studies. In the present study, the body weight change in the HF + taurine 20% group was significantly lower than that of the HF group. The weights of the spleen and kidney were lower in the HF + taurine 20% group than in the HF group. In a previous study (Tsuboyama-Kasaoka et al. 2006), 7-week-old female C57BL/6J mice were fed an HF diet or an HF diet with 5% taurine for 18 weeks, and fat cell size was compared. Blood taurine levels decreased and adipose tissue weight increased in the HF group, whereas body fat decreased and adipose tissue size decreased in the HF + taurine 5% group. Therefore, they hypothesized that taurine increased basal metabolism and contributed to the fat decrease. Although the size of the brown adipose tissue did not change, the white adipose tissue decreased in size. In the present study, total cholesterol and triglyceride levels were significantly decreased in the HF + taurine 20% group when compared to the HF group, supporting the results of previous studies (Huxtable 1992; Wasserhess et al. 1993; McKee 1999;

Park 2002), which verified the relationship between cholesterol and taurine. Previous studies (Wasserhess et al. 1993; Yoon et al. 2015) showed that taurine accelerated bile acid secretion and the conversion of cholesterol to bile acid. It was also reported that, since bile acid contains taurine, it increased the production and secretion of water-soluble bile salts from the gallbladder. In addition, Yokogoshi et al. (1999) reported that since taurine increases bile acid secretion and accelerates cholesterol biotransformation, bile acid discharge, and cholesterol removal, taurine degrades cholesterol and accelerates bile acid discharge. It has been reported that through these discharge processes, taurine aids in fat emulsification/absorption and decreases the levels of cholesterol and neutral fat (Huxtable 1992; Mochizuki et al. 1998; Murakaimi et al. 1999; Yoon et al. 2015). Diverse animal experiment-based studies (Kibe et al. 1980; Strasberg et al. 1983; Yoon et al. 2015) have shown that adding taurine to the diet increases bile acid synthesis/secretion. In addition, it was reported that long-term taurine intake significantly decreased cholesterol levels and neutral fat levels in the plasma/liver of mice fed a high-cholesterol diet (Gandhi et al. 1992; Park and Lee 1997; Seo 2005; Yoon et al. 2015). As for humans, in a study by Mizushima et al. (1996), young male subjects were fed a high-cholesterol diet and taurine for 4 weeks, and the changes in blood lipid level were examined. The high-cholesterol-induced increases in total cholesterol and LDL-cholesterol levels were significantly lowered by taurine. It was also reported that blood cholesterol levels started to decrease significantly in female adults who consumed taurine for more than 2 weeks (Chung et al. 2000). According to a study by Fain (1973), such a decrease is caused by the following process: taurine accelerates activation of adenylate cyclase through the β-adrenoceptor, which increases cAMP levels within the cells. This increase in cAMP activates hormone-sensitive lipase, which accelerates lipolysis. According to the study by Zhao and Jia (1997), the daily recommended intake of taurine for Chinese adults is 34–80 mg. According to a study by Kibayashi et al. (1999), the daily recommended intake of taurine for Japanese adults age 20–59 is 225.5 mg and 162.2 mg for males and females, respectively. In addition, according to a study by Park et al. (2001b), the daily recommended intake of taurine for Korean adults is 181–219 mg. Thus, the recommended taurine intake for Korean adults is higher than that for Chinese adults and similar to that for Japanese adults. In this study, although ALP, AST, ALT, and LDH, which are related to liver function, showed a tendency to increase following intake of an HF, no significant differences were observed among the groups. Such increased values reflect liver cell damage/necrosis, and are widely used as indicators of liver/heart diseases (Choi et al. 2016). In the present study, the addition of 20% taurine to an HF did not significantly improve liver function. However, blood insulin was significantly increased in mice fed an HF, and this pattern was very similar to that observed in previous studies (Jang and Choi 2003; Choi et al. 2015, 2016). Of the diet-related factors that increase blood insulin levels, high fat-diet intake was found to increase blood free fatty acids, and thereby decrease insulin sensitivity in the liver and induce hyperinsulinemia (Jang and Choi 2003; Choi et al. 2015). In addition, in the present study, insulin and leptin levels were significantly decreased in the HF + taurine 20% group in comparison

to the HF group. According to *in vitro* research, taurine is capable of binding the human insulin receptor. Accordingly, Maturo and Kulakowsik (1988) proposed that taurine regulates carbohydrate metabolism by interacting with the insulin receptor. In addition, it was reported that increased blood taurine levels decrease glycogenolysis (Lampson et al. 1983). In previous studies (Tokunaga et al. 1979; Chang 1999), it was reported that taurine decreases blood sugar levels, prevents diabetes mellitus, influences carbohydrate metabolism, and contributes to insulin secretion control in pancreatic cells to decrease blood sugar levels in streptozotocin-induced diabetic mice. It was also reported that since taurine can decrease blood sugar levels by binding to the insulin receptor with low affinity and replacing insulin, its action is similar to that of insulin (Maturo and Kulakowski 1988; Chang 1999). Comparison of our findings to previous research results shows that our data is good and it can be used to verify the relationship between taurine and blood sugar. According to previous research (Caro et al. 1996; Lu et al. 1998; Watson et al. 2000; Hong et al. 2001), leptin levels are highly correlated with body weight and body fat percentage, and an HF increases leptin expression and blood leptin levels. Leptin is the product of obese gene and it influences food intake, energy consumption, carbohydrate/fat metabolism, and metabolic gene expression (Barzilai et al. 1997; Friedman and Halaas 1998; Buettner et al. 2000; Woo et al. 2013). According to previous research (Havel et al. 1996; Havel 2000; Choi et al. 2016), blood leptin and insulin levels are correlated. Havel (2000) reported that insulin is a main factor controlling the production of leptin, since insulin increases blood leptin levels when injected into the human body, and that insulin accelerates the production of leptin in rodents and humans. According to a study by Hong et al. (2001), the blood leptin content was 2.3 times greater in the HF group than in the normal group after 2 weeks of high fat intake, and the HF increased blood leptin levels.

5 Conclusion

Blood HDL cholesterol levels were higher in the HF + taurine 20% group than in the HF group. Total cholesterol and triglyceride levels were significantly lower in the HF + taurine 20% group than in the HF group. In addition, ALP, AST, ALT, and LDH activity, which are related to liver function, did not differ significantly among the groups. However, insulin and leptin levels were significantly lower in the HF + taurine 20% group than in the HF group. We observed that the addition of 20% taurine to the HF significantly improved blood lipid levels and insulin and leptin levels, which control carbohydrate metabolism. Accordingly, the results of this present study verify previous research results. In addition, taurine intake through food or as a supplement is considered very beneficial for improving side effects some medications (hyperlipidemia enhancers, etc.), such as oxidative stress and insulin secretion.

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Effects of Replacement of Methionine in Diets with Taurine on Growth Performance and Blood Index in Broilers

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Abstract We studied effects of replacement of methionine with taurine on growth performance and blood index of AA+ broilers. Six hundred 1 day broilers were divided into 5 groups, with 3 replicates of 40 broilers in each. The experiment lasted for 42 days.

The control group were fed on formulated diets containing 2% methionine; the other groups were offered feed with equal nitrogen and calories to the control group, but contained 25, 50, 75 and 100% taurine in place of methionine.

Compared with the control group, no significant differences were observed in growth performance of 1–21 days broilers, or the serum LDL-C, TC, IgG and SOD of the experimental groups ($P > 0.05$). ADG and F/G from days 1–42, ADG, ADFI and F/G from days 22–42 were significantly different between the experimental groups and the control group ($P < 0.05$). ADFI and Mortality in 50, 75 and 100% taurine groups were significantly different compared with the control group ($P < 0.05$). IgM and GSH-PX of 50 and 75% taurine groups were significantly different compared with the control group ($P < 0.05$). Serum HDL-C, T-AOC levels in 50, 75 and 100% taurine groups were significantly different compared with the control group ($P < 0.05$). Based on the quadratic regression analysis, the best replacement ratios were 58%, 61% and 61% on days 1–21, 22–42, and 1–42, respectively. In conclusion, appropriate levels of taurine supplement can improve growth performance, immune system, T-AOC, and lipid metabolism.

Keywords Taurine • Methionine • Growth performance • Blood index • Broilers

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Abbreviations

ADFI	Average daily feed intake
ADG	Average daily gain
CSAD	Cysteine sulfinic acid decarboxylase
F/G	Feed conversion ration
GSH-Px	Glutathione peroxidase
HDL-C	High density lipoprotein cholesterol
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LDL-C	Low density lipoprotein cholesterol
NS	Non-significant
T-AOC	Total antioxidant capacity
TC	Total cholesterol
TG	Triglyceride

1 Introduction

Taurine, also known as 2-aminoethane sulfonic acid, is a sulfur-containing amino acid. It is synthesized from cystine, cysteine and methionine. Cysteine and methionine are metabolized to form the intermediate cysteine sulfinic acid, which in turn is decarboxylated by CSAD to form hypotaurine, which is oxidized to yield taurine. Such is the most important pathway to synthesize taurine (Lu 2000). Taurine can aid digestive, immune and antioxidant defense systems (Garbutt et al. 1971; Huxtable 1992; Takeuchi et al. 2001).

Methionine is one of essential amino acids in animals and also contains sulfur. As the first limiting amino acid for poultry it can improve growth performance, carcass quality, immune system and anti-oxidation (Qiao 2007). Animal nutritionists are dedicated to the replacement of methionine due to its higher and higher price and its toxicity for improper use. Recently more and more studies have been focused on MHA such as betaine, choline and cysteine, while taurine has seldom been reported in the substitute for methionine. Taurine is similar to methionine in methyl for biological function, so it is important to investigate how the replacement of methionine with taurine may affect growth performance of broilers.

The present experiment is to investigate effects of replacement of methionine with different levels of taurine on growth performance, lipid metabolism, immune and antioxidant systems of broilers AA+, and further to assess if taurine can possibly promote the balance of amino acids and save methionine as a result of methyl savings, thus providing a theoretical basis for animal nutrition study.

2 Methods

2.1 Animals and Study Design

Six hundred male and female 1 day AA+ broilers were randomly placed in five groups, one control group fed on a basal diet containing 0.2% methionine, and four treatments administered gradually reduced methionine concentrations (0.15, 0.1, 0.05 and 0%) and gradually increased taurine concentrations (0.05, 0.1, 0.15 and 0.2%) to a basal diet. In other words, the proportions of methionine replaced with taurine were 0 (the control group), 25, 50, 75 and 100%, with five replicates, and each replication was of 30 birds. The basal diet was recommended by GB-14924.3-2012 guidelines, and its chemical composition and nutrition were presented in Table 1.

2.2 Samples Collection

One male and one female 42 days birds were chosen at random in each replicate for blood samples. An amount of 8ml for each was placed into a tilted 10 mL centrifuge tube for the naturally clotted blood. In 0.5 h after blood coagulation, serum was harvested subsequently at 3000 r/m for 15 min in centrifuge machine. The top serum in the tube was collected and stored at −20 °C for analysis.

Table 1 Composition and nutrition of basal diet

Composition	Proportion (%)	Nutrition level	
Corn	62.00	ME	13.85 J/kg
Soybean meal-43	24.00	CP	18.00%
Oil	5.00	Crude ash	87.00%
DDGS Com distiller's grains with solubles	3.20	Water content	13.00%
Corn gluten meal	1.00	C-fiber	7.80%
Cottonseed meal	1.50	Ca	0.75%
Dicalcium phosphate	1.15	Total P	0.60%
Stone dust	1.00	Total Lys	1.20%
Lys-70	0.50	Total Met	0.45%
Thr	0.08		
Salt	0.30		
Compound-premix	0.27		
Met	0.20		
Total	100.00		

Provided following per kg of diets: calcium pantothenate 10.4 mg, VE 7.5 IU, VB_1 0.6 mg, VB_2 4.8 mg, VB_6 1.8 mg, VA 12,000 lU, VB_{12} 10 mg, VD_3 3000 IU, folic acid 0.15 mg, VK_3 1.50 mg, nicotinic acid 30 mg, Fe 100 mg, I 0.7 mg, Cu 10 mg, Zn 100 mg, Se 0.3 mg, Mn 100 mg.

2.3 Blood Index Measurement

Food intake, water, activity and mortality were observed on a daily basis in each pen. At 6:00 a.m. on the 42nd day, 15 broilers were selected randomly from each replicate for weight measurement. No food but free water was given for 12 h before weighing. ADG, ADFI, F/G, and Mortality were measured on days 1–21, 22–42, and 1–42, respectively.

TC, TG, HDL-C, LDL-C, IgA, IgG, IgM, GSH-Px, T-AOC and SOD were measured by kits produced by the Institute of Nanjing Jiancheng Biological Engineering, and the relevant indices were tested accordingly.

2.4 Data Analysis

Excel 2007 was used for data analysis and database, along with generalized linear models univariate variance by SPSS19.0 statistic software. Differences were considered statistically significant when the calculated P value was less than 0.05. Multiple comparisons were made by Duncan's new multiple range test (MRT), and each value expressed as the mean ± SEM.

3 Results

3.1 Results of Replacement of Methionine with Different Levels of Taurine on Growth Performance of Broilers

As replacement ratios increased in basal diet, NS differences were observed in terms of ADG, ADFI, F/G and Mortality in the control group and 25–100% taurine groups ($P > 0.05$) (Table 2).

Table 2 Effect of different levels of taurine in place of methionine on growth performance in 1–21 days broilers

	Taurine replacement proportion (%)				
Items	0	25	50	75	100
ADG (g)	42.17 ± 0.09^{ns}	42.24 ± 0.03	42.81 ± 0.24	42.61 ± 0.43	42.32 ± 0.23
ADFI (g)	65.15 ± 0.03^{ns}	65.22 ± 0.16	65.58 ± 0.11	65.46 ± 0.60	65.32 ± 0.09
F/G (%)	1.55±0.00^{ns}	1.54 ± 0.00	1.53 ± 0.01	1.53 ± 0.01	1.54 ± 0.01
Mortality (%)	4.46 ± 0.80^{ns}	4.30 ± 0.59	3.98 ± 0.70	3.89 ± 0.65	4.22 ± 0.57

Values are expressed as mean ± SEM. Values with different superscript indicators within the column are significantly different at $p < 0.05$ by Duncan's multiple range test; *ns* is not significant.

Quadratic polynomial was used to fit the correlations between ADG and taurine replacement proportion in 1–21 days broilers (Fig. 1). The regression equation of ADG was shown on the basis of parabolic regression, where $y = -0.00017x^2 + 0.01971x + 42.083$ ($R^2 = 0.69538$). The maximum ADG was at the highest point of the parabola, where the replacement proportion was 58%, which suggested the optimal percentage for 1–21 days broilers.

As taurine replacement proportions increased in basal diet, significant differences were noted in ADG, ADFI and F/G of 22–42 days broilers in the control group and the 25–100% taurine groups ($P < 0.05$), but NS in Mortality among the control group, 25%, 100% taurine groups ($P > 0.05$) (Table 3).

Quadratic polynomial was used to fit the correlations between ADG and taurine replacement proportion in 22–42 days broilers (Fig. 2). The regression equation of ADG was shown on the basis of parabolic regression, where $y = -0.00172x^2 + 0.21188x + 82.096$ ($R^2 = 0.93032$). The maximum ADG was at the highest point of the parabola, where the replacement proportion was 61%, indicating the optimal percentage for 22–42 days broilers.

As taurine replacement proportions increased in basal diets, there were significant differences in ADG and F/G of 1–42 days broilers between the control group and the 25–100% taurine groups ($P < 0.05$), likewise in terms of ADFI and Mortality between the control group and the 50–75% taurine groups ($P < 0.05$) (Table 4).

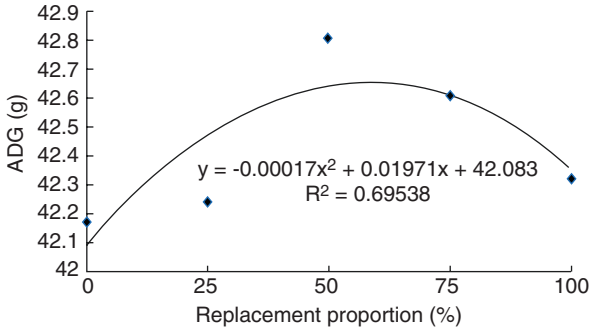

Fig. 1 Correlations between ADG and taurine replacement proportion in 1–21 days broilers

Table 3 Effect of different levels of taurine in place of methionine on growth performance in 22–42 days broilers

Items	Taurine replacement proportion (%)				
	0	25	50	75	100
ADG (g)	81.66 ± 0.62[a]	87.42 ± 0.96[b]	87.70 ± 0.72[b]	88.13 ± 0.70[b]	86.29 ± 0.92[b]
ADFI (g)	170.92 ± 0.21[a]	173.22 ± 0.20[b]	173.48 ± 0.12[b]	173.42 ± 0.38[b]	172.78 ± 0.06[b]
F/G (%)	2.09±0.01[a]	1.99 ± 0.02[b]	1.97 ± 0.02[b]	1.97 ± 0.02[b]	2.00 ± 0.02[b]
Mortality (%)	5.32 ± 0.01[a]	5.30 ± 0.00 [a]	4.81 ± 0.01[b]	4.79 ± 0.01[b]	5.30 ± 0.01[a]

Values are expressed as mean ± SEM. Values with different superscript indicators within the column are significantly different at $p < 0.05$ by Duncan's multiple range test.

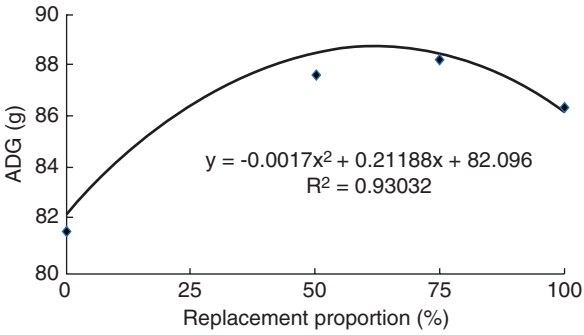

Fig. 2 Correlations between ADG and taurine replacement proportion in 22–42 days broilers

Table 4 Effect of different levels of taurine in place of methionine on growth performance in 1–42 days broilers

Items	Taurine replacement proportion (%)				
	0	25	50	75	100
ADG (g)	61.91 ± 0.27[a]	64.83 ± 0.50[b]	65.25 ± 0.32[b]	65.37 ± 0.14[b]	64.31 ± 0.44[b]
ADFI (g)	118.28 ± 0.28[a]	118.80 ± 0.08[ab]	119.08 ± 0.14[b]	119.15 ± 0.08[b]	118.66 ± 0.26[ab]
F/G (%)	1.91 ± 0.01[a]	1.83 ± 0.01[b]	1.82 ± 0.01 [b]	1.82 ± 0.00[b]	1.84 ± 0.01[b]
Mortality (%)	9.24 ± 0.11[a]	9.13 ± 0.02[ab]	8.97 ± 0.04[b]	8.97 ± 0.08[b]	9.19 ± 0.09[ab]

Values are expressed as mean ± SEM. Values with different superscript indicators within the column are significantly different at $p < 0.05$ by Duncan's multiple range test.

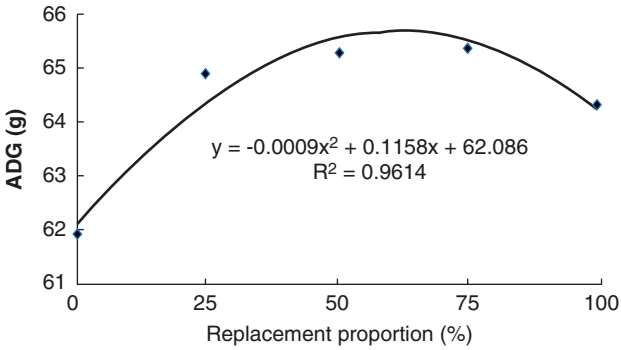

Fig. 3 Correlations between ADG and taurine replacement proportions in 1–42 days broilers

Quadratic polynomial was used to fit the interrelations between ADG and taurine replacement proportion in 1–42 days broilers (Fig. 3). The regression equation of ADG was shown by parabolic regression, where $y = -0.00094x^2 + 0.11576x + 62.086$ ($R^2 = 0.96145$). The maximum ADG was found at the highest point of the parabola, fitting the replacement proportion 61%, which indicated the best taurine replacement for 1–42 days broilers.

3.2 Results of Replacement of Methionine with Different Levels of Taurine on Lipid Metabolism

As taurine replacement proportions increased in diets, both TG and HDL-C fluctuated to some degree. TG levels were significantly different in the control group and the 25–75% taurine groups ($P < 0.05$), and HDL-C levels were significantly different between the control group and the 50–100% taurine groups ($P < 0.05$). Both LDL-C and TC showed reduction trend but NS difference in each group ($P > 0.05$) (Table 5).

3.3 Results of Replacement of Methionine with Different Levels of Taurine on Immunoglobulin

As taurine replacement proportion increased in diets, both IgA and IgM exhibited fluctuation to some degree (Table 6). There were significant differences both in IgA between the control group and the 25–75% taurine groups ($P < 0.05$), and in IgM

Table 5 Effect of different levels of taurine in place of methionine on lipid metabolism

	Taurine replacement proportion (%)				
Items	0	25	50	75	100
TG (mmol/L)	1.00 ± 0.12^{a}	0.95 ± 0.14^{b}	0.94 ± 0.01^{b}	0.92 ± 0.10^{b}	0.97 ± 0.07^{a}
HDL-C (mmol/L)	4.27 ± 0.34^{a}	4.50 ± 0.33^{a}	7.54 ± 0.65^{b}	7.81 ± 0.66^{b}	6.22 ± 0.33^{b}
LDL-C (mmol/L)	3.42 ± 0.34^{ns}	3.13 ± 0.09	2.73 ± 0.02	2.48 ± 0.06	3.31 ± 0.31
TC (mmol/L)	4.37 ± 0.31^{ns}	4.12 ± 0.07	3.48 ± 0.27	3.33 ± 0.33	4.17 ± 0.48

Values are expressed as mean ± SEM. Values with different superscript indicators within the column are significantly different at $p < 0.05$ by Duncan's multiple range test; *ns* is not significant.

Table 6 Effect of different levels of taurine in place of methionine on immunoglobulin in broilers

	Taurine replacement proportion (%)				
Items	0	25	50	75	100
IgA (g/L)	2.44 ± 0.34^{a}	3.44 ± 0.12^{b}	3.94 ± 0.26^{b}	4.18 ± 0.31^{b}	2.54 ± 0.30^{a}
IgM (g/L)	4.81 ± 0.36^{a}	5.49 ± 0.14^{ab}	6.09 ± 0.06^{b}	6.18 ± 0.10^{b}	5.51 ± 0.27^{ab}
IgG (g/L)	0.75 ± 0.03^{ns}	0.77 ± 0.04	0.80 ± 0.03	0.84 ± 0.05	0.78 ± 0.04

Values are expressed as mean ± SEM. Values with different superscript indicators within the column are significantly different at $p < 0.05$ by Duncan's multiple range test; *ns* is not significant.

between the control group and the 50–75% taurine groups ($P < 0.05$). IgG showed an increasing trend but no significant difference in the control group and the 25–100% taurine groups ($P > 0.05$).

3.4 Results of Replacement of Methionine with Different Levels of Taurine on Antioxidant Capacity

As taurine replacement proportions increased in basal diets, both T-AOC and GSH-PX fluctuated to some degree. There were significant differences both in T-AOC between the control group and 50–100% taurine groups ($P < 0.05$), and in GSH-PX between the control group and 50–75% taurine groups ($P < 0.05$). SOD showed an increasing trend but NS difference in the control group and 25–100% taurine groups ($P > 0.05$) (Table 7).

4 Discussion

4.1 Effect of Different Levels of Taurine in Place of Methionine on Growth Performance

Growth performance is critical for broiler feeding. It was found during the experiment that different levels of taurine in place of methionine had effects on growth performance of broilers.

Previous work revealed that 0.10% taurine supplementation in diet significantly improved weight in 6 days broilers and reduced F/G in 0–45 days broilers (He 1995); that ADG significantly increased and F/G significantly decreased in broilers (Huang et al. 2009); and that ADG increased and F/G decreased to some degree in ducks (Guo 2004). It was reported that taurine supplement significantly improved

Table 7 Effect of different levels of taurine in place of methionine on antioxidant capacity in broilers

Items	Taurine replacement proportion (%)				
	0	25	50	75	100
T-AOC (U/mL)	6.68 ± 0.33^{a}	7.11 ± 0.34^{a}	8.32 ± 0.32^{b}	8.74 ± 0.03^{b}	8.24 ± 0.22^{b}
GSH-PX (U/mL)	351.67 ± 3.84^{a}	359.00 ± 3.06^{a}	404.33 ± 2.85^{b}	414.73 ± 3.76^{b}	365.33 ± 7.31^{a}
SOD (U/mL)	97.68 ± 0.67^{ns}	99.78±1.99	102.01 ± 2.00	104.16± 3.51	100.68 ± 0.57

Values are expressed as mean ± SEM. Values with different superscript indicators within the column are significantly different at $p < 0.05$ by Duncan's multiple range test; *ns* is not significant.

FCR and growth performance (Lee et al. 2004), and that appropriate levels of taurine could reduce Mortality of sudden death syndrome (Blair et al. 1991). The present experiment was consistent with the previous studies.

As was observed by quadratic polynomial regression analysis in the experiment, the optimal growth performance occurred in the proportions of 58% for 1–21 day chicks, and 61% for both 22–42 and 1–42 days broilers. It was possibly because a part of methionine was converted to cystine for broilers when there was only methionine supplement in a basal diet. As taurine was added, the conversion of methionine and growth performance improved. However, it must be limited to a certain amount, or there would be a higher Mortality, which was in conflict with improvement in growth performance. Although methionine is used in the synthesis of taurine, it also serves other important roles, such as methyl transfer and protein synthesis. Without causing some pathological injury, We can control the amount of taurine to replace methionine.

4.2 Effect of Different Levels of Taurine in Place of Methionine on Lipid Metabolism

Cholesterol is an essential constituent of cell membranes. It is converted to bile salts in liver, promoting digestion and absorption of lipids, and also a precursor of other steroid compounds. TG levels in serum serve as an index of coronary heart disease, reflecting lipid metabolism in animals, along with cholesterol. Lipid in blood significantly increases when its transportation is blocked. Change in TG concentrations is thought to reflect whether lipid metabolism is normal or not.

Taurine was reported to have significantly lowered the levels of TG and TC with atherosclerosis (Zhang et al. 2010). Rabbits had significantly lower levels of cholesterol in serum when supplemented with taurine (Yokogoshi and Nanami 1999). A proper amount of taurine addictive significantly improved lipid metabolism and reduced TG and TC in broilers (Ji and Wang 2006). There was a significant increase in HDL-C and decrease in TG, TC and LDL-C in rats when taurine was added to diets (Hou et al. 1994). The current study conformed to the previous results.

In our experiment it was found that there was a significant decline in TG in the ratios of methionine replaced with taurine from 25 to 75%, and a significant increase in HDL-C in those from 50 to 75%. It was likely that taurine improved lipid metabolism in broilers as a component of Taurocholic acid, whose proportion increased along with taurine.

4.3 Effect of Different Levels of Taurine in Place of Methionine on Immunoglobulin

Immunoglobulin is globulin that has antibody activity, which is similar to antibody in chemical structure. It is an important immune molecule for mediated humoral immunity, which is produced by plasma cells differentiated by B cells after antigen

stimulation. Immunoglobulin works as follows. IgA is secreted from the mucosal immune system, occurring in part; IgM found in the initial infection as the earliest antibody; IgG produced by plasma cells in spleen and lymph nodes as the main antibody in mediated humoral immunity. IgG functions as the main anti-infection immunity, as well as the essential antibody for vaccination monitoring and serological diagnosis.

Taurine supplement improved the synthesis of IgM with IgG in monocytes (Ran and Guo 1993). Taurine may increase polyclonal antibody response and promote the proliferation of immune cells (He et al. 1995). A proper amount of taurine added to basal diets in mice significantly increased correction clearance index, clearance index and hemolysin antibody IgM; 3 weeks' drinks added with taurine was found to improved taurine concentrations in neutrophils, capacity for Escherichia coli phagocytosis, mobility and stability of neutrophil cell membranes by projecting light polarization elimination (Zhang et al. 1996). The current experiment was consistent with the previous reports.

It was indicated in our experiment that a significant increase was noted both in IgA from 25 to 75% groups, and in IgM from 50 to 75% groups. It was possibly because taurine significantly promoted synthesis of immunoglobulin in broilers, which was enhanced as a result of higher concentrations of taurine. In this way produced more immunoglobulin, thus increasing the concentrations of IgM and IgA and improving anti-infection capability for broilers.

4.4 Effect of Different Levels of Taurine in Place of Methionine on T-AOC

T-AOC in animal immune system closely interrelates with health condition, and many diseases may result from lower T-AOC (Yang et al. 2002). Taurine belongs to endogenous anti-injury substance, and its main mechanism is related to free radical scavenging and anti-lipid per-oxidation. It was reported that taurine functioned as anti-oxidative damage either directly or indirectly, that is to say, amino groups in taurine molecule combined with an oxidizing agent as anti-oxidative damage directly, or taurine may stabilize cell membrane, protect phosphoric acid membrane from degradation, and fight oxidant indirectly. One essential way to clear oxygen free radicals was via anti-oxidase system, including GSH-Px, T-AOC and SOD (Qian et al. 1995). Antioxidant enzyme system in organisms can work alone or collaboratively to clear oxygen free radicals and protect the integrity of cell structures and its function. Taurine is an antioxidant in the cell because it reduces mitochondrial superoxide generation or by scavenging hypochlorous acid.

Studies have been done on T-AOC in laying hens, carps, rabbits, broilers and ducklings. It has been unanimously reported that taurine supplementation significantly improved GSH-Px, SOD activity and T-AOC, inhibited hydroxyl radicals from accumulating, and reduced lipid peroxidation (Wang 2003; Qiu et al. 2008; Winiarska

et al. 2009; Li et al. 2010; Yang et al. 2011), indicating that taurine did help to improve T-AOC, strengthen the free radical scavenging, and reduce lipid per-oxidation damage. Similarly, in the current investigation, 50–100% taurine groups significantly improved T-AOC activity, whereas 50–75% ones showed better GSH-Px activity compared to control, which conformed to the studies mentioned above. In case of methionine deficiencies, taurine was added to improve T-AOC in broilers. It is possibly because cysteine is the first limiting substrate for synthesis of glutathione, and higher levels of cysteine are more conducive to the synthesis of glutathione. Therefore, different levels of taurine in place of methionine can improve T-AOC in broilers.

5 Conclusion

In the current experiment, the maximum replacement ratio of 75% had no effects on growth performance, lipid metabolism, immune response or T-AOC. Based on the quadratic regression analysis, the best replacement ratios were 58%, 61% and 61% on days 1–21, 22–42, and 1–42, respectively. In conclusion, appropriate levels of taurine supplement can improve growth performance, immune system, T-AOC, and lipid metabolism of broilers.

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Consumer Awareness and Preferences Related to Taurine-Containing Drinks in Korean Female High School Students

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Abstract The purpose of this cross-sectional study was to investigate awareness, consumption patterns, and preferences related to intake experience of taurine-containing drinks (TCD) in Korean female high school students. Study subjects were 335 female high school students residing in Incheon, Korea. Data were collected using self-administered questionnaires. A total of 249 students (74.3%) consumed TCD while 86 students (25.7%) did not. In the TCD consumption group, 66.7% of consumers drank TCD once every 2 or 3 months while most consumers consumed it during the examination period (57.4%). Regarding the effects of TCD intake, 45.8% of students cited 'relieve sleepiness' while 41.8% showed 'no effect'. According to self-reported school records, there were significant differences in intake time of TCD, effects of TCD intake, and degree of interest in TCD ($p < 0.05$). About 82.7% of all the subjects perceived TCD as an energy drink for fatigue recovery. Degree of interest in TCD ($p < 0.001$), degree of harmfulness to the body ($p < 0.01$), and improvement for learning-ability ($p < 0.001$) had significant effects on intake frequency of TCD. Regarding preferences, 'Vita 500' was preferred by 52.2% of subjects, 'Bacchas' by 20.1%, and 'Hot Six' by 17.3%. Although subjects preferred 'Vita 500' as a caffeine-free TCD, caffeine was perceived as the most abundant ingredients in TCD by 77.1% of subjects, taurine by 16.1%, and vitamin by 4.8%. These results show most Korean female high school students consumed TCD in order to stay awake during the examination periods. Therefore, nutritional education is needed to correct Korean high school students' consumption of TCD.

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Keywords Taurine-containing drinks • Awareness • Preferences • Consumer • Female high school students

Abbreviations

BMI Body mass index
TCD Taurine-containing drinks

1 Introduction

TCD are functional drinks containing taurine as well as other ingredients (e.g., caffeine, guarana extracts, sugar, and vitamin, etc.) (Gun 2013). Recently, it has been reported that TCD can improve cognitive ability, physical potential adaptability, and health (Barthel et al. 2001; Seidl et al. 2000). Consumption of TCD has continuously increased, and the global market of TCD was about 394 billion US dollars in 2014 while the domestic market of TCD was about 2 trillion Korean won in 2015 (Ko 2014; Nho 2016). Especially, consumption of TCD increases in Korean adolescents during examination periods due to its perceived stress relief and fatigue recovery functions.

In adolescents, standards for TCD selection are based on taste, advertising, and friend's drink preferences (Gun 2013; Lee et al. 2014). However, purchasing TCD without considering its ingredients may harm health due to excessive caffeine intake. Most TCD contain caffeine as an ingredient. When caffeine intake exceeds 500 mg per day, there is a risk of chronic toxicity (anxiety, insomnia, and headache, etc.) and disrupted calcium absorption (Heckman et al. 2010; Nawrot et al. 2003). Although many studies on consumption of TCD have been reported (Lee et al. 2013; Lee and Ra 2014; Park et al. 2015; Yang et al. 2014), studies on TCD consumption in Korean high school students have been limited. Therefore, the purpose of this study was to investigate awareness, consumption patterns, and preferences related to TCD in Korean female high school students.

2 Methods

2.1 Subjects

The subjects of this cross-sectional study were 335 female high school students residing in Incheon, Korea. This study was carried out using anonymous self-administered questionnaires about awareness, consumption patterns, and preferences related to TCD. This study was approved by the institutional review board of Inha University, Korea (150605-1A).

2.2 General Characteristic and Anthropometric Data

General characteristics of subjects were age, self-reported school record, and pocket money. The self-reported school record was divided into high, medium, and low categories by subjective judgment of each subject. The subjects' height and body weight were presented as self-reported estimates, and BMI was calculated as weight in kg divided by height in meters squared (kg/m^2).

2.3 Questionnaire

The survey in this study was conducted using a questionnaire based on previous studies (Ko 2013; Lee et al. 2014; Nho 2014). The questionnaires focused on awareness (six items), consumption patterns (five items), and preferences (selection of three among 24 TCD) related to TCD and nutrition education (two items) related to TCD intake. Items consisted of multiple-choice type on a 5-point Likert scale.

2.4 Statistical Analysis

Statistical analysis was conducted using the SPSS 18.0 program. The frequency (percentage) and Mean ± Standard error (SE) for each survey question were calculated. The chi-square test, one way ANOVA, and Student *t*-test were performed for determination or significant differences, and simple regression analysis was used to assess significant correlation between intake frequency of TCD and variables of awareness.

3 Results and Discussion

3.1 General Characteristics and Anthropometric Data

General characteristics and anthropometric data of subjects are shown in Table 1. There were no significant differences in general characteristics and anthropometric data between the TCD consumption group and non-TCD consumption group. The average age of subjects was 18 years (range 17–19 years), and among the total subjects, 249 students (74.3%) consumed TCD while 86 students (25.7%) did not. Compared with another study, consumption experience of TCD in this study was 70.4%, which was similar to the rate of TCD consumption by young people (average age: 16 years) surveyed recently in Bahrain (Nassaif et al. 2016). According to the self-reported school record, the high, medium, and low categories in the TCD consumption group showed percentages of 28.5%, 43.8%, and 27.7% whereas those

Table 1 General characteristics and anthropometric data of the subjects

Variables	TCD consumer (n = 249)	Non-TCD consumer (n = 86)
Age (years)	18.1 ± 0.1[a]	18.0 ± 0.1[NS]
Self-reported school record		
High	71(28.5)[b]	32(37.2)[NS]
Medium	109(43.8)	34(39.5)
Low	69(27.7)	20(23.3)
Pocket money (Korean won)		
<10,000	80(32.1)	32(37.2)[NS]
10,000~<20,000	85(34.1)	31(36.0)
≥20,000	84(33.7)	23(26.7)
Height	160.6 ± 0.3	160.9 ± 0.5[NS]
Body weight	54.8 ± 0.7	53.2 ± 0.8
BMI (kg/m^2)	21.3 ± 0.3	20.6 ± 0.3

NS Not significant
[a]Mean ± SE
[b]n (%)

of the non-TCD consumption group showed percentages of 37.2%, 39.5%, and 23.3%, respectively. For pocket money, 85 TCD consumers (34.1%) and 32 non-TCD consumers (37.2%) spent 10,000–<20,000 won and <10,000 won per week. Average height, body weight, and BMI were 160.6 cm, 54.8 kg, and 21.3 kg/m^2 and 160.9 cm, 53.2 kg, and 20.6 kg/m^2 in TCD consumption group and non-TCD consumption group, respectively.

3.2 Awareness of TCD by Consumption

Awareness of TCD according to consumption experience is shown in Table 2. The TCD consumption group showed a significantly higher degree of interest in TCD compared to the non-TCD consumption group ($p < 0.01$). In the TCD consumption group, degree of interest in TCD of the high school record subgroup was significantly higher than those of the medium and low sub-groups ($p < 0.001$). On the other hand, the non-TCD consumption group showed a significantly higher degree of perceived harmfulness related to TCD consumption than the TCD consumption group ($p < 0.001$). More than 80% of subjects were aware of TCD as a drink for fatigue recovery, which was similar to a previous result reporting that female high school students are aware of drinks for fatigue recovery (Nho 2014). The TCD consumption rate of female middle school students in Seoul for the fatigue recovery was 41.5% (Ko 2013), which was lower than TCD consumption rate of high school students. These results seem that high school students drink TCD to improve concentration and to recover fatigue for study of college entrance examination.

There was a significant difference in awareness of ingredients of TCD between the TCD consumption group and non-TCD consumption group ($p < 0.01$). Over

Table 2 Awareness of TCD by consumption

Variables	TCD consumer (n = 249)			Non-TCD consumer (n = 86)		
	High (n = 71)	Medium (n = 109)	Low (n = 69)	High (n = 32)	Medium (n = 34)	Low (n = 20)
Degree of interest in TCD*	2.9 ± 0.1$^{a>b***}$	2.5 ± 0.1^{b}	2.7 ± 0.1^{b}	1.0 ± 0.0	1.1 ± 0.1	1.1 ± 0.1
Degree of harmfulness to the body**	2.1 ± 0.1	1.9 ± 0.1	2.0 ± 0.1	3.8 ± 0.1	3.8 ± 0.1	3.8 ± 0.1
Improvement for learning ability	2.9 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	–	–	–
Types of drinks						
Carbonated	1(1.4)	3(2.8)	4(5.8)	0(0.0)	0(0.0)	0(0.0)
Ion drinks	2(2.8)	5(4.6)	6(8.7)	2(6.2)	0(0.0)	2(10.0)
Coffee and tea	9(12.7)	11(10.1)	2(2.9)	2(6.2)	6(17.6)	1(5.0)
Drinks for fatigue recovery	59(83.1)	90(82.6)	57(82.6)	28(87.5)	28(82.4)	17(85.0)
*Ingredients in TCD***						
Caffeine	53(74.6)	84(77.1)	55(79.7)	30(93.8)	31(91.2)	19(95.0)
Taurine	16(22.5)	17(15.6)	7(10.1)	1(3.1)	0(0.0)	0(0.0)
Vitamin	1(1.4)	5(4.6)	6(8.7)	1(3.1)	2(5.9)	1(5.0)
Sugar	1(1.4)	3(2.8)	1(1.4)	0(0.0)	1(2.9)	0(0.0)

Mean ± SE, n (%) *$p < 0.01$; **$p < 0.001$ (different between consumption and non-consumption groups by Student *t*-test)
***$p < 0.05$, $^{a, b}$(different superscript letters mean significant difference among groups by one-way ANOVA with Scheffe's post-hoc test)
****$p < 0.01$ (significance of consumption and non-consumption groups by chi-square test)

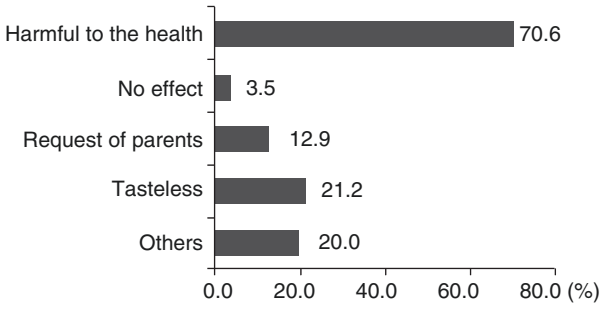

Fig. 1 Reasons for not consuming TCD in non-TCD consumption group by multiple responses

93.0% of non-TCD consumer group answered that ingredient in TCD was caffeine and the reasons for not consuming TCD in the non-TCD consumption group were harmful to health (70.6%) and tasteless (21.2%) (Fig. 1). Because excess caffeine intake can lead to side effects (nausea, anxiety, and insomnia etc.) (Malinauskas et al. 2007), it is considered that they were aware of that the drink intake was to harmfulness to health.

3.3 Consumption of TCD by Self-Reported School Record in TCD Consumption Group

Consumption of TCD according to the self-reported school record in the TCD consumption group is shown in Table 3. There was a significant difference in intake time of TCD among subgroups by school record ($p < 0.05$); 66.2% of high subgroup, 62.4% of medium subgroup, and 40.6% of low subgroup consumed TCD during examination periods. There was a significant difference in the effect of TCD intake among the high, medium, and low subgroups ($p < 0.01$). In Korea Consumer Agency (2013), 39.4% of middle school, high school, and university students who consume TCD experience showed that increasing the intake frequency of TCD for keeping awake during examination periods, and 44.0% of high school student in Gyeoungbuk region also drank TCD to fight off sleepiness (Lee et al. 2014). Main intake place of TCD was home, school, and

Table 3 Consumption of TCD by self-reported school record in the TCD consumption group

Variables	Self-reported school record		
	High (n = 71)	Medium (n = 109)	Low (n = 69)
Intake frequency of TCD			
Everyday	2(2.8)[a]	2(1.8)	1(1.4)
3~6 times/week	4(5.6)	2(1.8)	4(5.8)
1~2 times/week	4(5.6)	9(8.3)	3(4.3)
1~3 times/month	20(28.2)	20(18.3)	12(17.4)
1 times/2~3 months	41(57.7)	76(69.7)	49(71.0)
Intake place			
Home	45(63.4)	70(64.2)	36(52.2)
School or academy	17(23.9)	21(19.3)	17(24.6)
Reading room	9(12.7)	13(11.9)	12(17.4)
On the move	0(0.0)	5(4.6)	4(5.8)
Intake time			
Examination period	47(66.2)	68(62.4)	28(40.6)*
During exercise	0(0.0)	1(0.9)	3(4.3)
Fatigue	8(11.3)	17(15.6)	12(17.4)
Usual time	10(14.1)	15(3.8)	12(17.4)
Feeling sleepy	6(8.5)	8(7.3)	14(20.3)
Effects of intake			
Relieve sleepiness	41(57.7)	42(38.5)	31(44.9)**
Improve concentration	2(2.8)	0(0.0)	0(0.0)
Improve the physical strength	0(0.0)	2(1.8)	1(1.4)
Quench thirst	4(5.6)	9(8.3)	13(18.8)
No effect	24(33.8)	56(51.4)	24(34.8)

[a]n (%)

*$p < 0.05$ (by chi-square test)

**$p < 0.01$ (by chi-square test)

academy, which was similar to previous studies (Kim 2012; Lee et al. 2014). Since taurine intake has been reported to be effective for blood fatigue recovery (Kang and Kang 2015), these results seem that students may feel that TCD intake has the effect to relieve sleepiness.

For motivation to purchase, 58.2% and 18.9% of the TCD consumption group answered advertising and recommendation by friends, respectively (Fig. 2). According to previous studies, most high schools students from other areas in Korea are motivated to purchase TCD through advertising (Lee et al. 2014; Nho 2014).

3.4 *Effects of Awareness on Intake Frequency of TCD*

In Korean female high school students who consumed TCD, degree of interest in TCD ($p < 0.001$), degree of harmfulness to the body ($p < 0.01$), and improvement for learning ability ($p < 0.001$) had significant effects on intake frequency of TCD (Table 4).

3.5 *Preferences Related to TCD*

As for preferences, 'Vita 500' (52.2%), 'Bacchas' (20.1%), and 'Hot Six' (17.3%) were the most preferred (Table 5). Although the TCD consumption group preferred 'Vita 500' as a caffeine-free TCD (Table 5), they answered that the most abundant ingredient in TCD is caffeine (Table 2).

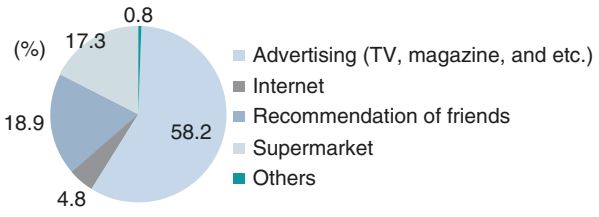

Fig. 2 Motivation to purchase TCD in consumers

Table 4 Effects of awareness on intake frequency of TCD

Independent variable	B	β	t(p)	R^2	F
Degree of interest in TCD	0.337	0.365	6.487**	0.146	42.084**
Degree of harmfulness to the body	0.269	0.206	3.302*	0.042	10.903*
Improvement for learning ability	0.190	0.237	3.825**	0.056	14.634**

*$p < 0.01$; **$p < 0.001$ (by simple regression analysis)

Table 5 Ranking preferences and ingredients of TCD

	Preferences		Ingredients in TCD		
Ranking	Items	n (%)	Taurine (mg)	Caffeine (mg)	Volume (mg)
1	Vita 500	130(52.2)	Included[a]	0.0	100.0
2	Bacchas	50(20.1)	2000	30.0	100.0
3	Hot Six	43(17.3)	1000	60.0	250.0
4	Red Bull	12(4.8)	1000	62.5	250.0
5	Volt Energy	6(2.4)	1000	60.0	250.0

[a]Taurine is present in the TCD, but the content is not shown

Table 6 Need for nutrition education for proper consumption of TCD

Variables	TCD consumer (n = 249)	Non-TCD consumer (n = 86)
Need for nutrition education	3.5 ± 0.1[a]	3.9 ± 0.1[*]
Nutrition education method		
Expert lectures	83(38.1)[b]	35(43.8)
Watching video	126(57.8)	49(61.3)
Attached poster	59(27.1)	19(23.8)
Leaflet	30(13.8)	11(13.8)
Homepage of school	4(1.8)	1(1.3)
Mobile phones (application)	31(14.2)	13(16.3)
Family letter	30(13.8)	7(8.8)
Others	2(0.9)	1(1.3)

[a]Mean ± SE; 5-point Likert scales ranging from 1 (strongly disagree) to 5 (strongly agree)
[b]n (%); multiple response
[*]$p < 0.001$ by Student *t*-test

3.6 Need for Nutrition Education

For proper consumption of TCD, need for nutrition education in the non-consumer group was significantly higher compared to the consumer group ($p < 0.001$). Most subjects answered that 'watching videos', 'expert lectures', and 'attached poster' would be a good method of nutrition education for proper consumption of TCD (Table 6). This result was similar to the previous result reported that the most preferred nutrition education method related to TCD of middle school students in Seo-gu Incheon was answered by the "watching video" lessons (53.4%) (Kim 2015). Therefore, it is considered that it is necessary to develop video educational materials about proper consumption method of TCD for middle and high school students.

4 Conclusion

Our study investigated awareness, consumption patterns, and preferences related to TCD in Korean female high school students. In this study, most of the TCD consumption group drank TCD in order to stay awake during their examination periods and cited the positive effect of less sleepiness. Most of the non-TCD consumption group did not consume TCD due to perceived harmfulness to health. The non-TCD consumption group needs more nutrition education about TCD intake. Therefore, it may be necessary to provide nutrition education and develop healthy drinks containing taurine for the mental and physical well-being of Korean high school students.

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Taurine Intake with Magnesium Reduces Cardiometabolic Risks

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Abstract WHO-CARDIAC (Cardiovascular Diseases and Alimentary Comparison) Study revealed the quintile analyses of 24-h urinary (24 U) taurine (T) and magnesium (Mg) excretions were inversely related with cardiometabolic risks (CMR) such as obesity, hypertension and hypercholesterolemia in 50 population samples in the world. To exclude the influence of ethnicity in the study, 24 U T and Mg excretions were analyzed for the association with CMR in one ethnicity, Japanese population.

24 U T/creatinine (C) ratios were divided into 5 quintiles and the ratios of Japanese to the total of each quintile were analyzed from CARDIAC Study samples. The highest 24 U T quintile consisted of 60% Japanese, indicating high seafood consumption in Japanese.

Over 600 Japanese aged 30–79 were invited to a health examination for blood pressure measurement and for fasting blood and 24 U samplings. Tertile analysis of 24 U T/C ratios in relation to CMR indicated the third tertile had significantly higher HDL cholesterol, 24 U potassium (K) and 24 U salt than the first (lowest) tertile. Tertile analysis of 24 U Mg/C ratios indicated the third tertile had significantly lower body mass index and significantly higher folic acid, 24 U isoflavones, K and salt than the first tertile after age and gender adjustment. The third tertile of both T/C and Mg/C had significantly lower body mass index, LDL/HDL and Na/K ratios, and

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significantly higher HDL cholesterol and folic acid than the first tertile, indicating seafood eaters taking Mg rich diets had lower risks of obesity, atherosclerosis, hypertension and higher folic acid, beneficial for healthy longevity.

Keywords Taurine • Magnesium • 24-h urine • Salt • Potassium • HDL cholesterol • Folic acid • Atherogenic index • Body mass index • Blood pressure

Abbreviations

24 U	24-hour urine
BP	Blood pressure
C	Creatinine
CMR	Cardiometabolic risks
HDL	High density lipoprotein
HOMA-IR	Homeostasis model of assessment of insulin resistance
K	Potassium
Mg	Magnesium
T	Taurine
WHO	World Health Organization

1 Introduction

Cardiovascular Disease and Alimentary Comparison (CARDIAC) Study coordinated by World Health Organization (WHO) covered 61 populations in 25 countries and revealed 24-h urinary (24 U) taurine (T), the biomarker of seafood intakes was inversely related with cardiovascular risks and the mortality rates of coronary heart diseases (CHD) (Yamori et al. 1996, 2001a, b, 2006, 2009, 2010a, b; Sagara et al. 2015). Since CHD mortality is inversely associated with the average of males' and females' life expectancy in the world (WHO statistics), Japanese world top longevity may be due to sufficient T intake. However, the association of T with low CHD mortality might be due to the ethnic characteristics of Japanese who are commonly eating seafood and low in CHD mortality because of the their possible genetic and/or environmental influence.

Therefore, in this study Japanese intakes of seafood were compared internationally by 24 U T data of CARDIAC Study and the association of 24 U T with CMR was studied in the Japanese themselves who took much or less seafood. Further, the association of 24 U magnesium (Mg) with CMR was analyzed and finally CMR were investigated in the Japanese who excreted both T and Mg sufficiently or not.

2 Methods

2.1 International 24 U T Comparison

The creatinine (C) ratios of 24 U T in 4470 males and females aged 48–56 from CARDIAC Study samples in the world were divided into quintiles. The ratio of Japanese to the total in each quintiles was calculated.

2.2 Association of 24 U T and/or Mg with CMR

Health examination for anthropometrical measurements, blood pressure measurements by an automated system (Omron HEM907), fasting blood sampling, 24 U collection by using aliquot cups (Yamori et al. 1984) were carried out for over 600 inhabitants (male ratio 42%) aged 30–79 of Hyogo Prefecture located in the middle of Japan after obtaining their informed consent. Blood was analyzed for total, HDL- and LDL-cholesterol, triglycerides, folic acid and 24 U samples were analyzed for sodium (Na) to calculate salt intakes, for potassium (K), T and Mg to estimate the intakes of vegetables seafood, grains, nuts, beans, and seaweeds.

2.3 Statistics Analysis

General linear models were used to estimate adjusted mean values of body mass index (BMI) blood pressure, and serum and urinary biomarkers and multiple linear regression models were used to estimate the linear trend in each analysis.

All analyses were done using IBM SPSS version 19. Two-tailed $P < 0.05$ was considered statistically significant.

3 Results

3.1 Japan's High Consumption of Seafood

The creatinine (C) ratios of T (μmol/mmol) were divided into quintiles I–V. The range of each quintile and (medium) were I: ≦27.5 (14.8), II: 27.6–53.6 (40.2), III: 53.7–89.7 (69.6), IV: 89.8–157.6 (117.5) and V: ≧157.8 (229.3) and the ratios of Japanese to the total of each quintile were I: 1.1%, II: 3.2%, III: 11.7%, IV: 29.5%, and V: 60.0% (Fig. 1).

Since the average ratio of Japanese in this CARDIAC Study samples was 21.1% of the total, the Japanese ratio, 60% in the highest V quintile of T, was far higher than the average, indicating Japanese intakes of seafood was high compared with other ethnic groups.

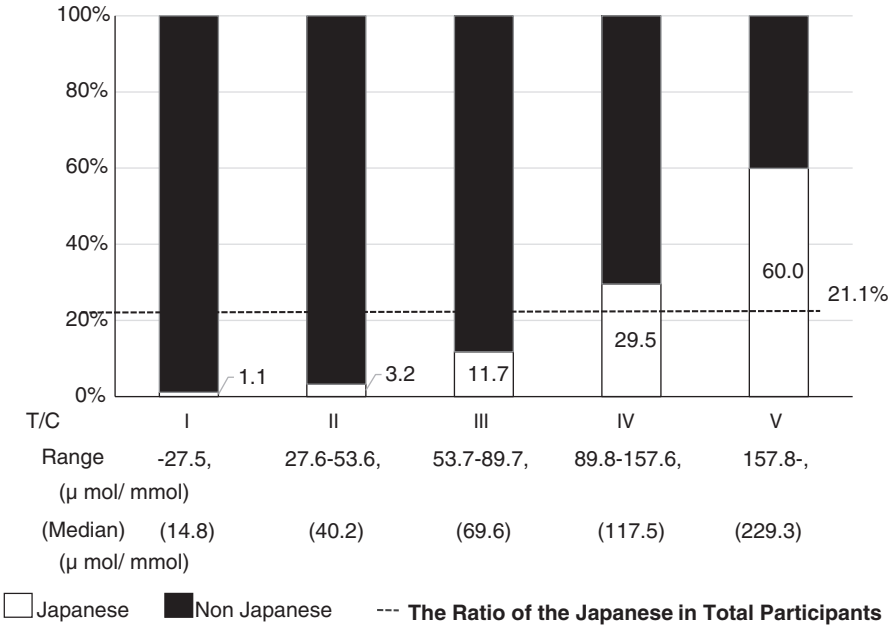


Fig. 1 The ratio of the Japanese to the total in each quintile of 24 U T/C ratio

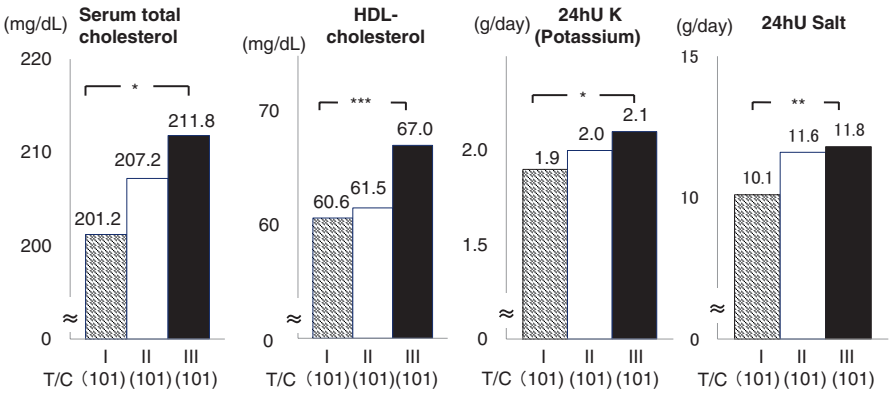
3.2 Seafood Intake and CMR

Male and female participants in the health examination from whom all data were available, were 303 in total and were divided into tertile I, II and III. The ranges and the medians of three tertiles of T/C ratios were in Fig. 2, in which only significantly different CMR were shown after age and gender adjustment.

T III was significantly higher than T I in total cholesterol, HDL cholesterol, 24 U K and 24 U salt.

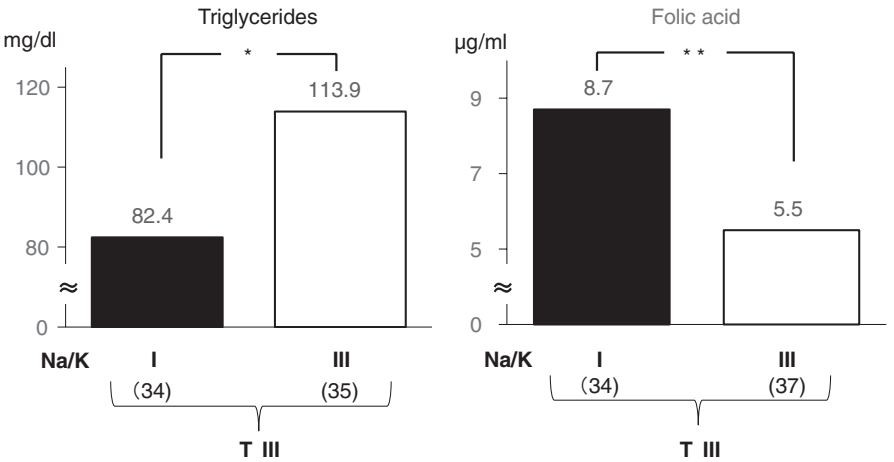
3.3 Effect of 24 U Na/K on CMR in High Seafood Consumers, TIII

T III showed significantly higher 24 U salt and K and K was known to reduce the adverse effect of salt. Therefore, the effect of higher K and less salt intake shown as low 24 U Na/K was investigated in T III. 24 U Na/K was divided into three tertiles, Na/K I–III, and CMR in Na/K I was compared with those in Na/K III among T III. As shown in Fig. 3, low Na/KI showed significantly lower triglycerides and significantly higher folic acid than high Na/K III.



24U T/C	I	II	III
Range (μmol/mmol)	18.8-93.5	96.2-159.2	159.3-1146.7
Median (μmol/mmol)	62.9	123.0	233.6

Fig. 2 CMR (Cardiometabolic risks) in the tertile of 24 U T/C ratio after age and gender adjustment



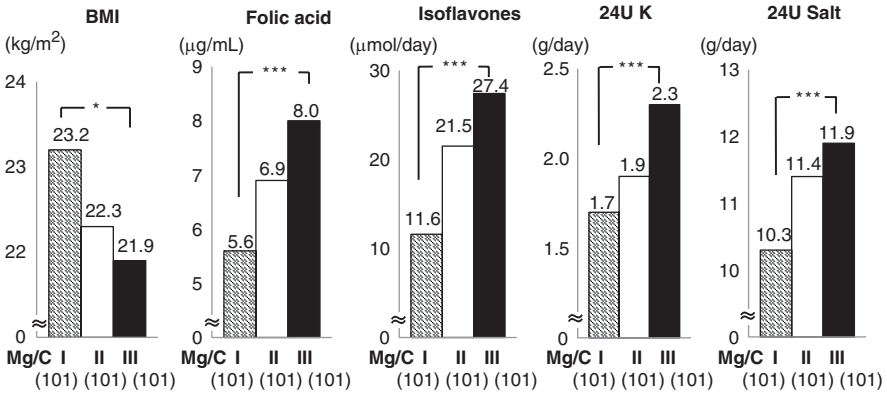
24U Na/K	I	II	III
Range	0.8-3.0	3.1-4.4	4.5-13.8
Median	2.5	3.7	5.8

Fig. 3 Effect of Na/K, high (III) and low tertiles (I) on CMR in high seafood consumers (T III)

3.4 Effect of 24 U Mg on CMR

As for 24 U M which was proved to be rerated to CMR by CARDIAC Study (Yamori et al. 2015), 24 U Mg/C ratios were divided into tertiles, M I, II, and III.

The range and median were shown in Fig. 4, which indicated after age and gender adjustment, significant reduction of BMI and significant elevation of folic acid,



24hU Mg/C	I	II	III
Range (mg/g)	15.5-62.1	62.2-90.9	91.1-286.3
Median (mg/g)	50.0	75.7	116.3

Fig. 4 CMR (Cardiometabolic risks) in the tertile of 24 U Mg/C ratio after age and gender adjustment

and 24 U isoflavones, K and salt in Mg III, compared with Mg I. Increased 24 U Mg was related to CMR reduction such as lower BMI and higher folic acid, isoflavones and 24 U K, except for higher 24 U salt.

3.5 Effect of High 24 U T with Mg on CMR

Since 24 U T and 24 U Mg were proved to be related to CMR reduction by CARDIAC Study (Sagara et al. 2015; Yamori et al. 2015), the highest tertile of both 24 U T and 24 U Mg (T-Mg III) were compared with the lowest tertiles of both (T-Mg I) in relation to CMR in Fig. 5. The highest tertiles of both 24 U T and 24 U Mg (T-Mg III) indicated significantly lower BMI and higher HDL, therefore, significantly lower LDL/HDL ratios (atherogenic index) and significantly higher folic acid than the lowest tertile of both 24 U T and 24 U Mg (T-Mg I). Although 24 U salt was higher significantly in the highest tertile of both 24 U T and Mg (T-Mg III) than in the lowest (T-Mg I), 24 U Na/K ratio was significantly lower in the both highest tertile than the lowest because of the significant elevation of 24 U K each in the both highest tertile of T and Mg, respectively (Figs. 2, 4).

4 Discussion

The present study based on 24 U biomarkers of nutrition confirmed the common seafood intakes of Japanese in comparison with other ethnic groups in the world as well as the beneficial CMR reduction of T and Mg intakes in the Japanese first shown by CARDIAC Study (Yamori et al. 2001a, b, 2006, 2009, 2010a, b, 2015;

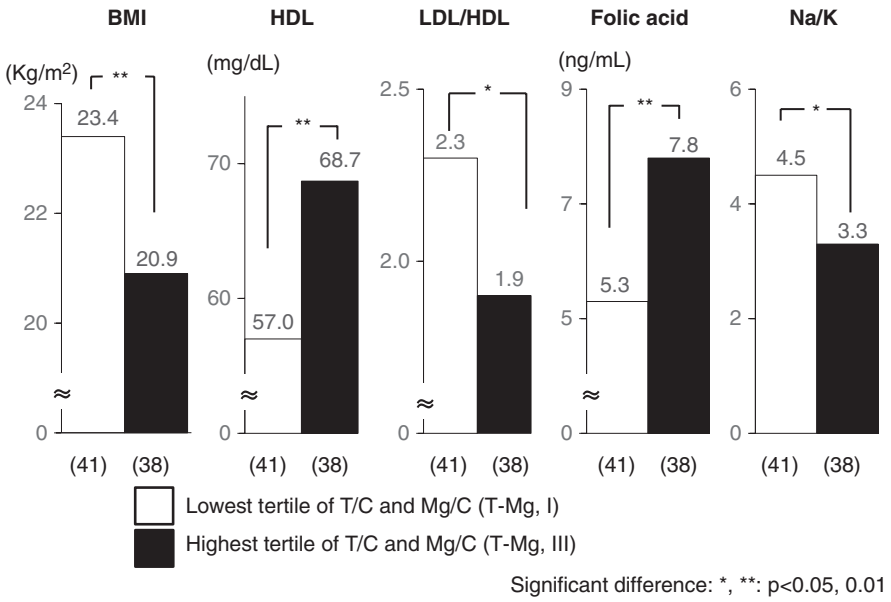


Fig. 5 CMR (Cardiometabolic risks) in the lowest and highest tertiles of combined T/C and Mg/C ratios after age and gender adjustment

Sagara et al. 2015). These CARDIAC Study data estimated the intake of Na, K, T and Mg by 24 U biomarker analyses. The objective comparative estimation of these nutrient intakes by 24 U excretion was the merit of the present study and the estimation of Na, K and T by 24U has been well documented by these CARDIAC study data and others. Urinary Mg was reported to be related with risk of hypertension (Joosten MM, et al 2013) and Yamori et al. (2015) revealed the significant inverse association of 24 U Mg with CMR in the world wide populations, indicating the preventive effect of Mg on cardiovascular diseases.

Despite CARDIAC data indicating BP reduction in relation to increased 24 U T and Mg in world-wide populations, the present tertile analysis of 24 U T and Mg in Japanese did not show any significant BP reduction in T III and Mg III compared with T I and Mg I. This is because dietary T and Mg intakes were significantly related to the high salt intake demonstrated by 24 U salt in T III and Mg III tertiles (Figs. 2, 4). The adverse effect of high salt intake has been well documented (Yamori et al. 2006; Yamori 2008; Strom et al. 2013) and this adverse effect is attenuated by K intake (Yamori 2006, 2008; Perez and Chang 2014). The reduced Na/K ratio in 24 U beneficially affected CMR in T III; triglycerides were significantly reduced and folic acid was significantly increased (Fig. 3). The importance of Na/K reduction is also suggested in the highest tertile of 24 U T and Mg, which Na/K ratio was significantly lower due to high 24 U K (T-Mg III) (Fig. 5).

The benefits of the combined highest intakes of T and Mg (T-Mg III) were demonstrated by this study: lower BMI, higher HDL, lower atherogenic index and higher folic acid than in T-Mg I. The association of BMI with CMR was extensively reviewed about its metabolic mediators such as BP, blood glucose and cholesterol

(Lu et al. 2014). High HDL and lowering of LDL/HDL ratio, that is atherogenic index, is a well documented factor for CMR reduction (Abbott et al. 1988; Millán et al. 2009). In addition, high folic acid is regarded as being beneficial for CMR reduction because folid acid intake is inversely associated with cardiovascular diseases in Japan (Cui et al. 2010) and the folic acid fortification of grains reduced cardiovascular diseases in the US (Eoin et al. 2003). High folic acid deceases homocysteine, oxidative stress causing atherosclerosis (Haynes 2002).

Lower BMI and higher folic acid were noted in the high 24 U Mg tertile, Mg III, in which 24 U isoflavones were also significantly higher than in Mg I. Since isoflavones are the biomarker of soy intakes. Mg is taken from soy products in Japanese. Isoflavones were proved to be inversely associated not only with the CHD mortalities but also with the mortality rates of breast and prostate cancers by CARDIAC Study (Yamori et al. 2001; Yamori 2006a, b, 2008).

Therefore, the merit of Mg intake is supposed to be derived partly from the intakes of soy, particularly isoflavones which reduce cardiovascular risks (Omoni and Aluko 2005; Sacks et al. 2006), and the elevation of folic acid in Mg III may be due to the intakes of soy and soy products which contain folic acid.

5 Conclusion

24 U T analysis of the world-wide population by WHO-CARDIAC Study proved Japanese daily diet contained T from seafood. The tertile analysis of 24 U T in the Japanese revealed significantly higher HDL and 24 U K and salt in the highest tertile than in the lowest. Among the highest T tertile lower 24 U Na/K ratio was associated with lower triglycerides and high folic acid in the blood. The tertile analysis of 24 U Mg revealed lower BMI, higher folic acid, higher 24 U isoflavones and K but higher 24 U salt. The highest tertile of both T and Mg had significantly lower BMI, higher HDL, lower atherogenic index, higher folic acid and lower 24 U Na/K than the lowest tertile of both T and Mg. These data indicate low salted seafood and Mg rich soy, nuts, unpolished grains, vegetables, seaweeds should be recommended for CMR reduction.

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Comparison of Toxicity of Taurine and GABA in Combination with Alcohol in 7-Day-Old Mice

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Abstract Previously, we described the combined toxicity of taurine and alcohol, and assumed hypoglycemia to be one reason of this toxicity. To understand whether taurine-ethanol combined toxicity is exclusively connected to taurine or whether other inhibitory amino acids may have similar effects when combined with ethanol, we tested different doses of gamma-aminobutyric acid (GABA) in combination with ethanol in 7-day-old mice. The minimal dose of GABA in combination with 5 g/kg ethanol which could kill a mouse was 2 g/kg. GABA combined with ethanol at doses of 3 g/kg, 4 g/kg, 6 g/kg induced lethality of 30%, 90% and 100%, correspondingly. Taurine at the doses of 4 and 6 g/kg combined with ethanol induced death in 60 and 100% of mice. Ethanol (5 g/kg), taurine (6 g/kg), GABA (4 g/kg) administered alone and the combination of ethanol (5 g/kg) with taurine (3 g/kg) have no lethal effects. GABA (6 g/kg) applied alone induced 90% lethality. Taurine or GABA alone decreased blood glucose in a dose-depending manner. Ethanol potentiated GABA- and taurine-induced decrease in blood glucose and in some animals it dropped from 8.8 (intact) to a hypoglycemic level 3.1–3.3 mmol/L (GABA 4 g/kg, taurine 6 g/kg), but this may not be considered a single reason of death. We conclude that the combination of GABA and ethanol has a lethal effect and this is stronger than the combined toxicity of ethanol and taurine.

Keywords GABA • Taurine • Alcohol • Toxicity • Hypoglycemia • 7-Day-old mice

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Abbreviations

E	Ethanol
G	GABA
GABA	Gamma-aminobutyric acid
HPLC	High performance liquid chromatography
OPA	Ortho-phthaldialdehyde
T	Taurine

1 Introduction

Taurine is a small simple molecule existing at high concentrations throughout the animal kingdom (Jacobsen and Smith 1968). A variety of taurine functions in mammalian body are known, such as osmotic regulation, bile acid conjugation, detoxification, membrane stabilization, regulation of intracellular calcium homeostasis, neuromodulation and cell protection (Huxtable 1992; Birdsall 1998; Lourenço and Camilo 2002; Roysommuti et al. 2003). The best proof of the importance of taurine for normal wellbeing is the health injury under taurine-deficiency (Hayes et al. 1975; Chesney et al. 2009). Every year appears proposals of the use of taurine for treatment of different human diseases (Birdsall 1998; Della Corte et al. 2002; Murakami 2015; De Luca et al. 2015; Prentice et al. 2015; Chen et al. 2016). A general belief of taurine being natural, safe and nontoxic even at high doses renders it attractive for clinical use (Nishizawa et al. 1991; Kihara et al. 1991; De Luca et al. 2015). We focused earlier on the role of taurine in protection of neurons under ischemic conditions (Taranukhin et al. 2008) and in acute ethanol intoxication (Taranukhin et al. 2009, 2010, 2012). However, a few years ago we got evidence of the toxic effect of taurine in combination with alcohol (Taranukhin et al. 2013). This finding prompted us to look at taurine from an opposite side and our interest was shifted to taurine toxicity. In experiments on 7-day-old, adult and old mice we have shown that the combined toxicity of ethanol and taurine is age-dependent. One possible mechanisms of lethality after co-administration of alcohol and taurine could be hypoglycemia (Taranukhin et al. 2013, 2015). We now studied whether combined toxicity of taurine and ethanol is a unique phenomenon or whether other inhibitory amino acids may exhibit have the same effect. Blood glucose was also assessed to see whether the putative toxicity of GABA and ethanol is related to hypoglycemia. To compare GABA and taurine we performed experiments with taurine in parallel to experiments with GABA using 7-day-old mice pups.

2 Methods

2.1 Animals and Experimental Procedures

Pregnant 8-week-old NMRI female mice were purchased from Harlan (Horst, the Netherlands). We used their 7-day-old pups (males and females). To compare the toxicity of GABA and ethanol with the toxicity of taurine and ethanol and to eliminate any possible influence of individual hereditary characteristics, mice from each litter were divided into experiments with GABA and taurine. Seven experimental groups were tested – intact, control, ethanol-treated, taurine-treated, GABA-treated, ethanol + taurine-treated and ethanol + GABA-treated. Ethanol (20% w/v solution in saline) was administered subcutaneously at a total dose of 5 g/kg (2.5 g/kg at time 1 h and 2.5 g/kg at 3 h). Taurine and GABA as 7% solution in saline were also injected subcutaneously in two half-doses (first at time zero and second at 4 h). The experimental groups receiving taurine or GABA with or without ethanol were divided into four sub-groups according to the different taurine or GABA doses (2, 3, 4 and 6 g/kg). The control animals were given saline injections equal to those in the ethanol + taurine-treated groups. The intact mice were used to establish a baseline in blood glucose. A part of animals from all experimental groups was monitored for 14 days to detect any sings of toxicity or lethality. The other animals were used for determination of taurine, GABA, ethanol and glucose in blood 30 min after the last injection.

2.2 Blood GABA and Taurine Measurements

The concentrations of GABA and taurine in blood serum were determined with high performance liquid chromatography (HPLC). After decapitation blood was collected and centrifuged to obtain blood serum samples. They were kept frozen (−20 °C) and thawed immediately prior to analysis. Taurine and GABA was measured using HPLC with fluorescent detection after precolumn derivatization with ortho-phthaldialdehyde (OPA) using the analysis equipment system of Shimadzu Scientific Instruments (Kyoto, Japan). The separation column was 4.6 × 250 mm Ultropac 8 Resin, lithium form (Farmacia, Denmark). Derivatization was done with OPA reagent (0.2% OPA, 0.1% mercaptoethanol and 1% ethanol in 1 M borate buffer, pH 10.4). Elution was done with lithium citrate buffers in the following order: (1) 0.2 M, pH 2.80, (2) 0.3 M, pH 3.00, (3) 0.5 M, pH 3.15, (4) 0.9 M, pH 3.50, and (5) 1.6 M, pH 3.30. The fluorescence of taurine and GABA derivatives was measured with an RF-10A detector using excitation and emission wavelengths set at 340

and 450 nm, respectively. The quantitation was finally effected using a commercial amino acid mixture (Pickering, UK) as an external standard and diamino-n-butyrate as an internal standard.

2.3 Blood Ethanol Measurements

For the determination of blood ethanol, two parallel trunk blood samples from each animal taken with a 10 μL capillary were instantly after decapitation blown into 190 μL of distilled water in 22 mL gas chromatography vials. The samples were stored at −20 °C until analysis. They were analyzed with headspace gas chromatography (Perking Elmer, GC 8410 gas chromatography with HS 40 headspace autosampler, Shelton, Connecticut, USA) as described elsewhere (Nurmi et al. 1994).

2.4 Blood Glucose Measurements

The tail blood samples (5 μL) were collected into HemoCue Glucose cuvettes and immediately analyzed in a HemoCue B-Glucose Analyzer (HemoCue AB, Ängelholm, Sweden). By reason of the small size of 7-day-old mice (4.5–6.0 g) we were not able to take blood samples twice from each animal—one time before the beginning of experiment (baseline) and second time at the end of experiment. The intact mice (at least one male and one female from each litter) were therefore used to establish a baseline for each litter.

2.5 Statistic Analysis

Statistical significance was estimated by Student's t-test. Each value was expressed as the mean ± SD. Differences were considered statistically significant when the calculated P value was less than 0.05.

3 Results

3.1 Comparison of the Combined Toxicity of Taurine and Ethanol Versus That of GABA and Ethanol

In all groups from each litter we used an equal number of males and females. Since there was no difference between the lethality of males and females, the results are

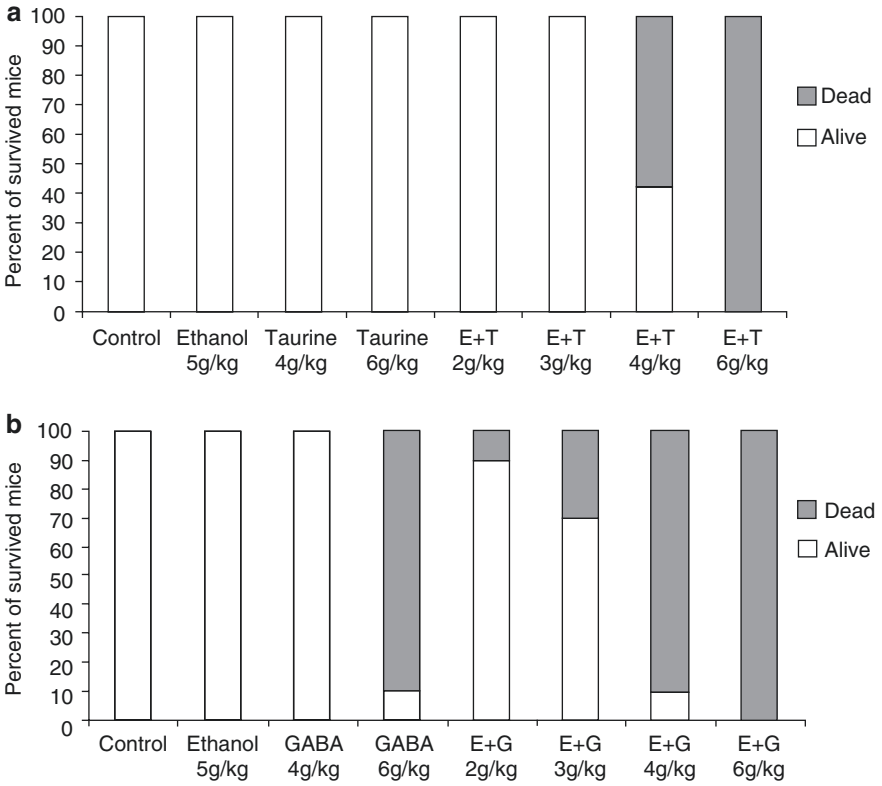

Fig. 1 (**a**) Lethality of the co-administration of ethanol and taurine in 7-day-old mice. (**b**) Lethality of ethanol and GABA co-administration in 7-day-old-mice. The number of animals in different groups varied from 6 to 12. *Abbreviations*: *E* ethanol, *T* taurine, *G* GABA

based on the lethality of both sexes (12 animals in the groups with any sign of toxicity, in the other six animals). Seven-day-old mice treated with ethanol alone at the total dose 5 g/kg, with taurine alone at the dose 6 g/kg, and the mice treated with ethanol (5 g/kg) in combination with taurine at the dose up to 3 g/kg survived the 14-day observation period and did not show any signs of toxicity (Fig. 1a). The combination of ethanol (5 g/kg) with taurine (4 g/kg) killed 58% of the treated mice. Hundred % of mortality was seen when the taurine dose was increased up to 6 g/kg co-administered with ethanol. To compare taurine and GABA toxicities alone and in combination with ethanol we tested the same doses of ethanol, GABA and taurine. GABA alone at a total dose 4 g/kg did not provoke any signs of toxicity during 14 days. However, unlike to taurine, GABA at the dose 6 g/kg induced 90% of mortality. Co-administration of ethanol with GABA induced mice mortality in a dose-dependent manner with GABA (Fig. 1b). Ethanol combined with GABA at doses of 2 g/kg, 3 g/kg, 4 g/kg and 6 g/kg induced 10%, 30%, 90% and 100% mortality, correspondingly.

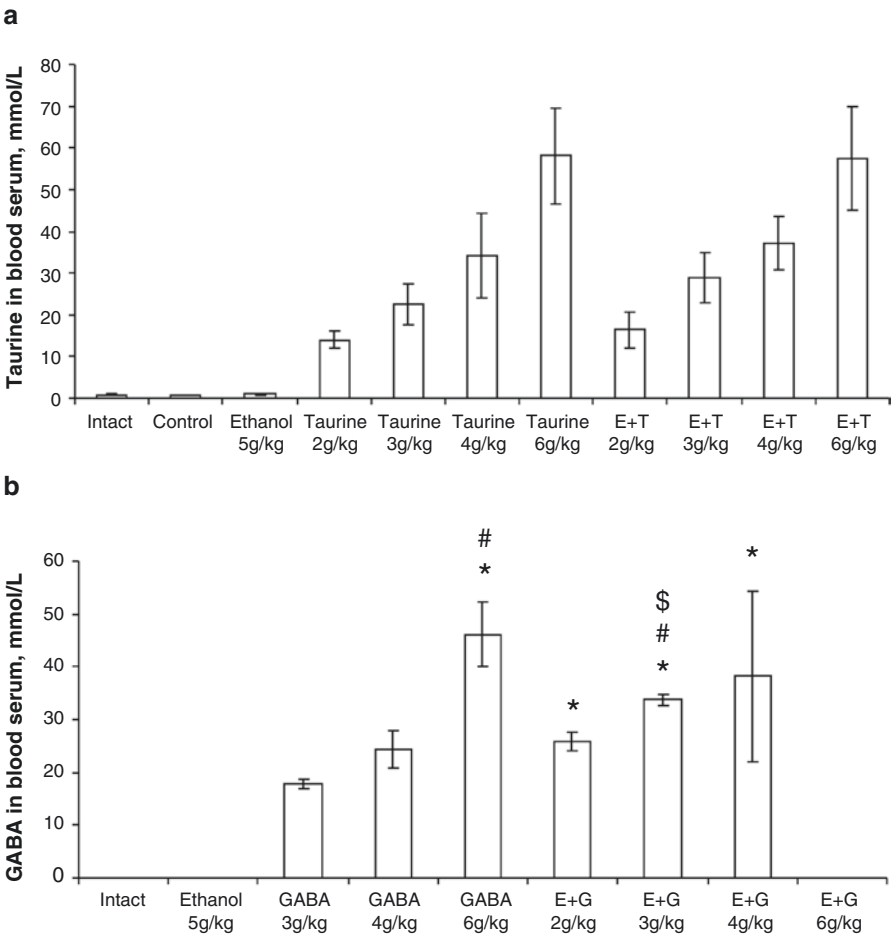

Fig. 2 (**a**) Taurine and (**b**) GABA in blood serum after taurine and ethanol and GABA and ethanol administrations. The number of animals in different groups varied from 4 to 8. The significance of differences compared to 3 g/kg GABA: *P < 0.05 and compared to 4 g/kg: GABA $^{\#}$P < 0.05. The significance of differences between the 2 g/kg E + G and 3 g/kg E + G groups: $^{\$}$P < 0.05. *Abbreviations*: *E* ethanol, *T* taurine, *G* GABA

3.2 Taurine, GABA and Ethanol Concentrations in Blood

To determine the dangerous concentrations of taurine and GABA which could alone or in combination with alcohol induce death of animals we measured the blood serum concentrations of taurine, GABA (Fig. 2b), and ethanol (Table 1). The same dose of ethanol (5 g/kg) was used in all ethanol-treated mice. The ethanol concentration in blood was about 100 mmol/L without significant variation between the experimental groups (Table 1).

Table 1 Ethanol in blood

Experimental groups	Ethanol (mmol/L)
Control (saline)	0.3 ± 0.2
Ethanol 5 g/kg	101.0 ± 8.9
Ethanol 5 g/kg + Taurine 2 g/kg	97.0 ± 10.2
Ethanol 5 g/kg + Taurine 3 g/kg	104.3 ± 3.8
Ethanol 5 g/kg + Taurine 4 g/kg	94.9 ± 3.0
Ethanol 5 g/kg + Taurine 6 g/kg	97.1 ± 3.2
Ethanol 5 g/kg + GABA 2 g/kg	106.0 ± 5.2
Ethanol 5 g/kg + GABA 3 g/kg	107.8 ± 7.9
Ethanol 5 g/kg + GABA 4 g/kg	104.1 ± 4.6
Ethanol 5 g/kg + GABA 6 g/kg	Not measured

Saline injections to the control group and ethanol alone did not alter the taurine level in blood compared to the intact mice. The taurine concentrations in these groups (intact, control and ethanol) were 0.93 ± 0.14, 0.76 ± 0.11 and 1.01 ± 0.26 mmol/L, correspondingly. Taurine injections alone or in combination with ethanol induced a gradual increase of taurine concentrations in blood according to applied taurine doses (Fig. 2a). Taurine at the highest dose of 6 g/kg increased the blood taurine level up to 58.12 ± 11.53 mmol/L which did not kill any mouse. There was no significant difference in the taurine concentrations in blood serum between the taurine and E + T groups with equal doses of taurine administered. The combination of 100 mmol/L of ethanol with 28.99 ± 6.06 mmol/L of taurine did not induce animal's death. The increase of taurine concentrations in blood to 37.22 ± 6.35 mmol/L (E + T 4 g/kg) and to 57.53 ± 12.35 mmol/L (E + T 6 g/kg) with 100 mmol/L of ethanol lead to 58 and 100% mortality (Figs. 1a, 2a, and Table 1).

The ethanol administration did not alter the GABA concentration in blood serum which was almost equal in the intact mice (0.01 ± 0.003 mmol/L) and in the mice treated with 5 g/kg of ethanol (0.01 ± 0.004 mmol/L). Administration of GABA alone or together with ethanol increased the GABA concentration in blood serum gradually with the applied doses of GABA (2, 3, 4 and 6 g/kg). However, the increase of GABA concentrations in GABA and GABA- and ethanol-treated mice was not equal. Two g/kg GABA co-administered with ethanol increased the GABA concentration to 25.94 ± 1.81 mmol/L, which was significantly higher than the GABA concentration 17.77 ± 0.87 mmol/L after the administration of GABA alone at the total dose 3 g/kg (Fig. 2b). Also the GABA concentration in the group E + G 3 g/kg (33.75 ± 1.05 mmol/L) was significantly higher than that in the group GABA 4 g/kg alone (24.11 ± 3.68 mmol/L). GABA alone at concentrations up to 24.11 ± 3.68 mmol/L was safe to animals. However, the combination of 25.94 ± 1.81 mmol GABA with 100 mmol/L ethanol had a lethal effect (Figs. 1b, 2b, Table 1). The GABA concentration of 46.11 ± 6.10 mmol/L after administration of GABA at the total dose of 6 g/kg was deadly dangerous and induced 90% death in the treated mice. Hundred mmol/L of ethanol with 33.75 ± 1.05 mmol/L and 38.25 ± 16.21 GABA induced death of 30% and 90% of mice, correspondingly. It was impossible to measure the GABA concentration in blood serum in the group E + G 6 g/kg with 100% lethality, because animals died very fast (often during few minutes after the second GABA injection).

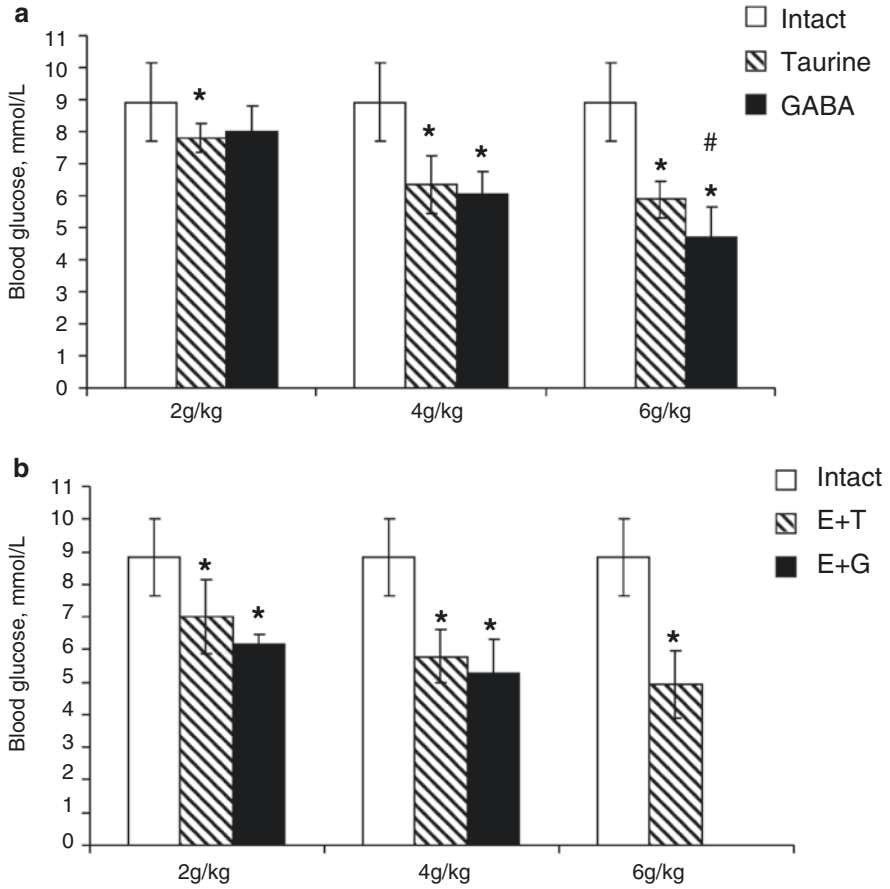

Fig. 3 (**a**) Decrease in blood glucose after taurine and GABA administration and (**b**) after taurine and GABA co-administration with ethanol. The number of animals in different groups varied from 5 to 18.The significance of differences compared to the intact group: *P < 0.05, and between the taurine 6 g/kg and GABA 6 g/kg groups: #P < 0.05. *Abbreviations*: *E* ethanol, *T* taurine, *G* GABA

3.3 Comparison of the Effects of GABA and Taurine Alone or in Combination with Ethanol on Blood Glucose

Taurine alone and GABA alone gradually decreased blood glucose when compared to the intact mice with the increase in the applied doses of taurine and GABA (Fig. 3a). At the dose of 6 g/kg GABA decreased significantly more blood glucose (4.69 ± 0.96 mmol/L) than taurine at the same dose (5.89 ± 0.57 mmol/L). Ethanol applied together with GABA and taurine enhanced the drop in blood glucose (Fig. 3b). No statistically significant differences were seen in blood glucose between taurine or GABA at doses of 2 and 4 g/kg in combination with ethanol. By the

reason of the very fast death of mice from the group E + G 6 g/kg we could not measure the blood glucose level in this group.

Wide variation in blood glucose between individual animals was seen in some experimental groups. For example, in the group E + T 6 g/kg blood glucose varied from 6.9 to 3.1 mmol/L, in the group E + G 4 g/kg from 6.8 to 3.3 mmol/L, and in the group GABA 6 g/kg from 6.1 to 3.3 mmol/L. Even the intact mice exhibited heterogeneity in blood glucose from 11.1 to 7.2 mmol/L. The significant drop in blood glucose from 11.1 to 3.1 mmol/L in some individual mice after the taurine + ethanol and GABA + ethanol treatments could be considered as a possible reason of hypoglycemic death.

4 Discussion

Taurine is an inhibitory amino acid whose actions mimic GABA actions, which is a major inhibitory neurotransmitter in the brain. Special taurine receptors have not been found yet, but taurine interacts with $GABA_A$ (Malminen and Kontro 1987; Quinn and Harris 1995) and $GABA_B$ (Kontro and Oja 1990; Kontro et al. 1990) receptors, acting as an agonist at both of them. Alcohol increases both GABA release and extrasynaptic $GABA_A$ receptor activity leading to motor impairment (Krystal et al. 2006). Both taurine (Winiarska et al. 2009; L'Amoreaux et al. 2010; Santos-Silva et al. 2015) and GABA (Gomez et al. 1999; Tian et al. 2011) are involved in blood glucose regulation.

It has been shown in Wistar rats, that taurine at a single dose of 7 g/kg (intravenously) or 5 g/kg (orally) has had no toxic effects (Kihara et al. 1991). In mice, however, a single subcutaneous injection of taurine at the doses of 7.5 g/kg and 6.0 g/kg has induced death of 100% and 50% of adult animals, respectively (Goldberg and Jefferies 1946). In our experiments taurine at the dose 6 g/kg administered to 7-day-old mice was not toxic, possibly because it was injected in two half-doses 4 h apart. Taurine from the first injection was then already partly incorporated into bile salts or secreted into urine, as reflected in the decrease of taurine levels in the blood (Lallemand and De Witte 2004; Taranukhin et al. 2010). It has been supposed that GABA is virtually without side effects, because GABA at the dose 5 g/kg did not cause any mortality in rats, indicating that 50% lethal dose of GABA must be more than 5 g/kg (Alternative Medicine Review 2007). In our experiments GABA at the dose 6 g/kg (even injected in two half-doses 4 h apart) induced 90% of mortality in 7-day-old mice. GABA is thus more toxic than taurine. The normal taurine concentration in blood of 7-day-old mice is about 0.9 mmol/L. There is GABA in blood significantly less (0.012 mmol/L) than taurine and the administration of GABA at the dose 6 g/kg increased GABA in blood 3843-fold. In other words, the organism is more accustomed to a high concentration of taurine than that of GABA. That is a simple explanation for the different sensitivity of taurine and GABA.

The co-administration of 4 g/kg of GABA with ethanol (5 g/kg) killed 90% of 7-day-old mice, whereas ethanol or GABA administered separately at the same doses did not induce any signs of toxicity. These data allow us to conclude that the combined toxicity of ethanol and GABA is more harmful than the combined toxicity of ethanol and taurine.

Previously, we have shown that hypoglycemia induced by the combined toxicity of taurine and ethanol could be one reason leading to death of old (Taranukhin et al. 2013) and 7-day-old mice (Taranukhin et al. 2015). We now found that both taurine and GABA alone or in combination with alcohol decrease glucose blood gradually according to the doses. The equal doses of GABA and taurine with or without ethanol induced similar drops in blood glucose. Only 6 g/kg GABA alone decreased blood glucose significantly more than taurine alone at the same dose. Ethanol enhanced the decrease of blood glucose induced by GABA and taurine. However, even the maximal drop in blood glucose found in the mice treated with GABA (6 g/kg) and the combination of taurine (6 g/kg) with ethanol were still far from the dangerous level to induce hypoglycemic death. In some mice the blood glucose decreased to 3.1–3.3 mmol/L, which is so hypoglycemic to be at least an additional reason for death. The similar heterogeneity in the blood glucose level after high doses of co-administration of taurine and ethanol was observed previously in old mice (Taranukhin et al. 2015).

By observing dying animals treated with ethanol and GABA or ethanol with taurine we noticed that they exhibited a marked decrease in the heart beat rate and suppression of respiration. We therefore suggest that they could be two further mechanisms involved in the lethality. Such a conception is corroborated by the fact that ethanol at high doses has a depressive effect in the central nervous system, resulting in lowered heart and breathing rates (Sahn et al. 1975; Lamminpää and Vilska 1990; Pagala et al. 1995). Ethanol can also induce death by respiratory depression (Church and Witting 1997; Paton 2005). Taurine reduces the heart rate (Bousquet et al. 1981; Paakkari et al. 1982; Wessberg et al. 1983) and respiration (Paakkari et al. 1982; Holtman et al. 1983; Wessberg et al. 1983) which is also in agreement with this hypothesis, which requires further experimental confirmation, of course.

Taurine is a common component of alcoholic and nonalcoholic energy drinks (Lutmer et al. 2009; Ayala et al. 2009; Higgins et al. 2010). The combined toxicity of taurine and ethanol may be a potential danger of taurine- and ethanol-containing energy drinks on human health, especially for pregnant women and adolescence. Both synthetic and natural GABA are available as dietary supplements against anxiety, sleep disorders and epilepsy (Alternative Medicine Review 2007; Bioclinic Naturals 2011). However, in view of our findings, it seems wise to avoid excessive alcohol consumption during GABA treatment.

5 Conclusion

We conclude that the combination of high doses of GABA with alcohol and the combination of high doses of taurine with alcohol have a lethal effect on neonatal mice. It seems that the combined toxicity of GABA and-ethanol is stronger than the

combined toxicity of ethanol and taurine. Even if applied alone, GABA is more toxic than taurine. The details of mechanisms of death induced by combined toxicity of ethanol with taurine or with GABA remain still unclear. We have shown that hypoglycemia may be considered as one reason at least in a part of treated animals. We assume that suppression of breathing and/or heart beating may also be reasons for lethality after combined administration of ethanol with GABA or with taurine. However, this assumption requires further experimental confirmation.

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Antioxidant Effects of Short-Neck Clam (*Tapes philippinarum*) Water Extract Containing Taurine Against AAPH-Induced Oxidative Stress in Zebrafish Embryos

Dong-Sung Lee, Seung-Hong Lee, You-Jin Jeon, and Sun Hee Cheong

Abstract The purpose of this study was to investigate the antioxidant activities of short-neck clam water extract (SNC-WE) enriched in taurine. In the present study, the half-maximal inhibitory concentration (IC_{50}) values of the SNC-WE for DPPH, superoxide, and alkyl radical scavenging activities determined by an electron spin resonance (ESR) spectrometer were 3.16, 1.54 and 0.58 mg/mL, respectively. Furthermore, we evaluated the inhibitory effect of taurine enriched SNC-WE against the oxidative stress induced by 2,2′-azobis dihydrochloride (AAPH) in zebrafish embryos. In the present study, we observed that taurine enriched SNC-WE significantly suppressed reactive oxygen species (ROS) production, lipid peroxidation as well as cell death in the zebrafish model. These results indicate that taurine enriched SNC-WE might have antioxidant effects in both *in vitro* and *in vivo* zebrafish model.

Keywords Short-neck clam (*Tapes philippinarum*) • Taurine • Zebrafish • AAPH Oxidative stress

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Abbreviations

4-POBN	Alpha-(4-pyridyl-1-oxide)-N-tert-butylnitrone
AAPH	2,2′-Azobis dihydrochloride
BHA	Butylated hydroxyl anisole
BHT	Butylated hydroxyltoluene
CAT	Catalase
Cys	Cysteine
DCF	Fluorescent 2′,7′-dichlorofluorescein
$DCFH_2$	Non-fluorescent 2′,7′-dichlorodihydrofluorescein
DMPO	5,5-Dimethyl-1-pyrroline N-oxide
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DPPP	Diphenyl-l-pyrenylphosphine
ESR	Electron spin resonance
GPx	Glutathione peroxidase
Met	Methionine
PG	Propyl gallate
ROS	Reactive oxygen species
SNC-WE	Short-neck clam water extract
SOD	Superoxide dismutase
TBHP	t-Butyl hydroperoxide

1 Introduction

Oxidative stress can be induced by an imbalance between the biological system's ability to detoxify the reactive intermediates and the reactive oxygen species (ROS) generation (Rush et al. 1985). Excessive oxidative stress can cause cell damage and induce numerous detrimental effects in the human body including organ and skin injury, cancer as well as aging (Sarma et al. 2010). In general, synthetic antioxidants including propyl gallate (PG), butylated hydroxyltoluene (BHT) and butylated hydroxyl anisole (BHA) have been used in several industries (Kang et al. 2013). However, recent research has focused on the antioxidants from various natural compounds such as herbal teas, seaweeds and phenolic compounds (Chen et al. 2009; Kang et al. 2013; Yin et al. 2015).

Taurine (2-aminoethanesulfonic acid), in general, synthesized from cysteine (Cys) or through conversion from methionine (Met). However, it also exist in the diet such as fish and seafood (Rosa et al. 2014). It has osmotic regulation, immune defense enhancement, cell membrane-stabilizing and antioxidant properties, as it inhibits lipid peroxides (Patrick 2006; Métayer et al. 2008). Taurine, a major amino acid in seafood, attenuates tissue damages and oxidative stress in several in vitro (Heidari et al. 2013) and animal models (Aly and Khafagy 2014; Maia et al. 2014;

Sirdah 2015). Although taurine is a well-established antioxidant and protects various organs from oxidative stress induced by several heavy metals (Das et al. 2009; Manna et al. 2009) drugs (Sinha et al. 2008) and chemicals (Das et al. 2008), little is known about its action in AAPH-stimulated oxidative stress in zebrafish embryos.

The zebrafish (*Danio rerio*) is a specific alternative animal model for development that is used in biomedical science. Some advantages of the zebrafish model such as its well-characterized embryonic development and the ease evaluation for toxic effects of several chemicals (Scholz et al. 2008). Therefore, the purpose of this study was to evaluate the protective role of short-neck clam water extract containing high concentrations of taurine against oxidative damage stimulated by AAPH using a zebrafish model.

2 Methods

2.1 Reagents

All chemicals were the highest grade available commercially. 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azobis (2-amidinopropane) hydrochloride (AAPH), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), and *alpha*-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) were purchased from Sigma Chemical Co. (St. Louis, MO USA).

2.2 Free Radical Scavenging Activities In Vitro

DPPH radical-scavenging activity was measured by the method of Nanjo et al. (1996). At each concentration, short-neck clam extract (60 mL) was added to 60 μL of DPPH (60 μM) solution. Superoxide radical was produced by ultraviolet irradiation of a riboflavin/ethylenediaminetetraacetic acid. The reactant mixtures supplemented with 20 μL of 1.6 mM EDTA, 20 μL of 0.8 mM riboflavin, 20 μL of 0.8 M DMPO, and 20 μL of sample were irradiated for 1 min under an ultraviolet lamp at 365 nm. Alkyl radical was produced by AAPH. Briefly, phosphate-buffered saline (pH 7.4) including AAPH (10 mM), 4-POBN (10 mM) and the sample were incubated at 37 °C for 30 min. The reaction mixtures were moved to a 100 μL Teflon capillary tube after mixing for 10 s, and the then DPPH, superoxide as well as alkyl radical scavenging activities were determined by an ESR spectrometer. The experimental conditions as follows; modulation frequency: 100 kHz, modulation amplitude: 2 G, central field: 3475 G, gain: 6.3×10^5, temperature: 298 K, microwave power: 5 mW for DPPH, 10 Mw for superoxide radical and 1 mW for alkyl radical, respectively.

2.3 Amino Acid Contents and Compositions

The crude protein, crude fat, and ash contents of the short-neck clam samples were analyzed by the methods of the Association of Official Analytical Chemists (AOAC 1990). To analyze amino acid composition, 80 mg of samples were mixed with of 6 N HCl (10 mL) and then N_2 gas was purged to the samples. All the samples were hydrolyzed at 110 °C for 24 h and evaporated, then added a sodium-distilled buffer (pH 2.2). After the filtering each sample by a 0.45 μm syringe filter, amino acids were analyzed and identified by an amino acid autoanalyzer (Pharmacia Biotech Biochrom 20, Ninhydrin Method) by absorbance at 440 and 570 nm.

2.4 Maintenance of Parental Zebrafish and Exposure to the AAPH of Zebrafish Embryos

Zebrafish were purchased from a Seoul aquarium (Seoul, Korea) and 15 fishes were maintained in an acrylic tank at 28.5 ± 1 °C, and were fed twice daily (Tetra GmgH D-49304 Melle, Germany). The previous day, one female zebrafish interbreed with two males. After collection of embryos from a natural spawning, the embryos (n = 15) were moved to 12-well plates containing 900 L embryo media from ~7–9 hpf and then incubated for 1 h. The embryos exposed for up to 24 hpf were added the AAPH solution (15 mM) and then washed with fresh embryo medium.

2.5 Intracellular ROS Production Induced by Oxidative Stress in Zebrafish

ROS generation by zebrafish was analyzed using the DCFH-DA. Zebrafish larvae were moved to a 96-well plate at 3 dpf, added with 20 g/mL DCFH-DA solution. After the incubation for 1 h at 28.5 ± 1 °C, zebrafish larvae were washed with fresh embryo medium and anesthetized by 2-phenoxy ethanol (1/500 dilution; Sigma). Zebrafish larvae were photographed using a CoolSNAP-Pro color digital camera (Olympus, Japan) and the fluorescence intensity was calculated by the Image-J software.

2.6 Lipid Peroxidation Induced by Oxidative Stress in Zebrafish

Lipid peroxidation can cause the damage of cell membrane in zebrafish. Zebrafish larvae were moved to a 96-well plate at 3 dpf, and added 25 μg/mL of DPPP solution. After the incubation for 1 h at 28.5 ± 1 °C, the zebrafish larvae were washed with fresh embryo medium, and then anesthetized using 2-phenoxy ethanol (1/500 dilution; Sigma). Zebrafish larvae were photographed using a CoolSNAP-Pro color digital camera (Olympus, Japan) and the fluorescence intensity was calculated by the Image-J software.

2.7 Cell Death Induced by Oxidative Stress in Zebrafish

Acridine orange staining method was used for cell death in zebrafish embryos. Zebrafish larvae were moved to a 96-well plate at 3 dpf, added 7 g/mL of acridine orange solution into the each well. After the incubation for 30 min, they were washed with fresh embryo medium and then anesthetized by 2-phenoxy ethanol (1/500 dilution; Sigma). Zebrafish larvae were photographed using a CoolSNAP-Pro color digital camera (Olympus, Japan) and the fluorescence intensity was calculated by the Image-J software.

2.8 Statistical Analysis

Values are expressed as means ± SEM. The differences between means were assessed by one-way ANOVA followed by Tukey-Kramer multiple comparison test, and statistical significance was defined at $P < 0.05$.

3 Results

3.1 Amino Acid Contents of Short Neck Clam Extracts

Dry matter, crude protein, crude lipids, and ash contents of short neck clam sample were 95.59%, 57.13%, 7.62% and 6.13%, respectively (data not shown). As shown in Table 1, the total amino acid content of SNC-WE was 8.53-fold higher than the

Table 1 Amino acid content of short-neck clam extracts

Amino acid content (nmol/mg)	SNC-WE	Lysis buffer extract
Cysteine	1.92	0.00
Aspartic acid	40.54	5.58
Glutamic acid	112.44	13.66
Asparagine	3.53	0.00
Serine	26.23	3.43
Glutamine	20.29	2.56
Glycine	604.28	69.65
Histidine	18.89	1.48
Arginine	88.11	11.78
Taurine	476.88	52.98
Threonine	28.91	3.74
Alanine	246.36	31.73
Proline	28.33	3.74
Tyrosine	11.60	1.81
Valine	27.63	3.29
Methionine	12.63	0.74
Isoleucine	16.72	1.73
Leucine	23.20	2.67
Phenylalanine	14.50	1.57
Tryptophan	5.50	0.53
Lysine	27.59	2.63
Total	1836.08	215.30

lysis buffer extract. Taurine was the second largest free amino acids following glycine, in particular, the taurine concentration of water extract was ninefold higher than that of lysis buffer. In the following experiments, therefore, we used SNC-WE to assess antioxidant potentials in the AAPH-stimulated zebrafish model.

3.2 Free Radical-Scavenging Activities of SNC-WE

Figure 1 shows the free radical scavenging activities against DPPH, superoxide and alkyl radicals of SNC-WE. The IC50 value, a concentration inhibiting 50% of free radical generation, against DPPH in SNC-WE was 3.16 mg/mL (Fig. 1a). In contrast, the superoxide and alkyl radical scavenging activities were higher (IC50 = 1.54, 0.58 mg/mL, respectively) (Fig. 1b, c).

3.3 Effect of Taurine Enriched SNC-WE on the AAPH-Stimulated Cell Death in Zebrafish

Figure 2 shows the AAPH-induced cell death and protective role of SNC-WE using in vivo zebrafish model. In the present study, the AAPH-induced cell death in

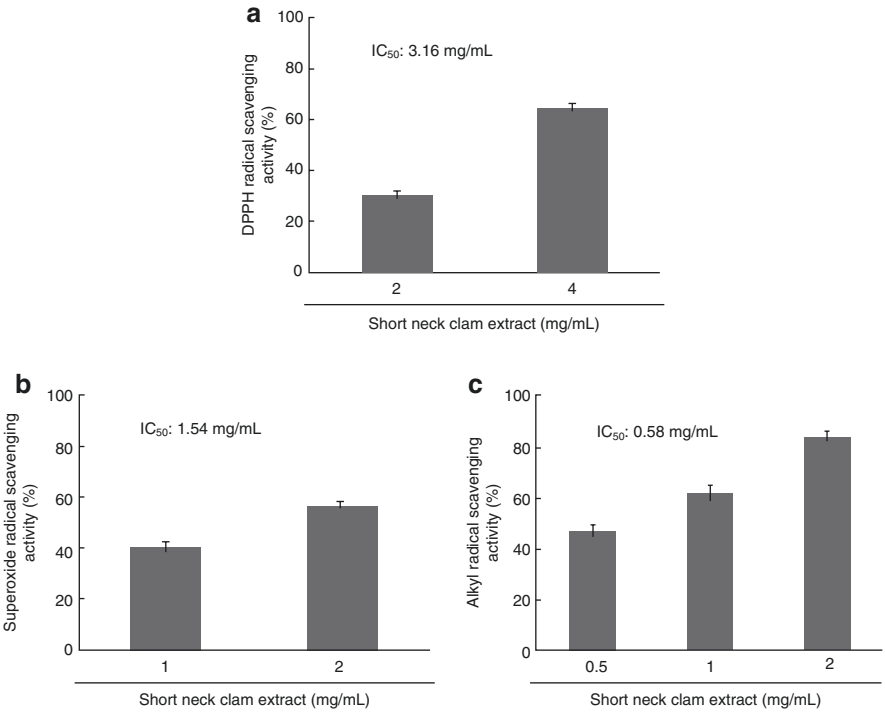

Fig. 1 Free radical scavenging activities of SNC-WE were determined using ESR. (**a**) DPPH, (**b**) superoxide, and (**c**) alkyl radical-scavenging activity. Data are presented as the mean values ± SD of three independent experiments

zebrafish was increased by 132% that of the control group. On the other hand, cell death induced by AAPH was significantly and dose-dependently decreased by SNC-WE with no toxicity ($P < 0.05$).

3.4 Effect of Taurine Enriched SNC-WE on AAPH-Induced ROS Production in the Zebrafish

In this study, we evaluated the inhibitory effect of taurine enriched SNC-WE on the ROS generation stimulated by AAPH treatment. DCFH-DA is oxidation-dependently converted to non-fluorescent 2′,7′-dichlorodihydrofluorescein ($DCFH_2$) and then fluorescent 2′,7′-dichlorofluorescein (DCF). The fluorescence intensity of DCF rises according to the generation of ROS (Walker et al. 2012). In AAPH-treated zebrafish, the ROS level was increased by 118% that of the control group. However, the zebrafish treated with SNC-WE showed dramatically decreased the generation of ROS (Fig. 3). These results suggest that taurine enriched SNC-WE may reduce the intracellular ROS production in the zebrafish model.

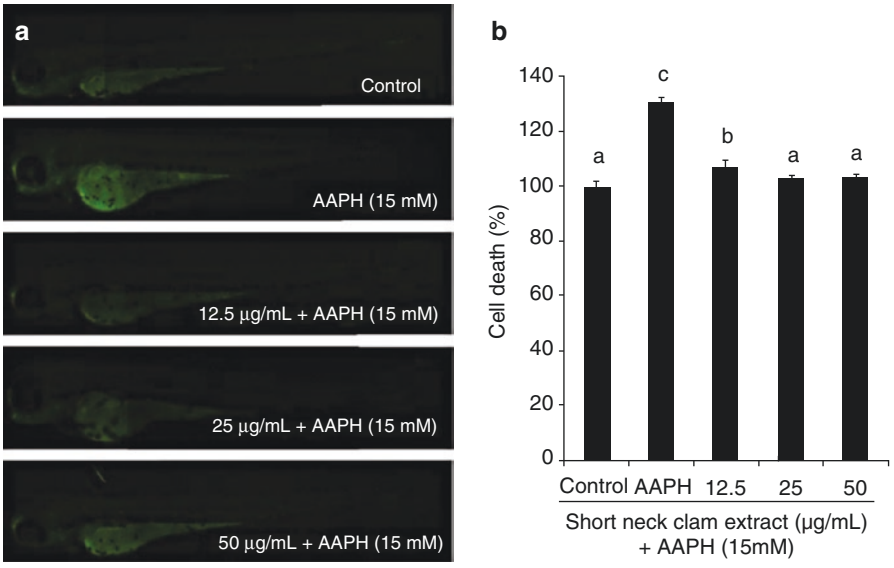

Fig. 2 Effect of the taurine enriched SNC-WE on cell death induced by AAPH in zebrafish. Data are presented as the mean values ± SEM of three independent experiments. Values with different letters are significantly different by Tukey-Kramer multiple comparison test at $P < 0.05$

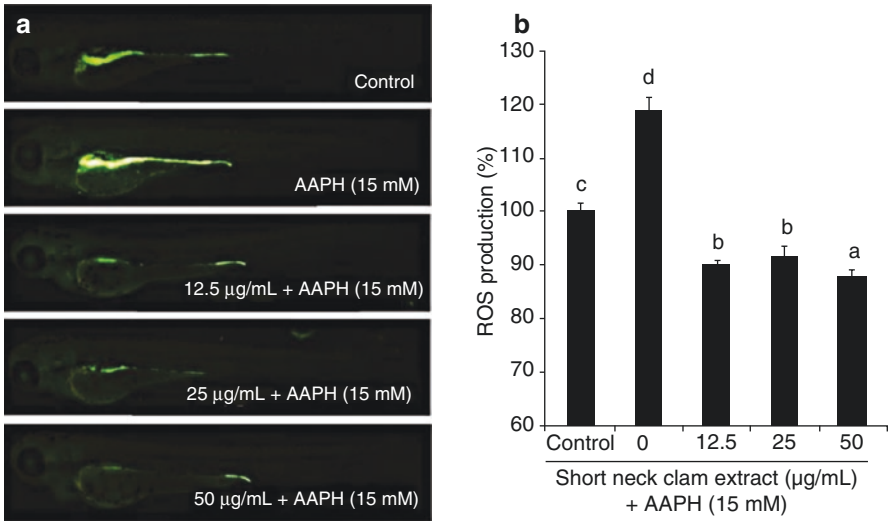

Fig. 3 Effect of taurine enriched SNC-WE against AAPH-induced ROS production in zebrafish. Intracellular ROS production was measured by image analysis and fluorescence microscopy (**a**), and the Image-J software (**b**), respectively. Data are presented as the mean values ± SEM of three independent experiments. Values with different letters are significantly different by Tukey-Kramer multiple comparison test at $P < 0.05$

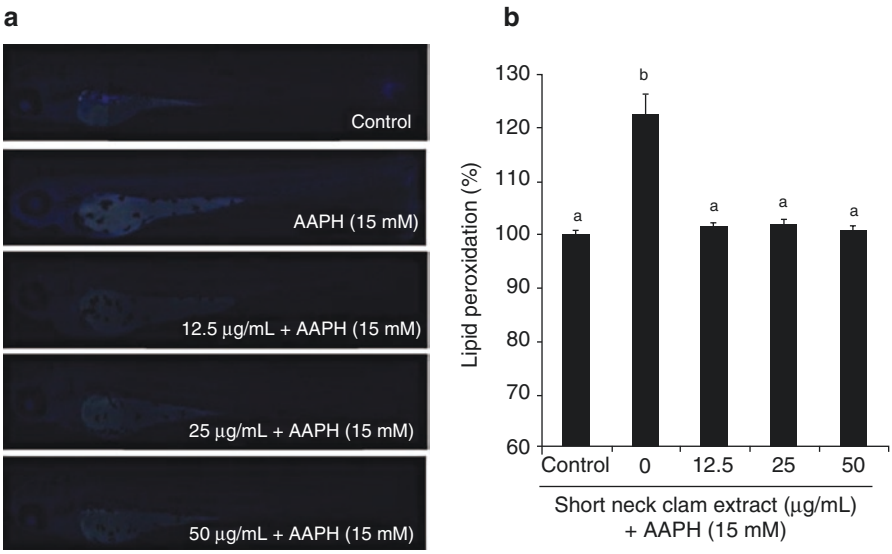

Fig. 4 Effect of taurine enriched SNC-WE on AAPH-induced lipid peroxidation in zebrafish. The degree of lipid peroxidation was measured by image analysis and fluorescence microscopy (**a**), and the Image-J software (**b**). Data are presented as the mean values ± SEM of three independent experiments. Values with different letters are significantly different by Tukey-Kramer multiple comparison test at $P < 0.05$

3.5 Effect of Taurine Enriched SNC-WE on AAPH-Induced Lipid Peroxidation in the Zebrafish

Diphenylpyrenylphosphine (DPPP) acts as a probe which can detect hydroperoxides, and DPPP oxide is strongly fluorescent (Akasaka et al. 1987). As show in Fig. 4, lipid peroxidation induced by AAPH. In the AAPH-treated zebrafish, lipid peroxidation markedly increased by 123.8% compared to the control group, whereas, lipid peroxidation was significantly decreased by treatment of SNC-WE to AAPH-treated zebrafish. These findings indicate that SNC-WE containing high concentrations of taurine can effectively reduce lipid peroxidation induced by AAPH.

4 Discussion

Reactive oxygen species (ROS)—such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$), and superoxide anions ($O_2^{\bullet -}$)—generally exist as by-products of several physiological processes in normal cells or due to exposure to environmental stimuli and resultant immune responses. ROS can be utilized in immune responses, cell

proliferation and differentiation as well as signal transduction (Aguirre et al. 2005). ROS production can increase oxidative stress and lead to cell death, lipid and protein oxidation, and breaks of DNA strand within the cells (Wang et al. 2006). Fish and shellfish protein hydrolysates are important natural sources of bioactive peptides. Peptides from fish and shellfish protein hydrolysates may have regulatory effects on immune defense, antihypertensive activities, antioxidant and anti-inflammatory activities (Pihlanto-Leppälä 2000). In the present study, taurine was the predominant amino acid of SNC-WE. Our data showed that taurine enriched SNC-WE has considerable free radical scavenging activities *in vitro*.

ROS is effectively controlled by the cellular antioxidant enzymatic defense systems including superoxide dismutase (SOD), peroxidase, glutathione peroxidase (GPx), and catalase (CAT). However, uncontrolled or excessive production of ROS can cause oxidative stress in tissues and damage cells (Ko et al. 2013). In general, lipid peroxidation of zebrafish embryo is easily induced by AAPH, which generates peroxyl radicals (ROO^{-}), which in turn attack lipids (Yoshida et al. 2004). Here, we investigated the protective efficacy of SNC-WE enriched in glycine, alanine as well as taurine against the oxidative stress induced by AAPH using zebrafish model. In our study, we also confirmed that the taurine enriched SNC-WE shows the suppressive effects against cell death, intracellular ROS generation and lipid peroxidation, and inhibited the oxidative stress induced by AAPH in zebrafish embryos. These results suggest that SNC-WE might have strong protective effects against oxidative stress-induced cellular damages in zebrafish embryos. Taurine has antioxidant properties, which can lead reduction of lipid oxidation, stabilize membrane lipids induced by oxidative stress (Hansen 2001; Cetiner et al. 2005). Roy and Sil (2012) demonstrated that taurine protects erythrocytes from the damaging of *t*-butyl hydroperoxide (TBHP) and reduces lipid peroxidation and improves the intracellular antioxidant enzymes activities. Although several studies reported the effect of taurine on antioxidant properties, none evaluated the inhibitory effects of short-neck clam enriched in taurine against oxidative stress-stimulated by AAPH *in vivo* in the zebrafish model.

Taken together, our results suggest that taurine enriched water extract from short-neck clam might have several beneficial effects against oxidative stress induced by AAPH *in vivo* as well as free radical-scavenging activities *in vitro* by inhibiting ROS generation, lipid peroxidation as well as cell death.

5 Conclusion

Our findings show that taurine enriched SNC-WE exhibited potent free radical scavenging activities *in vitro*. Moreover, our results indicated that AAPH can induce oxidative toxicity in zebrafish model, in contrast SNC-WE efficiently protects against the oxidative stress induced AAPH, by suppressing intracellular ROS production, lipid peroxidation as well as cell death in the zebrafish model.

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Role for Taurine in Development of Oxidative Metabolism After Birth

Kayoko Shimada-Takaura, Kyoko Takahashi, Takashi Ito, and Stephen Schaffer

Abstract The heart undergoes a major metabolic transition after birth, a change largely caused by alterations in substrate availability, hormone levels and transcription factor content. However, another factor that could contribute to the resulting upregulation of oxidative metabolism is the increase in taurine levels. We proposed that by increasing UUG decoding and the biosynthesis of mitochondria encoded proteins, elevations in taurine content enhance electron transport flux and increase oxidative metabolism. To test our hypothesis, the effect of reduced taurine content on oxidative metabolism of myocardial mitochondria and neonatal cardiomyocytes was examined. Taurine deficient neonatal mitochondria exhibited impaired oxidation of complex I specific- but not complex II specific-substrates, indicating that taurine deficiency regulates complex I activity. Taurine deficiency also reduced respiration of neonatal cardiomyocytes oxidizing carbohydrate (glucose, lactate and pyruvate). However, cardiomyocytes from 2–3 day-old hearts respiring either β-hydroxybutyrate, an important substrate in the neonatal heart, or palmitate, which is poorly metabolized during the early neonatal period, were resistant to the metabolic defects of taurine deficiency, These data support the hypothesis that taurine contributes to development of respiratory chain function after birth, which is required for oxidative metabolism of multiple substrates.

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Keywords ATP production • Development of oxidative metabolism • State 3 respiration • Complex I activity • Taurine deficiency

Abbreviation

PPARα Peroxisome proliferator-activated receptor α

1 Introduction

The taurine deficient adult heart is energy deficient (Schaffer et al. 2016). This property is largely caused by reduced respiratory chain flux secondary to inhibition of NADH dehydrogenase (complex I) activity. However, the reduction in complex I activity also leads to an elevation in mitochondrial NADH levels, which slows oxidative metabolism by inhibiting several key metabolic enzymes, including pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, isocitrate dehydrogenase and fatty acyl CoA dehydrogenase (Neely and Morgan 1974; Stanley et al. 2005). Although the normal heart is capable of utilizing a wide range of substrates for energy production, inhibition of the four key NADH-dependent enzymes restricts the production of NADH during aerobic metabolism. These results indicate that aerobic metabolism is depressed in the taurine deficient heart.

Fatty acids are the preferred substrate of the normal adult heart, however, a shift in metabolism in favor of glucose utilization occurs upon onset of mild heart failure (Stanley et al. 2005). The primary mechanism responsible for that metabolic shift is the downregulation of PPARα, a transcription factor that regulates the expression of several proteins involved in fatty acid metabolism (Campbell et al. 2002; Stanley et al. 2005). Although reductions in fatty acid metabolism of the failing heart is largely attributed to a decrease in PPARα, the mitochondria are often damaged, therefore, the shift in favor of glucose metabolism does not improve the energy status of the heart. While the resulting increase in glucose metabolism is associated with a rise in anaerobic metabolism, mitochondrial damage results in a decrease in glucose oxidation (Jaswal et al. 2011). Hence the failing heart becomes energy deprived.

Like mild heart failure, the taurine deficient heart of adult rodents undergoes a shift in metabolism favoring glucose utilization (Schaffer et al. 2016). The increase in glucose utilization and decrease in fatty acid metabolism by the taurine deficient adult heart is related in part to a decline in myocardial PPARα levels (Schaffer et al. 2016). Interestingly, the decrease in PPARα content of both the human failing heart and the taurine deficient adult heart is attributed to a phenotype transition from an adult to a fetal form (Stanley et al. 2005). In the non-diseased heart, the phenotype transition occurs because of changes in the dietary and hormonal status of the animal after birth. In the fetus, circulating levels of glucose, lactate and insulin are high while the levels of fatty acids are low. However, shortly after birth, carbohydrate

levels fall and fatty acids rise, as the neonate enters the suckling period (Lopaschuk and Spafford 1990; Onay-Besikci 2006). These changes in substrate content, coupled with genetic and hormonal alterations within the heart, cause a developmental shift in energy metabolism from preferential glucose utilization to preferential utilization of fatty acids. Thus, the post-neonatal and adult hearts are highly dependent on fatty acid oxidation and respiratory chain activity for energy.

Another pertinent change to the heart after birth is an elevation in taurine levels (Macaione et al. 1975). While taurine levels are low in the fetus and early neonate, they are significantly elevated a few weeks after birth and remain elevated in the adult mammalian heart. The taurine changes parallel the changes in aerobic metabolism. Because taurine affects respiratory function through the formation of 5-taurinomethy luridine-$tRNA^{Leu(UUR)}$ and the modulation of mitochondria encoded protein biosynthesis (Schaffer et al. 2014), it is logical to assume that the neonate is highly sensitive to changes in taurine content following birth. Thus, we began testing the hypothesis that the increase in myocardial taurine levels during the early neonatal period plays a role in the development of aerobic metabolic pathways. In the present study, O_2 consumption was monitored in neonatal cardiomyocytes from normal and taurine deficient rats at an age in which aerobic metabolic pathways begin to develop.

2 Methods

2.1 Preparation of Isolated Neonatal Rat Cardiomyocytes

Isolated neonatal rat cardiomyocytes were prepared according to the method described by Grishko et al. (2003). Cardiomyocytes were incubated with serum-substituted medium for 2 days before a 48 h treatment with either 0 mM (Control) or 5 mM β-alanine. The cells were then washed with PBS buffer prior to being incubated for 15 min with PBS buffer (37 °C) containing 120 mM K-MES, 10 mM NaCl, 20 mM imidazole, 8 mM $MgCl_2$, 5 mM K_2HPO_4 and 0.5 mM dithiothreitol supplemented with one of the following substrates: 5 mM pyruvate, 5 mM β-hydroxybutylate, 5 mM lactate, 10 mM glucose with 1 nM insulin or 0.1 mM palmitate with 33 mM bovine serum albumin. Cells were gently removed from the dish and put into a sealed chamber filled with buffer containing the desired substrate. Oxygen consumption was measured using a Clark electrode (Yellow Spring Instruments).

2.2 Measurement of Cardiomyocyte Taurine Content

Cardiomyocytes were scraped from the incubation dish. An aliquot was removed to determine protein content. The remaining cells were homogenized in ice cold 1 M perchloric acid and 2 mM EDTA. The homogenate was then centrifuged at 10,000*g* to collect the supernatant. After neutralization of the supernatant to pH ~ 5–6 with 2 M KOH, the samples were again centrifuged at 10,000*g*. The resulting supernatant

was used to measure cellular taurine content using the spectrophotometric procedure of Shaffer and Kocsis (1981).

To assay complex I activity, isolated cardiomyocytes were scraped from dishes and washed in 10 mM triethanolamine buffer (pH 7.6) containing 250 mM sucrose at room temperature. The cells were then lysed in ice cold isolation HEPES buffer (5 mM, pH 7.25 at 4 °C) containing 150 mM mannitol, 2 mM sucrose, 1 mM EDTA, protein protease inhibitors (1:100 dilution of protease inhibitor cocktail, Thermo Scientific), 1% PMSF and 100 mM Na_3VO_4. The homogenate was centrifuged at 800*g* for 6 min at 4 °C and the supernatant was again centrifuged at 12,000*g* for 4 °C. The pellet was defined as the mitochondrial fraction. To determine complex I activity, the pellet was suspended in Tris buffer (10 mM Tris-HCl, pH 7.4) and then an aliquot added to reaction buffer supplemented with 80 μM decylubiquinone, 1 mg/mL BSA, 0.25 mM KCN and 0.4 μM antimycin A and either 0 or 5 μM rotenone. Complex I activity was calculated from the rotenone sensitive change in NADH absorbance at 340 nm (Jong et al. 2012).

2.3 Determination of States 3 and 4 Respiration

Cardiomyocytes were suspended in buffer supplemented with either 4 mM glutamate and 2 mM malate or 5 mM succinic acid and placed in a 2 mL sealed chamber fitted with a Clark electrode to measure O_2 consumption. The cardiomyocyte cell membrane was permeabilized by addition of 20 μL of saponin (5 mg/mL). After obtaining a stable rate of state 2 respiration, 20 μL of 50 mM ADP was added to generate state 3 respiration. Following complete conversion of ADP to ATP via oxidative phosphorylation, a new slower rate of O_2 consumption was achieved, referred to as state 4 respiration. The P/O ratio was determined from the amount of ATP formed (ADP added) and the O_2 consumed during state 3.

2.4 Statistical Analyses

All results are reported as means ± SEM. Statistical significance was determined using the Student's t-test for comparison within groups or ANOVA followed by the Newman-Keuls test for comparison between groups. Values of $p < 0.05$ were considered statistically significant.

3 Results

Cardiomyocytes prepared from 3 day-old rats were placed in medium containing either 0 or 5 mM β-alanine, a taurine analogue that mediates a time-dependent decrease in taurine content. After a 48 h incubation with β-alanine, cellular taurine levels decreased from 189 ± 8 to 97 ± 9 μmol/mg protein.

Associated with the decrease in taurine content, we observed a 45% decrease in complex I (NADH dehydrogenase) activity. Removal of 5 mM β-alanine from the incubation medium one hour prior to the preparation of mitochondria did not change complex I activity, supporting the view that taurine loss rather than the increase in medium β-alanine *per se* was responsible for the decrease in complex I activity. The effect of taurine deficiency on the respiratory chain was specific for complex I, as taurine deficiency had no effect on the activities of complexes II and III.

Previous studies have shown that the PPARα content of neonatal rat cardiomyocytes is significantly decreased relative to that of the adult heart (Stanley et al. 2005). Therefore, the major mechanism contributing to altered aerobic metabolism in the taurine deficient, neonatal cardiomyocyte is impaired respiratory chain function. Flux of electrons through the respiratory chain involves two branches. In one branch, complex I accepts reducing equivalents from NADH and passes the electrons along to complex III. In the other branch, complex II accepts electrons from $FADH_2$ and passes them on to complex III and then on to complex IV. Based on the enzymatic assays of the respiratory chain complexes, we found that taurine deficiency only affected complex I activity. However, to provide information on the flow of electrons through the two branches of the respiratory chain (either complex I to III, IV or complex II to III, IV); we monitored O_2 consumption of control and taurine deficient cardiomyocytes incubated with either complex I or complex II specific substrates. We reasoned that if taurine deficiency caused a defect in either the complex I or complex II branches, one could bypass the metabolic defect caused by taurine deficiency using a substrate specific for the unaffected branch. To test that idea, isolated neonatal cardiomyocytes from control and taurine deficient rats were initially treated with saponin to permeabilize the cell membrane, allowing the cytosol to be replaced by the incubation medium. Taurine deficient and control permeabilized cells were then exposed to incubation medium supplemented with either the complex II specific substrate, succinate, or the complex I substrate combination, malate/glutamate. Mitochondrial oxygen consumption of the two experimental groups was determined before and following addition of ADP; ADP stimulates oxygen consumption to a new steady state level known as state 3. Upon complete conversion of ADP to ATP, a new steady state rate was established, which is known as state 4. The state 3 respiration rate of both taurine deficient and normal mitochondria was faster in the presence of succinate than when the mitochondria were exposed to the combination of 2 mM malate and 4 mM glutamate. To compensate for differences related to preparation variation and the faster rate of succinate oxidation, the data were normalized relative to the state 3 and state 4 rates of normal mitochondria. Figure 1 shows that taurine deficiency had no effect on either state 3 or state 4 respiration of mitochondria incubated with medium containing 5 mM succinate. However, in the presence of 4 mM glutamate and 2 mM malate, state 3 respiration of the taurine deficient mitochondria was significantly reduced (Fig. 1). By contrast, taurine deficiency had no significant effect on state 4 respiration in the presence of either complex I or complex II specific substrates. These data show that taurine deficient mitochondria exhibit a specific defect in complex I that is uncovered when respiration is stimulated with ADP.

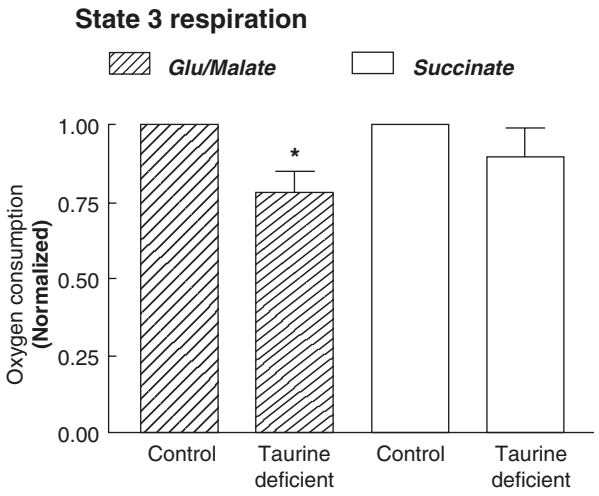

Fig. 1 Effect of taurine deficiency on activities of complexes I and II. The control and β-alanine-treated cardiomyocytes were incubated in medium containing either 4 mM glutamate + 2 mM malate (complex I substrates) or 5 mM succinate (complex II substrate). The cells were permeabilized to expose the mitochondria to the medium. After a constant rate of state 2 respiration was observed, 1 μmol of ADP was added and the rate of state 3 respiration determined. When ADP levels were depleted, state 4 respiration was determined. All of the control groups were normalized to 1.0. Values shown represent means ± SEM of 3–4 different experiments. Asterisks denote a significant difference between the control and β-alanine-treated groups ($p < 0.05$)

To examine the hypothesis that the taurine content of neonatal cardiomyocytes in culture influences the metabolic pattern of the cell, the effect of various substrates on respiration of taurine deficient and control cardiomyocytes was examined. Oxygen consumption of intact cardiomyocytes prepared from 2–3 day old rat neonates was highest in cells incubated with medium containing glucose and pyruvate, but lowest in cells incubated with medium containing palmitate (Fig. 2). Although lactate is a preferred substrate of the neonatal rabbit heart (Onay-Besikci 2006), the rate of lactate oxidation in normal neonatal rat cardiomyocyte was low. However, O_2 consumption was high in cells utilizing β-hydroxybutyrate, in agreement with the suggestion that the ketone body serves an important function in the neonatal rat heart (Cotter et al. 2011).

Taurine deficiency significantly reduced oxidative metabolism of all five substrates used in the present study, although it suppressed oxidative metabolism most when the cells were exposed to a carbohydrate substrate (Fig. 2).

Interestingly, the effect of taurine deficiency on the metabolism of β-hydroxybutyrate was minimal, in line with the importance of ketone bodies to the neonate. As expected, taurine deficiency had little effect on the metabolism of palmitate, whose metabolism is poorly developed during the early neonatal period. Particularly noteworthy was the similar rate of O_2 consumption of taurine deficient cells incubated with medium containing glucose, lactate or palmitate.

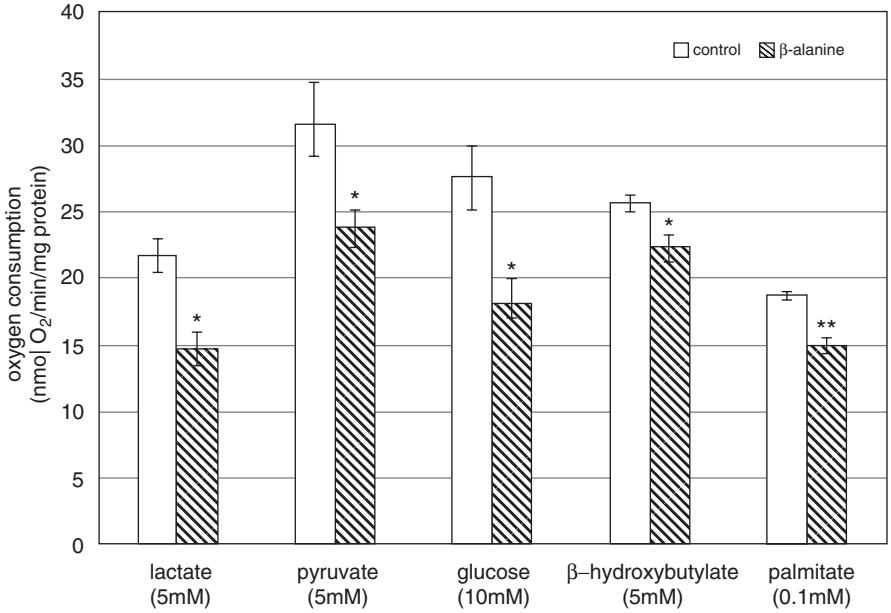

Fig. 2 Effect of taurine deficiency on aerobic metabolism of common cardiac substrates. Taurine deficient and control rat neonatal cardiomyocytes were incubated in MES medium (37 °C) supplemented with 5 mM pyruvate, 5 mM β-hydroxybutylate, 5 mM lactate, 10 mM glucose with 1 nM insulin or 0.1 mM palmitate with 33 μM bovine serum albumin. Values shown represent means ± SEM of 4–7 different preparations. The asterisks denote a significant difference between the control and taurine deficient cardiomyocytes ($p < 0.05$)

4 Discussion

4.1 Developmental Changes in Aerobic Metabolism

One of the major findings of the present study is that cellular taurine is required for normal oxidative metabolism by the early neonatal heart. The neonatal heart is an interesting model for studying developmental changes, as the heart undergoes a dramatic alteration in environment after birth, which promotes maturation and increases the availability of specific substrates. These changes occur immediately or within a few weeks of birth and lead to a significant shift in the metabolism of glucose and fatty acids. In the newborn rabbit heart, lactate and glucose are the preferred substrates (Onay-Besikci 2006). However, within the first 2 weeks after birth, a dramatic transition from the use of lactate to an increase in fatty acid oxidation takes place. While lactate oxidation is responsible for 49% of ATP produced by the 7 day-old rabbit heart, it only provides 15% of ATP production in the 14 day-old rabbit heart perfused with buffer containing 11 mM glucose, 0.8 mM palmitate, 0.5 mM lactate and 100 μU/L insulin (Itoi and Lopaschuk 1993). Glucose

metabolism also plays an important role in energy metabolism of the neonatal heart, but the relative importance of glycolysis and glucose oxidation toward ATP generation in the newborn heart remains controversial. Lopaschuk et al. (1992) reported that the rate of glycolysis provides over 50% of ATP production in the immediate newborn rabbit heart, as the rate of glucose oxidation is comparatively low. Indeed, pyruvate is preferentially diverted to lactate rather than being oxidized by pyruvate dehydrogenase in the newborn rabbit heart. In apparent contrast to that finding, Bartelds et al. (2000) showed that newborn lambs prefer glucose oxidation for ATP production.

Cardiomyocytes from 2–3 day-old rats were used in the present study. Like the neonatal rabbit, 2–3 day-old neonatal rat hearts are highly dependent on glucose for energy generation. According to Schroedl et al. (1982), the rate of glucose utilization by neonatal cardiomyocytes from 2 day-old rats is more than 2.7 times greater than that from 5 day-old rats. Yet, like the newborn rabbit heart, the rate of glucose oxidation of the 2–3 day-old rat heart is low, suggesting that the oxidative pathways are poorly developed at an early age. Interestingly, in the present study, O_2 consumption was highest for neonatal cardiomyocytes maintained on pyruvate. Thus, despite evidence that pyruvate utilization is limited by inadequate development of the oxidative machinery, neonatal cardiomyocytes from 2–3 day-old rats prefer pyruvate to many other substrates. Like glucose, ketone bodies also serve an important metabolic role in the neonatal rodent, as their oxidation appears to be required for postnatal survival (Cotter et al. 2011). In particular, the oxidation of ketone bodies assumes a critical role in ATP generation in the absence of carbohydrate. Thus, it is not surprising that O_2 consumption of the 2–3 day-old neonatal cardiomyocyte maintained on β-hydroxybutyrate is reasonably robust in the absence of carbohydrate (Fig. 2).

The metabolic status of the 2–3 day-old neonatal heart is least suited for the oxidation of fatty acids. Although the onset of the suckling period provides an abundant supply of fatty acids, the development of the oxidative machinery, which undergoes a major change shortly after birth, is inadequate by 2–3 days of age. Moreover, major changes in hormonal content, such as thyroid hormone, occur shortly after birth (Sugden et al. 2000). Another change that dramatically impacts fatty acid metabolism in the postnatal period is the elevation in the transcription factor, PPARα (Stanley et al. 2005). Among its many actions, PPARα regulates a key rate limiting step in fatty acid metabolism, the transport of long chain fatty acids into the mitochondria. Carnitine palmitoyltransferase I (CPT-1) catalyzes the formation of long chain fatty acyl carnitine from long chain fatty acyl CoA and carnitine. Because long chain fatty acyl carnitine, but not long chain fatty acyl CoA, can be transported via a translocase into the mitochondria, CPT-1 is required for the oxidation of long chain fatty acids. Not only is CPT-1 expression increased by PPARα, but myocardial content of the CPT-1 inhibitor, malonyl CoA, is diminished by PPARα (Campbell et al. 2002; Stanley et al. 2005). Thus, in the 2–3 day-old neonate, CPT-1 activity is reduced, dramatically slowing the utilization of palmitate.

The 2–3 day-old neonate is highly dependent on glucose for energy, but has begun transitioning from a newborn to an adult. In this study we tested the possibility that

taurine deficiency might affect the transition from anaerobic metabolism to oxidative metabolism in the neonate. The data in Fig. 2 argue that taurine deficiency may prevent or delay the transition toward oxidative metabolism, as taurine deficiency significantly reduced O_2 consumption of all substrates tested, with the greatest reduction in oxidation observed for pyruvate, lactate and glucose, which all share the need to oxidize pyruvate. This may relate to the dramatic suppression of pyruvate dehydrogenase by the elevation in NADH in taurine deficient mitochondria. Thus, taurine may facilitate the shift from anaerobic metabolism to oxidative metabolism in the neonatal heart by decreasing mitochondrial NADH levels, which in turn elevates the oxidation of pyruvate via pyruvate dehydrogenase.

Although taurine deficiency significantly narrowed the differences in oxidative metabolism between the five substrates examined, it exerted only small changes in O_2 consumption of cells that were utilizing β-hydroxybutyrate. During its metabolism, β-hydroxybutyrate is oxidized to acetoacetate, which directly enters the citric acid cycle. Thus, ketone body metabolism bypasses some of the limiting regulatory steps of fatty acid and glucose metabolism, such as carnitine palmitoyltransferase-1, the pyruvate dehydrogenase complex and fatty acyl CoA dehydrogenase (Neely and Morgan 1974; Stanley et al. 2005). Another substrate that is fairly resistant to the effects of taurine deficiency is palmitate. In comparison to the adult cardiomyocyte, the utilization of palmitate by the normal 2–3 day-old neonatal cardiomyocyte is significantly suppressed, an effect related to its low PPARα content. Although taurine deficiency further lowers PPARα content, the overall effect of taurine deficiency is small, as the fatty acid metabolic machinery already exists in a severely depressed state.

4.2 Effect of Taurine on Mitochondrial Function

The present study reveals that a major function of taurine in the heart is to ensure normal respiratory chain activity. In the mitochondria, several factors regulate respiratory chain function, one being the availability of subunits for assembly of the respiratory chain complexes (Schaffer et al. 2014; Vartak et al. 2015). Taurine regulates the biosynthesis and availability of one of the complex I subunits, ND6. ND6 is a mitochondria encoded protein that contains 19 leucine residues, 8 of which are dependent on base pairing between the UUG codon and the AAU anticodon of $tRNA^{Leu(UUR)}$. The anticodon of $tRNA^{Leu(UUR)}$ exists in two states, an unmodified form (AAU) and a modified form (AAU*), in which the wobble position uracil of the anticodon is converted from uridine (U) to 5-taurinomethyluridine (U*) (Suzuki et al. 2002; Schaffer et al. 2014). The taurine conjugation reaction significantly strengthens the interaction of the modified anticodon (AAU*) of $tRNA^{Leu(UUR)}$ with the UUG codon, thereby facilitating UUG decoding and the biosynthesis of ND6 (Suzuki et al. 2002; Schaffer et al. 2014). None of the other mitochondria encoded proteins tested were significantly altered by taurine deficiency. Two of those proteins, cytochrome b of complex III and ND3 of complex I, are dependent on UUG

for 3% of their leucine residues. All other mitochondria dependent proteins, with the exception of ATPase 8, are less dependent on UUG decoding than cytochrome b and ND3. Although ATPase 8 is dependent on UUG for 10% of its leucine residues, we were unable to obtain an antibody for that protein. However, it is noteworthy that ATPase 8, which is a subunit of complex V, does not appear to be very UUG dependent, as taurine deficiency has no influence on complex V activity.

Little is known about the regulation of the taurine conjugation reaction in the mitochondria. While large amounts of taurine are present in the mitochondria, the transporter responsible for the uptake of taurine by the mitochondria has not been characterized. Also unclear is the Km for taurine transport into the mitochondria and the relationship between the mitochondrial taurine pool and the cytosolic taurine pool. Finally, the enzyme responsible for the conjugation of the wobble position uracil by taurine has not been adequately identified. Although it has been shown that tetrahydrofolate serves as a cofactor for the conjugation reaction, the Km of taurine for 5-taurinomethyluridine-tRNA$^{Leu(UUR)}$ formation is unknown. Thus, much more information must be gleaned before formulating conclusions on the effect of development on mitochondrial taurine content, the regulation of mitochondrial 5-taurinomethyluridine-tRNA$^{Leu(UUR)}$ formation and the activity of complex I.

5 Conclusions

The heart undergoes dramatic changes after birth related to changes in substrate availability, hormonal content, transcription factor levels and the size of the taurine pool. Because the suckling period increases fatty acid content and the beginning of maturation modulates blood levels of thyroid hormone and insulin, the heart begins to preferentially utilize fatty acids rather than glucose for ATP production. Taurine levels also rise during the neonatal period, presumably to promote the utilization of reducing equivalents by the respiratory chain. Thus, the increase in taurine levels after birth not only ensures adequate generation of ATP from aerobic metabolism, but actually contributes to maturation of the neonate, which is dependent on oxidative metabolism. Taurine regulates the flow of electrons through the respiratory chain by stimulating the utilization of NADH by complex I. Because the accumulation of NADH by the mitochondria inhibits enzymes involved in fatty acid oxidation, pyruvate oxidation and citric acid cycle activity, increases in taurine levels after birth play a crucial role in the development of oxidative metabolism by the heart.

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Beneficial Function of Taurine on Bone Metabolism in Alcohol-Fed OVX Rat Model

Mi-Ja Choi, Kyung-Ja Chang, Jung-Won Lee, and Yun-Jung Jung

Abstract The present study investigates the effects of taurine on bone markers and bone mineral density (BMD) in alcohol-fed ovariectomized (OVX) rat model. We divided twenty four rats into Sham and OVX groups. These two groups were thereafter subdivided into two groups: control and experimental diet containing 2 g/kg of taurine. BMD and bone mineral content (BMC) were estimated by PIXImus. As bone markers, we measured serum calcium, phosphorus, ALP activity, osteocalcin and urine calcium, phosphorus and DPD crosslinks value. The results were as follows: weight gain showed no significant difference and serum calcium concentration was in normal range. Urine DPD crosslink value was significantly decreased in taurine-fed group ($p < 0.05$). Serum ALP activity and osteocalcin levels, and urine phosphorus concentration did not show any differences among groups. Also the mineral density and content of spinal and femural bone did not show any differences among groups. However, the femur BMD was significantly increased in taurine-fed group ($p < 0.05$). In conclusion, taurine supplemented diets may have positive results on bone metabolism in alcohol-fed OVX rat model.

Keywords Taurine • BMD • BMC • Alcohol • OVX rat

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Abbreviations

ALP Alkaline phosphatase
DPD Deoxypyridinoline
FER Food efficiency ratio

1 Introduction

Dietary status and calcium, vitamin D, protein, potassium, fruits, vegetables, and beverages such as alcohol are well known to function in bone metabolism. Alcohol beverage is not nutritious but almost universally consumed. Prolonged excessive alcohol consumption has harmful for the brain, liver, muscles, and heavy chronic alcohol drinking is detrimental in skeleton as well (Schuckit 2009).

In binge alcohol exposed rats (3 g/kg), BMD of tibia and vertebra was remarkably decreased with lowered vertebral compression strength (Lauing et al. 2008). Binge alcohol treatment in rats showed that bone loss was not after 1 week of treatment but after four binge alcohol cycles, with a 23% loss in cancellous BMD and 17% in vertebral compressive strength compared to controls (Callaci et al. 2009). It was reported that low consumption of 5% ethanol during 2 h per day improved bone mechanical properties and increased BMD in 4-week old female rats (Yamamoto et al. 1997). However, consumption of 5–20% ethanol increased BMD and trabecular thickness in alcohol-fed male rats in other study (Maurel et al. 2011). There are little animal models of chronic alcohol consumption in earlier studies. Also, it is complex and multifactorial for alcohol to effect on bones. Direct and indirect effects of alcohol cause various changes in quality of bones. The difference of effects may depend on the alcohol amount, period of alcohol exposure, and drinking patterns of alcohol.

It was reported that high concentration of taurine is found in bone cells, it enhances bone tissue formation, and it inhibits bone loss (Gupta et al. 2005; Yuan et al. 2007; Jeon et al. 2007). Taurine is well known to regulate the metabolism of osteoblast and increase osteoblast differentiation (Park et al. 2001). It also was reported that taurine inhibits formation and survival of osteoclast, and experimental bone resorption (Kum et al. 2003; Koide et al. 1999; VanBeek et al. 1999).

Recently, taurine has been in bone, but its function is not fully understood. Taurine stimulated proliferation and differentiation of osteoblast through ERK1/2 activation signal pathway (Park et al. 2001). Taurine inhibited formation of osteoclast induced by IL-1alpha, PGE2, and lipopolysaccharide (Koide et al. 1999). Cheong and Chang (2009) reported that taurine supplementation could prevent from bone loss in ovariectomized (OVX) rat model. These previous studies showed that taurine is necessary for bone metabolism. However, the mechanisms of taurine in bone metabolism have not been reported yet.

Therefore, we studied the combined effects of chronic alcohol consumption with taurine supplementation on bone markers and BMD in alcohol-fed OVX rat model.

2 Methods

2.1 Materials

We divided twenty-four rats into two groups: Sham and OVX. The two groups were thereafter divided into two subgroups: Control and Taurine. In the present study, Sham and OVX rats were fed an alcohol of 7.5% (v/v) over 9 weeks. Alcohol made up 22% of the total calories that were supplied, which is the same as drinking six bottles a day. The taurine group consumed 2 g/kg of taurine in diet. The rats were fed on powdered formula diets (Table 1).

2.2 Measurement of Bone Markers

We collected blood samples from the abdominal aorta after scarifying rats. Serums were separated using centrifuge (3000 rpm/20 min) and stored in −70 °C deep freezer. Serum and urine calcium and phosphate concentration were analyzed. As bone markers, we measured serum ALP and osteocalcin, and urinary DPD crosslinks value. We measured urinary DPD with an enzyme immunoassay and corrected it for creatinine excretion (DPD/Cr). We measured serum osteocalcin with ELISA assays using osteocalcin kit (IRMA, OSTEO-RIACT, France). We measured serum ALP activity with enzymatic assay using a kit (Prueauto S ALP).

Table 1 Diet composition of formula diets

Ingredients	Control (g/kg diet)	Taurine (g/kg diet)
Casein[a]	200	200
Corn starch	529.486	509.486
Sucrose	100	100
Soybean oil	70	70
α-Cellulose[b]	50	50
Min-mixture[c]	35	35
Vit-mixture[c]	10	10
L-Cystine[b]	3	3
Choline bitartate[b]	2.5	2.5
Tert-butylhydroquinone[d]	0.014	0.014
Taurine[e]	–	20

[a]Casein, Maeil dairy industry Co., South Korea
[b]α-Cellulose, L-Cystine, Choline bitartate, Sigma Chemical Co., USA
[c]AIN-93G Mineral-Mix, AIN-93G Vitamin-Mix, Teklad Test Diets, USA
[d]Tert-bultyl hydroquione, Sigma-Aldrich, Inc., USA
[e]Taurine, Dong-A Pharm Co., South Korea

2.3 Measurement of BMD and BMC

We measured BMD and BMC with PIXImus (GE Lunar Co., USA) in spine and femur after 9 weeks of feeding.

2.4 Statistical Analysis

We statistically analyzed all data with SAS package (version 9.2, Institute, Inc., USA). We used analysis of variance (ANOVA) to verify significance among the groups. If statistical significance appeared with ANOVA ($p < 0.05$), it was determined whether means were significantly different using Duncan's multiple comparisons test. Values with significance have p-values <0.05.

3 Results

3.1 Weight Change, Food Intake, and FER

Table 2 showed the effect of taurine on initial weight, final weight, food intake and FER in OVX rats fed alcohol. Initial weight, final weight and body weight change of rats were greater in OVX groups compared to Sham groups. Mean food intake of the OVX-control and OVX-taurine groups was 16.5 ± 1.6 g/day and 16.5 ± 2.4 g/day, respectively. FER of the OVX-control group was significantly greater than Sham group.

3.2 Concentrations of Calcium and Phosphorus in Serum

Table 3 showed the effect of taurine on concentrations of calcium and phosphorus in serum of alcohol-fed OVX rats. Mean concentrations of serum calcium were 9.16 ± 2.37 mg/dl, 9.75 ± 2.47, 7.58 ± 0.99 and 7.55 ± 1.32 for

Table 2 Effects of taurine diet on weight change and food intake in alcohol-fed OVX rat model

	Sham group		OVX group	
	Control	Taurine	Control	Taurine
Initial weight (g)	239.0 ± 6.8[1a2]	235.8 ± 8.5[a]	253.5 ± 16.7[a]	254.2 ± 20.1[a]
Weight change (g)	98.5 ± 19.7[a]	99.5 ± 16.2[a]	118.9 ± 24.3[a]	103.76 ± 42.8[a]
Food intake (g/day)	15.8 ± 1.6[a]	14.3 ± 1.0[a]	16.5 ± 1.6[a]	16.5 ± 2.4[a]
FER	0.11 ± 0.02[b]	0.11 ± 0.03[b]	0.14 ± 0.02[a]	0.13 ± 0.03[ab]

[1]Mean ± SD

[2]Different superscripts within the row show significant difference at $p < 0.05$ with Duncan's multiple range test

Table 3 Effects of taurine diet on concentrations of calcium and phosphorus in serum of alcohol-fed OVX rats

	Sham group		OVX group	
Variables	Control	Taurine	Control	Taurine
Serum calcium (mg/dl)	9.16 ± 2.37[1a2]	9.75 ± 2.47	7.58 ± 0.99[a]	7.55 ± 1.32[a]
Serum phosphorus (mg/dl)	3.41 ± 1.74[a]	4.35 ± 0.69[a]	5.76 ± 1.57[ab]	6.26 ± 2.39[b]

[1]Mean ± SD
[2]Different superscripts within the row show significant difference at $p < 0.05$ with Duncan's multiple range test

Table 4 Effects of taurine diet on concentrations of calcium and phosphorus in urine of alcohol-fed OVX rats

	Sham group		OVX group	
Variables	Control	Taurine	Control	Taurine
Urinary calcium (mg/day)	0.72 ± 0.73[1a2]	0.75 ± 0.83[a]	0.53 ± 0.30[a]	0.47 ± 0.27[a]
Urinary phosphorus (mg/day)	9.92 ± 3.08[a]	8.80 ± 2.08[a]	9.33 ± 2.90[a]	9.00 ± 3.56[a]

[1]Mean ± SD
[2]Different superscripts within the row show significant difference at $p < 0.05$ with Duncan's multiple range test

Sham-control, Sham-taurine, OVX-control and OVX-taurine, respectively, which showed no significant difference among groups. Mean serum phosphorus was 3.41 ± 1.74, 4.35 ± 0.69, 5.76 ± 1.57, 6.26 ± 2.39 mg/dl for Sham-control, Sham-taurine, OVX-control and OVX-taurine, respectively. The concentration of phosphorus in serum was significantly increased in OVX-taurine group than other groups.

3.3 Concentrations of Calcium and Phosphorus in Urine

Table 4 showed the effect of taurine on concentrations of calcium and phosphorus in urine of OVX rats fed alcohol. The urinary calcium and phosphorus excretion was not significantly different among all Sham and OVX groups.

3.4 Serum ALP Activity and Osteocalcin Concentration

Table 5 showed the effect of taurine on serum ALP activity and osteocalcin concentration in alcohol-fed OVX rat model. Serum ALP activity was not significantly different among all Sham and OVX groups. Concentrations of serum osteocalcin were 27.39 ± 7.80, 31.11 ± 7.44, 38.76 ± 10.00 and 27.19 ± 4.52 ng/ml for

Table 5 Effects of taurine diet on ALP activity and osteocalcin concentration in serum of alcohol-fed OVX rats

Variables	Sham group		OVX group	
	Control	Taurine	Control	Taurine
ALP activity (U/l)	136.6 ± 47.5[1a2]	124.0 ± 44.2[a]	132.8 ± 36.0[a]	106.8 ± 46.6[a]
Serum osteocalcin (ng/ml)	27.39 ± 7.80[a]	31.11 ± 7.44[a]	38.76 ± 10.00[b]	27.19 ± 4.52[a]

[1]Mean ± SD
[2]Different superscripts within the row show significant difference at $p < 0.05$ with Duncan's multiple range test

Table 6 Effects of taurine diet on DPD, creatinine, and crosslink value in urine of alcohol-fed OVX rats

Variables	Sham group		OVX group	
	Control	Taurine	Control	Taurine
Urinary DPD (nmol/l)	243.8 ± 118.7[1a2]	300.2 ± 171.9[a]	665.3 ± 432.0[a]	510.8 ± 540.0[a]
Urinary Creatinine (mmol/l)	4.3 ± 1.6[a]	4.5 ± 1.8[a]	3.7 ± 1.9[a]	4.3 ± 2.3[a]
Crosslinks value (nmol.DPD/ mmol. creatinine)	56.8 ± 24.5[a]	67.4 ± 35.[a]	170.6 ± 38.0[b]	101.9 ± 60.8[a]

[1]Mean ± SD
[2]Different superscripts within the row show significant difference at $p < 0.05$ with Duncan's multiple range test

Sham-control, Sham-taurine, OVX-control and OVX-taurine, respectively. Serum osteocalcin, a marker of bone formation, was significantly increased in OVX-taurine group compared to OVX-control group ($p < 0.05$).

3.5 Urinary DPD, Creatinine and Crosslink Value

Table 6 showed the effect of taurine on DPD, creatinine and crosslink value in urine of alcohol-fed OVX rats. Urinary crosslink values of OVX-control and OVX-taurine groups were 170.6 ± 38.0 and 101.9 ± 60.8 nM DPD/mM Cr, respectively. The bone resorption marker of crosslink value in urine was significantly decreased in OVX-taurine group compared to OVX-control group. Crosslink value also seemed to be greater in OVX group compared to Sham group.

3.6 BMD and BMC

Tables 7 and 8 showed BMD and BMC. Spine BMDs of Sham-control, Sham-taurine, OVX-control and OVX-taurine were 0.169 ± 0.010 g/cm^2, 0.1702 ± 0.011, 0.155 ± 0.010 and 0.150 ± 0.020 g/cm^2, respectively. Spine BMCs of Sham-control, Sham-taurine, OVX-control and OVX-taurine were 0.574 ± 0.067 g, 0.547 ± 0.007, 0.568 ± 0.070 and 0.527 ± 0.092 g, respectively. Taurine supplementation had no effect on the spine BMD and BMC. Spine BMD and BMC tended to be decreased in OVX group compared to Sham group (Table 7).

Femur BMDs of Sham-control, Sham-taurine, OVX-control and OVX-taurine were 0.205 ± 0.015, 0.209 ± 0.014, 0.194 ± 0.011 and 0.196 ± 0.011 g/cm^2, respectively. Femur BMCs of Sham-control, Sham-taurine, OVX-control and OVX-taurine were 0.414 ± 0.032, 0.419 ± 0.034, 0.416 ± 0.039 and 0.408 ± 0.052 g respectively.

Table 7 Effects of taurine diet on spine BMD and BMC in alcohol-fed OVX rat model

	Sham group		OVX group	
Variables	Control	Taurine	Control	Taurine
SBMD (g/cm^2)	0.169 ± 0.010[1a2]	0.170 ± 0.011[a]	0.155 ± 0.010[a]	0.150 ± 0.020[a]
SBMD (g/cm^2)/wt (kg)	0.525 ± 0.06[a]	0.511 ± 0.065[a]	0.420 ± 0.05[a]	0.433 ± 0.11[a]
SBMC (g)/wt (kg)	0.574 ± 0.067[a]	0.547 ± 0.007[a]	0.568 ± 0.070[a]	0.527 ± 0.092[a]
SBMC (g)/wt (kg)	1.794 ± 0.32[a]	1.725 ± 0.319[a]	1.530 ± 0.21[a]	1.513 ± 0.41[a]

[1]Mean ± SD
[2]Different superscripts within the row show significant difference at $p < 0.05$ with Duncan's multiple range test

Table 8 Effects of taurine diet on femur BMD and BMC in alcohol-fed OVX rat model

	Sham group		OVX group	
Variables	Control	Taurine	Control	Taurine
FBMD (g/cm^2)	0.205 ± 0.015[1a2]	0.209 ± 0.014 [a]	0.194 ± 0.011[a]	0.196 ± 0.011[a]
FBMD (g/cm^2)/wt (kg)	0.640 ± 0.07[a]	0.629 ± 0.774[a]	0.524 ± 0.05[b]	0.583 ± 0.10[a]
FBMC (g)	0.414 ± 0.032[a]	0.419 ± 0.034[a]	0.416 ± 0.039[a]	0.408 ± 0.052[a]
FBMC (g)/wt (kg)	1.294 ± 0.17[a]	1.258 ± 0.183[a]	1.123 ± 0.10[a]	1.115 ± 0.19[a]

[1]Mean ± SD
[2]Different superscripts within the row show significant difference at $p < 0.05$ with Duncan's multiple range test

Taurine supplementation had no effect on the femur BMD and BMC. Femur BMD and BMC tended to be decreased in OVX group compared to Sham group (Table 8).

4 Discussion

ALP is important in bone mineralization and is an early marker of osteoblast differentiation. Taurine significantly increased the mRNA expression and ALP activity (Zhou et al. 2014). Taurine also enhanced calcium deposits in dose-dependent manner, suggesting that taurine accelerated mineralization of hMSCs (human mesenchymal stem cells). These results showed that taurine promoted the osteogenic differentiation of hMSCs in early and late stages. However, there was not ant significant difference in serum ALP activity in this study.

Skeletal damage induced by ethanol seems to be dependent on negative effects on bone formation (Alvisa-Negrin et al. 2009). In alcoholic patients, slightly decreased osteocalcin, marker of bone formation, and slightly increased D-Pyr, marker of bone resorption were reported, which showed no significant difference (Kim et al. 2003). Also possible decrease in osteoblastic activity was mentioned (Turner 2000). Alcoholic patients had significantly higher ALP activity, bone-specific ALP activity, and lower serum osteocalcin and 25-hydroxyvitamin D_3 levels.

Estrogen deficiency reduced bone mass after menopause, on which taurine had effect (Chung 2001). Bone quality of the cortical bone and trabecular bone seemed to be weakened by heavy alcohol intake and ovariectomy, respectively, and to have an additive effect when they combined. The mineral level in the 1% taurine-fed OVX group was greater than that in the 0% taurine-fed OVX group even though the mineral level in OVX group was decreased compared to Sham group. This seems to be the mechanisms by which taurine increased BMD. In addition, taurine is very safe in terms of tissue toxicity and is used as a supplement in baby milk. Thus, taurine which is very safe in tissue toxicity might be useful in preventing osteoporosis and further investigation is needed to explore anti-osteoporotic effect of taurine in osteoporotic patients.

Our results were consistent with the previous result which showed a lower cross-link value in taurine supplemented group of OVX rat model (Choi and DiMarco 2009). Taurine supplementation in growing male rats had marked effects by increasing femur BMC per weight in the early growth stage (Choi and Seo 2013). In addition, a study by Choi and Jang reported that a taurine diet significantly increased the femur BMD at 9th weeks after supplementation of taurine in growing female rats. Moreover, the femur BMC per weight was significantly increased in the arginine- and taurine-supplemented groups compared to control groups (Choi and Chang 2013).

However, another previous study (Choi 2009) did not show any effect of taurine on BMD in the spine and femur. Our results were similar to those of

the previous study by Choi (2009) in which calcium, phosphorus, and cross-link value in urine, and serum osteocalcin were not significantly different among all the groups. In OVX rats fed calcium-deficient diet, the taurine-OVX group had lower femur BMC compared to the control-OVX group. These results indicated no significant difference in spine and femur BMD in 2% taurine-OVX group with 6 week calcium-deficiency (Choi 2009). The previous results suggested that it is required to verify the effect of taurine on bones with different calcium levels.

In this study, Sham and OVX rats were fed an alcohol of 7.5% (v/v) over 9 weeks, with the taurine group given a 2% taurine diet with normal calcium levels. The present results indicate that supplementation of taurine can increase femur BMC per weight in alcohol-fed OVX rats. Chronic alcohol consumption causes osteopenia which increases skeletal fractures (Bikle 1993). It has been reported that alcohol decreases the osteoblast number, osteoblast proliferation, and osteoid formation, which decrease the bone formation rate (Klein et al. 1996).

Positive effect of alcohol on BMD was reported (Felson et al. 1995). Recently co-twin control study in postmenopausal women from UK registry showed significant effect of intake of energy-adjusted alcohol on spine BMD but not on femoral BMD (Fairweather-Tait et al. 2011). A study by Mukamal et al. (2007) found significant protective effects of alcohol consumption with up to two drinks/day but a tendency to higher risk with >two drinks/day. It suggested that further confirmation is needed for relation between alcohol consumption and bone, and difference in age and sex.

In summary, the femur BMD per weight of OVX-taurine group was significantly increased compared to OVX-control group ($p < 0.05$). Out results did not show any significant difference in concentration of calcium and ALP activity in serum and concentrations of calcium and phosphorus in urine among all the groups. The concentration of osteocalcin in serum and crosslink value in urine of OVX-taurine group were significantly decreased compared to OVX-control group ($p < 0.05$). There was not any significant difference in spine and femur BMD, and BMC between OVX-taurine group and OVX-control group.

5 Conclusion

In conclusion, taurine-supplemented diets may have beneficial effects on bone in alcohol-fed OVX rat model. Therefore, taurine may act on bone metabolism in OVX rats fed alcohol. We concluded that a taurine-supplemented diet has positive effects on femur BMD per weight in OVX rats fed alcohol. The effect of taurine on bone markers seems to be small, but on a clinical basis, it may differ in health outcomes.

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Liver Antioxidant Enzyme Activities Increase After Taurine in Ovariectomized Rats

Young-Ju Kang and Mi-Ja Choi

Abstract The present study was performed to know the effects of taurine on the lipid level of plasma and liver, lipid peroxidation and antioxidative enzyme activities of liver tissue in ovariectomized (OVX) rats fed cholesterol. Twenty-four female SD rats (200 ± 5 g) were grouped; sham and ovariectomy groups, which were each randomly subgrouped; fed control and control supplemented with taurine (20 g/kg diet). The serum total cholesterol, TG (triglyceride), LDL-cholesterol, athrogenic index, and HDL-cholesterol of taurine diet group were not statistically different. Also the levels of liver total cholesterol, triglyceride were not considerably different in different diets. The lipid peroxidation of malondialdehyde concentration was considerably lower in taurine-feeding group than control-feeding group in ovariectomy group. The superoxide dismutase activity in liver tissue was significantly higher in rats fed taurine than in rats fed control diet in OVX rats. GSH-Px (glutathione peroxidase) activity was statistically greater at the rats fed taurine diets compared to rats fed control diet in ovariectomy group. Activity of catalase was higher in taurine group than in control group in ovariectomy group, but it was not significantly different. In conclusion, taurine supplementation was beneficial on antioxidative enzyme activities of liver tissue in ovariectomized rats fed cholesterol.

Keywords Taurine • OVX rat • SOD • GSH-Px • CAT

Abbreviations

CAT	Catalase
GSH-Px	Gluthatione peroxidase
MDA	Malondialdehyde
OVX	Ovariectomized
SHAM	Sham operated
SOD	Superoxide dismutase

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1 Introduction

Taurine is a typical component of the food and is ubiquitous in animal tissues (Bouckenooghe et al. 2006). Taurine is made either from cysteine via cysteine dioxygenase (CDO), or from cysteamine by cysteamine (2-aminoethanethiol) dioxygenase (ADO) (Ueki and Stipanuk 2009). Taurine is a key osmolyte, and can act as antioxidants (Lambert 2004; Jong et al. 2012). The postmenopausal deficiency of estrogen may be guilty for deteriorating antioxidant defenses, as well as lipid concentrations (Goudev et al. 2000). Both of them are involved in inducing cardiovascular disease (CVD) (Cooke et al. 2003). The potential for the intake of taurine to inhibit chronic disease is the topic of significant scientific search. Although many results have been reported for the favorable roles of taurine in diverse models of chronic disease (Ito et al. 2014; Kim and Kim 2013), the antioxidant properties of taurine are frequently cited (Ozden et al. 2013). A great deal of research has evaluated the antioxidant and biological activities of taurine *in vitro*. Much of the results supporting an antioxidant function for taurine are derived from experimenting their antioxidant activity *in vitro* (Askwith et al. 2012). Taurine prevented decrease in tissue glutathione (GSH) concentrations in animal studies. Clinical and experimental studies (Nakaya et al. 2000; Ueki and Stipanuk 2009) have shown the atherosclerosis protective effects of taurine. Taurine reduces blood pressure and blood lipids, which is one of the most important factors in preventing CVD (Maia et al. 2014). It is not decided yet if taurine affects oxidative stress. The beneficial effect of taurine on high blood lipid level was tested in estrogen deficiency induced rats that feed a high-cholesterol 10 g/kg.

However, evidence that taurine acts directly or indirectly as antioxidants *in vivo* is limited. No information is available concerning the antioxidant effects of taurine at OVX model with high cholesterol. Thus, if taurine is effective in depressing blood lipids, it may play a key role in preventing CVD. In fact, the goal of the current research was first to know taurine roles on the level of blood lipids and second its effect as antioxidant.

Here we tested how taurine (2%) acts on the plasma lipid profile, and liver antioxidant enzyme activities in hypercholesterolemic OVX rats.

2 Methods

2.1 Animal

Female SD rats (200 ± 7.7 g, mean ± SD) were acquired from Bio Genomics (Seoul, South Korea) and divided into experimental groups (n = 8 per group), and adapted in animal housing on the basis of an established guideline (National Research Council Institute 1996).

2.2 High Fat Diet

High fat diets was prepared as previously described somewhere (Choi et al. 2006). The diets were prepared weekly, freeze-dried and stored at 4 °C. Water was freely available from nipple drinkers. The diets lasted a period of 9 weeks. Final formula diet contained 20 g taurine in 1 kg diet. When various amounts of taurine, 0.25 to 50 g/kg diet were supplemented with a high-cholesterol diet for 2 weeks, serum total cholesterol dramatically decreased when diet intake is increase, but normalized at the dose of 10 g taurine/kg, compared with the control cholesterol free diet group (Yokogoshi et al. 1999). Therefore this study decided to employ 20 g taurine/kg for supplementation because of ovariectomized status.

Formula diets were stored at −15 °C. At the end of the experiment, the rats were deprived of food overnight. Plasma was prepared by centrifuging the heparinized blood (1,100 × g, 10 min). Liver was removed and immediately shock-frozen in liquid nitrogen and stored at −70 °C for later analysis.

2.3 Analytical Methods

The serum lipids were measured with commercial assay kits (Asan Pharmaceutical, South Korea). Serum LDL-cholesterol was calculated (Friedwald et al. 1972). Liver was prepared as mentioned previously (Choi et al. 2006). Briefly, a 1.0 g was homogenized in 10 mL volumes containing 50 mmol/L Tris 0.1 mmol/L EDTA (pH7.6). An aliquot was centrifuged (105,000 × g, 15 min, 4 °C) using an ultracentrifuge. Protein content was measured with Lowry et al. (1951). Spectrophotometric determination of enzyme activity was carried out using a spectrophotometer with temperature control set at 25 °C.

CAT activity was measured according to Aebi (Aebi 1974). GSH-Px was assessed by the method of Paglia and Valentine (1977). SOD activity was measured by the NADH oxidation procedure (Martin et al. 1987). Free radical damage was determined by specifically measuring malondialdehyde (MDA), as described by Ohkawa et al. (1979). One unit of catalase activity is defined as the amount consuming 1 μmol hydrogen peroxide/min. One unit of SOD activity is defined as the amount of enzyme required to inhibit the autoxidation of pyrogallol by 50%. One unit of GSH-Px activity is defined as 1 μmol NADPH oxidized/min. Calibration was performed using a standard curve. Enzyme activities of liver were expressed per milligram or gram protein.

2.4 Statistical Methods

With the use of SAS package (ver 9.12), we performed an ANOVA to compare data among the groups. Mean differences were considered significant when $p < 0.05$.

3 Results

3.1 Weight Gain and FER

Table 1 shows feed intake and FER (g gain/g food), and weight gain in the groups. There were no statistically differences in beginning weight among the rats fed different diet. As expected, OVX rats showed statistically higher body weight gain, feed intake and FER compared with the Sham group ($p < 0.05$). However, there were no significant differences in body weight between those with and without taurine supplementation.

In sham rats, control 17.5 ± 0.7 g vs. taurine 16.6 ± 1.5 g, there was no significant difference in food intake. In OVX group, control 20.1 ± 1.3 g vs. taurine 18.1 ± 1.6 g, they were significantly different; taurine group was significantly lower than control diet group. Even though feed intake was not different, rats fed the taurine showed statistically lower weight gain compared with rats fed the control diet.

3.2 Serum Lipid Concentrations

Table 2 showed the effect of taurine on serum lipid concentrations in OVX rats fed cholesterol diets. Taurine diet fed rats had lower total cholesterol level in their blood compared with control diet fed rats in Sham group (Table 2). Compared with control diet, total cholesterol, LDL-cholesterol, and atherogenic index (AI) in Sham rats were markedly decreased with taurine, and similar trends existed in total cholesterol and AI of OVX rats but there were no significant differences in triglyceride and HDL-cholesterol concentrations in OVX rats. AI was significantly higher in the OVX group, as expected. However, AI in the Sham group was significantly lower in taurine group ($p < 0.05$). The results showed that the taurine diet significantly lowered in blood total cholesterol about 13.7% compared to control diet.

Table 1 Beginning weight, food intake and weight gain in rats

	Sham		Ovx	
	Control	Taurine	Control	Taurine
Beginning weight (g)	208.3 ± 16.1[1a2]	209.2 ± 11.2[a]	205.5 ± 14.4[a]	207.5 ± 11.2[a]
Weight gain (g)	116.4 ± 22.0[b]	110.4 ± 27.6[b]	194.3 ± 11.8[a]	170.6 ± 35.5[a]
Mean food intake (g/day)	17.5 ± 0.7[b]	16.6 ± 1.5[b]	20.1 ± 1.3[a]	18.1 ± 1.6[b]
FER	0.09 ± 0.01[b]	0.10 ± 0.01[b]	0.15 ± 0.02[a]	0.14 ± 0.02[a]

[1]Mean ± SD

[2]Different superscripts are statistically different at $p < 0.05$

Table 2 Serum lipid concentrations in rats

	Sham		Ovx	
	Control	Taurine	Control	Taurine
Total cholesterol (mg/dl)	100.8 ± 14.0[1a2]	87.0 ± 3.5[b]	105.3 ± 6.0[a]	98.8 ± 7.3[ab]
Triglyceride (mg/dl)	65.1 ± 7.0[b]	64.5 ± 4.3[b]	72.0 ± 5.7[a]	69.7 ± 10.2[a]
HDL-cholesterol (mg/dl)	39.1 ± 2.6[ab]	43.2 ± 5.6[a]	33.6 ± 5.4[b]	35.5 ± 4.6[b]
LDL-cholesterol (mg/dl)	48.6 ± 12.4[a]	36.4 ± 12.6[b]	57.4 ± 11.0[a]	52.4 ± 12.2[a]
Atherogenic index	1.5 ± 0.3[b]	0.9 ± 0.5[c]	2.2 ± 0.6[a]	1.7 ± 0.6[ab]

[1]Mean ± SD
[2]Different superscripts are statistically different at $p < 0.05$

Table 3 Liver lipid content in taurine fed rats

	Sham		Ovx	
	Control	Taurine	Control	Taurine
Total cholesterol (mg/g)	51.8 ± 8.4[1ab2]	45.8 ± 7.8[b]	59.2 ± 5.0[a]	54.4 ± 2.6[a]
Triglyceride (mg/g)	43.7 ± 5.4[ab]	41.5 ± 3.0[b]	48.3 ± 2.6[a]	45.3 ± 2.8[ab]

[1]Mean ± SD
[2]Different superscripts are statistically different at $p < 0.05$

3.3 Liver Lipid Content

Table 3 shows the effect of taurine on liver lipid content in OVX rats fed cholesterol diets. Contents of total cholesterol and triglyceride in the liver tended to be less in the taurine-supplemented group than in the unsupplemented group, but lipid content was not significantly different between the two groups.

3.4 Contents of Malondialdehyde (MDA) in Liver Tissue

Table 4 showed the effect of taurine on liver MDA contents in OVX rats fed cholesterol diets. After taurine supplementation, liver MDA contents of OVX rats was 5.99 ± 0.32 and 4.70 ± 0.16 nmol/g in control and taurine-treated rats, respectively. In the sham group, MDA content was higher in the control group than in the taurine group. Liver MDA contents in OVX control group was significantly higher than the rest of the groups ($p < 0.05$). On the other hand, liver MDA contents in OVX taurine

Table 4 Effect of taurine diet on the liver malondialdehyde contents in rats

	Sham		Ovx	
	Control	Taurine	Control	Taurine
MDA (nmole/g)	5.18 ± 0.41[1b2]	4.64 ± 0.72[b]	5.99 ± 0.32[a]	4.70 ± 0.16[b]

[1]Mean ± SD
[2]Different superscripts are statistically different at $p < 0.05$

Table 5 The SOD, GSH-Px, CAT activities in rats

	Sham		Ovx	
	Control	Taurine	Control	Taurine
SOD (unit/mg protein/min)	1.43 ± 0.15[1c2]	1.72 ± 0.31[b]	1.15 ± 0.40[c]	2.29 ± 0.17[a]
GSH-Px (nmol NADPH/mg protein/min)	29.12 ± 7.19[a]	32.30 ± 2.81[a]	18.61 ± 2.52[b]	27.73 ± 6.04[a]
CAT (nmole H_2O_2 reduced/mg protein/min)	63.94 ± 16.90[b]	78.87 ± 11.75[a]	69.12 ± 2.52[ab]	78.63 ± 17.46[a]

SOD Superoxide dismutase, *GSH-Px* Glutathione peroxidase, *CAT* catalase
[1]Mean ± SD
[2]Different superscripts are statistically different at $p < 0.05$

group were significantly lower than the OVX control group ($p < 0.05$). Taurine supplementation significantly reduced nmole MDA/g by 21.5% in OVX rats. In contrast, the taurine did not affect the hepatic nmole MDA/g in Sham.

3.5 Antioxidant Enzyme Activity: SOD, GSH-Px, Catalase

Table 5 shows the activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) in liver of rats fed taurine diets containing cholesterol. Hepatic SOD activity was significantly higher in the taurine group than in the control group. The activity of liver SOD was significantly higher in the OVX taurine group than that of all the remaining groups of rats. Hepatic GSH-Px (nmol NADPH/mg protein/min) of OVX group was 18.61 ± 2.52 and 27.73 ± 6.04 in control and taurine-treated sham rats, respectively. The activity of GSH-Px (nmol NADPH/mg protein/min) did not differ in the sham groups, but taurine fed rats has greater GSH-Px (nmol NADPH/mg protein/min) activity than control rats in OVX. Although taurine supplementation did not significantly modify catalase activity, the hepatic catalase activity increased in the taurine group compared with the control group. Taurine treatment significantly enhanced SOD by 99%, and taurine treatment significantly increased GSH-Px activity by 49% relative to controls ($p < 0.05$).

4 Discussion

Recent studies reported that ovariectomized rats increased weight gain (Kalu et al. 1994) and also postmenopausal women increased weight (Criqui et al. 1988). The reason of increasing weight and feed intake in OVX-rats is a change in energy metabolism because of estrogen deficiency. However, weight gain and food intake were not affected by taurine and glycine and in humans (Nozaki et al. 1993).

Ovariectomy results increasing the blood total cholesterol in animals (Dodge et al. 1996; Lundeen et al. 1997). Taurine (5%) reduced blood cholesterol in OVX rats in Kishida et al. (2001) study and lowered VLDL and slightly increased HDL level (Yokogoshi et al. 1999). These results suggest that taurine diet has the beneficial effects to inhibit atherosclerosis. Taurine strongly expressed CYP7A1 gene and lowered VLDL level in a rat animal model (Yokogoshi et al. 1999). The study reported that favorable effects of 15 g cholesterol and 15 g taurine/kg diet compared with 15 g cholesterol/kg diet on liver lipids (Choi et al. 2006). There was no effect of taurine in growing rats with 0.5% cholesterol and 5% taurine, which is similar within this study. Kishida et al. suggested that both glycine and taurine help to ameliorate the ovarian hormone deficiency induced high blood cholesterol level in old OVX rats (Kishida et al. 2001). These results indicate that estrogen level affects serum cholesterol levels. After bile acid biosynthesis from cholesterol in the liver, bile acids are conjugated with taurine or glycine and secreted into bile. Its conjugation helps secrete bile acid in rats (Zouboulis-Vafiadis et al. 1982). Thus, taurine and glycine play a significant role in bile acid metabolism. They were also shown to be effective in lowering plasma cholesterol level in animal model fed high fat diet. However, the effects of taurine and glycine on the ovarian hormone deficiency-associated increase in plasma cholesterol concentration have not been evaluated in OVX aged rats (Sugiyama et al. 1986; Katan et al. 1982).

In addition, there were significant decreases in MDA concentrations in liver in OVX rats. Our finding of a decrease in total MDA after an intake of 2% of diet over 9 weeks suggests an improvement in antioxidant status and indicates that taurine decreases lipid oxidation. Diets supplemented with cystine and methionine have been shown to decrease plasma cholesterol concentrations in rats (Kawasaki et al. 1998). The hypocholesterolemic effect of taurine is greater in aged rats than in young rats (Kishida et al. 2001), which is similar in this study. In our study, taurine significantly lowered the MDA content in OVX rats fed cholesterol. Within the Sham group rats fed cholesterol diet, the taurine supplemented group had not lowered in MDA content than the Control group.

Thus, taurine prevented hypercholesterolemia in OVX rats (Kishida et al. 2001), recommending that intake of taurine is beneficial in preventing chronic disease in postmenopausal women. In agreement with former results (Deng et al. 2013), taurine treatment for 9 weeks at 5% in diet resulted in hepatic MDA. Meanwhile, taurine had no noticeable effect on lipid peroxidation. Atherosclerosis causes various cardiovascular diseases characterized with chronic low level inflammation and oxidative stress (Hansel et al. 2007). Menopause is related with hyperlipidemia in total

cholesterol and LDL-cholesterol. It increases the risk of coronary heart disease of postmenopausal women (Barrett-Connor 2013). Feeding taurine diet compared with control diet enhanced liver GSH concentrations. Many researches showed that taurine against hypercholesterolemia (Cai et al. 2014). Here, we hypothesized that food containing taurine with cholesterol supplementation, would alter antioxidant status and decrease susceptibility to lipid peroxidation.

Taurine acts antioxidant in the immune system. It helps to protect body from oxidative stress which induce inflammation (Schaffer et al. 2009). Its concentration is greatly increased in tissues stimulated with oxidants. Role of taurine is blocking oxidative stress indirectly (Oliveira et al. 2010). In fact, many reports showed that taurine is an effective antioxidant. The molecular mechanism by which it shows antioxidant activity remains elucidated. However, taurine significantly increases glutathione peroxidase, catalase, and superoxide dismutase (Jang et al. 2009).

In this study, taurine increases glutathione peroxidase and superoxide dismutase activities. This result indicates that taurine diet may beneficial for inhibiting oxidation stress. In addition, lipid peroxidation was statistically lower in livers of taurine fed rats compared with control rats. This study determined the combined effects of taurine with 20.0 g and 10 g cholesterol/kg diet on liver antioxidant enzyme activities and MDA production in OVX rats.

5 Conclusion

In conclusion, taurine inhibited hyperlipidemia and also decreases plasma total cholesterol level compared with control diet in OVX rats. Also, taurine supplementation was beneficial on antioxidative enzyme activities of liver tissue in OVX rats fed cholesterol. Dietary modification for the hyperlipidemia and oxidant stress related diseases call for increased taurine in the diet.

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Effects of Taurine and Vitamin D on Antioxidant Enzyme Activity and Lipids Profiles in Rats Fed Diet Deficient Calcium

Mi-Ja Choi and Yun-Jung Jung

Abstract Calcium deficiency is a worldwide problem affecting both developed and developing countries. The deficiency in calcium leads to a marked decrease of superoxide dismutase. It is known that vitamin D protects cells against oxidative damages while taurine plays an anti-inflammatory and antioxidant role. In this study, we examined whether vitamin D and taurine supplementation had a protective effect on oxidative stress in rats fed calcium deficient diet. Female SD rats (mean weight 60 ~ 70 g) were divided into four groups; control, taurine, vitamin D, taurine + vitamin D for 6 weeks (taurine: 2 g/100 g diet, vitamin D: 0.5 mg/100 g diet). We then analyzed the level of triglyceride (TG), total cholesterol (TC), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C) in serum and level of TC, TG in liver. We investigated antioxidative enzyme activities such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px). We observed that weight gain was not significantly different in the experimental groups. Food efficiency ratio (FER) was significantly higher in the normal control group than the taurine and vitamin D groups ($p < 0.05$). The level of liver TC was significantly lower in taurine, vitamin D, taurine + vitamin D groups than control group ($p < 0.05$). The concentration of malondialdehyde (MDA) was significantly lower in the taurine group than the control group. The activity of SOD was higher in taurine group than other experimental groups ($p < 0.05$), but GSH-Px and CAT were not significantly different. In conclusion, taurine has a positive effect on SOD activity but not on vitamin D. Also taurine and vitamin D have a protective effect as observed in liver TC in rats fed with a diet which lacks calcium.

Keywords Taurine • Vitamin D • Ca-deficient diet • TG • SOD

Abbreviations

CAT Catalase

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GSH-Px	Gluthatione peroxidase
MDA	Malondialdehyde
SOD	Superoxide dismutase
TC	Total cholesterol
TG	Triglyceride

1 Introduction

Calcium and vitamin D are the most involved essential factors regarding intake conditions of Koreans today. The average amount of calcium intake for men is approximately 520 ~ 584 mg, and 404 ~ 475 mg for women, which indicates women take in calcium about 100 mg less than men. These conditions suggest the general amount of calcium intake is caught short in Korea, except 1–2-year-olds. The proportion of people who are less consumed than Estimated Average Requirement (EAR) is 68.6%. Especially in 12–18-year-olds groups, the percentage of people who consume (Recommended Nutrient Intake, RNI) is less than 60%; that leads to the serious situation of nutritive conditions of adolescents in Korea (Ministry of Health and Welfare 2014).

The nutritive condition of vitamin D of elders is perceived as a poor state due to less functioning synthesis of vitamin D as aging, in fact, the conditions of adolescents and young people are the poor ones (Choi et al. 2011). Vitamin D is crucial not only for calcium metabolism, but also for internal accumulation of fat and blood lipid concentration. The correlation between blood vitamin D density and density of TG, and LDL-C is negative in studies of vitamin D and lipid metabolism of growing 12–13-year-olds students (Shin et al. 2012).

Taurine (2-aminoethylsulfonate) is important for physiological functions, such as antioxidation, membrane stabilization, calcium homeostasis, neuromodulation, anti-inflammatory, thermoregulation, conjugation of bile acids, cholestasis prevention, osmoregulation, and detoxification. In particular, taurine restrains lipid peroxidation of a membrane, protects cells against tissue damage, and reduces oxidation peroxide (Goodman et al. 2009). Oxidative stress results from unbalanced pro-oxidant versus antioxidant factors, which can lead to cellular and tissue damage.

The nutritive conditions of calcium in Korea are poor, regardless of life-cycle and gender, so supplement intake of vitamin D could compensate the lack of calcium. Since taurine is known to be involved in regulation of calcium homeostasis and antioxidation, the supplement of taurine is expected to help with blood lipid metabolism and antioxidation.

Therefore, this research performed the possible improvement effects of lipid profile and antioxidation when growing female rats that fed 50% calcium deficient diet with supplements of taurine and vitamin D at the same time.

2 Methods

2.1 Experimental Animals and Diet

Thirty-two female Sprague-Dawley rats (Biogenomics, Seoul, South Korea) with about 70 ± 5 g in weight were divided into four groups. The diet contained 50% calcium level in AIN-93G diet (Reeves et al. 1993). The basal calcium deficient diet mixture (g/kg experimental diet) contained: casein (Maeil dairy industry Co., Kyunggi-Do, South Korea), 200; alpha-cellulose (Sigma, USA), 50; sucrose (Samyang, Seoul, South Korea), 106.24; calcium deficient mineral mix (Teklad, USA), 13.76; Calcium carbonate (Sigma, USA), 6.25; Potassium phosphate (Sigma, USA), 8.75; vitamin mix (Teklad, USA), 10; L-cystine (Sigma, USA), 3.0; choline bitartrate (Sigma, USA), 2.5; soybean oil, 70. Female growing rats were divided into four groups; control, taurine, vitamin D and taurine + vitamin D for 6 weeks (taurine: 2 g/100 g diet, vitamin D: 0.5 mg/100 g diet). When various amounts of taurine, 0.25 to 50 g/kg diet were supplemented with a high-cholesterol diet for 6 weeks, plasma TC increasingly and significantly reduced in a dose-dependent manner and standardized at the dose of 1 g taurine/100 g, compared with the control-normal diet group (Yokogoshi et al. 1999). Therefore this research employed 20 g taurine/kg for supplementation because of calcium deficiency status. The level of taurine supplementation was 2.0 g/100 g diet. The relationship between vitamin D supplementation and vitamin D status was studied by several groups, and intakes exceeding the current UL (tolerable upper intake level) of 2,000 IU/day had no apparent adverse effects (Connie and James 2004). The level of vitamin D supplementation was 0.5 mg/100 g diet as 250% of normal range (Choi and Kang 2006).

Each rat was put in stainless steel wire cages in a room with temperature (23 ± 5 °C) and humidity (55 ± 5%). Experimental animals were maintained 12 h light and dark cycle.

2.2 Serum and Liver Lipids

Serum lipids were examined using commercial analysis kits (Asan Pharmaceutical). Concentrations of LDL-C were measured with the equation of Friedewald (Friedewald et al. 1972). Around 2 g of liver tissue were homogenized, and fats were came out with a methanol:chloroform mixture (1:2, v/v) as explained by Folch et al. (1957). The contents of TG and cholesterol in liver tissue were determined enzymatically with a commercial kit (Asan Pharmaceutical). The protein content of hepatic tissue was analyzed by the manner of Lowry et al. (1951).

2.3 Antioxidant Enzyme Activity

Catalase (CAT), Superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) activities of hepatic tissue was determined. CAT activity was measured according to Aebi (1974). SOD activity was measured by the NADH oxidation procedure (Martin et al. 1987). GSH-Px was assessed by the method of Paglia and Valentine (1967). Free radical damage was determined by specifically measuring malondialdehyde (MDA), as described according to the manner of Ohkawa et al. (1979). One unit of SOD activity is defined as the amount of enzyme required to inhibit the autoxidation of pyrogallol by 50%. One unit of GSH-Px activity is defined as 1 μmol NADPH oxidized/min. One unit of catalase activity is defined as the amount consuming 1 μmol hydrogen peroxide/min. Calibration was performed using a standard curve. Hepatic catalase activities were expressed per milligram protein.

2.4 Statistical Analysis

The statistical significance of variances among the experimental groups was examined by one-way ANOVA, using a SAS statistic package (ver. 9.4, SAS Institute, Inc., Cary, NC). Comparisons were made individually by Duncan's multiple range test using ANOVA. The variances were deemed to be statistical at $p < 0.05$. All data were expressed as means ± standard deviation.

3 Results

3.1 Effect of Taurine Supplementation in Body Weight Gain, Food Intake and Food Efficiency Ratio

Table 1 showed the effect of taurine and vitamin D on initial weight, final weight, weight gains in rats fed calcium deficient diets. There were no significant differences in body weight and weight gain among the groups. Table 2 showed the effects of taurine and vitamin D on mean food intake and food efficiency ratio (FER, g weight gain/g food intake) in rats fed calcium deficient diet. Mean food intake was 26.5 ± 2.9 mg/dl, 26.6 ± 2.4, 29.3 ± 2.1 and 23.9 ± 1.4 for control, taurine, vitamin D and taurine + vitamin D, respectively. They were significantly different; taurine + vitamin D group was significantly lower than the other diet group. There were no significant differences in FER among the groups.

Table 1 Effects of taurine and vitamin D on body weight and weight gain in rats fed diet deficient calcium

Variables	Control	Taurine	Vitamin D	Taurine + Vitamin D
Initial weight (g)	91.0 ± 11.5[1a2]	91.5 ± 10.4[a]	88.0 ± 9.9[a]	88.3 ± 9.9[a]
Final weight (g)	216.1 ± 8.7[a]	207.0 ± 11.4[a]	215.8 ± 16.3[a]	198.9 ± 18.8[a]
Weight gain(g)	125.0 ± 16.1[a]	115.5 ± 11.1[a]	127.8 ± 23.0[a]	110.6 ± 16.9[a]

[1]Mean ± SD
[2]Values with diverse superscript within row show statistical difference at $p < 0.05$ by Duncan's multiple range test

Table 2 Effects of taurine and vitamin D on FER in rats fed diet deficient calcium

Variables	Control	Taurine	Vitamin D	Taurine + Vitamin D
Mean intake (g/day)	26.5 ± 2.9[1a2]	26.6 ± 2.4[a]	29.3 ± 2.1[c]	23.9 ± 1.4[b]
FER	0.23 ± 0.01[ab]	0.21 ± 0.01[a]	0.21 ± 0.03[a]	0.22 ± 0.03[ab]

FER food efficiency ratio
[1]Mean ± SD
[2]Values with diverse superscript within row show statistical difference at $p < 0.05$ by Duncan's multiple range test

Table 3 Effects of taurine and vitamin D on serum lipid concentrations in rats fed diet deficient calcium

Variables	Control	Taurine	Vitamin D	Taurine + Vitamin D
Total cholesterol (mg/dL)	67.2 ± 9.4[1a2]	78.3 ± 11.3[ab]	75.7 ± 19.5[a]	93.6 ± 29.7[b]
Triglyceride (mg/dL)	79.2 ± 8.5[bc]	76.4 ± 6.0[c]	79.7 ± 8.0[bc]	88.5 ± 15.0[b]
HDL-cholesterol (mg/dL)	33.5 ± 13.9[a]	24.4 ± 4.9[a]	30.2 ± 8.2[a]	34.4 ± 12.0[a]
LDL-cholesterol (mg/dL)	27.2 ± 9.4[b]	38.5 ± 10.7[ab]	26.6 ± 9.2[b]	52.2 ± 16.3[a]
Atherogenic Index	1.26 ± 0.86[ab]	2.28 ± 0.61[c]	1.58 ± 0.65[bc]	1.84 ± 1.00[bc]

[1]Mean ± SD
[2]Values with diverse superscript within row show statistical difference at $p < 0.05$ by Duncan's multiple range test

3.2 Serum Lipid Concentrations

The TC, LDL-C, HDL-C and TG levels and atherogenic index are shown in Table 3. The TC and LDL-C levels were statistical higher in taurine + vitamin D group comparison with those in the other groups. The TG levels were significantly higher in the taurine + vitamin D groups than in the taurine groups. Atherogenic index were significantly higher in the taurine group than in the other groups. The LDL-C levels were significantly lower in the vitamin D group than in the taurine + vitamin D groups, while the HDL-C levels were not significantly different among the groups.

3.3 Liver Lipid Content

Table 4 shows the effect of taurine and vitamin D on liver lipid content in rats fed calcium deficient diets. Contents of total cholesterol were 134.2 ± 23.8 mg/g, 70.4 ± 26.8 mg/g, 75.1 ± 15.5 mg/g and 72.4 ± 20.5 mg/g for control, taurine, vitamin D and taurine + vitamin D, respectively. Compared with control diet, contents of total cholesterol in taurine or vitamin D treated rats were markedly decreased with experimental diets. Contents of triglyceride were 139.7 ± 38.1 mg/g, 150.5 ± 23.3 mg/g, 153.9 ± 29.1 mg/g and 120.8 ± 31.8 mg/g for control, taurine, vitamin D and taurine + vitamin D, respectively. Contents of triglyceride in the liver was significantly lower in taurine + vitamin D group than vitamin D group.

3.4 Contents of Malondialdehyde (MDA) in Liver Tissue

Table 5 showed the effect of taurine and vitamin D on liver MDA contents in rats fed calcium deficient diets. Liver MDA contents was 1.83 ± 0.08 nmole/g, 1.76 ± 0.04 nmole/g, 1.84 ± 0.10 nmole/g and 1.87 ± 0.14 nmole/g for control, taurine, vitamin D and taurine + vitamin D, respectively. Compared with control diet, liver MDA contents in taurine group were markedly decreased with taurine supplemented diets.

Table 4 Effects of taurine and vitamin D on the liver lipid contents in rats fed diet deficient calcium

Variables	Control	Taurine	Vitamin D	Taurine + Vitamin D
Total cholesterol (mg/g)	134.2 ± 23.8[1b2]	70.4 ± 26.8[a]	75.1 ± 15.5[a]	72.4 ± 20.5[a]
Triglyceride (mg/g)	139.7 ± 38.1[abc]	150.5 ± 23.3[bc]	153.9 ± 29.1[b]	120.8 ± 31.8[ac]

[1]Mean ± SD
[2]Values with diverse superscript within row show statistical difference at $p < 0.05$ by Duncan's multiple range test

Table 5 Effects of taurine and vitamin D on the liver contents malondialdehyde in rats fed diet deficient calcium

Variables	Control	Taurine	Vitamin D	Taurine + Vitamin D
MDA (nmole MDA/g)	1.83 ± 0.08[1a2]	1.76 ± 0.04[b]	1.84 ± 0.10[a]	1.87 ± 0.14[a]

MDA malondialdehyde
[1]Mean ± SD
[2]Values with diverse superscript within row show statistical difference at $p < 0.05$ by Duncan's multiple range test

Table 6 Effects of taurine and vitamin D on the liver activities of CAT, SOD, GSH-Px in rats fed diet deficient calcium

Variables	Control	Taurine	Vitamin D	Taurine + Vitamin D
SOD (unit/mg protein/min)	2.3 ± 1.0[1a2]	4.3 ± 2.2[b]	2.6 ± 1.0[a]	2.2 ± 0.7[a]
GSH-Px (nmol NADPH/mg protein/min)	3.5 ± 0.9[a]	3.9 ± 1.5[a]	3.6 ± 1.0[a]	3.6 ± 1.0[a]
CAT (nmole H_2O_2 reduced/mg protein/min)	25.7 ± 8.9[a]	29.8 ± 18.3[a]	18.5 ± 3.0[a]	22.0 ± 10.6[a]

[1]Mean ± SD
[2]Values with diverse superscript within row show statistical difference at $p < 0.05$ by Duncan's multiple range test

3.5 Antioxidant Enzyme Activity

Table 6 shows the effects of taurine and vitamin D on the liver activities of SOD, GSH-Px, CAT in rats fed calcium deficient diets. Hepatic SOD activity was 2.3 ± 1.0 unit/mg protein/min, 4.3 ± 2.2 unit/mg protein/min, 2.6 ± 1.0 unit/mg protein/min and 2.2 ± 0.7 for control, taurine, vitamin D and taurine + vitamin D, respectively. Hepatic SOD activity was significantly higher in the taurine group than in the other group. Taurine treatment significantly enhanced SOD by 86.9% relative to controls ($p < 0.05$). However, hepatic GSH-Px and catalase activity was did not differ in the experimental groups.

4 Discussion

This research analyzed the effects on growing rats regarding serum lipid profile, liver lipid content, and antioxidation, when growing rats are fed with 50% calcium deficient diet with supplements of taurine (2% of diet) and vitamin D (250% of diet), reflecting the current calcium deficient condition of adolescents in Korea. After six weeks of intake of the supplements, there were no improvements in serum lipid profile regarding total serum cholesterol, neutral lipid level, HDL-C, and LDL-C. However, TC of liver in taurine group, vitamin D group, and the taurine + vitamin D group was statistically lower than control group. The neutral lipid level of liver in taurine + vitamin D group was statistically lower than those in taurine group and the vitamin D group. The lipid peroxide content, MDA, in taurine group was statistically lower than control group, and there was no reducing effect of lipid peroxide due to the supplements of both taurine and vitamin D. Among antioxidant enzymes, catalase and GSH-Px were not affected by the supplements of both taurine and vitamin D. However, SOD in the taurine group was statistically higher than others diet. And there was no statistical difference in consuming both taurine and vitamin D.

The mechanism of reducing effect of serum cholesterol by calcium suggests when cholesterol is excreted into small intestine as part of bile liquid, diet calcium and fibers combine with bile salts, produce insoluble salts, which interrupt resorption of cholic acid, a precursor of cholesterol biosynthesis (Fleischman et al. 1966). Calcium is reported to reduce cholesterol and improve internal lipid metabolism (Jacqmain et al. 2003; Zemel et al. 2000).

When a person who has high serum LDL-cholesterol, ingests 1,200–2,200 mg of calcium per day, LDL-cholesterol rate is decreased (Reid et al. 2010; Sarkis et al. 2012). This positive effect of calcium on blood lipid metabolism suggests that the recommended intake of calcium or more intake is a very efficient way of prevention of disease (Lee et al. 2014). Therefore, calcium deficient condition is unfavorable for improvement of blood cholesterol.

Regarding the previous researches of taurine and vitamin D in lipid metabolism and antioxidation, low intake of calcium for 5-weeks-old male white rats increased blood lipid concentration. However, intake of vitamin D compensates calcium deficiency by increasing blood HDL-cholesterol concentration, which improves lipid metabolism. Therefore, to improve internal lipid metabolism, calcium intake is important, but also intake of vitamin D should be considered as well (Lee et al. 2014).

In the research of correlation between vitamin D and metabolic syndrome in menopausal women, vitamin D is closely related with neutral lipid (Song and Park 2013). Regarding the risk of cardiovascular disorders and correlations with 25(OH) D after calibrated ages, gender, seasons, and BMI, 25(OH)D was negatively correlated with total cholesterol, neutral lipid, and low-density cholesterol, but there were no correlations with high-density cholesterol (Shin et al. 2015). It suggests that vitamin D deficiency is correlated with cardiovascular disorders and metabolic syndromes (Anderson et al. 2010).

Whereas meta-analysis researches of vitamin D and cardiovascular system suggest that vitamin D has no significant effect on blood lipid metabolism (Elamin et al. 2011). The results of the effects of vitamin D on metabolic syndromes and blood lipid components were different, as they depend on subjects of study and institutions.

A few mechanisms were explained to describe the impact of vitamin D on fats, including its role in reducing fat absorption with the formation of insoluble Ca-fat complexes in the intestine. By reduced absorption of lipid, particularly saturated fatty acids, it is expected that blood concentration of TC and LDL-C will be lowered (Jorde and Grimnes 2011).

In this study, investigating supplements of vitamin D compensating for calcium deficient diet regarding blood lipid metabolism, there was no significant effect on improvement of blood lipid profile, but it significantly reduced total cholesterol in liver. Supplements of vitamin D only did not affect neutral lipid level in liver, but supplements of both taurine and vitamin D did significantly reduce neutral lipid level in liver. It shows regarding improving lipid level of liver, simultaneous intakes of taurine and vitamin D is more effective than intakes of taurine and vitamin D respectively.

Main function of taurine in the body is the conjugation of cholesterol into bile acids, changing solubility of cholesterol and enabling its excretion. The cholesterol

profiles of rats, mice, hamsters, guinea pigs, and rabbits have all been shown to be affected by taurine. For example, taurine supplementation of 0.25–5.0 g/kg for two weeks led to significant dose-dependent attenuation in the increase of serum cholesterol in rats fed a diet high in cholesterol compared to a group fed a high cholesterol diet without supplementation (Yokogoshi et al. 1999).

Adult SD rats fed with control rat chow with 3% β-alanine, 3% taurine, or water only from conception through ablactation (via maternal breeding) showed statistical differences in blood pressure normal and lipid peroxidation (Roysommuti and Wyss 2014). At the growing period, plasma MDA contents (a biomarker of oxidative degradation from lipoperoxidation) were statistically lower in taurine supplemented rats compared to β-alanine group.

In taurine research, overweight people who ingested 3 g taurine/day for 42 days had improvements in plasma triglyceride level, total cholesterol level, weight and atherogenic index (Zhang et al. 2004). Mizushima et al. (1996) studied, healthy young people ingested 6 g of taurine per day for 21 days, with high fat and cholesterol diet, and their TC level and LDL cholesterol level were decreased, that proved hypolipidemic effects of taurine supplementation. Mas et al. (2004) reported that taurine was effective on oxidation stress in experimenting with hepatic stellate cells. Parvez et al. (2008) showed taurine is effective in preventing oxidative damage of mitochondria by one of the anticancer medicines, tamoxifen, in rats. Previous study reported the increasing effect of glutathione, ascorbic acid, and tocopherol, and also the decrease of hepatic enzyme level and MDA in chronic ethanol administered rats (Balkan et al. 2002). Regarding taurine supplementation, Sener et al. (2005a, b) reported vitality of lipid peroxidation action and myeloperoxidase decrease, and glutathione is restored in nicotine administered rats. Cetiner et al. (2005) reported that there was a decrease of vitality of MDA and myeloperoxidase, and restoration of glutathione in rats administered methotrexate, one of antineopkstic drugs for leukemia.

However, specific mechanisms of antioxidation of taurine need to be more investigated. Previous study suggested that taurine does not have reactivity for superoxide radical, hydrogen peroxide, and hydroxyl radical (Aruoma et al. 1988). Shi et al. (1997) reported hydroxy radical was not effective eliminated in electron spin resonance.

In this study, with calcium deficient growing rats, the 2% taurine group had significantly lower lipoperoxide level in liver than other experimental groups, and vitality of SOD was significantly high among antioxidant enzymes. The vitality of antioxidant enzymes, a defending system against lipoperoxide, did not occur in supplementation of vitamin D only, and supplementation of both taurine and vitamin D.

In conclusion, there was an improvement of liver lipid level by intake of taurine and vitamin D in calcium deficient growing rats. Supplementation of both taurine and vitamin D decreased neutral lipid level in liver. However, vitamin D did not effect on the vitality of antioxidant enzymes in calcium deficient diet. Also, 2% of taurine supplementation significantly increased the vitality of antioxidant enzymes in calcium deficient growing rats. But there was no positive correlation between supplements of both taurine and vitamin D, and vitality of antioxidant enzymes. The amount of vitamin D in diet of this study is 2.5 times of regular amount, which in this level of supplementation, there was no effect on antioxidation. This study analyzed

the antioxidation by animal testing, the effect of internal antioxidation by supplementation of taurine and vitamin D can be different by various factors, like gender, life-cycle, level of intake, and duration of intake. So those factors should be considered, and it suggests later research should investigate internal positive correlations regarding different levels of taurine and vitamin D intake, respectively.

5 Conclusion

In conclusion, taurine and vitamin D intake worthily attenuate lipid levels and might protect oxidative stress risk factors due to calcium deficiency. However, taurine has a positive effect on SOD activity but not on vitamin D. Also taurine and vitamin D have a protective effect as observed in liver TC in rats fed a calcium deficient diet. From the clinical aspect of view, this research propose that taurine and vitamin D supplemented diets are more effective strategy to protect hyperlipidemia and calcium deficiency-related metabolic disorder.

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Taurine May Modulate Bone in Cholesterol Fed Estrogen Deficiency-Induced Rats

Mi-Ja Choi

Abstract Taurine is thought to affect bone in rats favorably. However, studies on the actions of this estrogen deficiency and high cholesterol diet factors on the bone metabolism are limited. In this study, the protective effect of taurine on bone was determined. Thirty-two 42 days old female SD rats were placed in individual stainless cages. Given to rats was fed to chow (Samyang Corporation, South Korea) and deionized water for a 4 days adaptation period. After the period of adaptation, Half of the rats were induced estrogen deficiency model by ovariectomy (OVX), and the left rats with sham-operated were used control (SHAM). For six weeks, the OVX and SHAM rats had separately a 2% taurine supplemented diet with *ad libitum* in both the water and the food. DEXA for small animals (PIXImus, GE Lunar co, Wisconsin) was used to determine spinal and femoral bone. The concentrations of serum calcium and phosphorus were also measured. The monitoring of bone formation was done by determining the serum ALP and osteocalcin. Urinary DPD the values were determined as index of bone resorption. Statistical measure was done with SAS (version 9.3). A lower overall intake of the daily food was observed in non-ovariectomized rats than in the OVX rats. At sacrifice, a much greater body weight was observed in ovariectomized group compare to non-operated group. That difference was absent in both fed taurine SHAM and OVX rats. Serum calcium and phosphorus were not statistically different by taurine supplementation. Urinary excretion of calcium was not effected by taurine supplementation. Serum ALP and was significantly decreased by taurine in OVX rats ($p < 0.05$). For the spine BMD and BMC, there was no difference among SHAM and OVX rats by taurine. Spine BMC per body weight of taurine groups were higher than control groups ($p < 0.1$). No significant difference was observed after taurine supplementation in femur BMD and BMC. The analysis of the results suggest that taurine supplementation modulates the bone mineral contents in postmenopausal model rats fed with high cholesterol diet.

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Abbreviations

ALP	Alkaline phosphatase
BMC	Bone mineral contents
BMD	Bone mineral density
DPD	Deoxypyridinoline
OVX	Ovariectomized
SHAM	Sham operated

1 Introduction

The global burden of osteoporosis continues to be one of the public health problems, with predictions that this will increase. The number of osteoporosis attributable fractures is likely to rise from 115,248 in 2010 to 273,794 in 2050, for a total of 8.1 million fractures mostly affecting women (78%) (Bleibler et al. 2013). Osteoporosis is a widespread disease. And osteoporosis is characterized by less bone mass. Osteoporosis is associated with deterioration of bone tissue, which leads to a higher risk of fracture (Nieves 2005). Increase in bone resorption is a chief destructive problem increasing the risk of fracture in elderly (Bonjour et al. 2012).

Taurine is present in animals with a higher concentrations in heart, brain, central nervous system, retina, olfactory bulb, and white blood cells (Ko et al. 2007). It is not an essential amino acid mostly found in animals nor a building block of proteins (Ko et al. 2006). Taurine has an immune function with anti-inflammatory properties (Ueki and Stipanuk 2009). Oxidative stress appears to enhance the differentiation of osteoclasts. And oxidative stress enhances function of osteoclasts. Also oxidative stress is directly contribute to bone degradation (Shen et al. 2013).

Taurine play an important role in bone health. A recent study found associations between taurine and bone status in rats (Choi and Seo 2013). Based on the results of animal studies, taurine supplementation has shown to benefit bone health as shown by decreasing bone loss, may due to taurine's antioxidant and anti-inflammatory properties. Taurine is thought to affect skeletal health favorably as well. Evidence suggests that taurine may protect bone, but more research is needed to understand possible interactions with other nutrients. Only limited studies investigated the actions of this estrogen deficiency with high cholesterol diet factors on the bone metabolism. The effects of taurine on the bone in high cholesterol fed OVX rats were determined.

Table 1 The composition of diet (g/kg diet)

Ingredients	Control	Taurine
Casein[a]	200	200
Corn starch	529.486	509.486
Sucrose	100	100
Soybean oil	70	70
α-Cellulose[b]	50	50
Min-mixture[c]	35	35
Vit-mixture[c]	10	10
L-cystine[b]	3	3
Choline[b]	2.5	2.5
Tert-butylhydroquinone[d]	0.014	0.014
Cholesterol[b]	10	10
Taurine[e]	–	20

[a]Casein from Maeil dairy industry Co., Seoul, South Korea
[b]α-Cellulose, L-Cystine, cholesterol, and Choline bitartate from Sigma Chemical Co., USA
[c]AIN-93G Mineral-Mix and AIN-93G Vitamin-Mix from Teklad Test Diets, USA
[d]Tert-bultyl hydroquione from Sigma-Aldrich Inc., USA
[e]Taurine from Dong-A Pharm Co., Seoul, South Korea

2 Methods

2.1 Animal Groups and Diet Composition

Female Sprague-Dawley rats (n = 32) aged 12 weeks were obtained from Bio Genomics, Oriental (Seoul, South Korea) and housed in an environment whose light and temperature are controlled. After acclimatization period (1 week), rats were divided into experimental groups by randomly: Sham control (n = 8), Sham cholesterol (n = 8), OVX control (n = 8), OVX cholesterol (n = 8). Operation were performed either sham operations or ovariectomies. Ovariectomies were conducted by ligating and excising the ovaries. The animals were fed either control diet with high cholesterol or a control + taurine supplemented diet (Table 1). All rats consumed diet *ad libitum* and deionized water was freely accessible. We measured food intake daily, and body weight weekly. After feeding period of 6-wks., serum collected for correspondent parameters measurement.

2.2 Bone Markers

Serum and urinary calcium concentrations were measured using spectrophotometer. The serum calcium and phosphorus, and calcium and phosphorus excretion in urine were determined in a Cobas 8000 (C702, Roche, Germany). Serum osteocalcin determined in duplicate by an immunoradiometric assay kit. Urinary DPD was determined with kit.

2.3 Measurement for Bone

The bone was determined at spine and femur. Bone mineral density (BMD) and bone mineral content (BMC) were measured by DEXA for small animal.

2.4 Statistical Method

Statistical measure was done using SAS software, version 9.3. Differences among four groups were compared using ANOVA followed by Duncan's multiple range test. Significance was set at $p < 0.05$. Results are expressed as means ± standard deviation.

3 Results

3.1 Effect of Taurine Supplementation on Body Weight Gain

Body weight gain of rats did not differ between the groups within in SHAM and OVX (Table 2). The beginning body weights remained similar among groups. Final body weight was shown higher ($p < 0.05$) in ovarietomized group than in sham group. The weight gain of OVX-control rats, 181.1 ± 11.8 g, significantly exceeded that of SHAM-control rats, 106.4 ± 22.0 g, during the 6 weeks experimental period ($p < 0.01$). Body weight gain was significantly higher in OVX-control rats (about 40%) than SHAM-control groups ($p < 0.05$).

3.2 Taurine Supplementation on Food Intake and FER

The mean food intake of OVX-control rats and OVX-taurine rats were 20.0 ± 3.2 g/day and 17.9 ± 2.6 g/day, respectively; exceeding the food intake of both

Table 2 Body weight and weight gain in rats fed taurine

Variables	Sham-Control	Sham-Taurine	Ovx-Control	Ovx-Taurine
Initial weight (g)	206.3 ± 15.2[1a2]	202.2 ± 8.3[a]	202.3 ± 13.1[a]	208.2 ± 8.5[a]
Final weight (g)	307.7 ± 21.2[a]	309.6 ± 33.1[a]	385.4 ± 26.6[b]	366.4 ± 34.6[b]
Weight gain (g)	106.4 ± 22.0[a]	102.5 ± 28.5[a]	181.1 ± 11.8[b]	158.6 ± 33.4[b]

[1]Mean ± SD

[2]Data were analyzed by ANOVA, followed by Duncan's multiple range test. Means in a row with different superscripts are significantly differ, $p < 0.05$

Table 3 Daily mean food intake and FER in rats fed taurine

Variables	Sham-Control	Sham-Taurine	Ovx-Control	Ovx-Taurine
Food intake (g/day)	16.2 ± 2.7[1a2]	16.1 ± 2.4[a]	20.0 ± 3.2[b]	17.9 ± 2.6[b]
FER	0.11 ± 0.01[a]	0.10 ± 0.01[a]	0.14 ± 0.02[b]	0.13 ± 0.02[b]

[1]Mean ± SD
[2]Data were analyzed by ANOVA, followed by Duncan's multiple range test. Means in a row with different superscripts are significantly differ, $p < 0.05$

Table 4 Taurine effect on ALP and osteocalcin in rats

Variables	Sham-Control	Sham-Taurine	Ovx-Control	Ovx-Taurine
ALP (U/L)	126.7 ± 20.3[1a2]	129.1 ± 22.7[a]	134.3 ± 28.2[a]	182.5 ± 36.1[b]
Osteocalcin (ng/ml)	21.8 ± 6.6[a]	23.9 ± 5.4[a]	30.3 ± 5.8[a]	28.5 ± 7.8[a]

[1]Mean ± SD
[2]ANOVA and Duncan's multiple range test were used for analysis. Means in a row with different superscripts are significantly differ, $p < 0.05$

Table 5 Taurine effect on urine deoxypyridinoline (DPD), creatinine and crosslinks value in rats fed with cholesterol diet

Variables	Sham-Control	Sham-Taurine	Ovx-Control	Ovx-Taurine
Deoxy-pyridinoline (nM)	459.4 ± 239.3[1a2]	562.0 ± 401.7[a]	1504.8 ± 572.5[b]	688.8 ± 636.3[a]
Creatinine (mM)	3.9 ± 1.8[a]	6.9 ± 6.2[a]	7.9 ± 2.1[a]	3.8 ± 2.6[a]
Crosslinks value (nM/mM)	115.1 ± 24.2[a]	80.8 ± 16.8[a]	190.9 ± 28.9[b]	180.8 ± 58.7[b]

[1]Mean ± SD
[2]ANOVA and Duncan's multiple range test were used for analysis. Means in a row with different superscripts are significantly differ, $p < 0.05$

SHAM-control rats, 16.2 ± 2.7 g/day, and SHAM-taurine rats 16.1 ± 2.4 g/day ($p < 0.05$) (Table 3). FER of the OVX-control group was remarkably higher than in the SHAM group and was not affected by taurine supplementation in OVX groups.

3.3 Bone Markers

Serum ALP was significantly higher in OVX-taurine rats than OVX-control rats. There were no differences between diet groups in serum osteocalcin concentrations (Table 4). Crosslinks values were significantly higher in OVX than in SHAM rats ($p < 0.05$) (Table 5).

Table 6 Effects of taurine on spine BMD and spine BMC in ovariectomized rats

Variables	Sham-Control	Sham-Taurine	Ovx-Control	Ovx-Taurine
SBMD (g/cm^2)	0.143 ± 0.009[1a2]	0.149 ± 0.012[a]	0.122 ± 0.002[b]	0.129 ± 0.010[ab]
SBMD /wt (kg)	0.51 ± 0.05[a]	0.53 ± 0.04[a]	0.40 ± 0.02[b]	0.47 ± 0.02[ab]
SBMC (g)	0.474 ± 0.015[a]	0.506 ± 0.069[a]	0.459 ± 0.039[a]	0.432 ± 0.047[a]
SBMC/wt (kg)	1.54 ± 0.16[a]	1.59 ± 0.14[a]	1.20 ± 0.19[b]	1.36 ± 0.23[ab]

[1]Mean ± SD
[2]Data were analyzed by ANOVA, followed by Duncan's multiple range test. Means in a row with different superscripts are significantly differ, $p < 0.05$

Table 7 Effects of taurine on femur BMD and femur BMC in ovariectomized rats

Variables	Sham-Control	Sham-Taurine	Ovx-Control	Ovx-Taurine
FBMD (g/cm^2)	0.171 ± 0.008[1a2]	0.184 ± 0.013[a]	0.166 ± 0.006[a]	0.169 ± 0.011[a]
FBMD/wt(kg)	0.62 ± 0.05[a]	0.61 ± 0.05[a]	0.411 ± 0.04[b]	0.47 ± 0.04[b]
FBMC (g)	0.360 ± 0.016[a]	0.367 ± 0.046[a]	0.363 ± 0.018[a]	0.360 ± 0.038[a]
FBMC/wt(kg)	1.10 ± 0.056[a]	1.10 ± 0.013[a]	0.97 ± 0.019[b]	1.01 ± 0.017[b]

[1]Mean ± SD
[2]Data were analyzed by ANOVA, followed by Duncan's multiple range test. Means in a row with different superscripts are significantly differ, $p < 0.05$

3.4 Bone Mineral Density and Bone Mineral Content

SHAM rats had higher spine BMD/wt and spine BMC/wt than both of the OVX groups ($p < 0.1$). Whereas the SHAM-control and SHAM-taurine rats did not differ and OVX-control and OVX-taurine rats did not differ, too (Table 6). SHAM rats had higher femur BMD/wt and BMC/wt ($p < 0.001$) than in the OVX groups. Femur BMD/wt and BMC/wt did not differ between the OVX-control rats and OVX-taurine rats (Table 7). Spine BMD/wt and femur BMD/wt were lower in OVX-control rats (spine BMD/wt: about 22%, femur BMD/wt: about 34%) than SHAM-control rats ($p < 0.05$).

3.5 Discussion

Estrogen deficiency in female has been known as risk factors for bone health, osteoporosis. The objectives of this study was to determine the role of estrogen deficiency and high cholesterol diet on bone health and to determine the association with bone markers.

The favorable effects of taurine, 2-amino ethane sulfonic acid on cholesterol metabolism by enhancing the effects that hypercholesterolemia exerts has been reported (Yokogoshi et al. 1999; Murakami et al. 2002). The most widely known functions of taurine are their antioxidant activities. In previous studies, taurine

supplementation restrained the synthesis of proinflammatory cytokines, interrupted oxidative stress damage, and enhanced antioxidant function in diverse animal studies (Kontny et al. 2000; Grimble 2006). Animal studies reveal that supplementation of taurine has positive influence on bone as shown by higher BMD (Choi and DiMarco 2009).

We examined whether the preventive role of taurine on estrogen-deficiency-induced bone loss differs with the cholesterol in ovariectomized (OVX) rats. Marked body weight increased among the OVX rats were previously reported (Choi and DiMarco 2009). The reduction of energy expenditure and metabolic rate in estrogen deficiency rats were reported. Estrogenic actions pathways are yet to be elucidated. It is known that bone-derived ALP is specific to bone, however it is not the case for ALP; giving a higher correlation ($r > 0.9$) (Rauch et al. 1997).

The concentrations are a little high to those observed in OVX rats. In this study, serum ALP was lower in the OVX-taurine group than OVX-control group. Results from the earlier study (Guo et al. 2009) showed that the marker of bone formation (serum osteocalcin) and the matker of bone resorption (urinary D-Pyr/Cr) were higher in ovarietomized control rats than in sham-operated rats. Our study did not show any difference on the osteocalcin concentration in all experimental groups. OVX treatment was remarkably decreased the femur BMD and the spine BMD ($p < 0.05$). These results are similar to the earlier study (Guo et al. 2009). Spine bone mineral density was remarkably reduced in the estrogen deficiency–induced, OVX rats compared to SHAM ($p < 0.05$). This finding is in agreement with previous observations showing that ovariectomy-induced estrogen deficiency was associated with lower spine BMD in OVX rats. (Choi and DiMarco 2009). In addition, Ovariectomy has an active role on spinal bone than on femural bone (Kalu 1991). On the other hand, trabecular bone mineral density is known to be negatively correlated with rat liver malondialdehyde concentrations (Dong et al. 2016).

Other studies have reported that bone mineral density increases with body fat mass, and that consequently obesity will have a protective effect against osteoporosis (Crepaldi et al. 2007).The weight gain is generally associated with hyperphagia induced ovariectomy (McElroy and Wade 1987). Consequently, body weight is has a positive factor on bone metabolism via the role of mechanical loading (Kim et al. 2010, Lee et al. 2011). However, recent studies reported the contrary: ovariectomy-induced hyperphagia did not affect bone content regardless of food intake (Zhang et al. 2007). Marked changes in bone mineral density in the ovariectomized rats were reported in previous research. After ovariectomy, the rats showed a significant reduction in spinal BMD. And OVX rats showed a remarkable increase in DPD level in urine and urinary Ca and P excretion (Zhang et al. 2007).

The study reported that taurine diet increased femoral BMC in ovariectomized rats (Choi and DiMarco 2009). The finding is in agreement with previous studies that have shown that estrogen deficiency reduced spine BMD. Results from other studies also showed that taurine plays a direct role in bone homeostasis by inhibiting osteoclastogenesis (Yuan et al. 2010). IGF-I can also exert anabolic effects on

bone mass (Rizzoli 2014). Trabecular thickness was improved by taurine and taurine increased serum IGF-1 levels, and tibia-growth plate (Moon et al. 2015). So the benefits of taurine may extend to the bone health. However taurine had not effect on femur BMC in OVX when calcium level is deficient (Choi 2009). The discrepancy between these results may be due to different calcium level or cholesterol. In aged rats, the formula diet with high fat and ovariectomy induce speedy bone resorption (Dong et al. 2016).

The function of taurine in bone is yet to be fully understood. Proposed mechanisms include calcium regulation. So dietary intakes are a primary modifiable environmental component that affects both bone accumulation and bone loss. Fatty fish diet is reduced fracture risk (Longo and Ward 2016). Health of bone and heart is associated with endocrine and metabolic mechanisms. It is including effects of parathyroid hormone which regulate minerals (Hill Gallant et al. 2016). Additionally, Zhao associates bone development is associated with the common precursor stem cell that leads to the differentiation of both adipocytes and osteoblasts, as well the secretion of adipocyte-derived hormones (Zhao et al. 2008). However, earlier studies suggest that high cholesterol diet may not protect against osteoporosis (You et al. 2011). Although being a harmful for health, obesity has traditionally been known good to bone because of its mechanical loading. This mechanical loading makes bone formation (Colaianni et al. 2014).

The western diet today include higher than recommended intakes of w-6 PUPA relative to w-3 PUPA that induce a chronic inflammation (Longo and Ward 2016). Taken together, these results support the hypothesis of a positive role of taurine on bone and heart. The associations between the consumption of taurine and the bone health are not well established, although taurine supplementation results a weakly protective effect for bone loss on optimal nutrition. Taurine is a safe and for protection and treatment of hormone-dependent bone loss. Future research is necessary to better define the optimal dose of taurine supplementation with cholesterol diet and to examine the potential interactions between taurine and another nutrient to bone health. To better understand the effects of taurine on bone at high risk of osteoporosis and fracture, more research is warranted. The function of taurine in bone is still poorly understood but is now the subject of ongoing investigations.

The spinal bone loss in OVX was a little restrained by taurine supplementation. A 2% taurine diet in OVX rats has a protective role against bone loss. However, the supplementation of taurine to the rats fed with a high cholesterol diet does not significantly reduce bone mineral density loss. More basic research and human studies are warranted for role of taurine in bone health.

4 Conclusion

Taurine supplementation shows a weakly positive protective trend for spine bone mineral content per weight in OVX rats fed cholesterol.

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Part IX
Interdisciplinary Fields

The Taurine Content of Japanese Seaweed

Azusa Kawasaki, Ayuko Ono, Shoshi Mizuta, Mitsunobu Kamiya, Takaaki Takenaga, and Shigeru Murakami

Abstract Japanese and South Koreans have a dietary habit of eating seaweed. Although it is known that some seaweed contains taurine, there have been few detailed analyses on the taurine content of seaweed other than the major types of edible seaweed. In the present study, we determined the content of free amino acids, including taurine, in seaweed obtained along the Sea of Japan coast. The taurine content in the seaweed varied according to the species. Among the 29 different types of seaweed that were studied, red algae contained relatively high concentrations of taurine. In contrast, the taurine content was low or undetectable in brown and green algae. The algal alanine level was relatively higher in brown sea algae, which was in sharp contrast to its taurine level. No clear trends were observed with regards to the distribution of the other free amino acids, including aspartic acid, glutamic acid, and phenylalanine. Considering the physiological role of taurine in cellular homeostasis, the algal taurine content may be associated with the growing environment. Taurine-rich red edible algae such as mafunori (*Gloiopeltis tenax*)/ fukurofunori (*Gloiopeltis furcata*), kabanori (*Gracilaria textorii*), and ogonori (*Gracilaria vermiculophylla*) may be used to create functional foods that are rich in naturally occurring taurine.

Keywords • Taurine • Seaweed • Red algae

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1 Introduction

Taurine is widely distributed in the animal kingdom; its tissue concentration reaches the millimolar range (Huxtable 1992). Fish and shellfish are especially rich in taurine, which is thought to be associated with the osmoregulatory action of taurine against the osmotic pressure of marine water. In contrast, taurine is rarely found in terrestrial plants. Some types of seaweed have been shown to contain taurine (Zhao et al. 1998; McCusker et al. 2014): however, aside from popular edible sea algae such as susabinori (*Pyropia yezoensis*), wakame (*Undaria pinnatifida*), and makonbu (*Laminaria japonica*), there is little information about the taurine content of different varieties of seaweed.

Japanese and South Koreans have the habit of eating seaweed, and seaweed as well as fish and shellfish are an important dietary source of taurine in these countries. In fact, a world-wide epidemiological study demonstrated that urinary taurine excretion, a marker of the dietary intake of taurine, was much higher in these countries than in other countries including countries in Europe and North America (Yamori et al. 2001). The epidemiological study also showed a strong and inverse relationship between the urinary excretion of taurine and mortality due to ischemic heart disease (Yamori et al. 2001; Sagara et al. 2015). This may be related to the lower risk of cardiovascular disease that is observed in Japan and South Korea.

Seaweed contains many health-promoting nutrient components, including minerals, amino acids, vitamins, omega-3 and omega-6 fatty acids (eicosapentanoic acid [EPA] and docosahexanoic acid [DHA]), carotenoids (astaxanthin and fucoxanthin), dietary fibers, and polysaccharides (alginic acid and fucoidan), in addition to taurine (Brown et al. 2014). Synthetic taurine is distributed internationally and is widely used in dietary supplements and as an ingredient in energy drinks (Higgins et al. 2010). In Japan, taurine has been clinically approved as a treatment for chronic heart failure and hepatic disorders (Azuma et al. 1985). Thus, taurine-containing seaweed is promising material for functional foods and health foods, since seaweed contains various health-promoting substances. In the present study, we determined the content of free amino acids, including taurine in seaweed obtained along the Sea of Japan coast, to search for seaweed that potentially has a high amount of taurine.

2 Methods

2.1 The Acquisition and Preparation of Algae

Seaweed samples were collected from Wakasa Bay, which is located in Fukui prefecture, in May. These samples included 13 red algae, 10 brown algae, 4 green algae, and 2 sea grasses (Table 1). The collected samples were rinsed with water, and stored at −20 °C until use. Some popular edible types of seaweed were purchased from grocery stores for use as controls and the taurine content was analyzed. These include susabinori (*Pyropia yezoensis*), hijiki (*Sargassum fusiforme*), and usuba-aonori (*Ulva linza*); these varieties are widely consumed in Japan (Table 1).

2.2 The Amino Acid Analysis

The free amino acids in hot-water soluble fractions were converted to phenylthiocarbamyl (PTC) amino acids by derivatization with phenyl isothiocyanate (PITC), and analyzed using a liquid chromatography system (600E, Waters, USA) equipped with a reverse-phase column (Cosmosil 5C18-AR-II, 4.6 mm ϕ × 250 mm, Nacalai Tesque, Kyoto, Japan). In addition to taurine, hydroxyproline and hydroxylysine, we analyzed the 17 amino acids (Asp, Glu, Ser, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val, Met, Cys, Ile, Leu, Phe, Lys) contained in a commercial amino acid standard mixture (Type H, Wako Pure Chemical Industries, Ltd.,

Table 1 A list of the types of seaweed analyzed in the present study

		Academic name	Japanese name
Red algae	1	Gelidium elegans	Makusa
	2	Gloiopeltis furcata/Gloiopeltis tenax	Fukurofunori/Mafunori
	3	Chondracanthus intermedius	Kainori
	4	Grateloupia lanceolata	Fudaraku
	5	Ahnfeltiopsis flabelliformis	Okitsunori
	6	Gracilaria parvispora	Shiramo
	7	Gracilaria textorii	Kabanori
	8	Gracilaria vermiculophylla	Ogonori
	9	Campylaephora hypnaeoides	Egonori
	10	Spyridia filamentosa	Ubugegusa
	11	Chondria crassicaulis	Yuna
	12	Palisada intermedia	Kurosozo
	13	Polysiphonia senticulosa	Shojokenori
Brown algae	14	Dictyota dichotoma	Amijigusa
	15	Hydroclathrus clathratus	Kagomenori
	16	Petalonia binghamiae	Habanori
	17	Scytosiphon lomentaria	Kayamonori
	18	Myagropsis myagroides	Joromoku
	19	Sargassum autumnale	Akiyoremoku
	20	Sargassum hemiphyllum	Isomoku
	21	Sargassum horneri	Akamoku
	22	Sargassum macrocarpum	Nokogirimoku
	23	Sargassum thunbergii	Umitoranoo
Green algae	24	Ulva linza	Usuba-aonori
	25	Ulva pertusa	Ana-aosa
	26	Ulva prolifera	Suji-aonori
	27	Codium fragile	Miru
Sea grasses	28	Zostera marina	Amamo
	29	Zostera japonica	Ko-amamo
Control algae			
Red alga	C1	Pyropia yezoensis	Susabinori
Brown alga	C2	Sargassum fusiforme	Hijiki
Green alga	C3	Ulva linza	Usuba-aonori

Osaka, Japan). Elution was performed using a linear gradient of two eluents, A and B, as follows: A, a mixture of sodium acetate buffer (140 mM sodium acetate, pH6.4) and acetonitrile mixed at a ratio of 94:6; B, a mixture of ultrapure water and acetonitrile mixed at a ratio of 4:6. The gradient program was set to 0–10 min, 0%; 10–30 min, 5–47%; 30–35 min, 47–90%; 35–47 min, 100%. The column temperature and flow rate were maintained at 43 °C and 0.8 mL/min, respectively, throughout each of the analyses. The concentration of each amino acid was calculated from the peak area by the external standard method. The data are expressed as the amino acid content (mg) per 100 g of dry seaweed. Each value represents the mean of three samples.

3 Results and Discussion

The taurine content of the three popular edible types of seaweed purchased from grocery stores showed characteristic tendencies (Table 1, C1–C3): the red alga (susabinori) had a higher taurine content, while the taurine content in brown (hijiki) and green (aosa) algae was low or undetectable (Fig. 1). Susabinori has previously been reported to contain high concentration of taurine (Hwang et al. 2013). Similarly, among 29 seaweeds collected in Wakasa Bay, the red algae contained relatively higher concentrations of taurine, especially mafunori (*Gloiopeltis tenax*)/fukurofunori (*Gloiopeltis furcata*), kabanori (*Gracilaria textorii*), and ogonori (*Gracilaria*

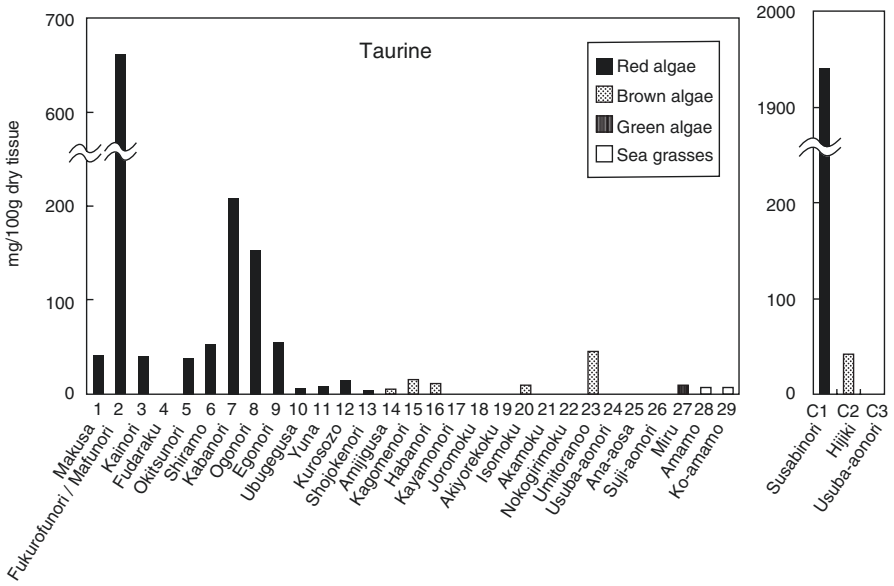

Fig. 1 The taurine content of seaweed. Refer to Table 1 for the numbers and names of the seaweed. Each value represents the mean of three samples

vermiculophylla) (Fig. 1, No 2, 7, and 8). In Japan, these red algae have long been processed and used in traditional foods such as agar (kanten) and gelidium jelly (tokoroten). In contrast, the taurine levels in brown and green algae were low or undetectable. A small amount of taurine was detected in sea grass, amamo (*Zostera marina*) and ko-amamo (*Zostera japonica*). These sea grasses are classified to angiosperm; they are flowering and seminiferous plants that are derived from terrestrial plants. Since terrestrial plants do not contain taurine, these algae might have acquired taurine during the moving process to the sea, suggesting a possible role of taurine as an osmoregulator (Schaffer et al. 2000). Thus, the present study revealed that the taurine content varied considerably in the different species of algae and that red algae had higher levels of taurine content than brown and green algae.

The reason why red algae contain higher levels of taurine is not known. However, the taurine content of algae may reflect their growing environment. Most of the red algae that were examined in this study grow in shallow water, where the sea level fluctuates according to the flux and reflux of the tides. These algae become dry and are subject to ultraviolet radiation during low tide. In contrast, they are in the sea with a high concentration of salt during high tide. Considering the crucial role of taurine in maintaining cellular physiological homeostasis, including osmoregulation (Huxtable 1992), the red algae in shallow waters may accumulate more taurine to counter these severe environmental variations.

The contents of the major free amino acids other than taurine are shown in Fig. 2. Seaweed generally contains higher levels of glutamic acid, aspartic acid, and alanine. It is interesting that the alanine content was higher in brown algae and lower in red algae, which is in sharp contrast to the concentration of taurine. Alanine is known to function as an osmolyte in mammals; however its role in seaweed is not known.

Red algae ogonori (*Gracilaria vermiculophylla,* No. 8) contained an extremely high level of arginine in comparison to other types of seaweed. A red alga, fudaraku (*Grateloupia lanceolate*, No. 4) contained a high level of threonine (Fig. 2). Algae contain various types of low-molecular bioactive compounds, such as sugar, sugar alcohol, and free amino acids including taurine (MacArtain et al. 2007). It is considered that these compounds play important roles in osmoregulation.

The taurine content in humans is approximately 0.1% of the body weight: a man who weighs 60 kg will contain 60 g taurine. Body taurine is supplied by both diet and biosynthesis. The synthetic activity of taurine in the body varies greatly depending on species (Hardison et al. 1977). For example, it is well known that taurine synthesis is extremely low in cats (Sturman 1992). Thus, the dietary supplementation of taurine is essential for cats. In the case of fish, most taurine seems to be obtained and accumulated in the body through the food chain. It is not known where the taurine in seaweed originates. However, an *in vitro* study suggested that seaweed can synthesize taurine. In this experiment, asakusanori (*Pyropia tenera*) was incubated with ^{35}S–labeled H_2SO_4 for 48 h, and the alga was the extracted with boiled water, and separated by column chromatography. ^{35}S–Labeled taurine was detected in the extract of asakusanori, indicating that algal taurine is at least partly derived from biosynthesis in the body.

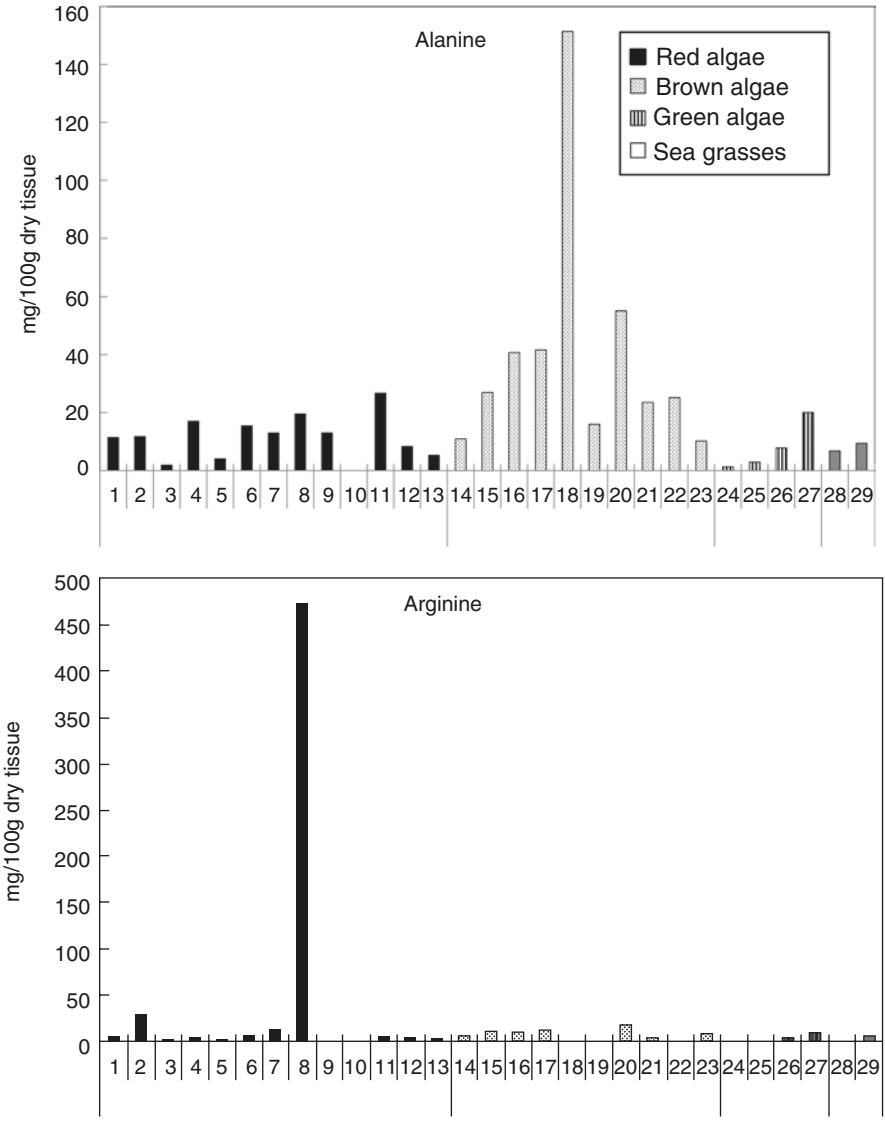

Fig. 2 Contents of alanine, arginine, and threonine in seaweed. Refer to Table 1 for the numbers and names of the seaweed. Each value represents the mean of three samples

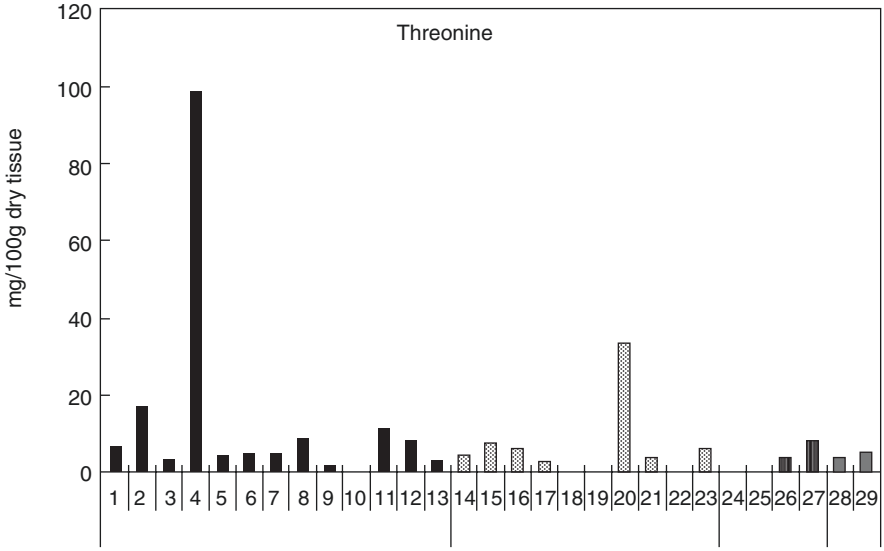

Fig. 2 (continued)

4 Conclusion

In conclusion, our study showed that the algal taurine content varies according to the species. Red algae, especially mafunori (*Gloiopeltis tenax*)/fukuronori (*Gloiopeltis furcata*), kabanori (*Gracilaria textorii*), and ogonori (*Gracilaria vermiculophylla*) contained higher levels of taurine. These red algae are edible and are available for the creation of functional foods that contain naturally occurring taurine.

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Analysis of Taurine's Anti-Down Syndrome Potential in *Caenorhabditis elegans*

Yun Jung Ko, Hyunsook Chang, and Dong Hee Lee

Abstract Down syndrome (DS) patients overexpress human DS critical region gene 1 (*hDSCR-1*), whose translational product inhibits calcineurin-dependent signaling pathways of genetic transcription. Compared to *hDSCR-1*, *C. elegans rcn-1* has 40% sequence similarity and its proteins share an analogous function with *hDSCR-1* in regulating calcineurin. Taurine has had a positive effect on DS patients. According to animal research studies, taurine reduces the expression of MCIP1, a calcineurin inhibitory protein, on C2C12 myotubes and fibroblast in mouse. This study utilizes two *C. elegans* models for DS: *rcn-1* overexpression model, displaying a calcineurin-deficient phenotype, and calcineurin loss-of function mutants. *C. elegans* larvae were treated with taurine to characterize its effect and mechanism in helping DS patients. *RCN-1* expression and behavioral changes were examined in *rcn-1* overexpression and calcineurin-deficient models at different concentrations of taurine. When treated with taurine, transgenic worms harboring an *rcn-1* reporter (RCN-1::GFP) showed a reduced level of *rcn-1* mRNA expression and improved behaviors that were comparable to those in the wild type. These results indicate that taurine exerts a down-regulating effect on the expression of *rcn-1* and, consequently, a positive effect on the expression of calcineurins. In summary, taurine may improve the DS symptoms by prompting a positive interaction between *RCN-1* and calcineurin. Furthermore, these results suggest that novel mechanisms may regulate interactions among taurine, *RCN-1* and calcineurin.

Keywords Down syndrome • Calcineurin • *C. elegans* • Taurine • Thermotaxis

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Abbreviation

CnA	Calcineurin A
DS	Down syndrome
DSCR-1	Down syndrome critical region 1
GFP	Green fluorescent protein
GOF	Gain of function
LOF	Loss of function
NGM	Nematode growth medium
RCN	Regulator of calcineurin

1 Introduction

Down syndrome (DS) patients have characteristic facial features and delayed physical development. In most cases, they also have mental retardation (Patterson 2009). They have three copies of chromosome 21. Approximately, 200–250 genes are located on the chromosome and are significantly overexpressed in cases of DS. Among the overexpressed genes, DS critical region gene 1 (*DSCR-1*) is highly expressed in the brain and interacts with calcineurin A, which is a Ca^{2+}/calmodulin-dependent protein phosphatase (PP2B) (Fuentes et al. 2000).

Calcineurin belongs to a class of serine/threonine protein phosphatases and its activity is greatly affected by Ca^{2+} and calmodulin. Calcineurin A has a catalytic phosphatase domain and a regulatory domain, which consists of three domains: calcineurin B binding domain, calmodulin-binding domain, and downstream autoinhibitory (AI) domain. The activity of Calcineurin A greatly depends on Ca^{2+}, which is incorporated into calmodulin (Klee et al. 1998). Calcineurin B regulates downstream genes via a regulatory subunit that contains four Ca^{2+}-binding EF-hand motifs. The activity of calcineurins is also regulated by *DSCR-1*. When *DSCR-1 is* overexpressed, calcineurin is inhibited by nuclear factor of activated T-cells (NFATc), which accelerates the dephosphorylation of calcineurin (Fuentes et al. 2000).

The *C. elegans* rcn-*1* gene shares 40% sequence similarity to *human DSCR-1*. Indeed, like human DSCR-1, RCN-1 interacts with calcineurins in *C. elegans* (Strippoli et al. 2000). When overexpressed, RCN-1 inhibits calcineurin activity and causes a phenotypic deficiency in calcineurin. The transcriptional level of *rcn-1* is far lower, both in calcineurin A loss-of-function (lof) mutants (CnA lof mutant/p675) and in calcineurin B loss-of-function mutants (CnB lof mutant/jh103) in comparison to the wild type worms. Those mutants show a slower growth, smaller brood size, shorter body length, clearer cuticles, and stronger resistance to serotonin during egg-laying (Lee et al. 2013).

Taurine, 2-aminoethanesulfonic acid, consists of sulfonic acid and amino functions, which are attached to each carbon backbone. Taurine protects the brain from various damage, reduces withdrawal syndrome, and help to enhance visual func-

tion. In addition, taurine is used to treat anxiety, epilepsy, and seizures. More importantly, it is effective in alleviating Down syndrome and muscular dystrophy in children. (Balch 2006) Taurine is synthesized by cysteine dioxygenase and cysteine sulfinic acid decarboxylase. Animal and human cysteine sulfinic acid decarboxylases are clearly different. The activity of the enzyme is very low in taurine biosynthetic capacity in humans compared to most mammals (Vitvitsky et al. 2011).

Considering that significantly lower levels of taurine are found in the cerebral cortex of fetuses with Down syndrome, a taurine deficiency causes abnormal brain development (Whittle et al. 2007). Accordingly, strengthened taurine can improve Down syndrome and is often used to reduce Down's syndrome in clinical treatment.

C. elegans is a widely used animal model because it can be maintained at a low cost to study human diseases. Its simple structure and biological applicability stems from a small number of cells, short life cycle (egg to adult in three days), and the features of its hermaphrodites. Cellular characteristics of the disease pathway are conserved for a variety of human diseases, including diabetes and aging. *C. elegans* is frequently used as very effective model in evaluating drug targets (Kaletta and Hengartner 2006).

In Down syndrome patients, the expression of the regulatory factor-1 DSCR of calcineurin is higher (Fuentes et al. 2000). The DSCR-1 and the RCN of *C. elegans* share a high homology (40%). *C. elegans* lof mutants CnA and CnB show a phenotype that is similar to the model of RCN-1 overexpression. This study investigated taurine processing in calcineurin lof mutants. Based on previous findings, we also tested whether the effect on the expression of RCN-1 improved the behavioral defects of calcineurin lof mutants. This study also aimed to clarify the nature of the positive effect of taurine on patients with Down syndrome through molecular biology and behavioral variations.

In Down syndrome patients, the level of DSCR-1 is typically high and this unusual level negatively affects calcineurin gene expression (Fuentes et al. 2000). RCN-1 shares over 40% similarity with DSCR-1 and serves as the counterpart of DSCR-1 in *C. elegans*. In addition, calcineurin lof mutants phenotypically resemble mutants that overexpress RCN-1. The present study utilized two types of calcineurin mutants to characterize the efficacy of taurine for the improvement of Down syndrome. Specifically, this study probed the effect of taurine on the expression of RCN-1 and on overall phenotypic expression. This study analyzed the basis for molecular and behavioral explanation of taurine's positive effect on Down syndrome pathology.

2 Methods

2.1 C. elegans *Strains and Maintenance*

For the experiment, three lines of *C. elegans* were used: Bristol N2, *cna-1(jh107)*, and *cnb-1(jh103)*. The former is for the wild type and the latter two strains are mutant strains. *Cna-1* is a gain-of-function (gof) mutant of calcineurin A and cnb-1

harbors calcineurin B lof mutation. Another calcineurin A-deficient mutant was used: *tax-6 (p675)*, which encodes an ortholog of calcineurin A. The mutant overexpressing RCN-1 was established by microinjection of a plasmid harboring an additional copy of *rcn-1* into the wild type worm. The transgenic worm was named N2 Ex[prcn-1::rcan::gfp]. All nematode strains were provided by the Hanyang University Developmental Biology Lab (Seoul, South Korea).

All nematodes were cultured at 20 °C on nematode growth medium (NGM) seeded with OP50. The developmental stages of the animals were synchronized by bleaching to generate eggs and, subsequently, by incubating the eggs on OP50-free media overnight. The worms were then fed for 48 h with OP 50. To fix media containing different concentrations of taurine, taurine was added to the OP50 stock by diluting a taurine stock solution of 10 mg/ml into double distilled water. The practical taurine stocks were 0, 1, 10, 100, and 1000 μg/ml in OP50, and these taurine stock solutions were spread onto the surface of NGM-agar plates.

2.2 Measuring GFP Intensity

To measure GFP intensity, worms were photographed under a fixed exposure and the GFP intensity was quantitated using Image J software. GFP intensity was quantitated per whole body and pharynx and was expressed as percentage of intensity in the taurine-free control.

2.3 RNA Isolation and cDNA Synthesis

2.3.1 RNA Isolation

After the worms were collected into 1 ml distilled water in an Eppendorf tube, they were washed twice and vortexed for 30 s in a steel bead tube (Gene Research Biotechnology) containing 400 μL Trizol (Molecular Research Center, Ohio, USA). The samples were incubated at room temperature for 10 min and centrifuged using a Bead Beater (Taco prep, Gene Research Biotechnology) for 4 min. The lysed samples were vortexed again in 600 μL Trizol for 30 s and incubated at room temperature for 10 min. Later, the samples were further vortexed in 250 μL chloroform for 15 s and incubated at RT for 5 min. After a 5 min centrifugation at 10000 rpm, the aqueous layer was removed to a new Eppendorf tube and agitated by pipetting in 550 μL isopropanol (MERCK, Darmstadt, Germany). Centrifugation was applied for 5 min at 14,000 rpm at RT and the resulting RNA pellet was washed in 1 ml 75% ethanol before a 5 min centrifugation at 9500 rpm. The pellets were air-died and dissolved in 20 μL distilled water. RNA concentration was measured according to the Nano drop protocol.

2.3.2 cDNA Synthesis

To synthesize cDNA, 2 μg of RNA sample was incubated at 70 °C for 5 min with 1 μL of 80 pmol random hexamer (Thermo Fishier Scientific) and, then, on ice for an additional 5 min. Synthesis was performed in a total volume of 20 μL. The reaction mixture included 4 μL (5X reaction buffer), 2.4 μL (25 mM $MgCl_2$), 1 μL (10 mM dNTP), 1 μL (reverse transcriptase, Promega) and 11.2 μL distilled water. The reaction mixture was then subjected to the following cycling conditions for cDNA synthesis: 25 °C for 10 min, 42 °C for 60 min and, 70 °C for 15 min.

2.4 Nested Reverse Transcriptase Polymerase Chain Reaction (Nested RT-PCR)

Using the synthesized cDNA as the template, a specific region was amplified by PCR. Initially, one-step PCR was performed in a reaction containing 10 μL 2X mastermix, 1 μL external primer, 3 μL cDNA (MGmed), and 2 μL double distilled water. The external pair was CCCGCGGCAGAATAAGCTCT (forward) and ACCCACACTCATGCACCATG (reverse). The internal sequence was amplified according to CACATGGAGATGAAGGGCGT (forward) and TCCCTTGATGGCCATGGCTA (reverse). The ACT-5 was amplified by CTGCTTGGAGATCCACAT (forward) and ACCCAGTTCTCCTTACCG (reverse). The amplification cycle was 95 °C (2 min)/95 °C (30 s) → 55 °C (30 s) → 72 °C (1 s). Thirty cycles were performed and were followed by an additional incubation at 72 °C for 5 min. After the one-step PCR was completed, the PCR product was diluted fivefold for two-step PCR. The amplification cycle was identical to that for one-step PCR. For RCN-1 amplification, two pairs of primer were used. The final amplified products were analyzed on a 1% agarose gel.

2.5 Lifespan Assay

Amp/FUDR plates were prepared by adding 33 μL of 150 mM FUDR (Sigma) and 100 μL of 100 mg/mL ampicillin (Gibco) into 100 mL NGM. OP50 was grown overnight at 37 °C and, then, was centrifuged at 3500 × g for 10 min. To prepare a 10 X OP50 stock, 90% of supernatant was discarded and the pellet was mixed in the remaining media. By diluting 10 mg/mL taurine stock solution into double distilled water, five different taurine concentrations were generated: 0, 1, 10, 100, and 1000 μg/mL. The prepared taurine solutions were mixed in the 10 X OP50 stock and spread onto amp/FUDR NGM plates. After 25 larva at L4 stage were moved to the plates, they were cultured at 20 °C. Worms were counted as dead or alive every other day until all worms died.

2.6 Behavioral Assays

2.6.1 Egg-Laying Assay

For the assay, L4 stage worms were grown for an additional 72 h at 20 °C on NGM and, then, they were treated for 6 h with different taurine concentrations. One worm was placed into each well of a 96-well plate containing 12.5 mM serotonin creatinine sulfate (Sigma) in M9 buffer. Worms were incubated for 60 min at 20 °C, and their eggs were counted.

2.6.2 Thermotaxis

Thermotaxis medium was prepared by mixing Bacto agar, 25 mM potassium phosphate pH 6.0, 1 mM $MgSO_4$, 1 mM $CaCl_2$, and 0.3% NaCl, and pouring the mixture into 10 cm petri dishes. After the gel solidified with lid open at room temperature, the plates were turned to the bottom-up position, and the center of the plate were gradually cooled with a 4 cm plate of ice for 20 min. The ice plate was replaced with a new one before half of the ice melted. Before the worms were placed onto the plate, they were collected and washed twice by centrifugation for 90 s. Worms were removed into an Eppendorf tube containing 1 ml NG buffer (25 mM potassium phosphate pH 6.0, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.3% NaCl). Once a temperature gradient was formed on the plate, the collected worms were placed with a circle of 5 cm, and excess of liquid were removed by Kimwipe. To maintain coolness in the center of the plate, ice was kept underneath within a 4 cm plate and replenished every 15 min. Worms were allowed to move for 30 min at 25 °C.

3 Results

3.1 Taurine Reduces RCN-1 Expression in an RCN-1 Overexpression Model

C. elegans expresses *RCN-1* in multiple tissues, including the side subcutaneous tissue, pharynx, vulva, and several ganglia neurons (Lee et al. 2003). Among the tissues, this study focused on the pharynx, in addition to the whole body (Fig. 1). To overexpress *RCN-1,* the strain N2 Ex[prcn-1::rcan::gfp] was cultured and the emitting GFP was photographed at 3, 6, 9, and 12 h after treatment with increasing concentrations of taurine. GFP intensity was quantitated using Image J software. The whole body showed little or no signal in animals treated with 0 μg/mL and 1 μg/mL of taurine. That is, no change in RCN-1 expression was evident at those taurine concentrations. When incubated with taurine at 10, 100, or 1000 μg/mL, RCN-1 expression decreased along the incubation time. The GFP intensity did not decrease further after 6 h incubation (Fig. 2a). In the pharynx, there was no detectable change in RCN-1 expression in the taurine-free control or in animals treated

with 1 μg/mL of taurine. When treated with 10, 100, or 1000 μg/mL, however, *RCN-1* expression decreased after 9 h treatment; at 100 μg/mL of taurine, RCN-1 expression significantly decreased after 12 h treatment (Fig. 2b).

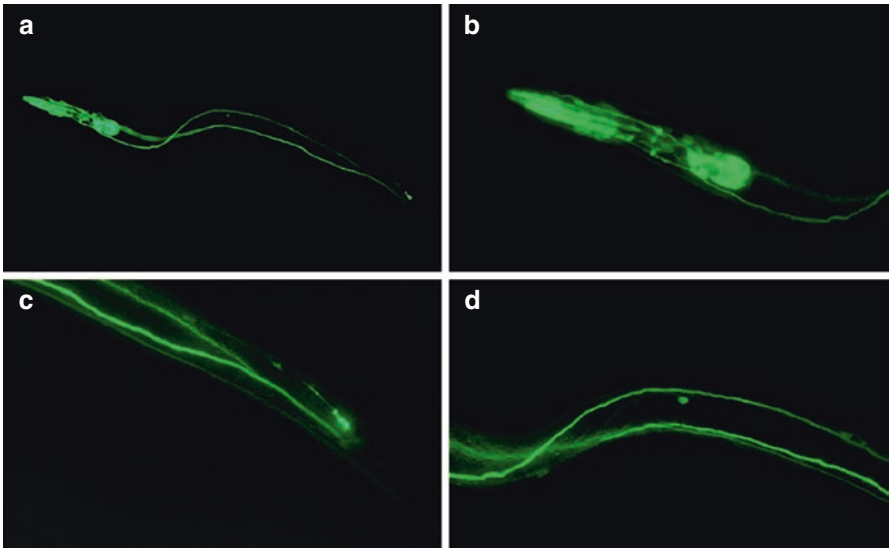

Fig. 1 *RCN-1* expression in N2 Ex[prcn-1::rcan::gfp]. After worms reached L4 stage, all worms were observed under a florescent microscope. To overexpress *RCN-1,* the strain N2 Ex[prcn-1::rcan::gfp] was cultured, the level of RCN-1 expression was determined according to GFP emission, and the animals were photographed at 3, 6, 9, and 12 h after taurine treatment. The GFP intensity was quantitated using Image J software. (**a**) Whole body (**b**) Pharynx (**c**) tail (**d**) Nerve cords and commissures

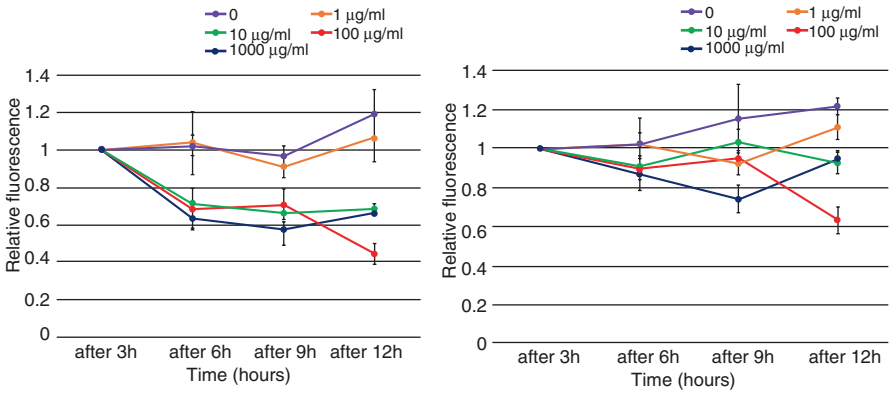

Fig. 2 GFP intensity of *RCN-1* expression on N2 Ex[prcn-1::rcan::gfp]. After taurine treatment (0, 1, 10, 100, and 1000 μg/mL) for 3, 6, 9, and 12 h, worms were observed using a fluorescence microscope. The levels of fluorescence were quantified using Image J software. (**a**) Whole body (**b**) Pharynx

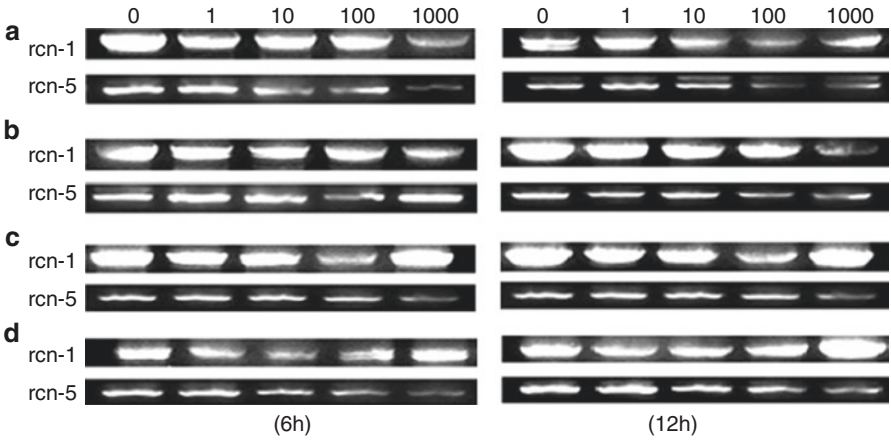

Fig. 3 RCN-1 expression. RCN-1 expression was detected via nested-PCR. After taurine treatment (0, 1, 10, 100, or 1000 μg/mL) for 6 and 12 h, worms were sacrificed for mRNA measurements. (**a**) Wild type (**b**) CnA gof mutant (**c**) CnA lof mutant (**d**) CnB lof mutant

3.2 Taurine Recovers RCN-1 Expression in Calcineurin Lof Mutants

The jh103 mutant, a gof calcineurin mutant (CnA gof mutant), shows 180% *RCN-1* expression when compared to wild type. p675, calcineurin lof mutant (CnA lof mutant), expresses approximately 30% of RCN-1 when compared to the wild type and jh103 (CnB lof mutant) expresses 80% (Lee et al. 2003). This experiment focused on whether taurine helps calcineurin mutants recover from reduced expression at the transcriptional level. Following taurine treatments for 6 h or 12 h, nested RT-PCR was performed and the expression was quantitated using Image J software. In the CnA gof mutant, RCN-1 expression decreased at 1000 μg/mL after both 6 h and 12 h. However, RCN-1 expression increased at 1000 μg/mL after both 6 h and 12 h in the CnA lof mutant, similar to that seen in the CnB lof mutant (Fig. 3). This set of data strongly indicates that calcineurin mutants affect the expression of RCN-1 within the range of taurine treatment, depending on the concentration and duration.

3.3 Taurine Increases Life Expectancy in Calcineurin Lof Mutants

The results from the experiment above confirmed that taurine affects *RCN-1* mRNA levels in calcineurin mutants according to the quantitative PCR. CnA gof mutants have life span that is similar to that of wild type, while CnA lof mutant and CnB lof mutant have longer life spans (Dwivedi et al. 2009; Mair et al. 2011).

When supplemented in culture media, taurine extends lifespan in fruit flies (Yang et al. 2012). In a Japanese study, taurine appeared to promote life expectancy in a supplement dependent manner (Yamori et al. 2010). Initially, the life expectancy of patients with Down syndrome was 12 years; however, the life expectancy was

extended to sixty years as a result of advancements in treatment of the syndrome. However, this lifespan is still much shorter than that of normal individuals (Esbensen 2010). Considering the data for humans, the calcineurin mutants were treated with taurine for the assay on life extension. When treated with as little as 10 or 100 μg/mL, lifespan increased in wild type. In the CnA gof mutant, there was no difference in the lifespans of treated and untreated animals. In both CnA lof and CnB lof mutants, however, lifespan significantly increased.

3.4 Taurine Recovers Serotonin Resistance in Calcineurin Lof Mutants

In nematodes, serotonin stimulates their muscle to lay eggs in a short period of time by prompting their motor nerves. Both CnA lof and CnB lof mutants are resistance to serotonin and lay fewer eggs in the presence of serotonin than wild type (Bandyopadhyay et al. 2002; Lee et al. 2003; 2013). Following treatment with taurine, serotonin assays were performed to determine whether taurine mediated resistance to egg laying in calcineurin lof mutants. CnA lof mutant recovered the resistance after treatment with 1, 100, or 1000 μg/mL taurine. CnB lof mutants recovered resistance after treatment with 10, 100, or 1000 μg/mL taurine.

3.5 Thermotaxis Behavior in Calcineurin Mutants

CnA lof mutants are thermophilic, while CnA gof mutants have cryophilicity (Kuhara et al. 2002). Thermotaxis assays were performed to determine whether taurine helps the worm to recover its thermophilicity. On one hand, CnA gof mutants showed a significant change in the recovery of thermophilicity, especially after treatment with 10 μg/mL of taurine (Fig. 6). On the other hand, CnB lof mutants showed significant movement at 10 μg/mL, despite its immobile nature. For both mutants, the taurine concentration was very effective in modulating the temperature specific responses (Table 1).

Table 1 Relative mRNA expression

Strain	Hour and [Tau]	0(μg/mL)	1	10	100	1000
Wild type	6	1.0	0.67 ± 0.14	0.86 ± 0.15	1.33 ± 0.33	3.43 ± 1.38
	12	1.0	0.86 ± 0.01	0.71 ± 0.09	1.76 ± 0.29*	1.43 ± 0.29*
CnA gof	6	1.0	0.92 ± 0.19	0.72 ± 0.27	1.22 ± 0.20	0.88 ± 0.10**
	12	1.0	0.80 ± 0.23	0.63 ± 0.20	1.70 ± 0.17*	1.13 ± 0.42
CnA lof	6	1.0	0.76 ± 0.11	0.87 ± 0.11	0.61 ± 0.11	1.89 ± 0.04**
	12	1.0	0.93 ± 0.02	1.00 ± 0.20	0.80 ± 0.07	1.30 ± 0.15**
CnB lof	6	1.0	0.94 ± 0.02	0.69 ± 0.28	1.30 ± 0.67*	1.51 ± 0.71
	12	1.0	0.89 ± 0.02	1.02 ± 0.18	0.73 ± 0.42	1.44 ± 0.08**

Each band of Fig. 2 was quantified by Image J software. All values were normalized to ACT-5 and were calculated as RCN-1/ACT-5. *$p < 0.05$; **$p < 0.01$

4 Discussion

Taurine is widely found in animal organs and accounts for 0.1% of the weight of humans (Huxtable 1992). Taurine is an extremely important dietary component, especially during the infant stage when its biosynthesis is very limited (Whittle et al. 2007). When mother cats are deficient in taurine during pregnancy, development of the kitten visual cortex is adversely affected (Palackal et al. 1986). In addition, taurine can prevent liver diseases, such as cirrhosis, and can reduce the chance of a fatty liver (Kerai et al. 1998). When C2C12 fibroblast myotubes and mouse fibroblasts were treated with taurine, the expression of calcineurin inhibitory protein, MCIP1, was specifically inhibited at the transcriptional level. Its transcription was significantly reduced regardless of the presence or absence of the Ca^{2+} chelator, nifedipine (Miyazaki 2013). In addition, taurine is an important element that controls muscle maturation during the developmental period. Both Ca^{2+} and Ca^{2+}-dependent calcineurin signaling play a key role in this process (De Arcangelis et al. 2006; Semsarian et al. 1999).

Calcineurin is a Ca^{2+}-calmodulin-dependent serine/threonine protein phosphatase that is regulated by Ca^{2+} and calmodulin. Calcineurin also serves as protein phosphatase 2B (PP2B) (Klee et al. 1979; Stewart et al. 1982; Klee et al. 1998). Calcineurin interacts with *DSCR-1* in the presence of Ca^{2+}. Similarly in *C. elegans*, RCN-1 interacts with calcineurin in a Ca^{2+}-dependent manner: the more *RCN-1* is expressed, the less calcineurin activity lowers (Fuentes et al. 2000; Lee et al. 2003). Based on these results, we hypothesized that *C. elegans* would be positively affected by taurine treatment, in terms of both behavior and biochemical properties.

When the RCN-1 overexpressing worms (N2 Ex[prcn-1::rcan::gfp) were treated with taurine at different concentrations and treatment periods, RCN-1 expression significantly decreased, especially at 10 and 100 μg/mL for 6 h (Fig. 2a). This result strongly suggested that the taurine treatment may be greatly affected by taurine concentration and treatment period. Under those circumstances, an RT-PCR experiment was performed to quantify the effect of taurine on RCN-1 expression using calcineurin lof mutants. When mutants were treated with taurine for 12 h, RCN-1 expression was restored to levels that were comparable to that in wild type. The data showed that the RCN-1 overexpression may inhibit calcineurin activity, whereas, RCN-1 expression was greatly recovered in mutants lacking calcineurin activity after taurine treatment (Lee et al. 2003).

Based on the data from molecular expression studies, the present study also examined the behavioral impact of taurine. The assays focused on three behaviors: life span, egg-laying, and thermotaxis. Calcineurin lof mutants have longer lifespan than the normal counterpart (Dwivedi et al. 2009). With an emphasis on these facts, worms were treated with a consistent amount of taurine across age group. As a result, calcineurin lof mutants showed the longest lifespan under taurine treatment. They respond promptly to taurine treatment (Fig. 4c, d).

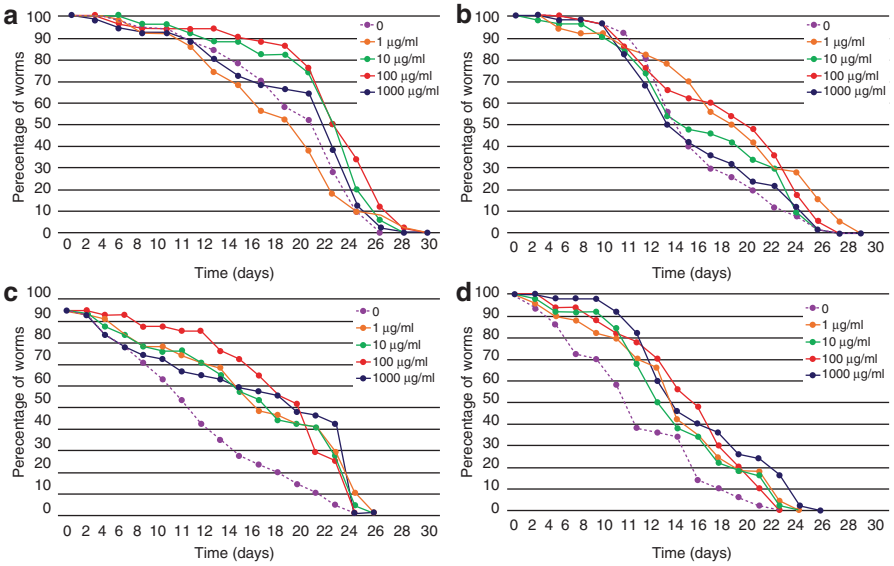

Fig. 4 Life span assay. Worms were grown on media containing taurine (0, 1, 10, 100, or 1000 µg/mL). The number of living and dead worms was recorded once every two days until all worms died. (**a**) Wild type (**b**) CnA gof mutant (**c**) CnA lof mutant (**d**) CnB lof mutant

The effect of taurine on egg-laying was assayed according to the change mediated by serotonin, which affects contraction of egg-laying muscles. Calcineurin lof mutants are resistant to serotonin and less fertile than the normal worms. Egg-laying is associated with G-protein mediated signaling, whose regulators also exert a significant effect on the activity of calcineurin (Bandyopadhyay et al. 2002). Calcineurin lof mutants showed a reduced resistance to serotonin when treated with taurine at 100 or 1000 µg/mL (Fig. 5). Considering mouse myoblast are greatly affected by taurine during differentiation and muscle maturation, calcineurin lof mutants are resistant to serotonin and less responsive to muscle nerve signaling. The present data indicated that taurine may have helped the mutants to recover the deficiency (Miyazaki 2013).

Calcineurin lof mutants are less sensitive to temperature change; however, they became temperature sensitive after taurine treatment. When the thermotaxis experiment was performed on mutant treated with 10 µg/mL taurine, the mutants significantly recovered sensitivity to temperature (Fig. 6).

The present study confirmed that lifespan, expression of RCN-1, egg-laying potential, and thermotaxis are significantly affected in calcineurin lof mutants after taurine treatment. The extent of the impact of taurine depends on the concentration and duration of the treatment. Although taurine's effect on Down syndrome cannot be easily assayed in humans, such assays can be performed in *C. elegans* with ease. The present study provided meaningful insight into understanding the biomolecular mechanism of this effect, even though the experiments were performed in nematodes.

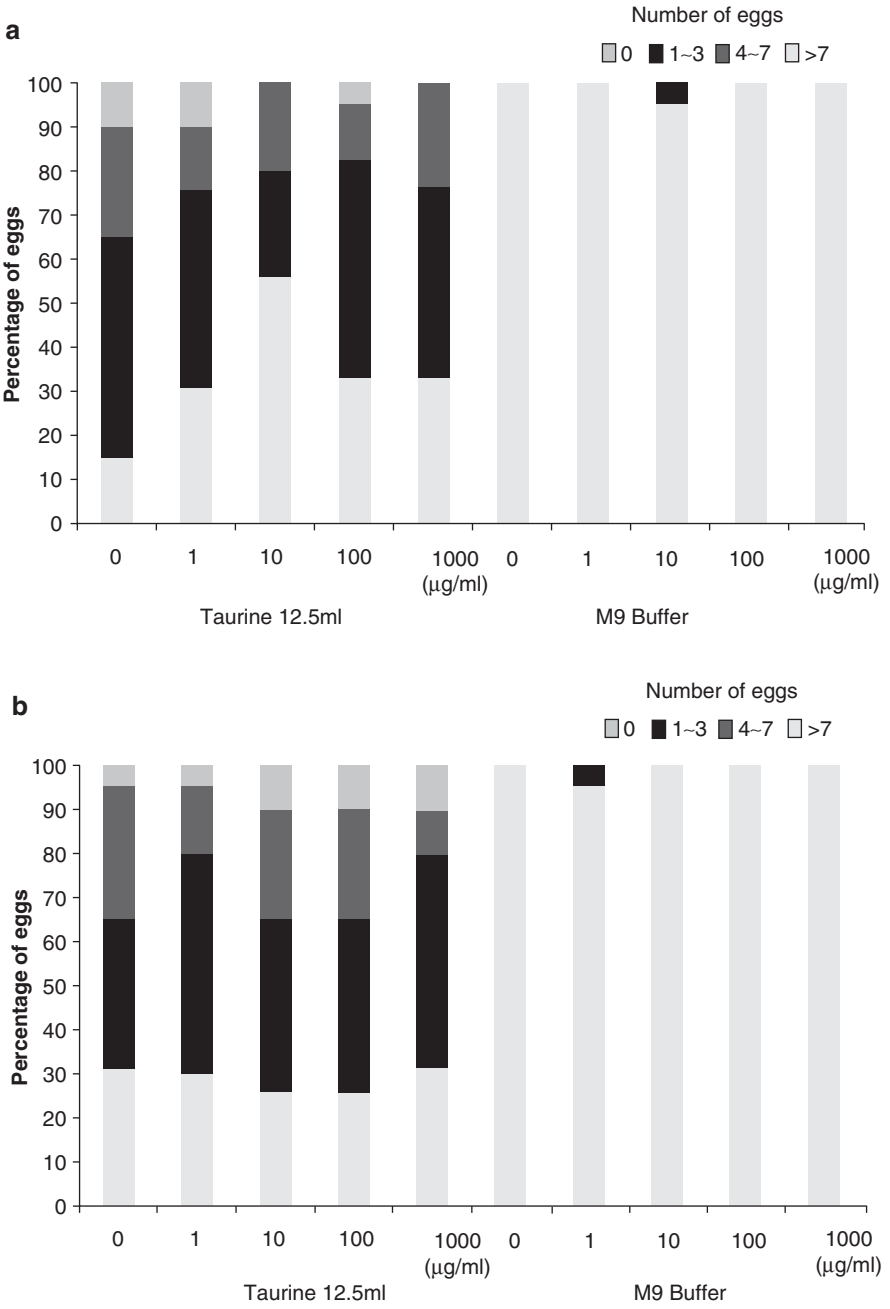

Fig. 5 Serotonin-mediated egg-laying assay. After taurine treatment (0, 1, 10, 100, or 1000 μg/mL) for 6 h, eggs were counted after worms laid eggs in 12.5 mM serotonin and M9 buffer for 1 h. The number of eggs laid in M9 buffer was used as the value for the positive control. (**a**) Wild type (**b**) CnA gof mutant (**c**) CnA lof mutant (**d**) CnB lof mutant

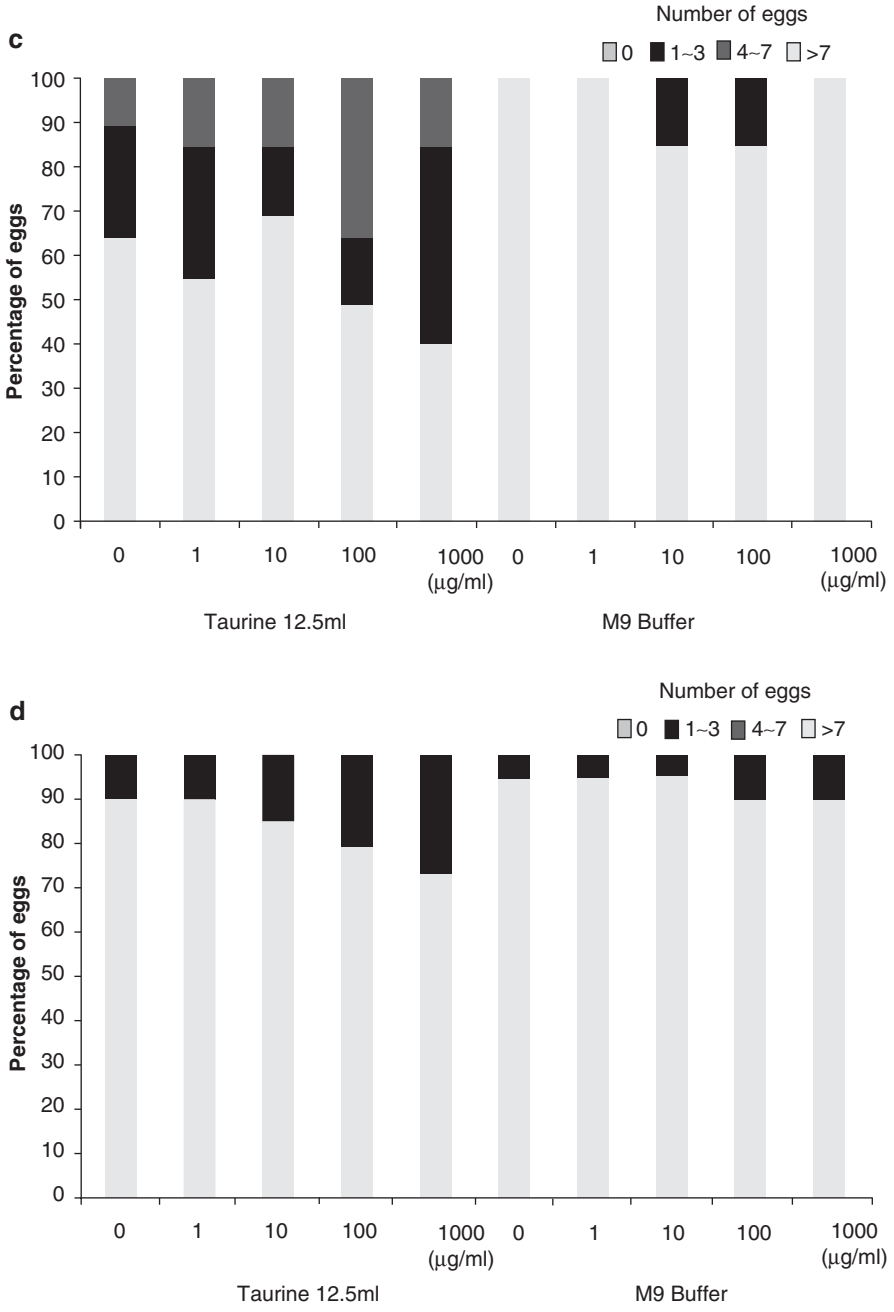

Fig. 5 (continued)

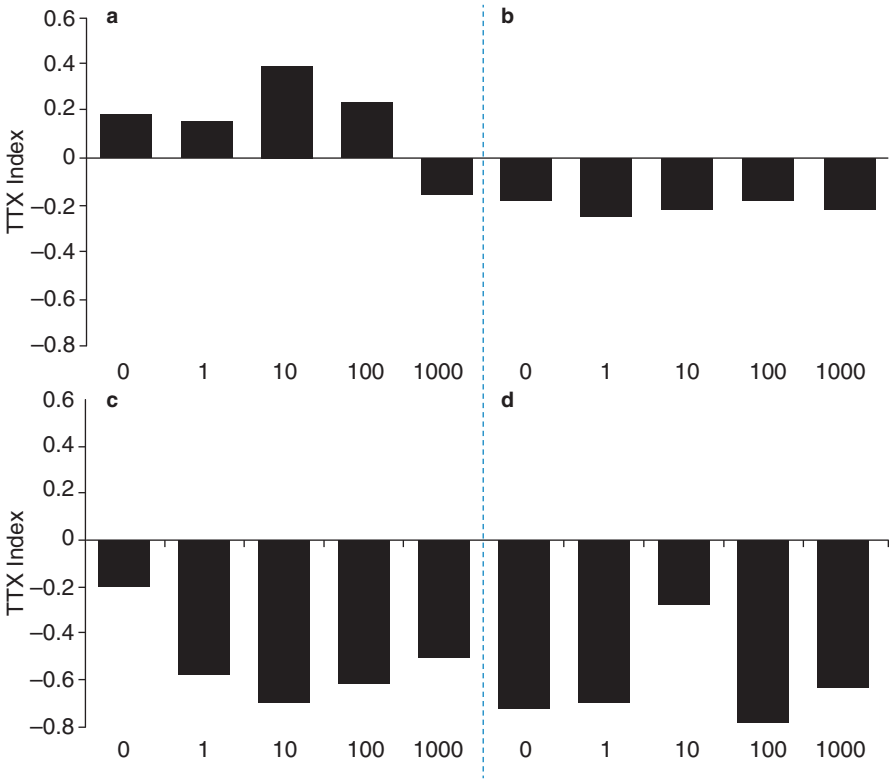

Fig. 6 Thermotaxis. After various taurine treatment (0, 1, 10, 100, or 1000 μg/mL) for 6 h, worms moved freely on thermotaxis medium for 30 min. The thermotaxis index (TTX) was calculated using the following equation: (# worms at W) − (# worms at C)/(# worms at W) + (# worms at C), where W indicates inside a 10 cm radius and (**c**) indicates inside a 4 cm radius. (**a**) Wild type (**b**) CnA gof mutant (**c**) CnA lof mutant (**d**) CnB lof mutant

These data may be helpful in elucidating the range of taurine to be used for treating Down syndrome. In further experiments, locomotion/movement, defecation, and other behavioral anomalies can be studied using calcineurin lof mutants under the treatment of taurine. Additional experiments are needed to understand the potential interactive mechanisms among sensory neurons, *RCN-1*, and calcineurin.

5 Conclusion

This study shows that Down syndrome can be alleviated by supplementation of taurine. In the nematode model of Down syndrome, typical Down syndromes were significantly improved. Based on the data in this study, taurine can be further pursued as treating agent against Down syndrome. In addition, RCN-1 can be utilized as the target for drug development for the disorder.

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Role of 5-HT_2 and 5-HT_7 Serotonin Receptors, and Protein Kinases C and A on Taurine Transport in Lymphocytes of Rats Treated with Fluoxetine

María Colmenares-Aguilar and Lucimey Lima

Abstract Fluoxetine, an antidepressant and selective serotonin reuptake inhibitor, modulates immune cells in vitro. The present study investigates the influence of pharmacological agents which acts as agonist and antagonist of serotonin receptors *ex vivo* over taurine transport in lymphocytes of rats treated with fluoxetine by one week. The treatment with fluoxetine increase taurine transport and the incubation with the agonist of 5-HT_2 receptor, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) counteract this effect, and ketanserin provoked no change in fluoxetine effect. While the agonist of 5-HT_7 receptor, 4-[2-(methylthio)phenyl]-N-(1,2,3,4-tetrahydro-1-naphth alenyl)-1-piperazinehexanamide hydrochloride (LP44) had no significant effects, however the differences between Control and Fluoxetine groups were not observed, the antagonist (R)-3-[2-[2-(4-methylpiperidin-1-yl)ethyl]pyrrolidine-1-sulfonyl]phenol hydrochloride (SB269970) had no differences. Preincubation of cells with the diacylglycerol analogue, 1-oleoyl-2-acetyl-sn-glycerol (OAG) caused inhibition of fluoxetine treatment effect but this not occurred in presence of the PKC inhibitor, 1-O-hexadecyl-2-O-methyl-rac-glycerol (AMG-C_{16}). Forskolin counteracted the effect of fluoxetine on taurine transport, since at the concentrations used, the rate of taurine transport in Fluoxetine group, returned to Control rate. No significant differences were observed with the PKA inhibitor. Although it is not possible to attribute a definitive role of 5-HT_2 receptors in fluoxetine effect on taurine transport, its signaling might affect the function of it. Participation of PKC and PKA have an apparently relevant role in lymphocyte taurine transport.

Keywords Fluoxetine • Lymphocytes • Taurine transport • Serotonin receptors

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1 Introduction

Fluoxetine, selective serotonin reuptake inhibitors (SSRIs), belong to the most frequently prescribed drugs worldwide (Anderson 2004). However, there are a limited number of studies that have addressed the effects of fluoxetine on immune cells (Pellegrino and Bayer 1998). It is clear that SSRIs act powerfully to inhibit serotonin (5-hydroxytryptamine, 5-HT) uptake centrally and peripherally (Anderson 2004) through the 5-HT transporter. 5-HT and other transmitters modulate immune cells activity in autocrine manner, for instance monocytes and lymphocytes contain and synthesize 5-HT (Urbina et al. 1999; Lima et al. 2005; Resler et al. 2008).

The 5-HT transporter (5-HTT) is widely distributed in various peripheral tissues such as platelets, placenta, pulmonary endothelium, mastocytes (Mössner and Lesch 1998), retina (Osborne et al. 1982; Lima et al. 1992; Lima and Schmeer 1994) and is present in a certain subtype of blood peripheral T lymphocytes (Faraj et al. 1994; Urbina et al. 1999; Cedeño et al. 2005; Lima et al. 2005).

Taurine and its transporter (TauT) are present in lymphocytes (Lima et al. 2003; Fazzino et al. 2006; Iruloh et al. 2007), some authors consider that taurine in white blood cells protect these cells against oxidative stress (Lubec et al. 1997). Many of the biological functions of taurine rely upon its intracellular concentration, which is determined for the capacity of cell to synthetize this amino acid and to transport it from the extracellular medium (Tappaz 2004). Previous studies in our laboratory have shown effects of fluoxetine treatment on taurine transporter, but the involved mechanisms are unknown. The amino acid sequence of TauT have several putative consensus sites for phosphorylation through Ca^{2+}/ diacyglycerol-dependent protein kinase C (PKC) (Mollerup and Lambert 1996; Han et al. 1999) and cAMP-dependent protein kinase A (PKA) (Loo et al. 1996) within the intracellular domains. This observation is consistent with the data showing that taurine transport may be regulated by phosphorylation (Tappaz 2004). Several studies have demonstrated that T and B lymphocytes are functionally responsive to 5-HT, implicating a role of this neurotransmitter in the generation of adaptive immune responses (Aune et al. 1994; Mossner and Lesch 1998; Meredith et al. 2005). We hypothesized that since fluoxetine treatment blockade 5-HT transporter, the concentration of 5-HT that surround the cell increase and could act on autocrine form over serotonin receptors present in lymphocytes such as 5-HT_2 and 5-HT_7 receptors (Aune et al. 1994; Sempere et al. 2003; Leon-Ponte et al. 2007; Urbina et al. 2014) which activate signaling pathways related to PKC and PKA. As we previously mentioned the activity of TauT could be regulated by these kinases. The aim of this work was to determine if serotonin receptors, specifically 5-HT_2 and 5-HT_7, and kinases related to these receptors, such as PKC and PKA have a role in the effect of fluoxetine treatment in taurine transport in lymphocytes of rats.

2 Methods

2.1 Reagents

DOI(1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane;ketanserin;4-[2-(Methylthio) phenyl]-N-(1,2,3,4-tetrahydro-1-naphth alenyl)-1-piperazinehexanamide hydrochloride (LP44); (R)-3-[2-[2-(4-Methylpiperidin-1-yl)ethyl]pyrrolidine-1-sulfonyl]phenol hydrochloride (SB269970).

2.2 Animals

Male Sprague-Dawley rats (180–200 g) from the animal housing at Instituto Venezolano de Investigaciones Científicas (IVIC). The animals were housed individually in a room controlled for temperature, humidity and lighting. Commercial rat food and water were available ad libitum. Fluoxetine was administered in a dose of 10 mg/kg ip in saline, daily between 8:30 and 9:30 h (Fazzino et al. 2009). The injections were continued for 1 week. Controls received the vehicle. All manipulations followed international ethical guide (Jayo and Cisneros 1996; Guide for the Care and Use of Laboratory Animals 1996).

2.3 Preparation of Blood Peripheral Lymphocytes

The rats were anesthetized with ether and the blood samples were taken by intracardiac puncture, between 8:00 and 9:00 am, in tubes with heparin, 1000 U/ml. The blood was centrifuged at 1000 rpm with a vasculant rotor for 10 min at room temperature. The plasma was discarded and the layer of white cells plus some red blood cells was taken and transferred to tubes with 10 ml of Tyrode buffer pH 7.4 composed (in mM) of 135 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 5.6 glucose, 0.1% bovine serum albumin (BSA) and washed twice by centrifugation at 1000 rpm. The suspensions were placed on Ficoll/Hypaque (1077 g/l) proportion ratio 2:1. After centrifugation at 2000 rpm for 30 min peripheral mononuclear cell layer was taken, washed twice with Tyrode buffer and centrifuged at 1200 rpm for 10 min. To achieve enriched lymphocyte preparation with a minimal monocyte contamination, the resulting pellet was diluted with Roswell Park Memorial Institute Medium 1640 (RPMI) free of bovine serum albumin (BSA) and incubated in a plastic flask for 45 min at 37 °C and 5% of CO_2. After the incubation, lymphocytes, which are non-adherent cells (80–90% of cells), were dislodged from adherent monocytes, transferred to plastic tubes and washed twice. The integrity of isolated lymphocytes was determined by Trypan blue exclusion test, and was greater than 90%.

2.4 *Effect of Agonist and Antagonist of 5-HT$_2$ and 5-HT$_7$ Receptor on [^{3}H]Taurine Transport*

The [^{3}H]taurine transport was determined on lymphocytes of rats treated with fluoxetine in presence of: DOI (1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane) at variable concentrations (in μM) 0.001, 0.01 and 1, or ketanserin at variable concentrations (in μM) 0.01, 0.1, 1 and 10, and in presence of agonist and antagonist of 5-HT$_7$: 4-[2-(Methylthio)phenyl]-N-(1,2,3,4-tetrahydro-1-naphth alenyl)-1-piperazinehexanamide hydrochloride (LP44) at variable concentrations (in μM) 0.01, 0.05, 1 and 10 and (R)-3-[2-[2-(4-Methylpiperidin-1-yl)ethyl] pyrrolidine-1-sulfonyl]phenol hydrochloride (SB269970) at variable concentrations (in μM) 0.03, 0.1, 0.3 and 1. All experiments were performed in duplicates, using~500,000 cells per tube, later than the preincubation, the cells were incubated for 5 min at 37 °C with [^{3}H]taurine 800 nM. After incubation, the process was stopped by rapid vacuum filtration through fiber glass filters (Whatman GF/C), followed by two washed with 5 ml of cold Tyrode buffer. The filters were placed in scintillation vials, dried and counted in 4 ml of Aquasol® (PerkinElmer) in a Packard scintillation counter Tricarb 1900TR Model (efficiency 60–62%) (Guerra et al. 2000; Marquez et al. 2014).

2.5 *[^{3}H]Taurine Transport in Lymphocytes of Rats Treated with Fluoxetine, Effect of Activator and Inhibitor of PKC and PKA*

The transport of [^{3}H]taurine was determined as previously described, in presence of 1-Oleoyl-2-acetyl-sn-glycerol (OAG) a diacylglycerol analogue at variable concentrations (in μM) 10, 20, 30 and with an PKC inhibitor 1-O-hexadecyl-2-O-methyl-rac-glycerol (AMG-C$_{16}$) at variable concentrations (in μM) 5, 10, 20. Incubations with forskolin at variable concentrations (in μM) 10 and 20 were realized, and with an PKA Inhibitor (PKI) at different concentrations (in nM) 1.2, 2, 4 and 8.

2.6 *Statistic Analysis*

Data is expressed as mean ± standard error of the mean, unless otherwise indicated. Statistical comparisons are based on Student t test. Analysis of variance was performed. Results were considered statistically significant if p-values were <0.05.

3 Results

3.1 Effect of DOI and KTS on [^{3}H]Taurine Transport in Lymphocytes of Rats Treated with Fluoxetine

Fluoxetine treatment caused significant increase of taurine transport. DOI, 5-HT_2 receptor agonist, blocked the increase of taurine transport exerted by fluoxetine at 0.01 and 1 μM without effect in Control group. A ANOVA analysis in the Control shows that $F_{(3,27)} = 2.63$ $p > 0.05$ and Fluoxetine $F_{(3,27)} = 6.33$ $p < 0.05$ (Fig. 1). KTS, 5-HT_2 receptor antagonist, did not affect taurine transport in lymphocytes obtained from rats treated with fluoxetine (Fig. 2).

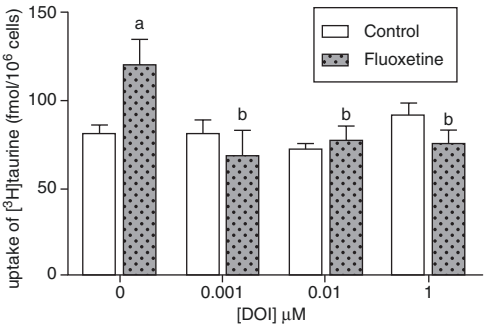

Fig. 1 [^{3}H] Taurine transport in lymphocytes of rats Control and treated with Fluoxetine, in presence of DOI, agonist of 5-HT_2 receptor, at variable concentrations. Each bar represents the mean ± standard error of seven experiments. [a]$p < 0.05$ respect control 0 μM of DOI; [b]$p < 0.05$ respect fluoxetine μM of DOI. Control, $F_{(3,27)} = 2.63$ $p > 0.05$; Fluoxetine, $F_{(3,27)} = 6.33$ $p < 0.05$

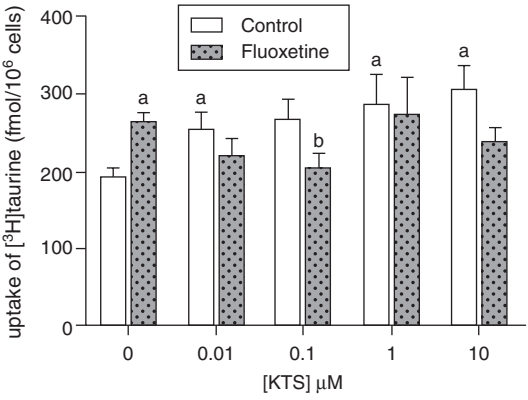

Fig. 2 [^{3}H] Taurine transport in lymphocytes of rats Control and treated with Fluoxetine, in presence of Ketanserin, antagonist of 5-HT_2 receptor, at variable concentrations. Each bar represents the mean ± standard error of six experiments. [a]$p < 0.05$ respect control 0 μM of KTS; [b]$p < 0.05$ respect fluoxetine μM of KTS. Control, $F_{(4,29)} = 2{,}41$ $p > 0.05$, Fluoxetine, $F_{(4,29)} = 1{,}23$ $p > 0.05$

3.2 *Effect of Inhibitors and Activators of Protein Kinase C on Taurine Transport in Lymphocytes of Rats Treated with Fluoxetine*

Differences in taurine uptake between Control and Fluoxetine group was not observed in presence of diacylglycerol analogue at the employed concentrations (Fig. 3). AMG, a PKC inhibitor, did not affect taurine transport in lymphocytes of rat treated with fluoxetine, however, at 20 μM did not offset the fluoxetine effect observed (Fig. 4).

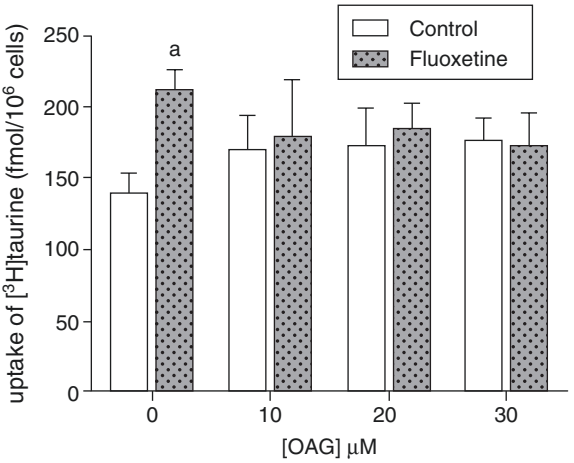

Fig. 3 [^{3}H] Taurine transport in lymphocytes of rats Control and treated with Fluoxetine, in presence of OAG, diacylglycerol analogue, at variable concentrations. Each bar represents the mean ± standard error of five experiments. $^a p < 0.05$ respect control 0 μM of OAG. Control, $F_{(3,19)} = 0.72$ $p > 0.05$, Fluoxetine, $F_{(3,19)} = 0.47$ $p > 0.05$

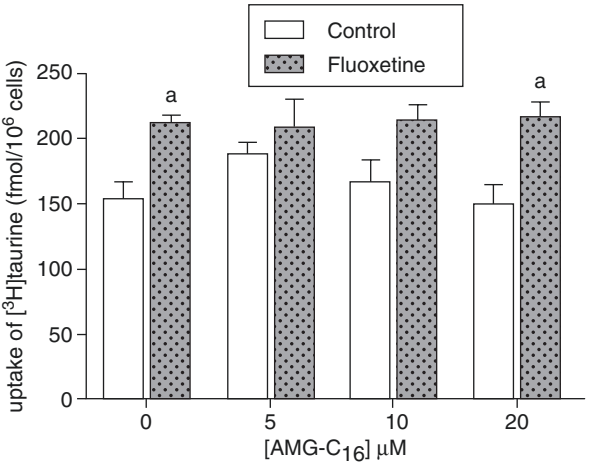

Fig. 4 [^{3}H] Taurine transport in lymphocytes of rats Control and treated with Fluoxetine, in presence of AMG-C_{16}, PKC inhibitor, at variable concentrations. Each bar represents the mean ± standard error of five experiments. $^a p < 0.05$ respect its own control. Control, $F_{(3,19)} = 1.43$ $p > 0.05$, Fluoxetine, $F_{(3,19)} = 0.05$ $p > 0.05$

3.3 *Effect of LP44 and SB269970 on [^{3}H]Taurine Transport in Lymphocytes of Rats Treated with Fluoxetine*

Fluoxetine treatment caused a significantly increase of taurine uptake. In presence of LP44 there are not differences between Control and Fluoxetine Group (Fig. 5). Taurine uptake did not show differences between Control and Fluoxetine in presence of different concentrations of selective antagonist of 5-HT$_7$ receptor, SB269970 (Fig. 6).

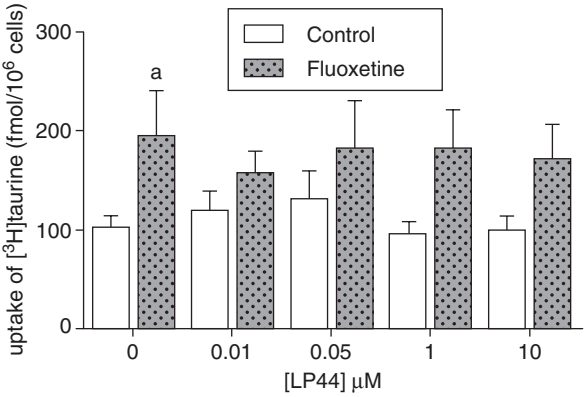

Fig. 5 [^{3}H] Taurine transport in lymphocytes of rats Control and treated with Fluoxetine, in presence of LP44, agonist of 5-HT$_7$ receptor, at variable concentrations. Each bar represents the mean ± standard error of ten experiments. $^a p < 0.05$ respect control 0 μM of LP44. Control, $F_{(4,49)} = 0.63$ $p > 0.05$, Fluoxetine, $F_{(4,49)} = 0.08$ $p > 0.05$

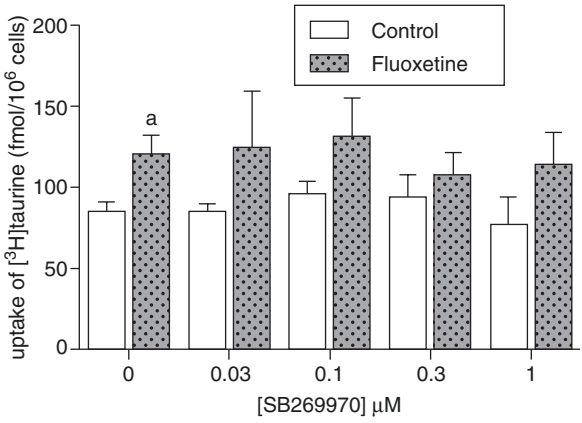

Fig. 6 [^{3}H] Taurine transport in lymphocytes of rats Control and treated with Fluoxetine, in presence of SB269970, antagonist of 5HT$_7$ receptor, at variable concentrations. Each bar represents the mean ± standard error of six experiments. $^a p < 0.05$ respect control 0 μM of SB269970. Control, $F_{(4,29)} = 0.48$ $p > 0.05$, Fluoxetine, $F_{(4,29)} = 0.19$ $p > 0.05$

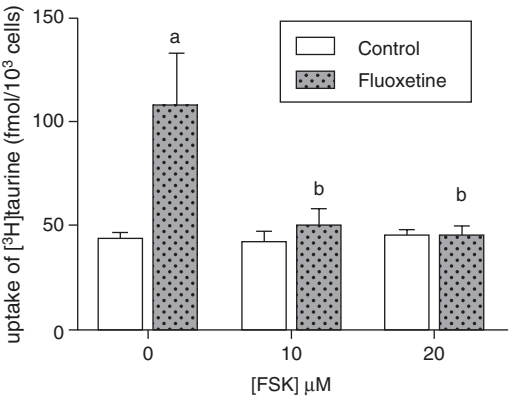

Fig. 7 [^{3}H] Taurine transport in lymphocytes of rats Control and treated with Fluoxetine, in presence of Forskolin, activator of the enzyme adenylyl cyclase, at variable concentrations. Each bar represents the mean ± standard error of five to six experiments. $^a p < 0.05$ respect control 0 µM of Forskolin; $^b p < 0.05$ respect fluoxetine 0 µM of Forskolin. Control, $F_{(2,14)} = 0.22$ $p > 0.05$, Fluoxetine, $F_{(2,15)} = 7.15$ $p < 0.05$

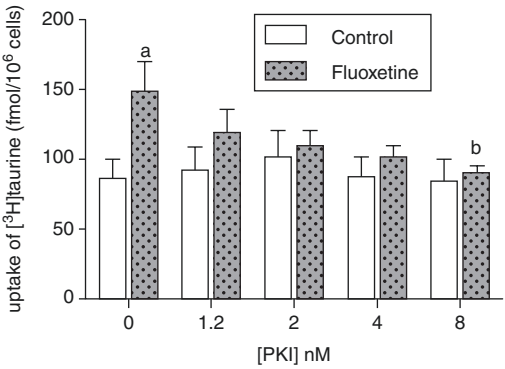

Fig. 8 [^{3}H] Taurine transport in lymphocytes of rats Control and treated with Fluoxetine, in presence of PKI, PKA inhibitor, at variable concentrations. Each bar represents the mean ± standard error of four to six experiments. $^a p < 0.05$ respect control 0 µM of PKI; $^b p < 0.05$ respect fluoxetine 0 µM of PKI. Control, $F_{(4,29)} = 0.16$ $p > 0.05$, Fluoxetine, $F_{(4,27)} = 2.37$ $p > 0.05$

3.4 Effect of Inhibitors and Activators Related to Signaling Pathway of Protein Kinase A on Taurine Transport in Lymphocytes of Rats Treated with Fluoxetine

To test the effect of activation of PKA on taurine transport we used Forskolin at 10 and 20 µM. Forskolin counteract the effect of fluoxetine treatment, since the values of transport capacity in its presence was similar to Control values (Fig. 7). Besides, in presence of the peptide inhibitor of PKA, there was no difference between Control and Fluoxetine group, although at 8 nM taurine uptake was decreased in Fluoxetine group (Fig. 8).

4 Discussion

Our results concerning of lymphocyte [^{3}H]Taurine uptake, as a measure of taurine transport in lymphocytes, in presence of DOI indicate that serotonin 5-HT_2 receptor would be involved in the increase caused by fluoxetine treatment. The presence of ketanserin not modified the effect of fluoxetine treatment on taurine transport, it

seems that 5-HT_2 receptor has not housekeeping activity in lymphocytes. In general, the rate of transport in this group was minor than in other conditions, nevertheless the increase in the capacity of uptake of taurine transporter caused by taurine treatment was maintained in an equivalent rank. KTS has been considered as a pure antagonist, yet several authors report differential effects. Ketanserin has showed biphasic curves in some regions and monophasic curves in others regions of the rat brain (López-Gimenez et al. 2013), it is related with the existence of different subtypes of 5-HT_2 receptors recognized in differential manner by KTS. Some authors refer KTS exert some effects on α1-adrenergic receptors, which are present in lymphocytes (Li et al. 2009), this could be influencing our results.

PKC activation caused diminution on the rate of taurine transport, such as has been reported in other tissues (Kulanthaivel et al. 1991; Han et al. 1996; Lambert and Hansen 2011), this support the hypothesis that the activation of PKC, probably, through 5-HT_2 receptors. Some conditions such as preemclampsia and obesity in pregnancy decrease the activity of TauT in the sincitiotrofoblasto, this has been associated with increase of neuropeptide Y, which activates PKC, it in turn phosphorylates TauT and decrease its activity (Desforges et al. 2013). The presence of OAG in lymphocytes counteracted the effect of fluoxetine in lymphocytes, Fluoxetine group exhibit values similar to Control values. We suggest the use of other PKC activators to evidence the role of this kinase and distinguish the PKC group that participate, considering the existence of three subgroups of PKC, with different structural characteristics (Keenan et al. 1997). Furthermore, has been reported that this DAG analogue could have inhibitory effects independently of PKC and of phosphatidylinositol 3-Kinase (PI3K) in other study models (Brénchard et al. 2009). Other kinases have a role on taurine effects mediated by its transporter, in example taurine inhibit apoptosis of osteoblasts induced by serum deprivation through TauT/ERK signaling pathway (Zhang et al. 2011).

The agonist and antagonist of 5-HT_7 receptors, LP44 and SB269970, respectively (Figs. 5 and 6), no modified the rate of taurine transport. The lack of outcome of the presence either agonist and antagonist would indicate that 5-HT_7 receptors did not participate in the mediation of the effect of fluoxetine treatment on taurine transport in the conditions of this experiments. T cells express predominantly the 5-HT_7 receptor, either naïve and activated T cells (León-Ponte et al. 2007; Urbina et al. 2014). Cellular functions can be influenced by crosstalk between different signaling pathways including protein kinases (Keenan et al. 1997). For this reason, despite of the observed effects with DOI, agonist of 5-HT_2 receptor, a unique role cannot be attributed to this receptor in the modulation of TTAU in fluoxetine treatment. The crosstalk in signaling pathways involving 5-HT_2 and 5-HT_7 receptors in specific conditions such as tumor cells and transfected cells (Raymond et al. 2001; He et al. 2010; Pozzi et al. 2010). Also, there are others serotonin receptors in lymphocytes, such as 5-HT_{1a} receptor (Aune et al. 1993) and 5-HT_3 receptor (Meyniel et al. 1997). As resume of the obtained results we schematize the participation of receptors and kinases evaluated in the modulation of TauT by effect of fluoxetine treatment (Fig. 9). However, as mentioned above this is not the attribution of a definitive role to one of these receptors, but is a key to the continuity of research. The study of other elements of intracellular signaling pathways and the set of agonists and antagonists and the combination of specific drugs is suitable to further assays.

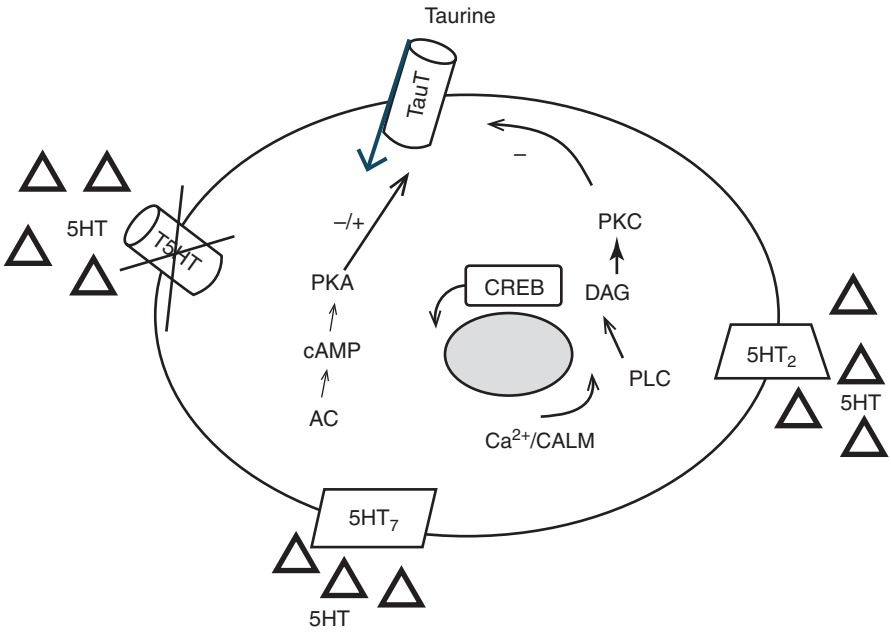

Fig. 9 Schematic representation of potential effect of fluoxetine treatment on TAUT, based on obtained results

5 Conclusion

In summary, this study shows that 5-HT receptors, present in lymphocytes, and their signal pathways would be involved in lymphocyte taurine transport modified by fluoxetine treatment. These relations between different molecules of central nervous system and immune cells and the effect of an antidepressant is a confirmation of the interrelation between both systems, and encourage the continuity of this research.

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Fermented *Asterina pectinifera* with *Cordyceps militaris* Mycelia Induced Apoptosis in B16F10 Melanoma Cells

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Abstract This prime objective of this study was to explore the anti-cancer activity of fermented *Asterina pectinifera* with *Cordyceps militaris* mycelia (FACM) in B16F10 murine melanoma cells. The effect of FACM on cell viability was assessed using MTT assay. Furthermore, the effect of FACM was compared with unfermented *A. pectinifera* on cell viability. The results demonstrated that the fermented FACM extract has a higher inhibitory activity on the proliferation of B16F10 murine melanoma cells than unfermented *A. pectinifera*. In addition, FACM also promoted the expression of pro-apoptotic protein Bax leading to stimulate apoptosis in B16F10 cells. Therefore the present study demonstrates that the FACM might be a potential effective anti-cancer agent, as a result of its stronger anti-proliferative effect and apoptosis inducing effect than *A. pectinifera* or *C. militaris* on melanoma cells.

Keywords *Asterina pectinifera* • *Cordyceps militaris* mycelia • B16F10 murine melanoma • Apoptosis

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1 Introduction

Since a long time, many kinds of mushrooms such as *Phellinus linteus*, *Hericium erinaceus, Ganoderma lucidum*, *Cordyceps militaris* are famous and widely utilized for medicinal preparations in Asian countries especially in China and South Korea (Chen et al. 2016). *Cordyceps militaris* has been the subject of a number of previous studies and they have revealed that *Cordyceps militaris* possesses multiple pharmacological properties such as antioxidant activity (Chen et al. 2004), anti-inflammatory (Won and Park 2005), anti-hyperlipidemia (Yu et al. 2004), improving insulin resistance and insulin secretion (Choi et al. 2004).

Recently, marine animals have been recognized as a rich source of biologically active compounds. Marine polysaccharides isolated from invertebrates have been reported to possess antioxidant activities due to their potential reactive oxygen species (ROS) scavenging capacity (Zhang et al. 2013). *Asterina pectinifera* (*A. pectinifera*) is commonly named as starfish or sea stars due to star-shaped echinoderms and it belongs to the class Asteroidea. Starfish is an extremely rich source of different steroid compounds including free sterols and polar steroid compounds such as polyhydroxy steroids, related mono- and biosides, and oligoglycosides named as asterosaponins (Ivanchina et al. 2013).

In our previous report, we revealed that fermented *A. pectinifera* with *C. militaris* mycelia showed a significant increase in various radical scavenging activities (Kim et al. 2016). We hypothesized that increased radical scavenging activity could result in anti-cancer effect in fermented *A. pectinifera* with *C. militaris* mycelia.

In the present study, we compared the anti-cancer effect of fermented *A. pectinifera* with *C. militaris* mycelia (FACM) with unfermented *A. pectinifera* using B16F10 murine melanoma cells. Moreover, cell cycle analysis, mitochondrial membrane potential, and apoptotic protein expression were evaluated and compared. Finally, it was observed that FACM's increased anticancer activity was a result of the fermentation process of *A. pectinifera* with *C. militaris* mycelia.

2 Materials and Methods

2.1 Reagent

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Industries Inc. (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Missouri, USA). All other chemicals and reagents used in the experiments were of analytical grade. Starfish, *A. pectinifera*, was collected during the breeding season from near Wooduri, Yeosu, South Korea.

2.2 Extract Preparation

First, *A. pectinifera* was inoculated with *C. militaris* mycelia and the inoculated culture was stored in an incubator maintained at 25 °C for 20 days for the fermentation process. After fermentation process, fermented *A. pectinifera* culture was dried in a freeze dryer. Then, the fermented freeze dried culture was dissolved in ten times of distilled water and it was boiled for 2 h. Next, the boiled water extract was filtered through a Whatman No. 41 paper. Finally, the filtrate was evaporated in a rotary evaporator and lyophilized. The same procedure was followed to obtain the unfermented *A. pectinifera* extract except for fermentation step. All extracts were stored at −20 °C until use.

2.3 Chemical Analysis and Amino Acid Composition

Crude protein, crude fat (ether extract) and total ash content of FACM were determined by adapting to the methods of the Association of Official Analytical Chemists (AOAC 1990). Amino acid composition was analyzed using following steps. First, FACM was mixed with 10 mL of 6 N HCl. After that, all the sample test tubes were purged with N_2 gas and then the samples were hydrolysed in a dry oven maintained at 110 °C for 24 h. The hydrolysed samples were then evaporated and added with sodium-distilled buffer (pH 2.2). Samples were then filtered through a syringe filter (0.45 μm) and amino Acid composition was determined by measuring the absorbance at 440 and 570 nm.

2.4 Cell Culture

B16F10 cell line was obtained from American Type Culture Collection (ATCC CRL-6475). It was cultured in Dulbeco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified incubator of 5% CO_2.

2.5 Measurement of Cell Viability Using MTT Assay

B16F10 cells were cultured in 96-well plates at a density of 4×10^3 cells/well with 200 μL of the medium and incubated at 37 °C for 24 h. After the incubation period, the cells were treated with different concentrations of FACM and again incubated at 37 °C for 24 h. Then MTT stock solution was added and incubated for another 3 h

at 37 °C. Next, 150 μL of DMSO was added to dissolve the dark blue formazan crystals. The absorbance at 540 nm wavelength was measured using a microplate reader (Tecan, Austria). Each cell viability data of treatment groups was expressed as a percentage of corresponding control groups.

2.6 Determination of Morphological Changes

B16F10 cells were seeded in 6-well plates at a density of 1.0×10^5 cells per well and incubated with DMEM at 37 °C under 5% CO_2 for 24 h. Then, cells were subjected to treatments of FACM different concentrations (0.05, 0.1, and 0.2 mg/mL) and incubated for another 24 h at 37 °C. Morphology of cells was observed using a phase-contrast microscope (Nikon, Japan). Images were recorded at 200× magnification.

2.7 Cell Cycle Analysis

Distribution of cell cycle was analyzed using a flow cytometer**.** The B16F10 cells were seeded in 6-well plates at a density of 1.0×10^5 cells per well and incubated for 24 h at 37 °C. After 24 h adherence, cells were treated with three different concentrations of FACM (0.05, 0.1, and 0.2 mg/mL) and incubated for another 24 h at 37 °C. Next, the cells were harvested by trypsin and washed with cold PBS (pH, 7.4). Subsequently, the cell pellet was resuspended in 300 μL of PBS, fixed with 1 mL of 70% ice-cold ethanol and stored at −20 °C for overnight. Cells were stained with 50 μg/mL propidium iodide solution containing 10 μg/mL of RNase, and 0.5% Tween 20 in PBS at 37 °C for 30 min in the dark environment. DNA fluorescence was measured using a Becton Dickinson (BD Biosciences, Madrid, Spain) FACScalibur flow cytometer. Cells were analysed for 10,000 events, and the relative distribution of cells in each cell cycle phase (Sub-G1, G0/G1, S, and G2/M) was displayed in histograms.

2.8 Measurement of Mitochondrial Membrane Potential (MMP)

Mitochondrial membrane potential was measured using Rhodamine 123 fluorescent dye. After cell treatment with different concentrations of FACM, Rhodamine 123 (20 μg/mL) was added to the treated cells and incubated at 37 °C for 30 min in the dark. Subsequently, the cells were washed twice with PBS and the fluorescence was measured using Flow cytometer. Data were analysed using FlowJo v7.6.1 software (Ashland, OR, USA).

2.9 Western Blot Analysis

B16F10 cells were seeded in 100 mm culture plates and incubated at 37 °C for 24 h prior to 24 h treatment with three different concentrations of FACM. After 24 h treatment of FACM, cells were harvested and lysed using PRO-PREP lysis buffer. The protein concentration was analyzed by Bradford assay method (Bio-rad) using bovine serum albumin (BSA) as the standard. The cell lysate was subjected to 8–12% SDS-PAGE to separate proteins and separated proteins in the gel were transferred to PVDF membrane. Then the membrane was blocked by non-fat dry milk (5% w/v) in TBST for 1 h at room temperature. Next, the membrane was incubated with specific primary antibodies at 4 °C overnight. After primary antibody incubation, the membrane was washed with TBST and incubated with the species appropriate HRP-conjugated secondary anti-bodies (at 1:1000 dilution) at room temperature for 1 h. The blots were developed by using ECL chemiluminescence detection reagent and blots were visualised using a charge-coupled device system (LAS-3000; Tokyo, Japan).

2.10 Statistical Analysis

Data were analyzed by using one-way ANOVA followed by Dunnett's tests. Values were expressed as the mean ± SD. When the calculated probability values of $P < 0.05$ were considered statistically significant.

3 Results

3.1 Chemical Composition of FACM

The chemical compositions of FACM and *A. pectinifera* are shown in Table 1. The amounts of dry matter, crude protein, crude carbohydrates, and ash of FACM were 99.1%, 83.7%, 7.2%, and 7.4% respectively. The amounts of dry matter, crude protein, crude carbohydrates, and ash of *A. pectinifera* were 80.5%, 72.2%, 3.53%, and 2.9% respectively.

Table 1 Chemical composition of FACM (%)

Chemical composition	*A. pectinifera*	FACM
Dry matter	80.5 ± 0.49	99.1 ± 0.10
Crude protein	72.2 ± 1.08	83.7 ± 0.02
Crude carbohydrates	3.53 ± 0.04	7.2 ± 0.05
Ash	2.9 ± 0.04	7.4 ± 0.41

Table 2 Amino acid composition (μg/mL)

Amino acid composition	*A. pectinifera*	FACM
Taurine	77.65	43.92
Aspartic acid	37.82	17.54
Glutamic acid	70.59	52.69
Proline	27.11	12.03
Glycine	156.81	12.11
Alanine	56.74	17.60
Valine	49.97	12.50
Cysteine	7.78	10.30
Methionine	25.83	8.058
Isoleucine	38.22	10.41
Leucine	80.90	28.55
Tyrosine	46.12	16.06
Lysine	82.67	34.75
Histidine	16.23	8.88
Arginine	73.76	61.22
Serine	37.04	16.84
Phenylalanine	56.40	19.41
Threonine	35.23	13.76
Total	977.87	396.70

3.2 Amino Acid Composition of FACM

The amino acid composition of FACM and *A. pectinifera* are shown in Table 2. According to the experimental results, total amino acid contents of *A. pectinifera* and FACM were 977.87 μg/mL and 396.70 μg/mL. Glycine, Lysine, Leucine, Taurine in *A. pectinifera* were 156.81 μg/mL, 82.67 μg/mL, 80.90 μg/mL, and 77.65 μg/mL, respectively. Arginine, Glutamic acid and Taurine were the most plentiful free amino acids available in FACM. It is clear that after fermentation of *A. pectinifera,* total and free amino acid contents have been decreased except Cysteine.

3.3 FACM Attenuates the Cell Viability of B16F10 Cells

The percentage cell viability of B16F10 after treatment with FACM is shown in Fig. 1; FACM inhibited B16F10 cell proliferation in a concentration-dependent manner. B16F10 cells were incubated with FACM extracts of different concentrations (0.25–0.2 mg/mL) for 24 h and then cell viability was assessed by MTT assay. The cell viability results of unfermented *A. pectinifera* were compared with FACM findings. Comparison results suggested that treatment of unfermented *A. pectinifera* and *C. militaris* mycelia increased cell viability, whereas treatment of FACM decreased cell viability. Consequently, we propose that FACM can exert an anticancer effect on B16F10 cells.

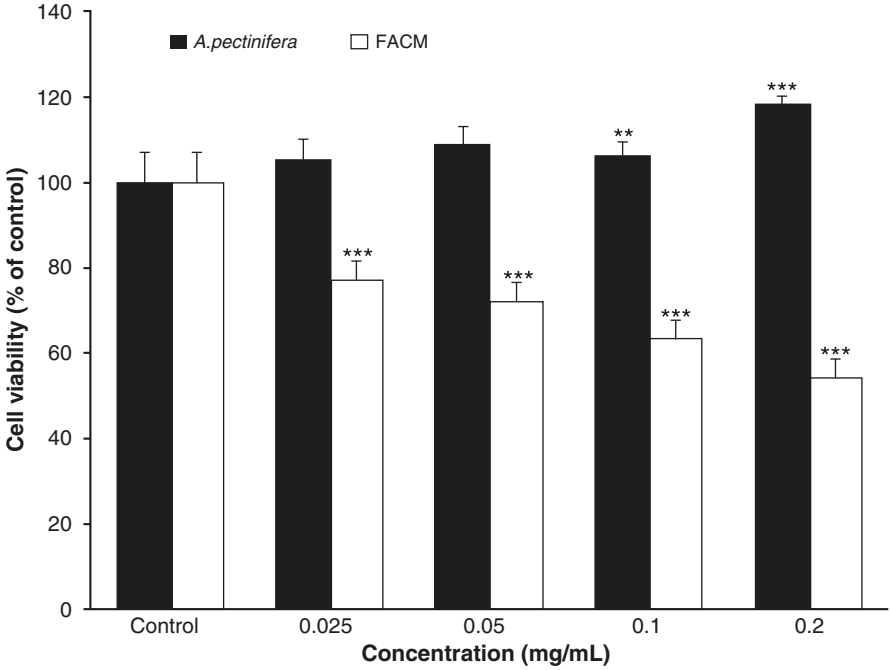


Fig. 1 FACM decreases the cell viability. Values are expressed as mean ± SD from three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control

3.4 FACM Changes the Morphology of B16F10 Cells

Microscopic inspection by an inverted contrast microscope revealed that FACM influenced the morphology of B16F10 cells. After 24 h, the cells were round and well attached to the bottom of the plates. The different concentrations of FACM changed the morphology of cells.

After 24 h, the cells were confluent and round in the control culture, whereas FACM treated cells were detached, irregular shapes, and low density (Fig. 2).

3.5 FACM Increases Sub-G1 Phase

The distribution of B16F10 cells in different phases of the cell cycle was analyzed after treatment with three different concentrations of FACM (0.05, 0.1, and 0.2 mg/mL) at 37 °C for 24 h. The FACM promoted the number of B16F10 cells in sub-G1 phase in a dose-dependent manner. The sub-G1 phase of control group was 3.43 ± 1.20%, and 0.05, 0.1, and 0.2 mg/mL FACM treated groups showed 4.83 ± 2.14%, 8.95 ± 2.42%, and 10.22 ± 2.26%, respectively (Fig. 3).

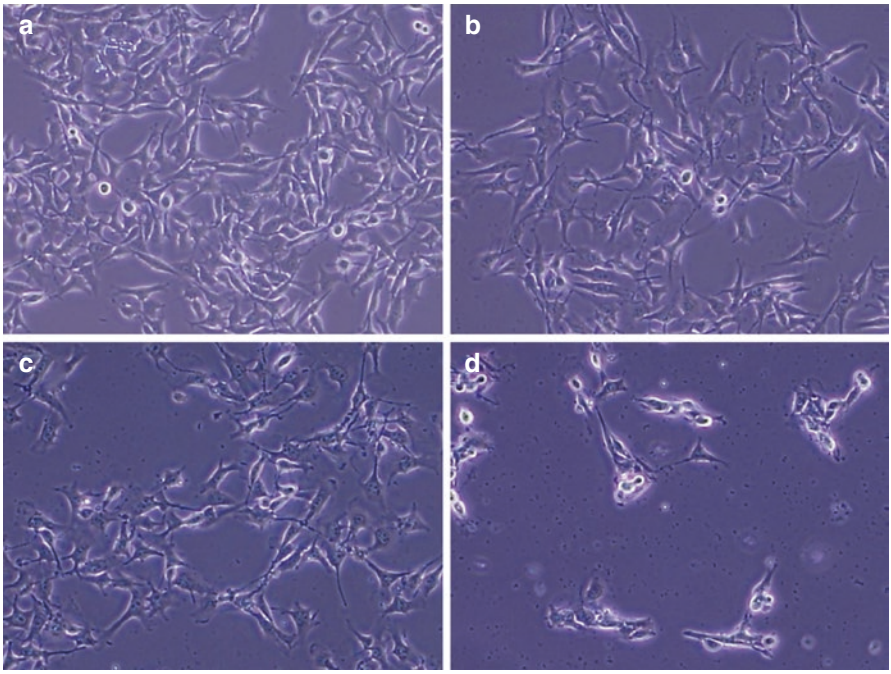


Fig. 2 FACM changes the morphology of B16F10 cells. Cells were incubated with three different concentrations of FACM for 24 h, and images were taken by a phase-contrast microscope. (**a**) Control, (**b**) FACM (0.05 mg/mL), (**c**) FACM (0.1 mg/mL), (**d**) FACM (0.2 mg/mL)

3.6 FACM Induces Mitochondrial Membrane Potential Depolarization

Changes in the mitochondrial membrane potential were evaluated using flow cytometry analysis. Figure 4 depicts the percentage of cells with a decrease in fluorescence intensity, which is directly related to the loss of mitochondrial membrane potential. The FACM resulted in the loss of MMP in concentration dependent way. The treatment of FACM induced mitochondrial membrane potential (MMP) depolarization in B16F10 cells. The control group showed 9.40 ± 2.32% of MMP and FACM (0.05 mg/mL, 0.1 mg/mL, and 0.2 mg/mL) induced MMP depolarization by resulting 28.35 ± 3.36%, 33.31 ± 4.24%, and 40.43 ± 3.65%, respectively. This data support the fact that FACM induces B16F10 cell death through mitochondrial apoptotic pathways.

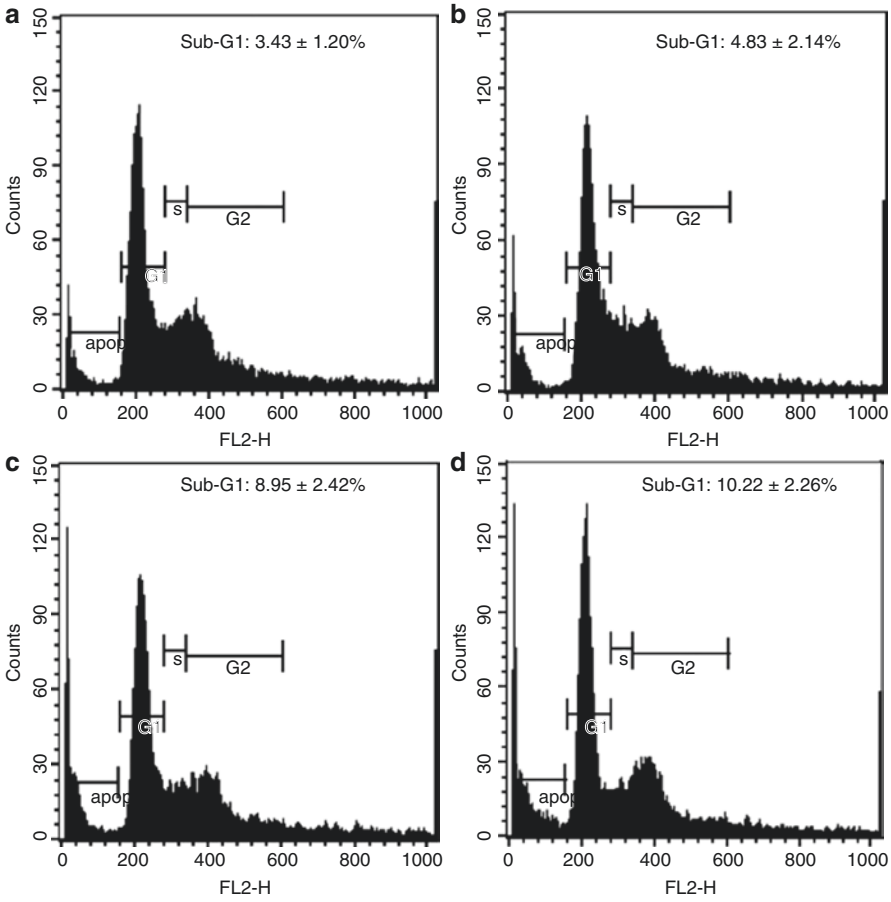


Fig. 3 FACM promotes Sub-G1 phase in B16F10 cells. After treatment of FACM for 24 h, the cell cycle distribution was analyzed by flow cytometer. (**a**) Control, (**b**) FACM (0.05 mg/mL), (**c**) FACM (0.1 mg/mL), (**d**) FACM (0.2 mg/mL)

3.7 Effect of FACM on Apoptotic Protein Expression

Furthermore, the expression levels of anti-/pro-apoptotic proteins were evaluated using the western blotting technique to figure out whether the apoptosis was mediated by the mitochondrial apoptotic pathways. The FACM treatments increased the levels of the pro-apoptotic Bax and reduced the levels of anti-apoptotic Bcl-2 protein (Fig. 5). The results suggested that the regulators of mitochondrial outer membrane permeabilization, including Bax and Bcl-2, were modulated by FACM treatments.

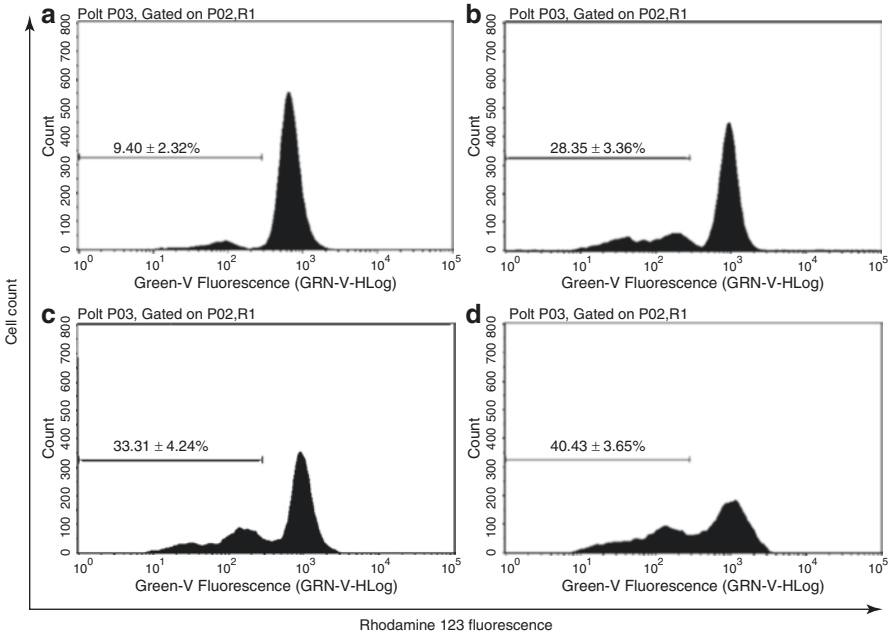
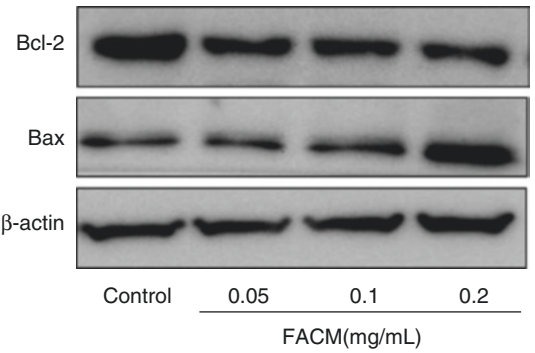


Fig. 4 FACM induces mitochondrial membrane potential (MMP) depolarization in B16F10 cells. After treatment of FACM for 24 h, B16F10 cells were stained with rodamine 123 for 30 min, and then immediately subjected to flow cytometric analysis. (**a**) Control, (**b**) FACM (0.05 mg/mL), (**c**) FACM (0.1 mg/mL), (**d**) FACM (0.2 mg/mL)

Fig. 5 Effect of FACM on apoptotic protein expression in B16F10 cells



4 Discussion

The last couple of decades, searching of bioactive secondary metabolites from microorganisms, plants and marine organism organisms has been a subject of many scientific investigations. As a result, a considerable amount of secondary metabolites has been identified from above mentioned sources (Choi et al. 1999).

Some of the secondary metabolites are found to possess strong biological activities such as antifungal, anti-inflammatory and antiviral activities and those compounds have become excellent sources for new and effective drug designs. Especially, many marine organisms are found to have bio active materials with inhibitory effects on a variety of microorganisms (Choi et al. 1999). Marine organisms have become such a strong source of potential bioactive secondary metabolites due to their developed mechanisms to survive in harsh environments and still every year a considerable number of new materials are being discovered (Proksch et al. 2002).

In this study, we also attempted to discover a new potential compound from the marine natural product, *A. pectinifera* after fermentation with mushroom mycelia, *C. militaris*. Interestingly, after fermentation of *A. pectinifera* (FACM), it showed a decrease amino acid composition compared to unfermented *A. pectinifera.* Moreover, taurine was the most abundant amino acid in both unfermented *A. pectinifera* and fermented *A. pectinifera* (FACM).

Cell viability studies revealed that FACM significantly decreased the proliferation of B16F10 cells. This decrease of proliferation was accompanied by increasing the incidences of apoptosis in B16F10 cells.

The group of proteins known as Bcl-2 family members is the central player of apoptosis. Because they form the interface between the early signalling events engaging the cells into the apoptotic process and the later events confer their apoptotic characteristics to the cells, leading to their elimination by the immune system (Adams and Cory 2007). Classically, the Bcl-2 family has been divided into 3 subfamilies: anti-apoptotic proteins (e.g. Bcl-2, Bcl-xL), pro-apoptotic proteins (Bax, Bak, Bok), and BH3-only proteins (e.g. Bid, Bad, Puma, Bim) (Renault et al. 2016). Our results revealed that FACM decreased Bcl-2 (anti-apoptotic protein) and increased Bax (pro-apoptotic proteins).

Bcl-2 family members regulate the apoptosis through a network of protein signalling pathways that lead to loss the integrity of outer mitochondrial membranes (Renault et al. 2016). FACM induced mitochondrial membrane potential depolarization in a dose-dependent manner. Furthermore, FACM promoted apoptosis through the regulation of Bcl-2 family proteins in B16F10 cells.

5 Conclusion

In summary, the present study shows that the compound (FACM) obtained from the fermentation of starfish with *C. militaris* mycelia resulted in a significant decrease in B16F10 cells viability compared to unfermented starfish. Taken together, these results showed that FACM could induce apoptosis via the regulation of Bcl-2 family proteins. Moreover, we figured out that fermentation of *A. pectinifera* using mushroom mycelia could enhance its bioactivity. Further studies are required to understand the molecular mechanisms underlying the function of FACM and identification of bioactive substances of FACM.

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Antioxidant Activity of Extract from the Cephalothorax of *Fenneropenaeus chinensis*

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Abstract We investigated the antioxidant activity of taurine rich water extract from the cephalothorax of Fenneropenaeus chinensis (FCC). The antioxidant potency of water extract from FCC was assessed using various assay methods, such as DPPH (1,1-diphenyl-2-picrylhydrazyl), alkyl radical scavenging activity, ABTS (2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonic acid ammonium salt)) radical scavenging activity and Ferric reducing antioxidant power (FRAP) assay. The DPPH and alkyl radical scavenging activities of FCC were dose-dependently increased. The lipid peroxidation was estimated using ferric thiocyanate (FTC) assay and thiobarbituric acid (TBA) methods. However, a higher lipid peroxidation activity was observed in TBA method than FTC method. The results of the present study suggested that the FCC extract potentially scavenged the free radical and reduced oxidative stress. Therefore, the present study is concluded that the FCC extract could be a potential source of antioxidant activity.

Keywords Antioxidant activity • *Fenneropenaeus chinensis* • Lipid peroxidation

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Abbreviations

ESR Electron spin resonance
FCC Cephalothorax of *Fenneropenaeus chinensis*
ROS Reactive oxygen species

1 Introduction

Oxidative stress is defined as an imbalance between antioxidants and free radicals can potentially lead to cellular damage. Excessive reactive oxygen species (ROS) generation disrupts the cellular antioxidant defense system and may lead to oxidative stress (Kim et al. 2016). A large number of studies have been supported the hypothesis that oxidative damage to the macromolecules such as DNA, lipids, and proteins may lead to the development of cardiovascular disease, cancer, and neurodegenerative diseases (Suzek et al. 2015). Bioactive compounds derived from natural resources play a vital role in the development new and promising drugs to overcome these problems. Taurine, a sulfur-containing β-amino acid is found in millimolar concentrations animal tissues. It has various biological activities such as maintaining calcium homeostasis, osmoregulation, removal of hypochlorous acid, and stabilizing the cell membranes. Some of the recent studies have been demonstrated that taurine could act as direct antioxidant by scavenging the ROS and/or as an indirect antioxidant by protect the membrane integrity from various oxidant injuries (Gürer et al. 2001). Chinese shrimp (*Fenneropenaeus chinensis*) is an important migratory marine species of Bohai Sea and Yellow Sea. It has significant commercial value to the Chinese aquaculture and fishing industries (Wang et al. 2016). Chinese shrimp (*F. chinensis*) is rich in nutraceutical content including the amino acids taurine (Laidlaw et al. 1990). Nowadays, there is a general concern related to the environmental pollution by seafood wastes represent due to their improper management. In this regard, every year about 18 to 30 million tons of these wastes are dumped around the world which creating a serious environmental pollution (Salazar-Leyva et al. 2016). We could overcome this problem by isolation of potential bioactive compounds from these wastes. Therefore, the present study was estimates the amino acid taurine and investigates its antioxidant and lipid peroxidation effect of water extract of cephalothorax *F. chinensis* (FCC).

2 Methods

2.1 Reagents

2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) as diammonium salt (ABTS), 2,2-azobis(2-amidinopropane) hydrochloride (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and (4-pyridyl-1-oxide)-N-tert-butylnitrone (4-POBN),

linoleic acid, ammonium thiocyanate, ferric chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of the highest grade available commercially.

2.2 *Preparation of Extracts from the Cephalothorax of* F. chinensis

F. chinensis purchased from complex fish market in 12, Soraeyeok-ro Namdong-gu Incheon, South Korea. To eliminate salt, *F. chinensis* washed with water and cut cephalothorax. On 500 g of cephalothorax added to 2 L of distilled and extracted for 1 h at 95 °C. The extraction was filtered with whatman No. 41 filter paper at room temperature (R.T). The filtrate was then evaporated by an rotary flash evaporator (EYELA, Tokyo, Japan) at 40 °C and then lyophilized in a freeze-dryer (Samwon, Busan, South Korea).

2.3 *Chemical Analysis and Amino Acid Composition*

Proximate compositions of FCC, including moisture, crude protein, crude carbohydrates, crude fat and crude ash were analyzed according to AOAC methods (1990). Carbohydrate content was obtained by phenol–H_2SO_4 methods. For amino acid analysis, FCC samples (80 mg) were combined with 10 mL of 6 N HCl. After N_2 gas was used to purge the samples in Anti-oxidant Effect of FCC extract in the test tube, the samples were hydrolysed in a dry oven at 110 °C for 24 h. The hydrolysed samples were then evaporated and a sodium-distilled buffer (pH2.2) was added. Samples were then filtered through a syringe filter (0.45 μm) and analyzed using an amino acid autoanalyzer (Pharmacia Biotech Biochrom 20, Ninhydrin Method). Amino acids were determined by absorbance at 440 and 570 nm.

2.4 *Radical Scavenging Activity by ESR Measurement*

2.4.1 DPPH Radical Scavenging Activity

The DPPH radical scavenging activity was measured using an ESR spectrometer (JES-FA machine; JOEL, Tokyo, Japan) according to the technique described by Kim et al. (2008). Sixty microliters of each sample (or ethanol itself as control) was added to 60 μL of DPPH (60 μM) in ethanol. After 10 s of vigorous mixing, the solutions were transferred to Teflon capillary tubes and fitted into the cavity of the ESR spectrometer. The spin adducts were determined by the ESR spectrometer exactly 2 min later under the following measurement conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2G, microwave power 5 mW, gain 6.3 × 105, and temperature 298 K.

2.4.2 Alkyl Radical Scavenging Activity

Alkyl radicals were generated by AAPH. The PBS (pH7.4) reaction mixtures containing 40 mM AAPH, 40 mM 4-POBN and indicated concentrations of tested samples were incubated at 37 °C in a water bath for 30 min and then transferred to 100 μL Teflon capillary tube. The spin adducts were recorded by an ESR spectrometer. The measurement conditions were as follows: central field 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2G; microwave power, 1 mW; gain, 6.3 × 105; and temperature, 298 K.

2.5 ABTS Radical Scavenging Activity

For ABTS assay, the procedure followed the method of Erkan et al. (2008) with some modifications. The stock solutions included 7.4 mM $ABTS^{\cdot+}$ solution and 2.6 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowed them to react for 14 h at R.T in the dark. The mixture was diluted and its absorbance was adjusted to 0.70 ± 0.02 at 734 nm. To determine the scavenging activity, 0.9 mL of ABTS reagent was mixed with 0.1 mL of extracts and the absorbance was measured at 734 nm after 6 min of reaction at R.T, using ethanol as a control. The antioxidant activities of FCC extracts were expressed by Trolox equivalents antioxidant capacity (TEAC), as mM Trolox equivalents/mg extract.

2.6 Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP assay was carried out by the method of Benzie and Strain (1996) with minor modification. The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}-TPTZ). To conduct the assay, a 3 mL aliquot of a FRAP reagent, a mixture of 0.3 M acetate buffer, 10 mM TPTZ in 40 mM HCl, and 20 mM ferric chloride (10:1:1 v/v/v), were combined with 1 mL of FCC extract. To determine the antioxidant capacity of the samples, the absorbance values were compared with those obtained from the standard curves of $FeSO_4$ (0–5 mM). The antioxidant capacity values were expressed as mM $FeSO_4$ equivalent in mg extract (mM $FeSO_4$ eq./mg extract).

2.7 Ferric Thiocyanate (FTC) Method

The FTC method was used to determine the amount of peroxide at the initial stage of lipid peroxidation. The method was followed as previously described. A reaction solution, containing extracts (4 mL, 1.0 mg/mL), 2.51% linoleic acid emulsion (4 mL), phosphate buffer (8 mL, 0.05 M, pH 7.0) and D.W (3.9 mL) was placed in a glass vial with a screw cap and mixed with a vortex mixer. The reaction mixture was incubated at 40 °C in the dark and the degree of oxidation was measured according to the thiocyanate method. To 0.1 mL of reaction mixture, 9.7 mL of 75% ethanol

and 0.1 mL 30% ammonium thiocyanate were added. Exactly 3 min after the addition of 0.1 mL of 0.02 M $FeCl_2$ in 3.5% HCl, the peroxide value was determined by recording the absorbance at 500 nm every 2 days until the absorbance of the control reached a maximum. The positive and negative controls were subjected to the same procedures as the sample, for the negative control, only the D.W was added instead of sample, and for the positive control, sample was replaced with α-tocopherol.

2.8 Thiobarbituric Acid (TBA) Method

The samples prepared for FTC method were used for this assay. To 1 mL of the sample solution in a 10 mL tube, was added 2 mL of 20% aqueous trichloroacetic acid and 2 mL of 0.67% aqueous thiobarbituric acid as previously described. The mixture was placed in a boiling water bath for 10 min. After cooling, it was centrifuged at 3000 rpm for 20 min. Absorbance of the supernatant was measured at 532 nm. Antioxidant activity was based on the absorbance of the final day of TBA assay.

2.9 Cell Culture

Chang liver cells purchased from American Type Culture Collection (ATCC CCL-13™) were cultured at 37 °C in humidified 5% CO_2, 95% air mixture in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated 10% fetal bovine serum (FBS), 100 U/mL of penicillin and 100 μg/mL of streptomycin. Adherent cells were detached by trypsin-EDTA and plated onto 6- or 48-well plates at 70–80% confluence.

2.10 Cell Viability

The cell viability was estimated by MTT assay. Chang liver cells were seeded in 48-well plate at a concentration of 7.0×10^3 cells/well. After 20 h, the cells were treated with different concentrations of various FCC extract, and incubated at 37 °C for 24 h. Thereafter, a 200 μL of MTT stock solution (0.5 mg/mL) was added and incubated for 2–4 h. Then, the supernatants were aspirated and the formazan crystals in each well were dissolved in 150 μL of DMSO. Absorbance was measured by spectroflurometer (SpectraMax M2/M2e, CA, USA) at a wavelength of 540 nm. Relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The optical density of the formazan formed in the control cells was taken as 100% viability.

2.11 Statistical Analysis

Statistical significance was determined by Student's t-test. Each value was expressed as the mean ± SEM. Differences were considered statistically significant when the calculated P value was less than 0.05.

3 Results

3.1 Chemical Composition of Water Extracts from FCC

The chemical composition of water extracts of FCC is shown in Table 1. In the present study, the content of dry matter, crude protein, Crude carbohydrates, Crude fat and ash content were recorded as 97.04%, 78.2%, 3.19%, 7.83% and 12.3%, respectively.

3.2 Amino Acid Composition of Water Extracts from FCC

A list of 18 amino acids of FCC extracts were identified and given in Table 2. The highest content of amino acid is recorded as arginine (183.46 µg/mL) followed by glycine (154.65 µg/mL), lysine (150.84 µg/mL) and taurine (130.57 µg/mL). Among the amino acids recorded, taurine is observed as fifth highest content of the extracts.

Table 1 Chemical Composition of FCC extract

Chemical composition (%)	FCC extract
Dry matter	97.04 ± 0.85
Crude protein	78.2 ± 0.78
Crude carbohydrates	3.19 ± 0.13
Crude fat	7.83 ± 0.81
Ash	12.3 ± 0.61

Table 2 Amino acid composition of FCC extract

Amino acid composition (µg/mL)	FCC extract
Taurine	130.57
Aspartic acid	42.03
Threonine	43.10
Serine	41.20
Glutamic acid	70.10
Glycine	154.65
Alanine	148.49
Valine	70.03
Cysteine	7.85
Methionine	45.17
Isoleucine	55.22
Leucine	117.85
Tyrosine	94.89
Phenylalanine	91.85
Lysine	150.84
Histidine	21.88
Arginine	183.46
Proline	114.02
Total	1583.19

3.3 Radical Scavenging Activity by ESR Measurement

DPPH has been widely used to evaluate the free radical scavenging potential of various antioxidant substances (Kim et al. 2014). The FCC extract showed scavenging activity of DPPH radicals. The highest IC_{50} value was 333.49 ± 0.11 μg/mL (Table 3). The alkyl radical spin adduct of 4-POBN/free radical was generated from AAPH, and a decrease the ESR signals was observed in FCC water extracts. The FCC extracts showed alkyl radical scavenging activities 215.42 ± 0.03 μg/mL.

3.4 ABTS Radical Scavenging Activity

The trolox equivalent antioxidant capacity (TEAC) assay is widely used to assess the amount of radicals that can be scavenged by an antioxidant, which is based on the antioxidant ability to react with $ABTS^{\bullet+}$ generated in the system. The TEAC value is assigned by comparing the scavenging capacity of trolox. An increased TEAC value indicates a higher level of antioxidant activity (Fan et al. 2012). The $ABTS^{\bullet+}$ free radical scavenging capacity of the FCC are shown in Table 4. The TEAC value of the FCC is 0.502 ± 0.009 mM Trolox eq. per mg extract.

3.5 (Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay measures the antioxidant effect of any substances in the reaction medium. The potential of reducing power is evaluated based on the reducing ability of sample from Fe^{3+} to Fe^{2+} ions (Uchegbu 2015). The antioxidant capacities of FCC is 0.021 ± 0.004 mM $FeSO_4$ eq./mg extract (Table 4).

Table 3 DPPH and Alky radical scavenging activity of the FCC extract by ESR measurement

Sample	DPPH radical scavenging activity (IC_{50} μg/mL)	Alkyl radical scavenging activity (IC_{50} μg/mL)
FCC	333.49 ± 0.11	215.42 ± 0.03

Means ± SD of determinations were made in triplicate experiments

Table 4 Values for ABTS radical scavenging, FRAP from the FCC extract

Sample	TEAC (mM Trolox eq./mg extract)[a]	FRAP (mM $FeSO_4$ eq./mg extract)[a]
FCC	0.502 ± 0.009	0.021 ± 0.004

TEAC Trolox equivalent antioxidant capacity, *FRAP* ferric reducing antioxidant power
[a]Means ± SD of determinations were made in triplicate experiments

3.6 *Lipid Peroxides Assay*

Effect of FCC extract on peroxidation of linoleic acid emulsions was evaluated using FTC and TBA methods and compared with vitamin C and Vitamin E as standard (Hwang et al. 2012). The effects of FCC extract and vitamin C and Vitamin E are shown in Fig. 1. The FTC and TBA assay methods were used to estimate the initial and final stages of lipid peroxidation of reactions respectively (Jegadeesh

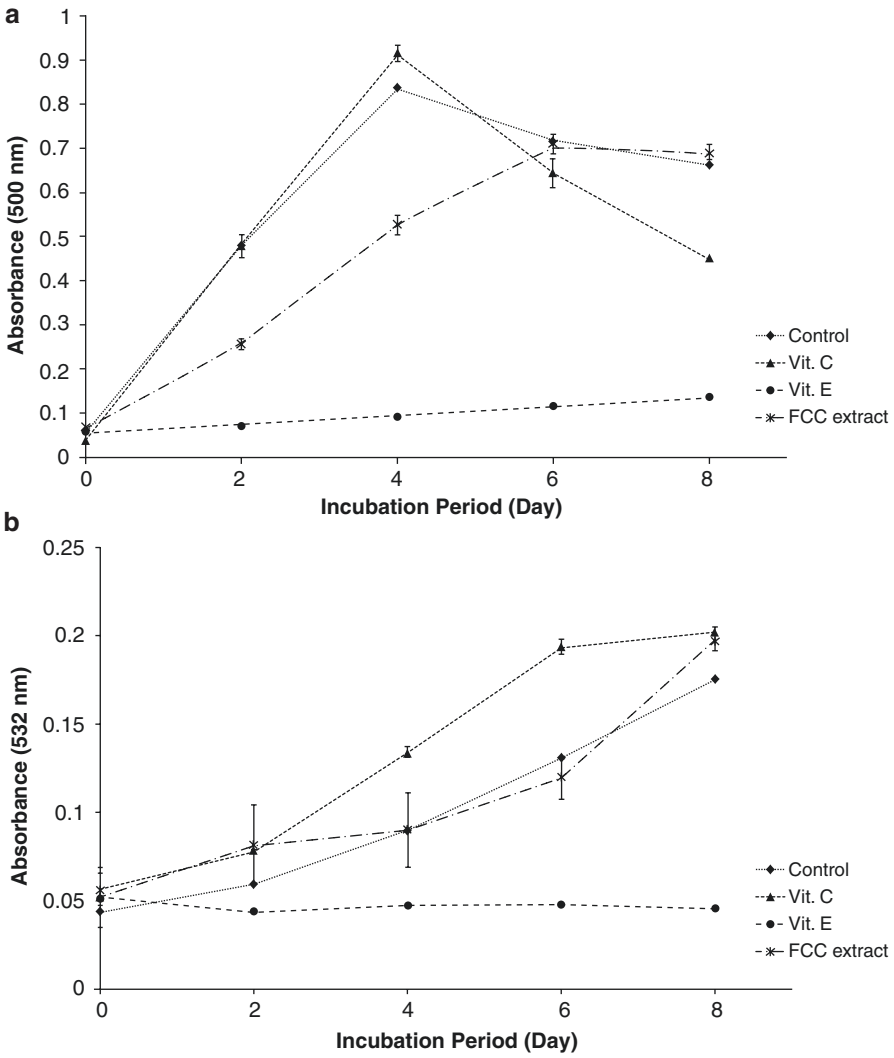

Fig. 1 Inhibitory effect of the FCC extract on lipid peroxidation using FTC (a) and TBA (b) methods at the concentration of 1 mg/mL

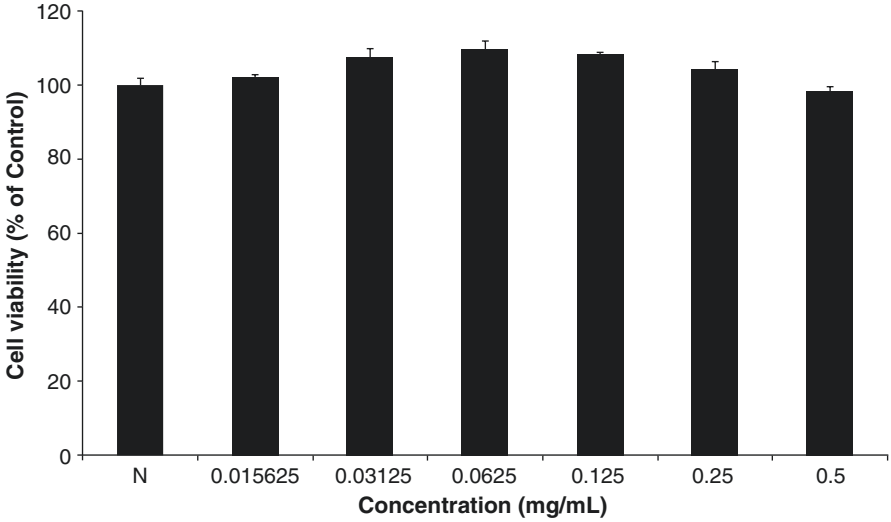

Fig. 2 Cytotoxic effect of FCC extract in Chang cells

et al. 2014). The results indicate that FCC has better (low) lipid peroxidation than vitamin C until almost day 6. The FTC lipid peroxides activity was observed in descending order of vitamin C > FCC extract > Vitamin E (Fig. 1a). Similarly, the TBA assay results also showed low levels of lipid peroxidation in vitamin E. however, the lipid peroxidation activity of vitamin C and FCC extracts gradually increased (Fig. 1b).

3.7 Cytotoxic Effect of FCC Extract

The cytotoxicity of FCC extract was analyzed by MTT assay. The cultured Chang cell were incubated with increasing concentrations of FCC extract up to 0.5 mg/mL (Fig. 2). The results showed that extracts from FCC were not toxic at any of the concentrations tested.

4 Discussion

The results of proximate composition and amino acid profiles are emphasis the nutraceutical value of cephalothorax of *F. chinensis*. The extract contains a high amount of protein (78.2%) followed by fatty acid (7.83%), carbohydrate 3.19%). Almost 18 amino acids are recorded in this study including taurine. The present

study was observed a moderate amount of taurine (130.57 μg/ml) from the cephalothorax of *F. chinensis*. Taurine is an important amino acid presents at higher concentrations in various animal tissues, especially heart, retina, skeletal muscle, brain, large intestines, plasma and blood cells. It has been involved in various biological and physiological functions such as antioxidant neurotransmission, osmoregulation, membrane integrity and so on (El-Sayed 2014). The FCC water extract was potentially scavenged the free radicals that generated by DPPH and AAPH and measured by ESR spectrophotometers. The increased radical scavenging activity of the extracts is highly attributed to its elevated antioxidant activity and reducing power (Gordon 1990; Natarajan and Kandasamy 2016). Lipid peroxidation is a molecular mechanism involved in the oxidative damage to cell structure and lead to cytotoxicity. In pathological conditions, reactive oxygen and nitrogen species (ROS and RNS) generated at higher than normal rate lead to elevated lipid peroxidation (Halliwell and Gutteridge 1984). In this study, the treatment of FCC water extract significantly prevented the lipid peroxidation and cytotoxicity due to antioxidant rich FCC extract. Therefore, reduction of lipid peroxidation and cellular protection are highly attributed to the effect of taurine rich extract.

5 Conclusion

Fish wastes are creating a big issue to manage a healthy environment. Further utilization of this waste is a possible way to avoid this problem. This study confirmed the presence of taurine in the water extract from cephalothorax of *F. chinensis*. The bioactive potential of FCC extracts is confirmed by its antioxidant capacity and inhibitory efficient of lipid peroxidation. Therefore the present study concluded that the shrimp, *F. chinensis* is a good source of natural bioactive compounds and it is highly deserved for further study.

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Antioxidant Effect of Taurine-Rich *Paroctopus dofleini* Extracts Through Inhibiting ROS Production Against LPS-Induced Oxidative Stress *In Vitro* and *In Vivo* Model

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Abstract Taurine is an essential amino acid to improve the function of cardiovascular, skeletal muscle, retina, and central nervous system. It also plays a role as an antioxidant agent against reactive oxygen species (ROS) generated by various substances. The aim of the current study was to examine the antioxidant capacity of water extracts of *Paroctopus dofleini*. Radical scavenging activity of *P. dofleini* extracts was performed using an ESR spectrophotometer. Protective effects of *P. dofleini* extracts against lipopolysaccharide (LPS)-induced oxidative stress in RAW264.7 cells were evaluated using flow cytometry. The *P. dofleini* extracts showed a potent antioxidant activity against LPS-induced oxidative stress on RAW264.7 cells. Furthermore, the *in vivo* antioxidant activity of *P. dofleini* extract on LPS-induced oxidative stress was assessed using zebrafish embryos. *P. dofleini* successfully scavenged the LPS-induced intracellular ROS and prevented lipid peroxidation in zebrafish embryos. The results obtained in this study clearly demonstrate that the *P. dofleini* significantly scavenge the ROS and prevent lipid peroxidation in both *in vitro* and *in vivo* models.

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1 Introduction

Taurine is a free amino acid abundantly present in animal tissues and some algae. It is chemically known as 2-aminoethanesulphonic acid, was first found in ox bile. Taurine is the non-essential and biologically inert β amino acid which usually occurs in the end-product of sulphur metabolism (Jacobsen and Smith 1968). Several *In-vivo* studies have been demonstrated that antioxidant potential of taurine and its contribution to growth and development (Huxtable 1992; Bass et al. 1983). Oxidative stress has been recognized as one of the major reasons responsible for tissue damage in various conditions such as infection, inflammations, cancer and ageing. Oxidative stress plays an important role in inflammation which is generally mediated by reactive oxygen species (ROS). The ROS are generated by activated leukocytes such as neutrophils, macrophages and eosinophils. Perhaps ROS favourably work towards the host defence against pathogens, but they are also responsible for tissue damage (Weiss 1989; Smith 1994). Reactive oxygen species (ROS) or higher levels of free radicals are highly responsible for oxidative stress and leads to macromolecular degradation such as DNA, proteins, lipids, and other cellular constituents (Fang et al. 2002; Lopaczynski and Zeisei 2001). Consequently, the oxidative stress leads to various human diseases including cancer, atherosclerosis, rheumatoid arthritis, muscular dystrophy, cataracts, ageing and some neuro-disorders (Kovatcheva et al. 2001; Ruberto et al. 2001). Despite Taurine is not directly involves to scavenging the ROS however, it seems to stimulate other cellular antioxidant functions (Kettle et al. 1997; Schaffer et al. 2003). Marine invertebrates especially the species coming under the class cephalopods such as nautilus, cuttlefish, squids, and octopus are rich in bioactive compounds which are responsible for antioxidant, antimicrobial, antitumor and antileukemic activities (Rajaganapathi et al. 2000; Jayaraj et al. 2008; Jesy et al. 2014; Sudhakar and Nazeer 2015). In this study, we evaluated the antioxidant activities of octopus *P. dofleini* (PD) against lipopolysaccharide (LPS)-induced oxidative stress, activation of macrophages, *in vitro* and *in vivo* anti-inflammatory effects on RAW264.7 cell as well as zebrafish animal model.

2 Methods

2.1 Preparation of the Extract

P. dofleini was purchased from Incheon, South Korea during the summer season of 2015. The *P. dofleini* (PD) was washed, extracted by hot water and lyophilized. The dried powder of PD (100 g) was extracted for 2 h by 1 L of hot water at 90 °C. The filtrate was concentrated using an evaporator (EYELA, Tokyo, Japan) at 50 °C. After evaporation, the water extracts were lyophilized and stored at −20 °C until use.

2.2 Radical Scavenging Activity by ESR Measurement

2.2.1 DPPH Radical Scavenging Activity

The DPPH radical scavenging activity was performed using an ESR spectrometer (JES-FA machine; Jeol, Tokyo, Japan) adapting to the method outlined by (Kim et al. 2008). Each experimental sample (30 μL) was added to ethanol (30 μL) containing DPPH (60 μM) and ethanol alone was used as the control. After vortexing for around 10 s, the properly mixed solutions were carefully transferred to Teflon capillary tubes and evaluated by ESR spectrometer. Exactly 2 min later, the spin adducts were evaluated by the ESR spectrometer under a set of appropriate experimental conditions.

2.2.2 Hydroxyl (OH) Radical Scavenging Activity

The Hydroxyl radical scavenging activity was determined by adapting to the method reported by Rosen and Rauckman (1984). OH radicals were produced through the Fenton reaction, and they were reacted rapidly with the nitrone spin trap DMPO. The resulted DMPO-OH adducts were detected by an ESR spectrometer. Samples (20 μL) were added to the reaction mixtures contained 20 μL of 0.3 M DMPO, 20 μL of 10 mM $FeSO_4$, 20 μL of 10 mM H_2O_2, reacted for 2.5 min and then transferred to a Teflon capillary tube for the determination of spin adduct formation.

2.3 Ferric Thiocyanate (FTC) Method

The lipid oxidation inhibitory activity of samples was measured using ferric thiocyanate (FTC) method described by Kikuzaki and Nakatani (1993). A reaction mixture contained 4 mg of sample in 99.5% ethanol, 2.51% linoleic acid in 4.1 mL of 99.5% ethanol, 8 mL of a 0.05 M phosphate buffer at pH7.0, and 3.9 mL of distilled water was mixed in a screw cap container. The reaction mixture was kept in the dark at 40 °C. Then, 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate were added to 0.1 mL of this solution. After 3 min, 0.1 mL of 3.5% hydrochloric acid containing 0.02 M ferrous chloride was added to the reaction mixture. The absorbance of the resultant red solution was measured at 500 nm. The reaction mixture without sample was considered as a negative control and Vitamin C and α-tocopherol were used as positive controls.

2.4 Cell Culture

The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (ATCC, TIB-71™) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine

serum (FBS) and antibiotics (100 units/mL of penicillin and 100 μg/mL of streptomycin) at 37 °C in a humidified incubator containing 5% CO_2.

2.5 Determination of Cell Viability Assay

The effect of PD extract on cell viability was evaluated using the MTT colorimetric assay. The RAW 264.7 cells seeded in 96-well plates at a density of 1×10^4 cells per well were incubated at 37 °C for 18 h. Then, the cells were treated with different concentrations (125, 250, and 500 μg/mL) of the PD extract or *N*-Acetyl-L-cysteine (positive control). After the treatments, cells were stimulated with or without medium containing LPS (100 ng/mL) at 37 °C for 24 h. After the incubation period, the medium in each well was replaced by DMEM medium (100 μL) containing MTT (500 μg/mL) and incubated at 37 °C for 4 h. The MTT solution was then removed and DMSO (200 μL) was added to each well and shaken for 5 min to dissolve intracellular formazan product. The absorbance at 540 nm was measured using a microplate reader (Tecan, Grödig, Austria). Cell viability values were expressed by comparing treatments against control.

2.6 Determination of Nitric Oxide (NO) Production

The nitric oxide concentration in the culture medium was measured using the Griess reagent. The RAW 264.7 cells seeded at a density of 1×10^4 cells per well into 96-well plates were pre-treated with different concentrations (2.5, 5, 10, and 20 μg/mL) of PD extract or *N*-Acetyl-L-cysteine. Then, the cells were stimulated with medium containing LPS (100 ng/mL) at 37 °C for 24 h. The culture supernatant (100 μL) was mixed with Griess reagent (100 μL, 1% sulfanilamide, 0.1% *N*-1-naphthylethylenediamine) and it was incubated at room temperature for 10 min. The absorbance of the assay mixture was observed at 540 nm using a microplate reader. A standard curve derived from a serial dilution of sodium nitrite was used to calculate the amount of nitrite present in each sample.

2.7 Determination of Intracellular ROS Using Flow Cytometry

The level of intracellular ROS was determined using flow cytometry by measuring the fluorescent intensity of cells as outlined by Bass et al. (1983). RAW 264.7 cells seeded at a density of 1×10^6 cells per well in 6-well plates were pre-treated with different concentrations (5, 10, and 20 μg/mL) of PD extract or *N*-acetyl-L-cysteine (NAC, 20 mM) for 1 h. Then, the cells were stimulated with the medium containing LPS (100 ng/mL) at 37 for 30 min. The Cells were washed with PBS and incubated

with $DCFH_2$-DA (10 μM) at 37 °C for 30 min in the dark. The cells were washed with PBS again and gently scraped. The fluorescent intensity was analyzed at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a FACS Calibur flow cytometer (Becton & Dickinson Co., Franklin Lakes, NJ, USA).

2.8 Origin and Maintenance of Zebrafish

Adult zebrafish were purchased from a commercial supplier (Seoul Aquarium, South Korea) and ten fish were maintained in a 3 L acrylic tank kept under 28.5 °C, with a 14/10 h light/dark cycle. The fish were fed on live brine shrimp (*Artemia salina*) three times per day up to 6 days in a week. Embryos were obtained from naturals pawning and induced by switched on the light in the morning. The embryos collection was completed within 30 min.

2.9 Estimation of Oxidative Stress-Induced Intracellular ROS, Lipid Peroxidation

The ROS generation and lipid peroxidation in zebrafish embryos were determined using the method described by Kang et al. (2013). Zebrafish embryos' intracellular ROS production was detected using an oxidation-sensitive fluorescent probe dye, $DCFH_2DA$ and generation of lipid peroxidation was determined using a fluorescent probe called diphenyl-1-pyrenylphosphine (DPPP; Dojindo Laboratories, Kumamoto, Japan) which detects cell membrane lipid peroxidation. DPPP is a non-fluorescent chemical agent, but it becomes fluorescent when it is oxidized. At 3–4 hpf, embryos were treated with the sample for 1 h and then 10 μg/mL LPS was added to the plate. After the 24 h treatment with LPS, the embryo medium was changed and the embryos were developed up to 2 dpf. The embryos were then transferred into 96-well plates and treated with $DCFH_2$-DA (20 μg/mL), DPPP (0.05 mM). Subsequently, the plates were incubated for individual reaction times in the dark at 28.5 °C. After incubation, the embryos were rinsed with fresh embryo media and the stained embryos were observed by a fluorescence microscope (Zeiss AX10, Carl Zeiss, Göttingen, Germany).

2.10 Statistical Analysis

The data were analyzed statistically using Analysis of variance (ANOVA), together with Turkey's test and Dunnett's test (GraphPad Prism 5). The results were expressed as the mean ± standard deviation. The values are considered significant at the probability value of $p < 0.05$.

3 Results

3.1 Radical Scavenging Activity by ESR Measurement

DPPH is a deep purple colored pro-oxidant which generates stable free radicals. It could be neutralized by the reaction with other radicals, electrons, or hydrogen atoms and it is observed by the color loss. This technique has been broadly employed as a method for evaluating antioxidants' free radical scavenging activities (Schaich et al. 2015). The water extracts of PD showed significant DPPH and hydroxyl radical scavenging activities with IC_{50} values of 0.849 ± 0.23 mg/mL and 0.741 ± 6.12 mg/mL respectively (Table 1). These results confirmed that PD extracts have potential DPPH and hydroxyl radical scavenging activities which are highly attributed to the effect of antioxidant and anti-inflammatory activities.

3.2 Cell Viability and Production of NO

RAW 264.7 cells were pretreated with various concentrations (125, 250, and 500 μg/mL) of PD extract for 1 h prior to stimulation with LPS (100 ng/mL) for 18 h. The LPS treated cells showed a marked increase in NO release as compared to the non-treated cells. The administration of PD extract significantly inhibited the levels of NO in a dose-dependent manner (Fig. 1b). However, the results of the MTT assay indicated that PD extract was not cytotoxic to RAW 264.7 cells at the concentrations up to 500 μg/mL (Fig. 1a). The results demonstrated that 20 μg/mL showed a significant reduction in NO production (Fig. 1b).

3.3 Inhibitory Effects of PD on ROS Generation

The water extracts of PD have significant inhibitory activity on LPS-induced intracellular reactive oxygen species (ROS) production. It was estimated by fluorescence spectroscopy using DCFH-DA as a fluorescent probe. The increased production of intracellular ROS was observed in LPS-induced cells however, it was decreased in PD treated cells by dose independent manner (Fig. 2).

Table 1 Various radical scavenging activities of PD extract (IC_{50}, mg/mL)

Sample	DPPH radical	Hydroxyl radical
Water extract	0.849 ± 0.23	0.741 ± 6.12

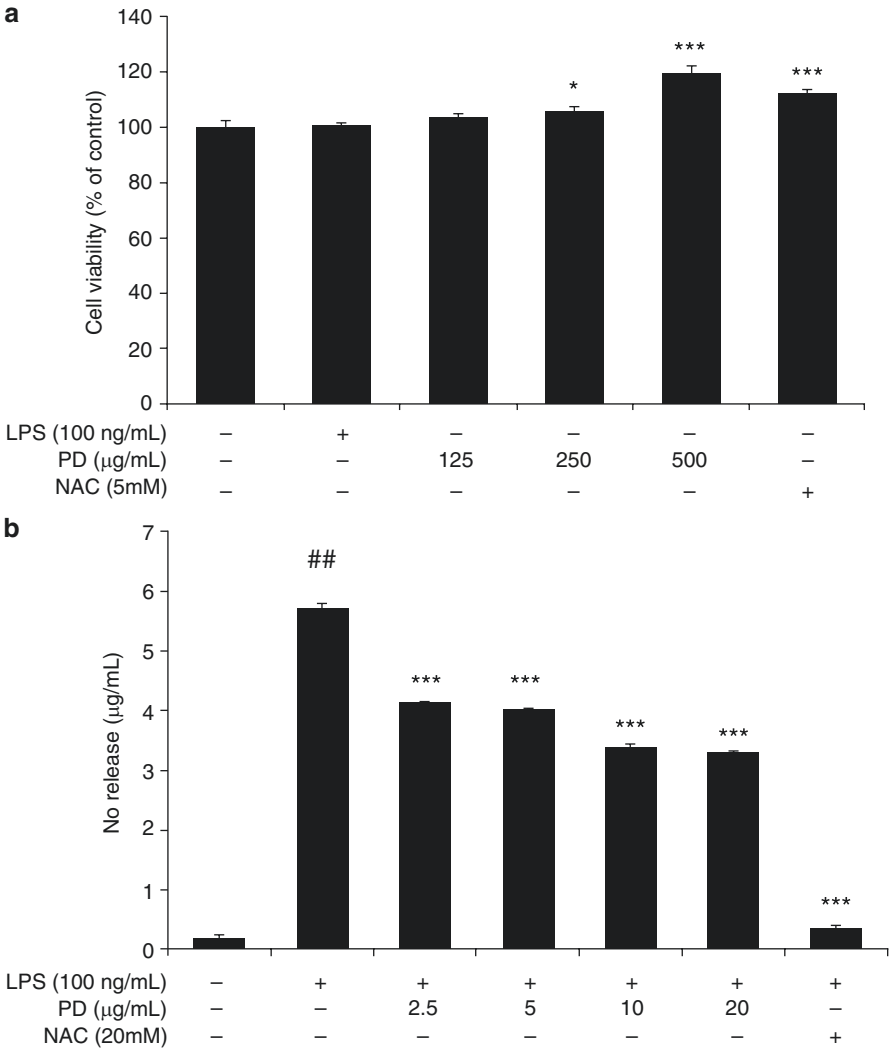

Fig. 1 Cell viability and inhibition of nitrite production (**a**) RAW 264.7 cells were pretreated with PD for 1 h (at 125, 250 and 500 μg/mL), and treated with LPS (100 ng/mL) for 18 h. The data represent the mean ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with non-treated cells. (**b**) RAW 264.7 cells were pretreated for 1 h with PD and treated with LPS (100 ng/mL) for 18 h. Data are represented as mean ± SD ($n = 3$). $^{\#\#\#}p < 0.001$ vs. control, $^{***}p < 0.001$ vs. LPS.

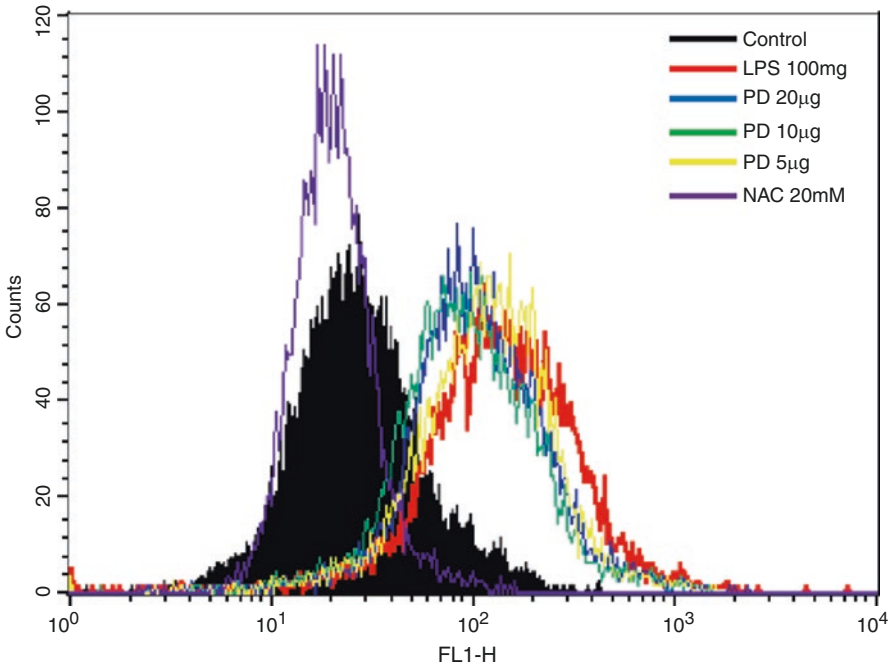

Fig. 2 Determination of intracellular ROS using DCF-DA on the LPS-stimulated RAW cells. (*Black*) Control; (*Red*) LPS; (*Purple*) LPS + NAC (20 mM); (*Yellow*) LPS + PD (5 μg/mL); (*Green*) LPS + (10 μg/mL); (*Blue*) (LPS + 20 μg/mL)

3.4 Lipid Peroxidation Inhibition Assay

Lipid peroxidation inhibitory effects of samples were assessed by FTC assay method using 10 mg/mL linoleic acid. The inhibitory potential of extracts was assessed based on the ability to prevent the peroxidation formation. The effect of samples was compared with the commercial anti-oxidants vitamin C and vitamin E at 1.0 mg/mL as a positive control. The FTC assay has been used for the measurement of peroxide levels during the early oxidation of linoleic acid (Hariprasath et al. 2015). The untreated control group of linoleic acid underwent auto-oxidation attained rapid increases in the peroxide value after day 2. It was also reached a maximum level on day 8, followed by a decline on day 10 (Fig. 3). However, different concentration of PD extract and vitamin E had a significant inhibitory effect on lipid peroxidation than the controls.

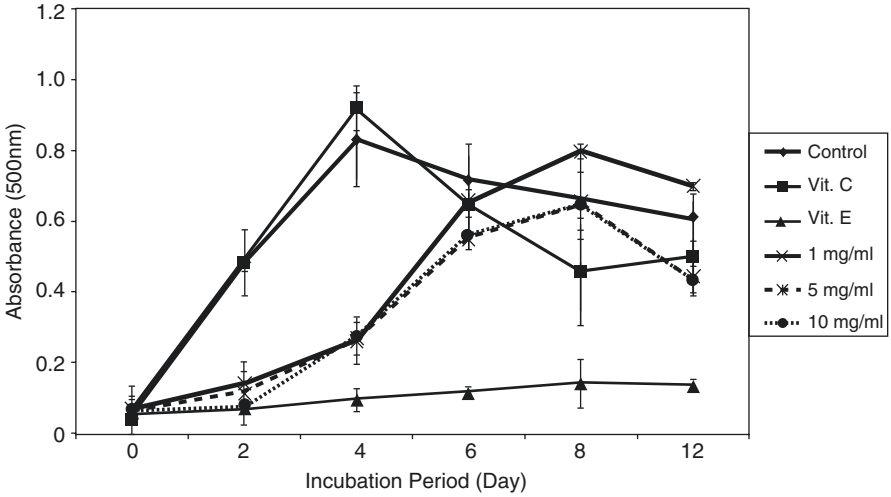

Fig. 3 Antioxidative activity of PD extracts by the FTC method

3.5 Measurement of PD Against LPS-Induced Oxidative Stress In Vivo Zebrafish Model

The effect of PD extract on LPS-induced oxidative stress was also evaluated on *in vivo* zebrafish model. When compared to control group, an increased ROS level was observed in LPS-treated zebrafish. In contrast, it was significantly reduced in zebrafish exposed to LPS and PD at different concentrations (5, 10 and 20 µg/mL) (Fig. 4). LPS-induced lipid peroxidation by DPPP fluorescent dye is shown in Fig. 5. The LPS-treated zebrafish showed the high value of lipid peroxidation, however, it was significantly decreased by different concentration of PD extracts.

4 Discussion

The antioxidant and free radical scavenging potential of PD extracts indicate the presence of active water soluble compounds in it. The PD extracts showed the scavenging activity of DPPH and hydroxyl radicals in a dose-dependent manner. Antioxidant and radical scavenging activity of PD extracts are mainly attributed to the presence of redox properties in the extract which can neutralize the free radicals. LPS-stimulation in macrophages is disrupted the balance of intracellular oxidation

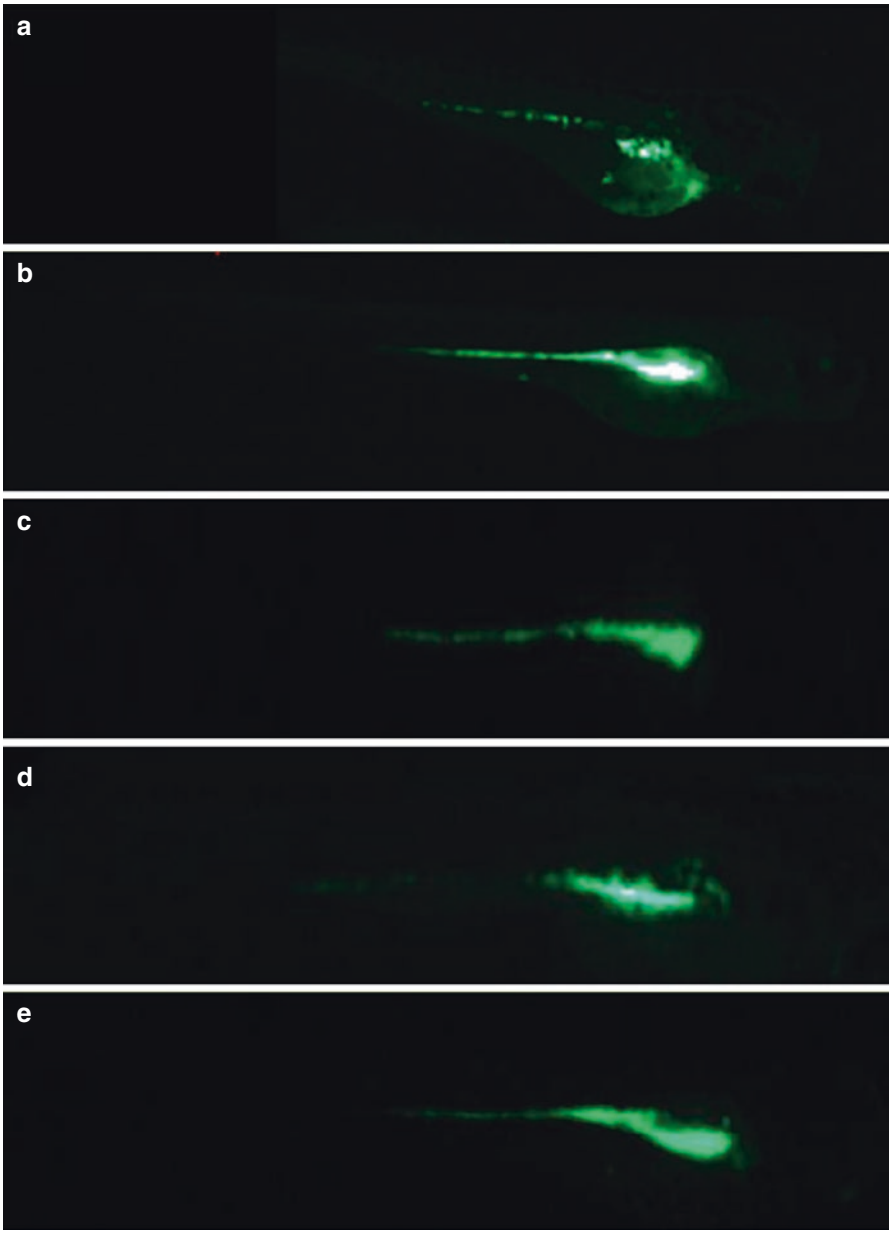

Fig. 4 Effect of *Paroctopus dofleini* on LPS-induced ROS level in zebrafish embryos. ROS levels were measured by image analysis and fluorescence microscopy. (**a**) Control; (**b**) LPS (10 μg/mL); (**c**) LPS + *P. dofleini* (5 μg/mL); (**d**) LPS + *P. dofleini* (10 μg/mL); (**e**) LPS + *P. dofleini* (20 μg/mL)

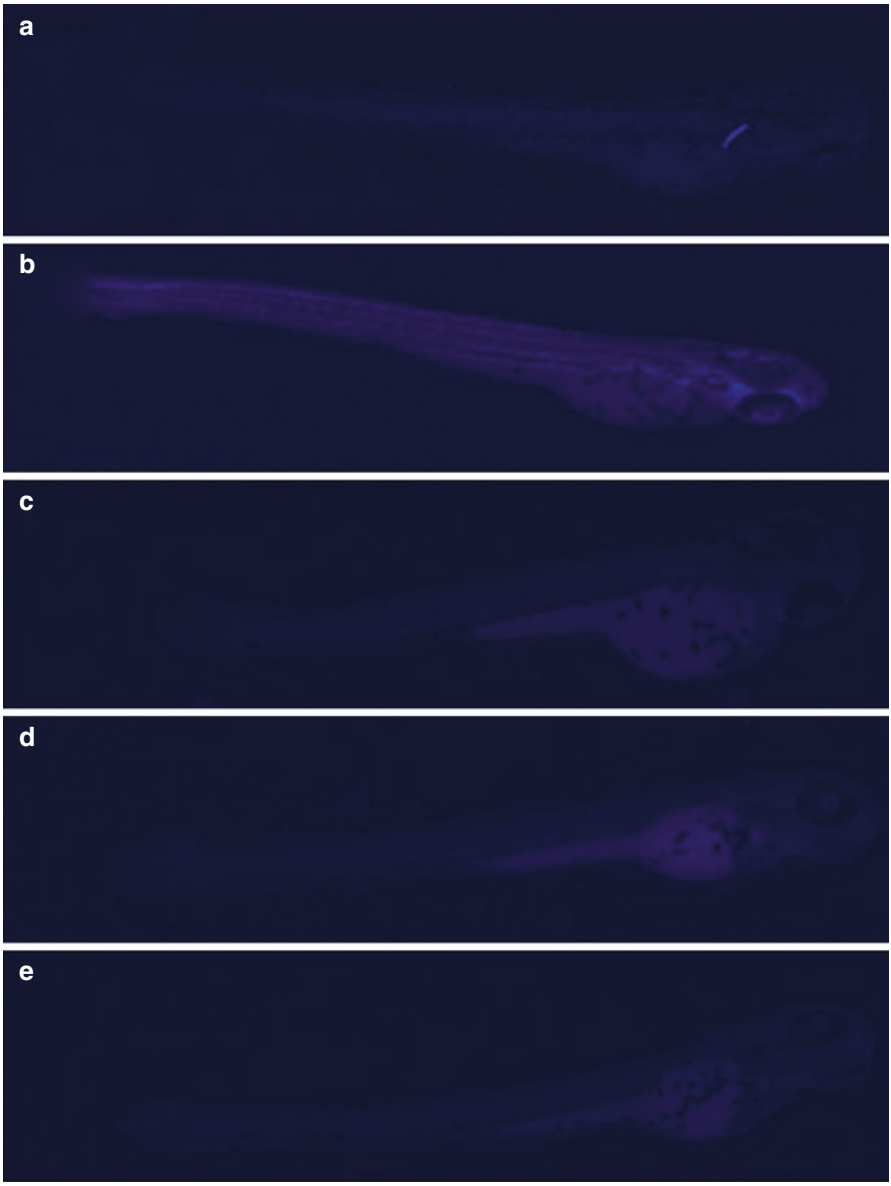

Fig. 5 Effect of *Paroctopus dofleini on* LPS-induced lipid peroxidation in zebrafish embryos. The lipid peroxidation levels were measured by image analysis and fluorescence microscopy. (**a**) Control; (**b**) LPS (10 μg/mL); (**c**) LPS + *P. dofleini* (5 μg/mL); (**d**) LPS + *P. dofleini* (10 μg/mL); (**e**) LPS + *P. dofleini* (20 μg/mL)

and reduction state, which leads to oxidative stress (Chen et al. 2007). NO can regulate the functions of various types of cells including T lymphocytes, macrophages, synovial fibroblasts, osteoclasts, and endothelial cells at the site of inflammation (Korhonen et al. 2005). Therefore, scavenging activity of NO is highly important to avoid the cellular abnormality, inflammation and other diseases. In this study, PD extracts exhibited a higher antioxidant activity including scavenging the NO production. Therefore the PD extract was chosen for further study to evaluate the effects of LPS stimulated ROS generation in RAW264.7 cells. Measurement of inhibitory activity of the *P. dolfleini* extract depends on the inhibitory rate of hydroperoxides formation. The present study showed that PD extracts effectively inhibited the formation of hydrogenperoxidase. The scavenging activity of *P. dolfleini* also tested on LPS-stimulated ROS and lipid peroxidation in a zebrafish model. Accumulation of ROS was assessed by DCFH-DA and lipid peroxidation was evaluated by DPPP. DCFH-DA is converted the non-fluorescent 2′,7′-dichlorodihydrofluorescein (DCFH2) to fluorescent 2′,7′-dichlorofluorescein (DCF) by oxidation-dependently. Thus, the intensity of fluorescence is directly proportion to increased production of ROS (Walker et al. 2012). Likewise, Diphenylpyrenylphosphine (DPPP) is a probe that also detects the hydroperoxides by strong fluorescent during increased oxidation (Akasaka et al. 1987). This result indicates the treatment of PD significantly inhibited the lipid peroxidation production in zebrafish animals. The results also emphasis that zebrafish could be a good animal model to screen the various antioxidant substances. The overall results indicate that extraction of octopus *Paroctopus dofleini* is highly deserved for many therapeutic applications.

5 Conclusion

In this study, the extracts of PD exhibits NO scavenging activity, DPPH radical scavenging activity and LPS induced intracellular ROS scavenging activity in RAW cells *in vitro*. In addition, the PD extracts also showed potent inhibitory activity on LPS-induced ROS, lipid peroxidation productions in zebrafish *in vivo* model. Therefore, our studies clearly demonstrated that PD extract is potential for therapeutic application and highly deserved for further study.

Acknowledgements This work was supported by a special grant from Konkuk University in 2016.

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Taurine Attenuates Doxorubicin-Induced Toxicity on B16F10 Cells

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Abstract This study aimed to investigate the effect of doxorubicin co-treatment with taurine on B16F10 melanoma cells. Frequently, Doxorubicin is used in the treatments of many different kinds of cancers, some of which are soft tissue sarcomas, hematological malignancies and carcinomas. However, the clinical application of doxorubicin is compromised by its severe adverse effects, including cardiotoxicity. In the present study, the efficacy of doxorubicin co-treatment with taurine was investigated. B16F10 cell viability was evaluated using MTT assays, trypan blue dye exclusion assays, and fluorescent staining technique. Apoptotic cells were detected by flow cytometry and the proteins associated with apoptosis and cellular differentiations were assessed by immunoblotting. Doxorubicin inhibited cell growth and induced cell death in B16F10 cells. Interestingly, doxorubicin co-treatment with taurine inhibited apoptosis in B16F10 cells. These results indicate that doxorubicin co-treatment with taurine attenuates doxorubicin-induced cytotoxicity and reduces ROS production in B16F10 cells.

Keywords Doxorubicin • B16F10 cells • Reactive oxygen species

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1 Introduction

Doxorubicin is a common chemotherapy derived by chemical semisynthesis from a bacterial species that is commonly used in cancer. Doxorubicin is useful in treating many different types of cancers such as various carcinoma, soft tissue sarcomas, and haematological malignancies (Das et al. 2011). However, its use is problematic due to dose-limiting cardiotoxicity and the development of multidrug resistance (Awasthi et al. 2002). Previous studies have shown that the doxorubicin treatment in different cancer patients results in myocardial antioxidant reduction which leads to cardiac oxidative stress and cardiotoxicity (Iliskovic and Singal 1997). The serious side effects of doxorubicin treatment are irreversible and cumulative cardiomyopathy, leading to congestive heart failure (Haq et al. 1985: Shan et al. 1996).

Taurine which is also known as 2-aminoethanesulfonic acid is a sulphur containing amino acid which is largely found in different animal tissues (Sochor et al. 2014). The major taurine source for the body is the diet, even though it can be synthesized endogenously. Taurine is found to play a role in bile acid formation which helps in fat digestion. It is not only an ingredient of bile acid, it is found to involve in different biological and physiological functions including enhancing proliferation and differentiation, scavenging of free radicals, and regulating membrane excitability (Chen et al. 1998; Godfrey et al. 2000; Hanna et al. 2004). Several studies reported that taurine has a neuroprotective function by inhibiting extracellular calcium influx and the outflow of calcium from intracellular pools, basically the balance of neurotransmitters (Chen et al. 2001; Zhang et al. 2010). Moreover, taurine is reported to possess anti-inflammatory properties (Marcinkiewicz 2009), involve in different physiological activities since it has a role in stabilizing cell membranes (Condron et al. 2010), help to manage fatty tissues metabolism (Ueki and Stipanuk 2009) and regulate calcium ion levels in the blood (Ribeiro et al. 2010). Taurine has potent anti-inflammatory effects in a variety of systemic inflammation models, including spinal cord injury (Nakajima et al. 2010), hepatic ischemia reperfusion (Zhang et al. 2010), lung injury (Abdih et al. 2000), and ischemic stroke (Sun et al. 2012). Taurine also attenuates oxidative stress (Redmond et al. 1996), reduces proinflammatory cytokines (Kontny et al. 2000), inhibits apoptosis (Haunstetter and Izumo 1998; Hsu et al. 2008), controls blood pressure (Racasan et al. 2004) and calcium homeostasis (Harada et al. 2004). Although the protective effects of taurine have been extensively investigated, the effect of co-treatment of taurine with doxorubicin remains poorly understood in B16F10 cells. Therefore, the purpose of this study was to evaluate the underlying protective role of taurine against doxorubicin-induced cytotoxicity.

2 Materials and Methods

2.1 Reagent

Taurine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Trypan blue, and Propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hoechst 33342 was purchased from Invitrogen (Eugene, OR, USA). All other reagents used in experiments were the highest commercially available grade.

2.2 Cell Culture

The murine melanoma B16F10 cell line was obtained from American Type Culture Collection (Rockville, MD). It was maintained in Dulbeco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco), penicillin (100 U/ml) and streptomycin (100 μg/mL) at 37 °C in a humidified incubator under 5% CO2.

2.3 MTT Assay

Cell viability was evaluated by adapting to MTT assay. Briefly, B16F10 cells were seeded in 96-well culture plates at a density of 2×10^4 cells per well. Cells were treated with doxorubicin (500 nM) alone and with a combination of three different concentrations of taurine (5, 10 and 20 mM) and incubated at 37 °C for 24 h. Next, MTT solution was added to each well, and the cells were incubated again at 37 °C for 4 h. After discarding of the medium, a 150 μL DMSO solution was added to each well and shook for 10 min to dissolve intracellular formazan product. Absorbance at 540 nm was measured using a microplate reader (Tecan, Austria), and the cell viability was expressed as a percentage of the control.

2.4 Trypan Blue Exclusion Assay

Cell viability was analyzed by adapting to the trypan blue dye exclusion method. Briefly, B16F10 cells (1×10^5 cells/well) were seeded in 6-well plates and allowed overnight attachment. Then, cells were treated with doxorubicin (500 nM) alone and

combination with three different concentrations of taurine (5, 10 and 20 mM) and incubated at 37 °C for 24 h. After treatment, cells were stained with trypan blue (0.4% in phosphate buffered saline) and counted using a hemocytometer (Jin et al. 2008).

2.5 Cell Cycle Analysis

The cell cycle analysis was conducted using flow cytometer. Cellular DNA content was measured through this experimental technique. Briefly, cells were seeded in six-well plates at a density of 1×10^5 cells per well and allowed to attach the cells overnight. Cells were then treated with doxorubicin (500 nM) alone and combination with three different concentrations of taurine (5, 10 and 20 mM) and incubated them at 37 °C for 24 h. The cells were then harvested with trypsin, washed with cold phosphate-buffered saline (PBS, pH7.4) and stained with PI solution (50 μg/mL of propidium iodide with 10 μg/mL RNase, and 0.5% Tween 20 in PBS). The stained cells were analyzed using a flow cytometer (FACSCalibur, BD Bioscience) to obtain DNA histograms and cell cycle profiles. Data from 10,000 cells per sample were collected and analyzed using CellQuest software (Becton Dickinson).

2.6 Reactive Oxygen Species (ROS) Assay

ROS production was determined by flow cytometry method using 2′,7′-dichlorodihydrofluorescin diacetate ($DCFH_2$-DA), which is a stable nonpolar dye which can easily diffuse into the cell and hydrolyzed by intracellular esterase yielding 2′,7′-dichlorodihydrofluorescin (DCFH) within the cell. $DCFH_2$-DA is non-fluorescence, but when this reagent is oxidized by ROS, it becomes green fluorescence. Briefly, B16F10 cells seeded at a density of 1×10^5 cells per well in 6-well plates were treated with doxorubicin (500 nM) alone and the combination with three different concentrations of taurine (5, 10 and 20 mM) and incubated at 37 °C for 24 h. Thirty minutes before the endpoint of each experiment, the cell culture was treated with $DCFH_2$-DA (10 μM) and stored in the dark. Cells were then harvested, centrifuged, resulted cell pellet was washed with 1 mL of PBS and centrifuged as previously described. The pellet was resuspended in 500 μL of PBS and fluorescence was measured using a flow cytometer (FACSCalibur, BD Bioscience).

2.7 Observation of Morphologic Changes

B16F10 cells seeded in 6-well plates at a density of 1×10^5 cells per well were maintained in DMEM at 37 °C under 5% CO_2 for 24 h. Then, B16F10 cells were treated with doxorubicin (500 nM) alone and combination with three different concentrations of taurine (5, 10 and 20 mM) and incubated at 37 °C for 24 h. After the

incubation period, cellular morphology was observed using a phase-contrast microscope (Nikon, Japan). Images developed at 200× magnification were analyzed for finding morphological changes.

2.8 Hoechst 33342 Staining

Fluorescent stain, Hoechst 33342 was used to observe the effects of taurine treatments on nuclear morphological changes in doxorubicin-treated B16F10 cells. B16F10 cells plated in 6-well plates at a density of 1×10^5 cells per well were managed in DMEM at 37 °C under 5% CO_2 for 24 h. Then the cells were treated with doxorubicin (500 nM) alone and the combination with three different concentrations of taurine (5, 10 and 20 mM) and incubated at 37 °C for 24 h. After treatment incubation, the cells were fixed in PBS containing 4% formaldehyde for 30 min at room temperature. The fixed cells were washed with PBS containing 0.02% tween 20 and stained with 1 μg/mL Hoechst 33342 (Sigma, St. Louis, MO, USA) for 20 min at room temperature. The cells were then washed twice with PBS and photographed under a fluorescence microscope (Zeiss AX10; Carl Zeiss, Göttingen, Germany).

2.9 Statistical Analysis

The data were expressed as the mean ± standard deviation (SD). Experimental data were analyzed statistically using Student's t-test for paired data and one-way analysis of variance (ANOVA) followed by post-doc Duncan's multiple range tests. Graph Pad Prism software version 4.00 (Graph Pad Software, Inc., San Diego, CA) was employed for analysis. A probability value of $p < 0.05$ was considered statistically significant.

3 Results

3.1 Effect of Co-treatment with Taurine and Doxorubicin on Cell Viability

In this experiment, cell viability was measured using MTT and trypan blue exclusion assays. MTT assay data revealed that doxorubicin (500 nM) inhibited cell viability by 58% (Fig. 1). However, co-treatment of doxorubicin (500 nM) with taurine (5, 10, and 20 mM) increased cell viability. Co-treatment with 20 mM of taurine significantly increased cell viability compared to treatment with doxorubicin alone ($p < 0.05$). Similar results were observed with trypan blue exclusion assays (Fig. 2). These results indicate that co-treatment of doxorubicin with taurine reduces the cytotoxicity involved with doxorubicin.

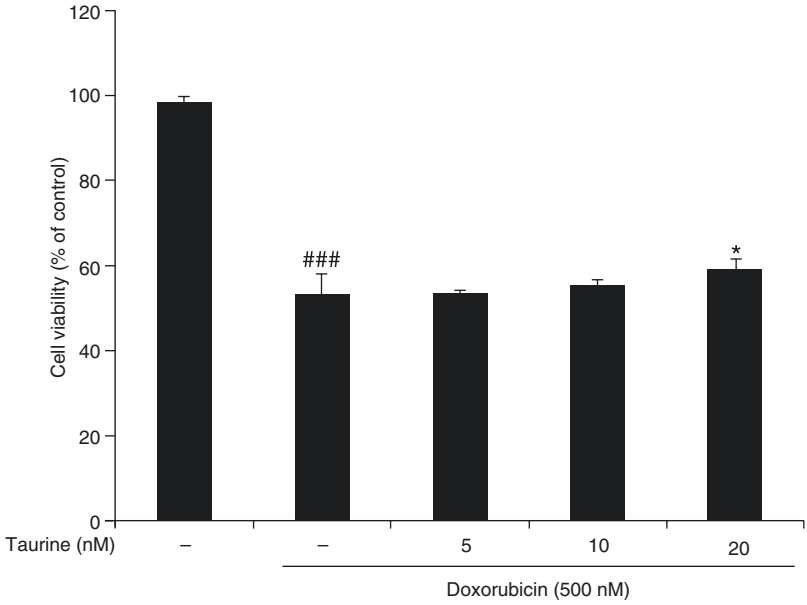

Fig. 1. Effect of co-treatment of doxorubicin with taurine on the viability of B16F10 cells using MTT assay. Results are expressed as mean ± SD from three independent experiments. One-way ANOVA was used for comparisons of multiple groups means followed by Dunnett's test $*p < 0.05$ compared to control

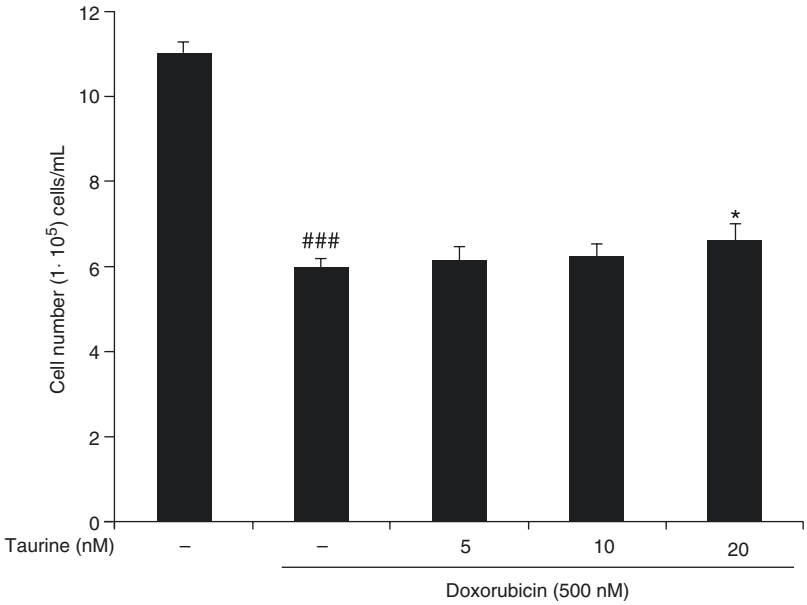

Fig. 2. Effect of co-treatment of doxorubicin with taurine on the viability of B16F10 cells using trypan blue exclusion assay. Results are expressed as mean ± SD from three independent experiments. One-way ANOVA was used for comparisons of multiple groups means followed by Dunnett's test $*p < 0.05$ compared to control

3.2 *Effect of Co-treatment with Taurine and Doxorubicin on the Cell Cycle Distribution in B16F10 Cells*

Apoptosis and cell-cycle arrest are considered as the major mechanisms underlying the antiproliferative effect of doxorubicin (Hsiao et al. 2007). To investigate whether the doxorubicin-mediated inhibition of cell proliferation is a result of cell cycle arrest or apoptosis, the distribution of the cell population in each phase of the cell cycle (sub-G1, G0/G1, S or G2/M) was analyzed by measuring cellular DNA content. The amounts of sub-G1 (apoptotic) and G2/M cells were significantly increased after treatment with doxorubicin for 24 h (Fig. 3, Table 1). However, co-treatment with taurine reduced the percentage of cells in sub-G1 phase in a dose-dependent manner. These results suggest that co-treatment with taurine reduces doxorubicin-induced apoptosis.

3.3 *Intercellular ROS Production*

Rudolfová et al. (2014) reported doxorubicin-induced ROS production and hepatotoxicity in vitro. The present study showed that doxorubicin-induced ROS production by 8.4-fold in B16F10 cells (Fig. 4). In contrast, co-treatment of doxorubicin (500 nM) with taurine attenuated ROS production by 13.15% (5 mM), 51%

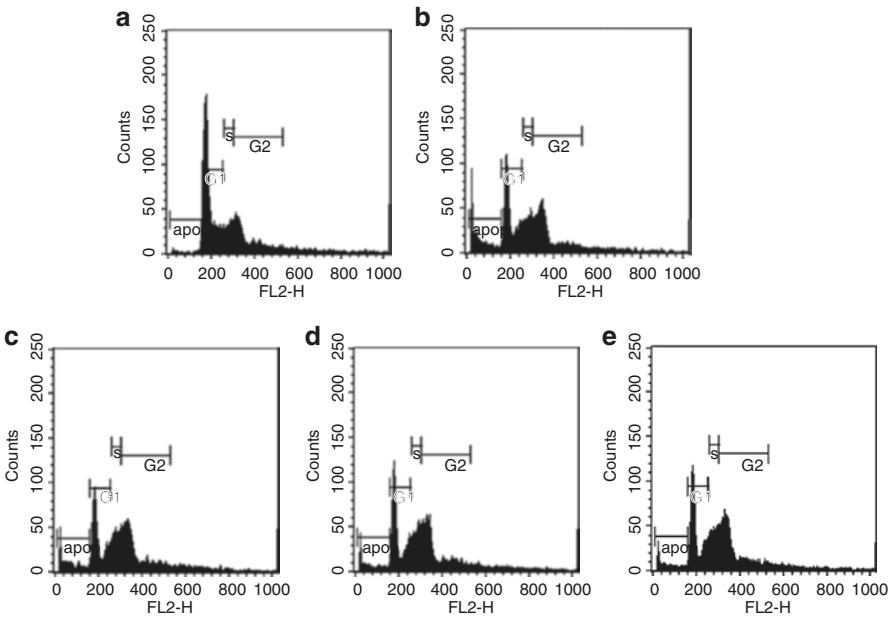

Fig. 3. Cell cycle analysis by flow cytometry. (**a**) control, (**b**) doxorubicin (500 nM), (**c**) doxorubicin (500 nM) + taurine (5 mM), (**d**) doxorubicin (500 nM) + taurine (10 mM), (**e**) doxorubicin (500 nM) + taurine (20 mM)

Table 1. Effects of co-treatment of doxorubicin with taurine on cell cycle distribution in B16F10 cells

Group	Number of cell (%)			
	Sub G1	G1	S	G2/M
Control	4.39 ± 0.52	50.43 ± 2.15	12.36 ± 1.36	24.87 ± 2.21
Doxorubicin (500 nM)	11.89 ± 2.23	32.23 ± 3.15	13.93 ± 2.21	33.58 ± 2.24
Doxorubicin (500 nM) + taurine (5 mM)	8.40 ± 1.02	28.39 ± 2.24	16.96 ± 1.23	36.48 ± 3.23
Doxorubicin (500 nM) + taurine (10 mM)	5.48 ± 1.11	30.56 ± 2.18	17.87 ± 1.21	36.09 ± 2.22
Doxorubicin (500 nM) + taurine (20 mM)	4.86 ± 1.21	30.91 ± 3.25	18.30 ± 2.14	37.85 ± 2.12

The cells were treated with below-indicated concentrations of Doxorubicin and taurine for 24 h and stained with PI for flow cytometry analysis. The cell percentage in each cell cycle phase was determined using three independent experiments

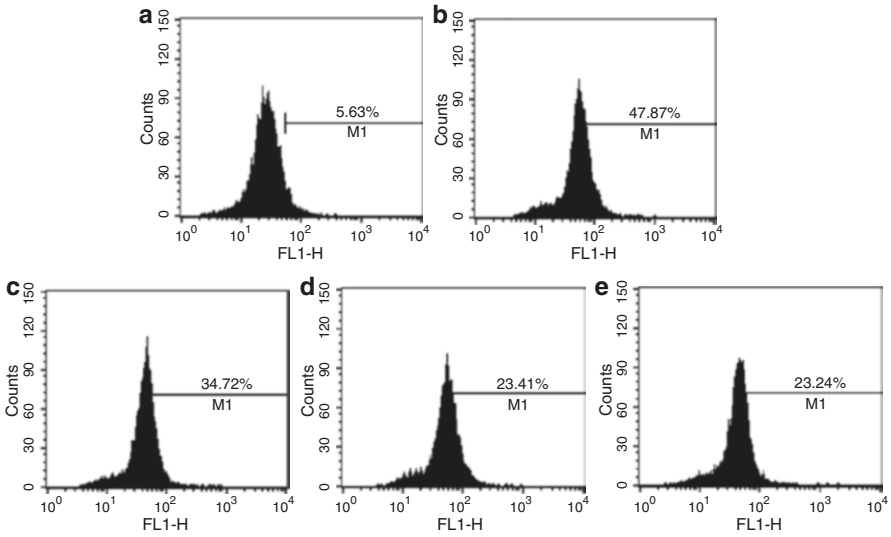

Fig. 4. Effect of co-treatment of doxorubicin with taurine on ROS generation in B16F10 cells. (**a**) control, (**b**) doxorubicin (500 nM), (**c**) doxorubicin (500 nM) + taurine (5 mM), (**d**) doxorubicin (500 nM) + taurine (10 mM), (E) doxorubicin (500 nM) + taurine (20 mM)

(10 mM), and 51.4% (20 mM), respectively. Doxorubicin might generate ROS by more than one mechanism and taurine might prevent excessive ROS production in mitochondria by altering the respiratory chain function. These data indicate that the protective effect of taurine against doxorubicin-induced cytotoxicity damage is mainly due to its ability to reduce ROS production.

3.4 Morphological Changes in B16F10 Cells

The cell morphological changes are analyzed using a phase-contrast microscope. Doxorubicin alone reduced the size and density of cells, whereas co-treatment with taurine increased cell density (Fig. 5). In addition, the nuclear morphological characteristics of apoptotic cells were detected by Hoechst 33342 staining. As shown Fig. 6, It was observed that nuclei were stained in a weak and homogeneous blue color in control cells, whereas doxorubicin-induced bright chromatin condensation and nuclear fragmentation. Co-treatment with taurine reduced the extent of doxorubicin-mediated nuclear apoptotic morphology.

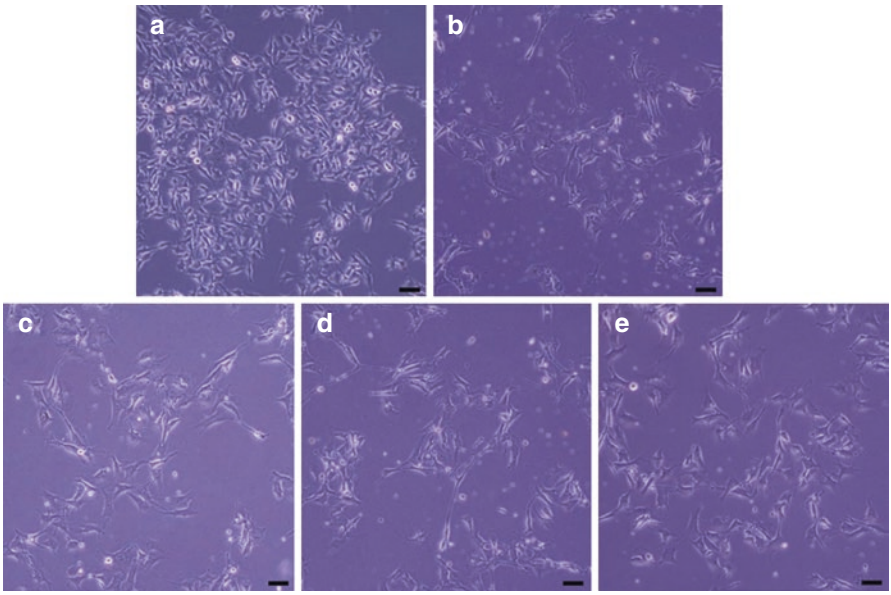

Fig. 5. Morphological change in B16F10 cells. The Cells were incubated with relevant treatment concentrations for 24 h, photographs were taken using an invert microscope. Scale bar: 10 μm. (**a**) control, (**b**) doxorubicin (500 nM), (**c**) doxorubicin (500 nM) + taurine (5 mM), (**d**) doxorubicin (500 nM) + taurine (10 mM), (**e**) doxorubicin (500 nM) + taurine (20 mM)

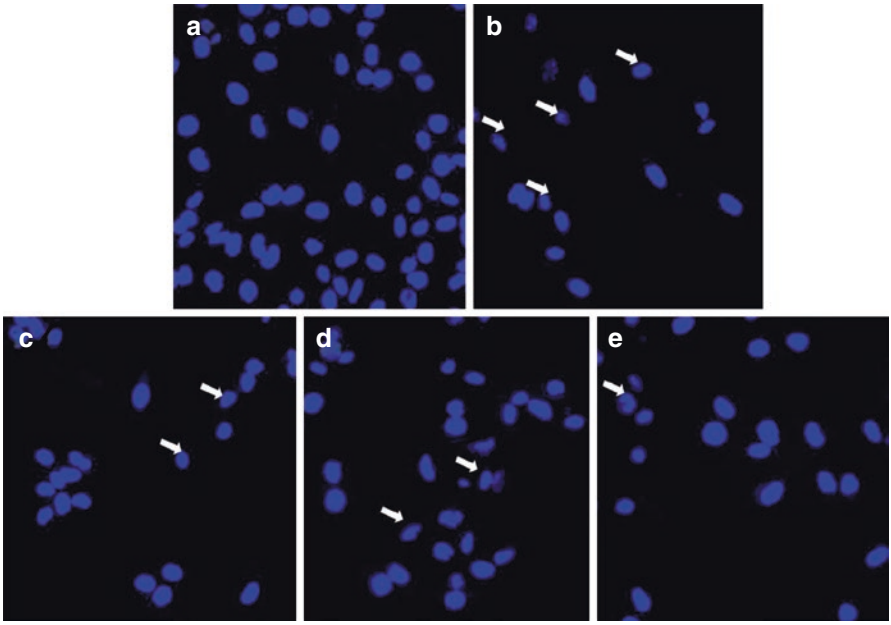

Fig. 6. Nuclear morphological changes in B16F10 cells. The Cells were incubated with relevant treatment concentrations for 24 h, fixed with 4% paraformaldehyde and stained with Hoechst 33342. Photographs were taken by using a fluorescent microscope. (**a**) control, (**b**) doxorubicin (500 nM), (**c**) doxorubicin (500 nM) + taurine (5 mM), (**d**) doxorubicin (500 nM) + taurine (10 mM), (**e**) doxorubicin (500 nM) + taurine (20 mM)

4 Discussion

Oxidative stress is a condition resulted from the imbalance of ROS generation and depletion. Therefore, naturally, there are multiple cellular defence mechanisms in the human body to eliminate excess free radicals and preventing intracellular damage caused by harmful effects of ROS (Rahman 2007). ROS can damage cells leading to cell death by harming to different cellular components such as damaging DNA, oxidizing the cell membrane lipids, and/or direct activation of genes and proteins which are responsible for apoptosis (Buttke and Sandstrom 1994). Therefore, reducing ROS production is critical for cell survival. Taurine is an amino acid having many biological functionalities including antioxidant, anti-inflammatory and immunomodulatory properties (Stapleton et al. 1998). Moreover, different experimental models showed that taurine could reduce oxidant-induced lung damage (Timbrell et al. 1995; Gordon et al. 1998; Gurujeyalakshmi et al. 2000). Venkatachalam et al. (2014) reported that taurine protects lungs from ROS production-induced lung damage by augmenting the function of antioxidants. The LPS-induced generation of ROS and activation of MARK and Bax in MRC-5 cells were reduced by co-treatment with 20 mM taurine (Jeon et al. 2009).

In the present study, the effects of co-treatment of doxorubicin with taurine were investigated in murine melanoma B16F10 cells using various molecular methods. Co-treatment with taurine decreased doxorubicin-induced cell death in B16F10 cells. The protective effect of taurine against doxorubicin-induced cytotoxicity could be attributed to the cell cycle regulation, cell proliferation and reduction of ROS production. In conclusion, this study suggests that taurine co-treatment could attenuate doxorubicin-induced cytotoxicity by decreasing ROS production in B16F10 cells.

Acknowledgements This work was supported by a special grant from Konkuk University in 2016.

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Protective Effect of Taurine on Mice with Doxorubicin-induced Acute Kidney Injury

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Abstract Nephrotic syndrome is still a therapeutic challenge because an effective treatment has not been developed. Evidence suggests that multidrug therapy is more effective than monotherapy in amelioration of renal injury. Therefore, we examined if taurine exerts a protective effect on doxorubicin-induced acute kidney injury in mice. Eight-week-old male Balb/c nude mice were used in this study. Taurine was orally administered at a dose of 50 mg/kg and 100 mg/kg body weight for 5 days. In the meantime, the mice were administered intraperitoneal injections of doxorubicin at 15 mg/kg body weight. At 24 h after the doxorubicin challenge, the response in the taurine-treated mice was compared with that in the vehicle-treated control mice. The doxorubicin-induced acute kidney injury model displayed a significant increase in the renal expression of apoptosis-related proteins (p53, phospho-p53, caspase 9, and caspase 3), whereas in the taurine-treated mice, the augmented expression of renal inflammation-related

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mRNAs such as NF-kB, COX-2, and iNOS was down-regulated. These results suggest that taurine acts as a renoprotective agent by inhibiting apoptosis and inflammation in the kidney of mice with doxorubicin-induced renal injury.

Keywords Taurine • Doxorubicin • Kidney

Abbreviations

COX-2	Cyclooxygenase-2
iNOS	Inducible nitric oxide synthase
NF-κB	Nuclear factor kappa-B

1 Introduction

Doxorubicin is the most effective anticancer drug available today (Kufe et al. 2003). However, doxorubicin-induced renal toxicity overshadows its anticancer effects (Hassan et al. 2014). Organ toxicity after doxorubicin infusion is a major cause of treatment disruption. However, whether different doxorubicin infusion rates induce different toxicities is still not known. Current clinical practice uses doxorubicin in combination with novel targeted therapeutics to maximize response (Rayson et al. 2008). Doxorubicin exerts its effects via a combination of several different mechanisms that account for its high efficacy (Minotti et al. 2004). These mechanisms include intercalation between complementary bases in the DNA, which prevent replication of rapidly growing cancer cells (Sinha et al. 1984). Unfortunately, doxorubicin efficacy is limited by its nephrotoxicity, cardiotoxicity, and hepatotoxicity (Taskin and Dursun 2012; Wang et al. 2000; Van den Branden et al. 2002; Deman et al. 2001).

Cyclooxygenase-2 overproduction has been demonstrated in nephrotoxicity (Honma et al. 2013; Rios et al. 2012). Interestingly, a product of COX-2, is shown to enhance production of cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) (Nukatsuka et al. 1996), which are critical mediators of inflammatory disorders (Akira and Kishimoto 1996; Shima et al. 2005; Suematsu et al. 1989; Tomiyama-Hanayama et al. 2009).

Taurine, a semi-essential amino acid, has several important functions in the mammalian central nervous system such as osmoregulation, neuromodulation, membrane stabilization, and cell proliferation (De Luca et al. 2015; Oja and Saransaari 2007). In addition, taurine protect cardiac toxicity results from doxorubicin which stimulates reactive oxygen spices (ROS), and attenuate the risk of heart failure (Ito et al. 2014; Wang et al. 2013; Pansani et al. 2012; Beyranvand et al. 2011). However, there are very few studies on its renoprotective effects in prostate cancer models.

Therefore, in this study, we determined whether taurine protected against doxorubicin-induced renal injury in prostate cancer model and explored the molecular mechanisms underlying the taurine-mediated suppression of pro-inflammatory cytokine expression.

2 Methods

2.1 Materials

Balb/c nude mice were obtained from Samtaco Bio Korea (Osan, South Korea). Taurine was purchased from Sigma-Aldrich. (St. Louis, MO, USA). The TRIzol reagent for RNA extraction was obtained from Invitrogen (Carlsbad, CA, USA). Primary antibodies and the peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). All other reagents were of the highest grade commercially available at the time of the study.

2.2 Animal Study

Six-week-old male Balb/c nude mice were randomized into four groups, each consisting of 6–8 animals. The mice were inoculated subcutaneously with 2×10^6 PC-3 human prostate cancer cells suspended in PBS. When the xenograft tumors reached a volume of approximately 100 mm^3, the mice were randomly assigned to four groups: (1) CON, (2) DOX, (3) DOX + T50 (doxorubicin +50 mg/kg/day taurine), and (4) DOX + T100 (doxorubicin +100 mg/kg/day taurine). Doxorubicin (0.2 mL at 15 mg/kg) was administered intraperitoneally and taurine was administered by oral gavage (0.2 mL). Control animals were administered normal saline orally. Doxorubicin was injected in a single dose on the nineth day after xenograft inoculation. Tumor size was measured using digital calipers and was calculated as $V = 1/2 \times (a \times b^2)$, where a is the largest superficial diameter and b is the smallest superficial diameter (Zhang et al. 2015).

2.3 Analysis of mRNA Expression

For the reverse transcription-polymerase chain reaction (RT-PCR), total cellular RNA was isolated from the kidney tissue of each treatment group using TRIzol according to the manufacturer's protocol. The first-strand complementary DNA (cDNA) was synthesized using Super-script II reverse transcriptase (Invitrogen). The RT-PCR conditions were similar to the ones described in related studies.

The mixture was incubated for 15 min at 95 °C, before 40 cycles of amplification as follows: denaturation for 30 s at 95 °C, annealing at a transitional temperature range from 58 to 62 °C with an increase of 0.5 °C per cycle; and then extension for 30 s at 72 °C. The expression levels of the analyzed genes were normalized to that of GAPDH for each sample and presented as relative mRNA level.

2.4 Western Blot

After the indicated treatments, kidney tissue was harvested in PBS and lysed in RIPA buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% nonidet P-40, 0.1% SDS, and 50 mM Tris) containing protease inhibitors (50 mg/mL phenylmethylsulfonyl fluoride, 10 mg/mL aprotinin, 5 mg/mL leupeptin, 0.1 mg/mL NaF, 1 mM DTT, 0.1 mM sodium orthovanadate, and 0.1 mM β-glycerophosphate). Total cellular proteins were quantified by the Bradford method, equal amounts of protein were mixed with loading buffer (25% glycerol, 0.075% SDS, 1.25 mL of 14.4 M 2-mercaptoethanol, 10% bromophenol blue, and 3.13% stacking gel buffer) and fractionated by gel electrophoresis on gradient gels (Novex, CA, USA). Rainbow marker (Novex, CA, USA) was used as the molecular weight standard. Proteins were transferred to nitrocellulose membranes (Novex, CA, USA) and blocked for 1.5 h with Clear Milk (Thermo Scientific, IL, USA). Blots were subsequently incubated with primary antibodies in 1X TBST for 1.5 h. Goat anti-rabbit or goat anti-mouse horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology, TX, USA) were used at 1:5000 dilution in 1X TBST. Blots were treated with Western Lightning Western Blot Chemiluminescence Reagent (Advansta, CA, USA) and the reactive protein bands were detected by autoradiography (Fujifilm, Japan). Protein levels were normalized using β-actin antibodies.

2.5 Statistical Analysis

All data are sourced from at least three experiments and presented using the mean ± SE. Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC, USA). Treatment effects were analyzed using one-way analysis of variance, followed by Dunnett's multiple range tests. $p < 0.05$ was considered statistically significant.

3 Results

3.1 Effect of Taurine on Prostate Cancer in the Doxorubicin-Induced Renal Injury Model

The anti-proliferative activity of taurine was evaluated by measuring the tumor volume. As shown in Fig. 1, doxorubicin significantly inhibited tumor growth. Moreover, taurine exhibited a dose-dependent anti-proliferative effect at 50 and 100 mg/kg/day.

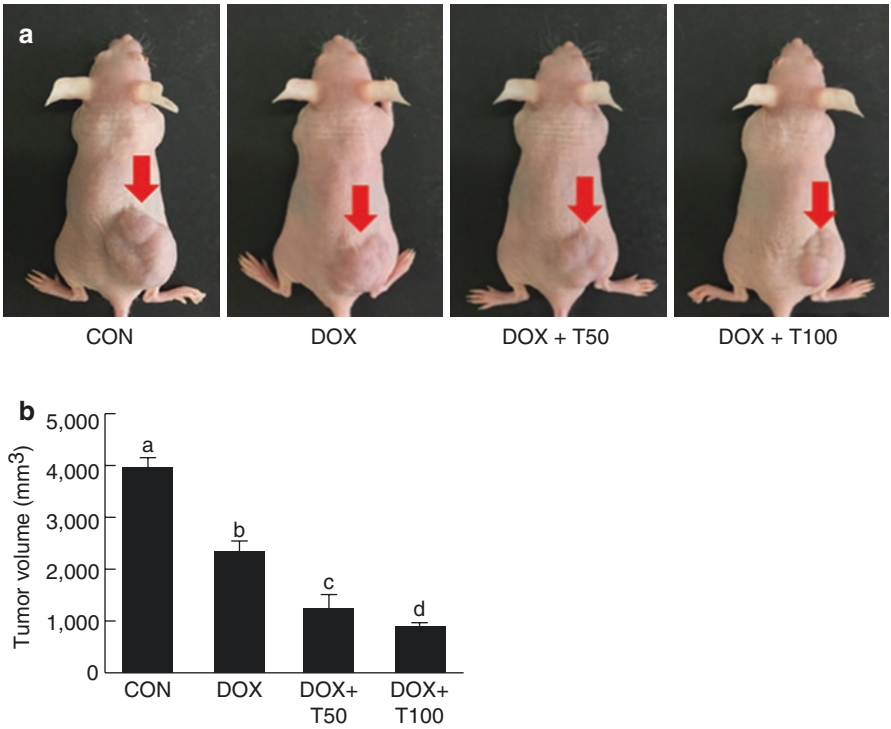

Fig. 1 *In vivo* tumorigenicity of PC-3 cells and effectiveness of drug treatment at 5 days after doxorubicin injection. CON (saline), DOX (15 mg/kg doxorubicin), DOX + T50 (doxorubicin +50 mg/kg/day taurine), DOX + T100 (doxorubicin + 100 mg/kg/day taurine). (**a**) Representative images of tumors in PC-3–inoculated mice on Day 5 after doxorubicin-treatment (n = 8/group) (**b**) Volume of tumors in each treatment group. Values not sharing a common letter are significantly different at $P < 0.05$ by Dunnett's multiple range test

3.2 *Effect of Taurine on Pro-inflammatory Cytokine Expression in the Kidneys of Doxorubicin-Induced Renal Injury Model*

To examine the effect of taurine on doxorubicin-induced renal injury, we performed RT-PCR using RNA from kidney tissue. To compare the differences in gene expression, images of amplicons were captured at the same size, and their relative density was determined. Figure 2 shows that doxorubicin induced a significant increase in the expression of pro-inflammatory cytokines such as COX-2, IL-6, IL-1β, and TNF-α. However, a significant dose-dependent reduction in the expression of these genes was observed in the taurine-treated group.

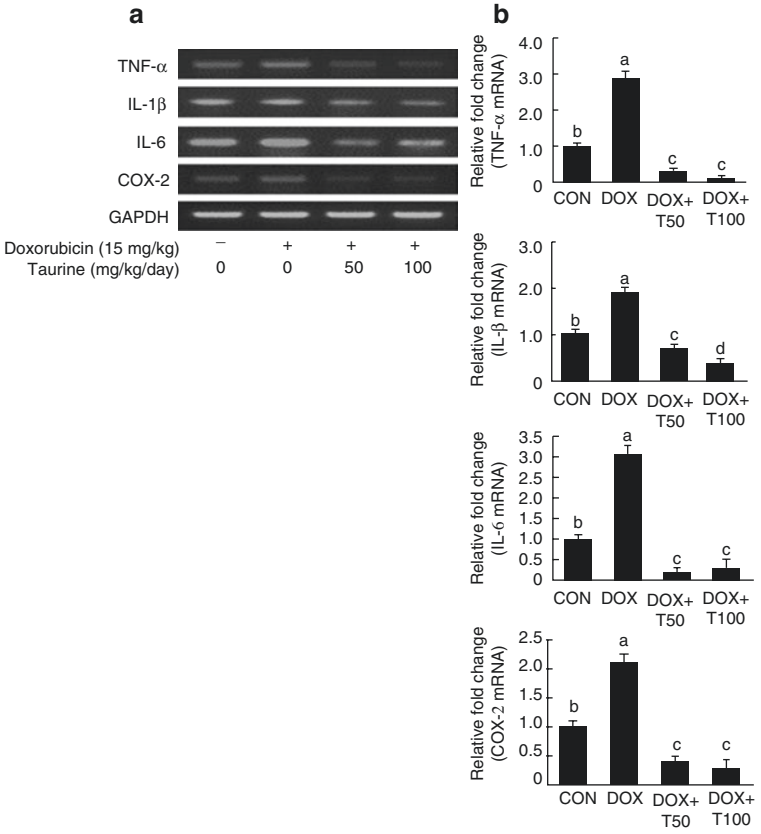

Fig. 2 Effect of taurine on the expression of pro-inflammatory genes in the kidneys of doxorubicin-induced renal injury model. (**a**) After 24 h of taurine treatment, cells were harvested and total RNA was isolated. Expression of TNF-α, IL-1β, IL-6, COX-2, and GAPDH genes was measured by RT-PCR. (**b**) Fold changes in the expression of each analyzed gene. mRNA levels of the analyzed genes were normalized to that of GAPDH for each group. Values not sharing a common letter are significantly different at $P < 0.05$ by Dunnett's multiple range test. CON (saline), DOX (15 mg/kg doxorubicin), DOX + T50 (doxorubicin +50 mg/kg/day taurine), DOX + T100 (doxorubicin + 100 mg/kg/day taurine)

3.3 Effect of Taurine on the Expression of Apoptosis-Related Genes in the Kidneys of Doxorubicin-Induced Renal Injury Model

Expression of apoptosis-related factors in the kidneys of the doxorubicin-induced renal injury model following taurine treatment was examined by western blot. Oral administration of taurine significantly decreased levels of cleaved-caspase-3 and cleaved-caspase-9, which had increased with doxorubicin treatment (Fig. 3). These

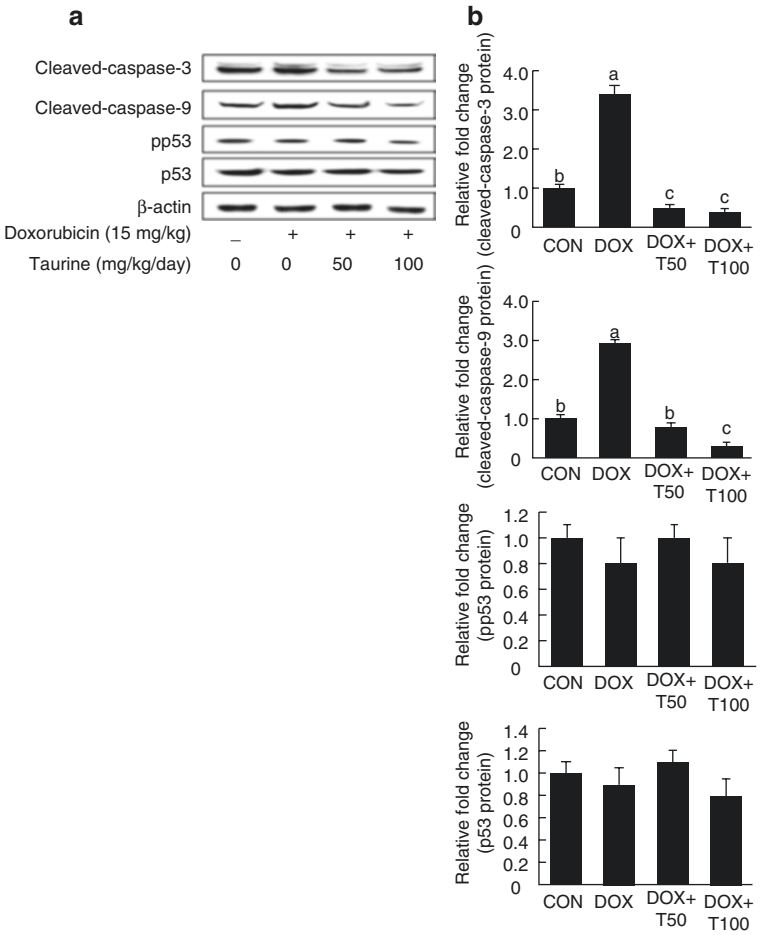

Fig. 3 Effect of taurine on the expression of apoptosis-related genes in the kidneys of doxorubicin-induced renal injury model. (**a**) Expression of apoptosis-related genes (cleaved-caspase-3, cleaved-caspase-9, pp53, and p53) was measured by western blot. (**b**) Fold changes in the expression of each analyzed genes. Protein levels were normalized to that of β-actin for each sample. Values not sharing a common letter are significantly different at $P < 0.05$ by Dunnett's multiple range test. CON (saline), DOX (15 mg/kg doxorubicin), DOX + T50 (doxorubicin +50 mg/kg/day taurine), DOX + T100 (doxorubicin + 100 mg/kg/day taurine)

data suggest that taurine suppresses kidney injury by regulating caspase activation in the kidney tissue. In addition, we also estimated p53 levels. As shown in Fig. 3, taurine did not significantly affect the expression of p53 or pp53. These data suggest that taurine suppresses the doxorubicin-induced renal injury by regulating the activation of caspase-3 and caspase-9.

4 Discussion

Doxorubicin is one of the most important anthracycline drugs used to treat carcinomas, leukemias, and sarcomas. The use of doxorubicin in the treatment of human tumors is strictly limited due to its strong cardiotoxicity, leading to dilated heart and cardiomyopathic failure in patients (Grenier and Lipshultz 1998; Volkova and Russell 2011). The cytotoxic action of doxorubicin involves DNA intercalation by formation of additional bonds between nitrogen bases of the complementary strands, leading to disruption of DNA replication and transcription in affected cells.

Since its development in the early 1960s, doxorubicin remains among the most effective anticancer drugs. Several mechanisms have been proposed to explain the drug's antitumor activity, poisoning of topoisomerase II, and the generation of free radicals, which causes DNA and cell membrane damage (Gewirtz 1999; Torres and Simic 2012). These effects are associated with increased production of pro-inflammatory cytokines (Ujhazy et al. 2003). However, doxorubicin has proven to be a "double-edged sword" because it also causes severe organ damage that is refractory to common medications (Abbas 2011; Shafik et al. 2011).

Doxorubicin-induced increase in superoxide anion production elevates the level of pro-inflammatory cytokines including TNF-α, IL-1β, IL-6, and COX-2 (Mohamed et al. 2011). In addition, there are several reports on the antioxidant activity of taurine (Adedara et al. 2017; Zhu et al. 2016; Rosa et al. 2014). In this study, pro-inflammatory cytokines were up-regulated by doxorubicin in renal tissue, whereas taurine suppressed their expression.

In experimental animal models, doxorubicin exhibited acute toxicity followed by chronic progressive toxicity, mainly affecting kidney and heart (Comereski et al. 1994; Mazue et al. 1995). Doxorubicin accumulation in the kidney of rodents leads to severe nephrotoxicity, which mirrors human chronic kidney disease, and has been extensively studied as a model of renal injury (Ayla et al. 2011; Mihailovic-Stanojevic et al. 2009). In this study, doxorubicin stimulated caspase 3 and caspase 9 in the doxorubicin-induced kidney injury model. In contrast, taurine suppressed the expression of cleaved-caspase 3 and cleaved-caspase 9. These results support the hypothesis that taurine exerts a protective effect on doxorubicin-induced renal injury by regulation of the expression of pro-inflammatory and apoptosis-related genes.

5 Conclusion

This study was aimed at assessing the protective effects of taurine on doxorubicin-induced nephrotoxicity in mice. Doxorubicin induced a significant decrease in tumor volume and kidney weight. Doxorubicin also stimulated a significant increase in inflammatory cytokines in the renal tissue. However, administration of taurine with doxorubicin mitigated all doxorubicin-induced effects. Taurine ameliorated doxorubicin-induced renal injury by inhibiting inflammatory cytokine production and the activities of caspase-3 and caspase-9.

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Taurine Attenuates Epithelial-Mesenchymal Transition-Related Genes in Human Prostate Cancer Cells

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Abstract Prostate cancer is the most common non-cutaneous cancers among men and the second leading cause of cancer-related deaths among men. Aberrant activation of the epithelial to mesenchymal transition (EMT) has been exhibited to be one of the most common causes of treatment failure and death in cancer patients. In cancer cells with metastatic competence, the E-cadherin switch is a well-established hallmark. Suppression of E-cadherin through its transcriptional repressor SNAIL is thus a determining factor for EMT. TWIST1 is an important transcription factor in EMT, which is present under both physiologic (embryogenesis) and pathologic (metastasis) conditions, and enhances the invasiveness and migration ability of cells. In this study, we investigated the inhibitory effects of taurine on EMT-related

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genes, such as E-cadherin, N-cadherin, TWIST1, ZEB1, SNAIL, and vimentin. EMT markers were detected by RT-PCR and western blotting. The results showed that taurine down-regulated the expression of N-cadherin, TWIST1, ZEB1, SNAIL, and vimentin. In contrast, taurine increased E-cadherin expression. Our findings indicate that taurine has EMT inhibitory effects on human prostate cancer cells.

Keywords Taurine • Prostate cancer • Epitherial to mesenchymal transition

Abbreviations

EMT	Epithelial to mesenchymal transition
PSA	Prostate-specific antigen
SNAIL	Snail family zinc finger 1
TWIST1	Basic helix-loop-helix protein 38
ZEB1	Zinc finger E-box binding homeobox 1

1 Introduction

Taurine, also known as 2-amino ethane sulfonic acid, is synthesized endogenously from cysteine and methionine in the presence of vitamin B6 and is supplied by the diet, particularly from fish and meat. Taurine is present in most organs, showing the highest abundance in the heart and brain. Numerous studies have reported that taurine can function as an anticancer agent (Zhang et al. 2014; El-Houseini et al. 2013).

Prostate cancer is the second leading cause of cancer-related mortality and is one of the most common cancers in men (Ferlay et al. 2013). The incidence of this cancer has recently increased, after which the mortality of prostate cancer also increased (Tormey 2014). In Asia, the incidence of prostate cancer has increased more rapidly than in other developed countries (Tang et al. 2015). We previously showed that taurine inhibited prostate-specific antigen (PSA) levels and migration in human prostate cancer cells (Tang et al. 2015).

In prostate cancer, epithelial-mesenchymal transition (EMT) plays a key role in prostate cancer metastasis (Fu et al. 2016). EMT first occurs during embryogenesis and has been investigated to determine its effects on tumor formation and metastatic growth (Garg 2013). It is a naturally occurring process that drives the transformation of adhesive and non-mobile epithelial-like cells into mobile cells with a mesenchymal phenotype that have the capacity to move to anatomically distant locations (Kalluri and Weinberg 2009). EMT is orchestrated and triggered by various EMT-activating transcription factors, including the basic helix-loop-helix factors (Twist1 and Twist2); zinc-finger E-box-binding homeobox (ZEB)1; the SNAIL family of zinc-finger transcription factors: SNAIL1 (SNAIL), SNAIL2 (SLUG), and SNAIL3 (SMUC); vimentin; and N-cadherin (Perez-Moreno et al. 2001; Nieto 2002; Yang

et al. 2004; Rodriguez et al. 2008). In addition, one of the hallmarks of EMT is the functional loss of E-cadherin, which is thought to be a metastatic suppressor of tumor progression (Hanahan and Weinberg 2011). SNAIL is a prominent inducer of EMT and strongly represses E-cadherin expression (Barrallo-Gimeno and Nieto 2005).

Several studies have reported that the progression of EMT in cancer can be retarded by using plants and chemical substances (Ge et al. 2016; Shankar et al. 2011; Scarpa and Ninfali 2015). However, little is known about the inhibition of EMT in prostate cancer cells by taurine.

Therefore, in the present study, we investigated the effects of taurine on EMT in prostate cancer cells.

2 Methods

2.1 Materials

LNCaPs, which are androgen-dependent human prostate cancer cells, were obtained from the Korean Cell Line Bank (Seoul, South Korea; KCLB numbers: 21740). Taurine and dihydrotestosterone (DHT) were purchased from Sigma-Aldrich. (St. Louis, MO, USA). TRIzol reagent for RNA extraction was obtained from Invitrogen (Carlsbad, CA, USA). Antibodies for primary antibodies and peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other reagents were of the highest grade commercially available at the time of the study.

2.2 Cell Culture

The human prostate cancer cell line, LNCaP, was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a 5% CO_2 atmosphere at 37 °C. The cells were seeded at a density of 3.5×10^5 cells per well in a 13-cm culture dish. After 24 h, the cells were treated with 250, 500, and 1000 μM taurine in the medium. Cells were treated with taurine for 24 h and then harvested.

2.3 Analysis of mRNA Expression

For reverse transcription-polymerase chain reaction (RT-PCR), total cellular RNA was isolated from the cells of each treatment group using TRIzol according to the manufacturer's protocol (Kim et al. 2014). First-strand complementary DNA was

synthesized using Superscript II reverse transcriptase (Invitrogen). The conditions for RT-PCR were the same as those described in a previous study (Kim et al. 2014). The mixture was first incubated for 15 min at 95 °C for initial denaturation, followed by 40 cycles of the amplification step. The following protocol was followed: denaturation for 30 s at 95 °C, annealing at a transitional temperature range from 58 to 62 °C with an increase of 0.5 °C per cycle; and then extension for 30 s at 72 °C. The expression levels of the analyzed genes were normalized to that of GAPDH for each sample and presented as relative mRNA levels.

2.4 Western Blotting

After the indicated treatments, the cells were harvested in PBS and lysed in RIPA buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% nonidet P-40, 0.1% SDS, and 50 mM Tris) containing protease inhibitors (50 mg/mL phenylmethylsulfonyl fluoride, 10 mg/mL aprotinin, 5 mg/mL leupeptin, 0.1 mg/mL NaF, 1 mM DTT, 0.1 mM sodium orthovanadate, and 0.1 mM β-glycerophosphate). Total cellular proteins were quantified using the Bradford procedure and equal amounts of proteins were mixed with loading buffer (25% glycerol, 0.075% SDS, 1.25 mL of 14.4 M 2-mercaptoethanol, 10% bromophenol blue, and 3.13% stacking gel buffer) and fractionated by gel electrophoresis on gradient gels (Novex, CA, USA). Rainbow marker (Novex, Thermo Fisher, Waltham, MA, USA) was used as the molecular weight standard. Proteins were transferred to nitrocellulose membranes (Novex) and blocked for 1.5 h with clear milk (Thermo Scientific). Blots were subsequently incubated with primary antibodies in 1X TBST for 1.5 h. Goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at a 1:5000 dilution in 1X TBST. Blots were treated with Western Lightning Western Blot Chemiluminescence Reagent (Advansta, Menlo Park, CA, USA) and the proteins were detected by autoradiography (Fujifilm, Tokyo, Japan). β-Actin was used as a loading control.

2.5 Statistical Analysis

All data are presented as the mean ± SE from at least three experiments. Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC, USA). Treatment effects were analyzed using one-way analysis of variance, followed by Dunnett's multiple range tests. For the results, $p < 0.05$ was considered to indicate significance.

3 Results

3.1 Effect of Taurine on the Expression of EMT-Related Genes

In a previous study, we reported that taurine significantly stimulated prostate cancer cell death in a dose-dependent manner at concentrations of 125–1000 μM (Tang et al. 2015). Based on these results, we investigated the effects of taurine (0, 250, 500, and 1000 μM) on EMT in prostate cancer cells. DHT-stimulated human prostate cancer cells, LNCaPs, were treated with media supplemented with taurine for 24 h. After 24 h, the cells were harvested, and total RNA and protein were isolated. The gene expression and protein levels of EMT-related genes including E-cadherin, N-cadherin, TIWST1, ZEB1, SNAIL, and vimentin were measured by RT-PCR and western blotting, respectively. Treatment of LNCaP cells with taurine significantly increased the mRNA expression of the epithelial gene E-cadherin (Fig. 1a and b). In addition, mesenchymal genes were estimated. As shown in Fig. 1a and b, taurine

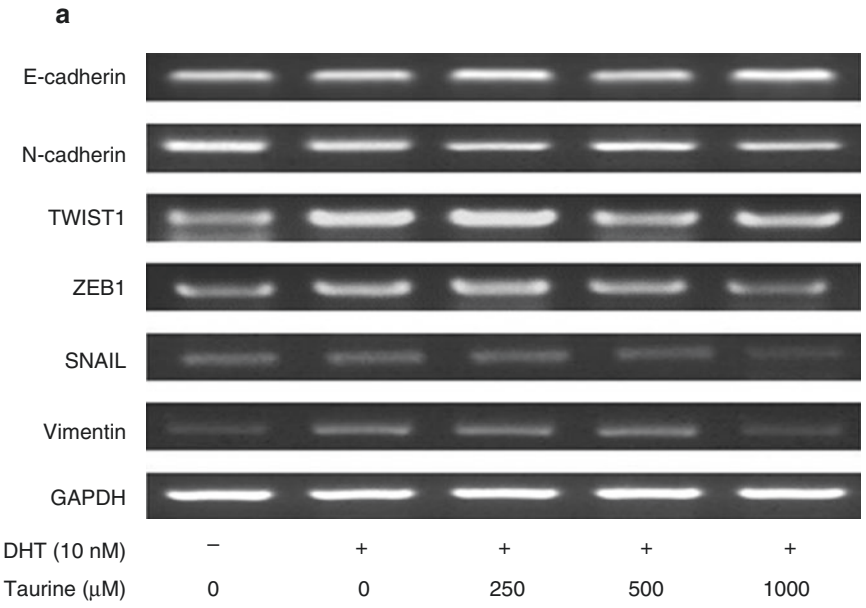

Fig. 1. Effect of taurine on EMT-related gene mRNA levels. LNCaP cells were incubated with or without DHT (10 nM) or taurine (250, 500, and 1000 μM). (**a**) After 24 h, cells were harvested and total RNA was isolated. Gene expression levels of EMT-related genes (E-cadherin, N-cadherin, TWIST1, ZEB1, SNAIL, and vimentin) were measured by RT-PCR. (**b**) The mRNA levels of the analyzed genes were normalized to that of GAPDH for each sample. Means with different letters (a–c) within each graph were significantly different from other samples at $p < 0.05$. CON, control; DHT, dihydrotestosterone

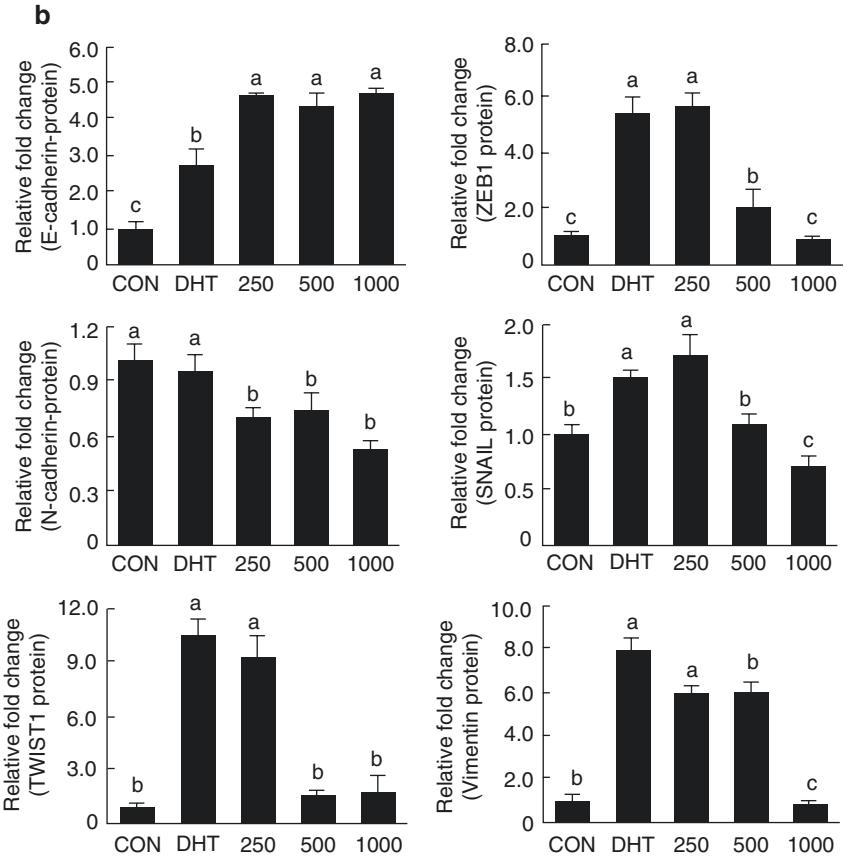

Fig. 1. (continued)

significantly suppressed the mRNA expression of EMT-related genes such as N-cadherin, TIWST1, ZEB1, SNAIL, and vimentin in LNCaP cells. These data suggest that taurine suppresses the mesenchymal condition by regulating the mRNA levels of E-cadherin, N-cadherin, TIWST1, ZEB1, SNAIL, and vimentin in the androgen-dependent human prostate cancer cells LNCaPs.

In addition, we examined the effect of taurine on the protein expression of EMT-related genes. As shown in Fig. 2a and b, taurine significantly suppressed the protein expression of EMT-related genes such as N-cadherin, TIWST1, ZEB1, SNAIL, and vimentin in LNCaP cells. These data suggest that taurine suppresses the mesenchymal condition by regulating the protein and mRNA levels of E-cadherin, N-cadherin, TIWST1, ZEB1, SNAIL, and vimentin in LNCaPs.

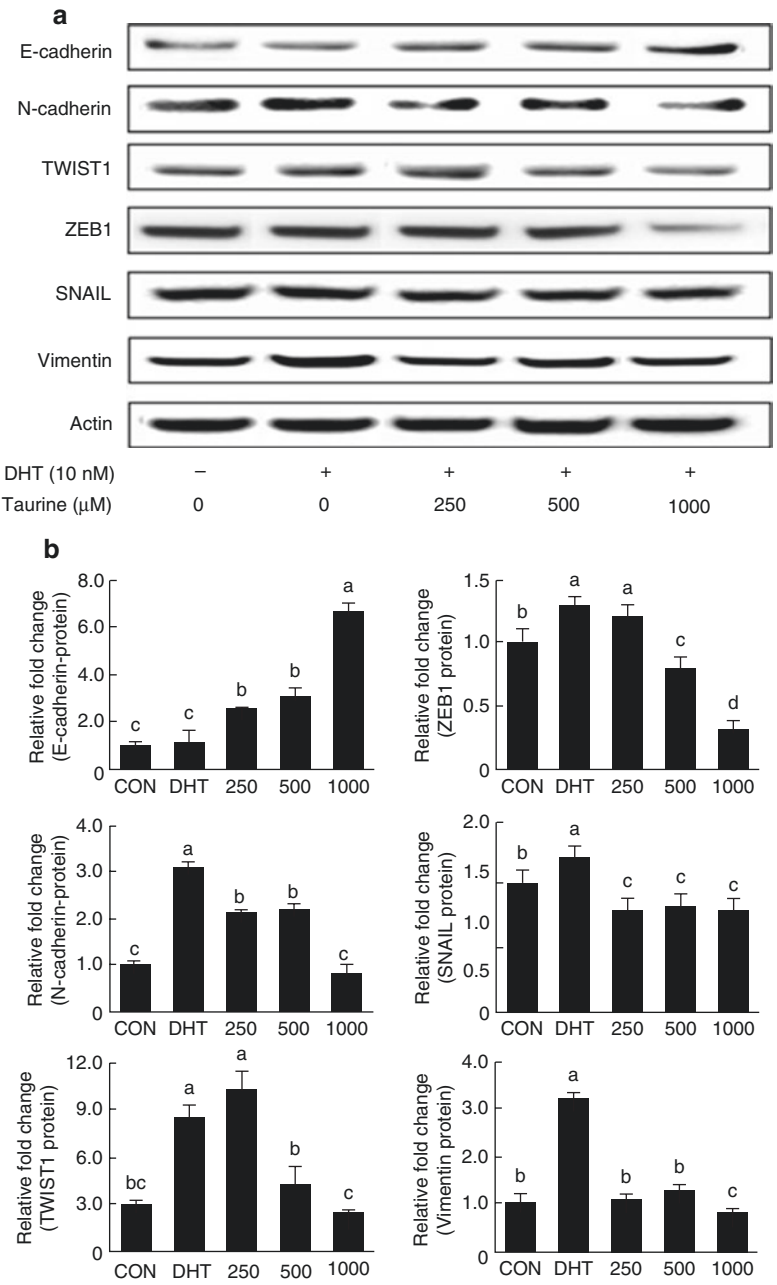

Fig. 2. Effect of taurine on EMT-related gene protein levels. Taurine LNCaP cells were incubated with or without DHT (10 nM) and taurine (250, 500, and 1000 μM). (**a**) After 24 h, the cells were harvested and total protein was isolated. Gene expression levels of EMT-related genes (E-cadherin, N-cadherin, TIWST1, ZEB1, SNAIL, and vimentin) were measured by western blotting. (**b**) The protein levels of the analyzed genes were normalized to that of β-actin for each sample. Means with different letters (a–d) within a graph are significantly different at $p < 0.05$. CON, control; DHT, dihydrotestosterone

4 Discussion

EMT is naturally occurring phenomenon and plays a key role during embryogenesis as well as adult tissue repair and maintenance (Garg 2013). It is represented by specific gene expression pattern changes and the loss of adherent tight junctions that maintain contact of epithelial cell with their neighbors. These factors are related to epithelial genes including E-cadherin, gain of mesenchymal phenotype, including fibro-blastoid morphology, and increased mobility potential to distant sites, which involves mesenchymal genes such as N-cadherin, TIWST1, ZEB1, SNAIL, and vimentin (Fig. 3). Recent increasing evidence has demonstrated the vital process of EMT-activating transcription factors in oncogenic transformation.

EMT is characterized by the downregulation of epithelial markers/tight junction components, desmosomes, cytokeratins, and gain of mesenchymal markers such as reorganization of the cytoskeleton (e.g., switch from cytokeratins to vimentin), and the synthesis of extracellular matrix components and metalloproteases (Thiery et al. 2009). Loss-of-function mutations and promoter hyper-methylation may downregulate E-cadherin expression and function in a number of carcinomas, but modulation of EMT during embryogenesis and cancer progression primarily involves EMT-activating transcription factors (Scheel et al. 2011). In our previous study, we demonstrated that taurine inhibited metalloproteases in human prostate cancer cells (Tang et al. 2015). Moreover, we demonstrated that taurine stimulated the expression of an epithelial markers/tight junction component, E-cadherin. In addition to a mesenchymal switch, these factors control the entire EMT program and endow cancer cells with stem-like characteristics. These migrating cancer stem cells are not only important in the genesis of primary tumors, but also enhance metastasis and possibly the root cause of tumoral chemoresistance and recurrence (Scheel et al. 2011). We also found that taurine suppressed the migration of human prostate cancer cells (Tang et al. 2015).

Binding of TWIST1 to other transcriptional regulators, post-translational modifications, and choice of partner for dimerization regulate the expression of target genes. Classical EMT-inducing pathways, such as TGFβ, Wnt, hypoxia, and ligand

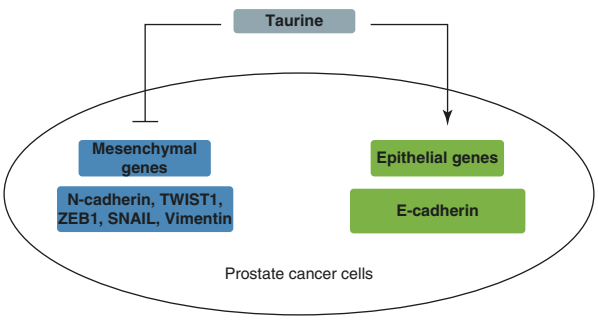

Fig. 3. Schematic illustration of taurine-mediating signaling pathways during epithelial-to-mesenchymal transition in prostate cancer cells

binding activation of receptor tyrosine kinases and inflammatory cytokine receptors, activate TWIST1 and have significant implications in tumor invasion and angiogenesis (Qin et al. 2012; Yang et al. 2008). In addition, the binding of TWIST1 to the H4K20 methyltransferase SET8 represses E-cadherin and activates N-cadherin (Fu et al. 2011; Yang et al. 2012). In this study, taurine inhibited N-cadherin and TWIST1 expression in DHT-stimulated human prostate cancer cells.

The ZEB family consists of the zinc finger/homeodo-main proteins-ZEB1 and ZEB2, which are well conserved among species. These proteins interact with other transcriptional factors and their expression is modulated by post-translational modifications such as SUMOylation by Pc2 or acetylation by p300/pCAF and phosphorylation. These proteins trigger EMT by inhibiting epithelial markers and activating mesenchymal properties (Garg 2013). Additionally, SNAIL-mediated histone modifications are induced to repress E-cadherin. Signals, including TGFβ, Notch, tumor necrosis factor-α, EGF, FGF, Wnt, Shh, and estrogens, regulate SNAIL proteins, not only during development but also in cancer cells (Thiery et al. 2009). In the present investigation, taurine suppressed the levels of ZEB1 and SNAIL as well as vimentin, which is a mesenchymal marker.

5 Conclusion

Our results demonstrated that taurine inhibited human prostate cancer cell metastasis. Our findings also indicate that taurine possesses EMT inhibitory effects in the androgen-dependent human prostate cancer cells, LNCaPs. These *in vitro* results provide a foundation for future studies of this novel therapeutic regimen and suggest that combined use of taurine with other agents can be used to prevent and treat prostate cancer in humans.

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