Oxidative Stress in Human Reproduction

Shedding Light on a Complicated Phenomenon

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Foreword

Since the introduction of the concept of "oxidative stress" introduced into redox biology and medicine and coining of the term by Helmut Sies more than three decades ago in 1985 [1], it became very clear that oxidative stress is involved not only in the pathogenesis of many conditions ranging from aging, infections, inflammations, obesity, and cancer, but also in male and female factor infertility. A multitude of almost 157,000 PubMed entries in September 2016 for a general search of "oxidative stress" highlights the importance of research in this field. If one is only thinking of the significant impact oxidative stress has on sperm function, prevalence rates between 30 and 80% have been reported. One feature of this condition is that it is caused by either an excessive production of reactive oxygen species (ROS) and/or a deficiency in the antioxidative defenses in the body. Therefore, it is important to understand not only the pathophysiology of the respective medical condition but also the biochemistry behind as well as consequences and treatment options. Alongside with a better understanding of the implications of oxidative stress, particularly in an infertility clinical setting, the diagnostic tests of oxidative stress have evolved from very costly and insensitive tests into cheaper and more sensitive assays. These assays allow proper statistical evaluation with Receiver Operating Characteristics (ROC) curve analyses, providing useful information such as the ROC plot and full sensitivity/specificity reports, which are important in optimal counseling and advising patients in terms of options of treatment, be it options of assisted reproductive techniques or antioxidant treatment.

Oxidative Stress in Human Reproduction: Shedding Light on a Complicated Phenomenon is an excellent and up-to-date summary of the efforts made to understand the contribution of oxidative stress to male and female infertility and relevant treatment options. The authors of this book are well suited to report on this topic as they are affiliated to the World's Number One Andrology laboratory of infertility care. The lead authors (Drs. Ashok Agarwal, Rakesh Sharma, and Sajal Gupta) are the authorities in the field who have contributed to the advancement of knowledge on various aspects of oxidative stress in male and female infertility in more than 750 scientific articles (includes original articles, reviews and invited book chapters) and have assembled renowned authors elaborating on the sources of reactive oxygen

species (ROS) and methods to measure ROS. Further chapters review the physiological and pathological roles of ROS and oxidative stress in both the male and female reproductive systems as well as lifestyle, general health, and environment as extrinsic factors causing oxidative stress. In addition, various therapeutic options for the treatment of infertility as an important part of patient care are adequately dealt with. Finally, the current knowledge is summarized in a compendium of studies published on oxidative stress by the Cleveland Clinic in the past 24 years. The book is clearly structured, well written, and appropriately referenced.

For all these reasons, I am convinced that this book will be of great value to researchers, embryologists, infertility specialists, urologists, and practitioners involved in human infertility assessment and treatment. I am sure the reader will learn a lot from this book and I recommend it strongly.

Ralf Henkel
Bellville, South Africa

Dr. Henkel studied Biology and Chemistry at the Philipps University of Marburg, Marburg, Germany, and obtained his PhD in Zoology also from the same university. After his further training in Andrology with Wolf-Bernhard Schill, MD, at the Justus-Liebig University of Giessen, Giessen, Germany, Thinus Kruger, MD, and Daniel Franken, PhD, at Tygerberg Hospital, Tygerberg, South Africa, he obtained his Habilitation (Second PhD) in Reproductive Biology at the Justus-Liebig University of Giessen. Dr. Henkel is a member of the German Society of Andrology. He is a Professor and the Head of the Department of Medical Bioscience at the University of the Western Cape, Bellville, South Africa. He developed and evaluated new tests for andrological diagnosis. Furthermore, Dr. Henkel extensively investigated the impact of oxidative stress on sperm function and DNA fragmentation. He has published 94 original research papers and review articles as well as 28 book chapters and graduated 49 MSc and PhD students. He is also member of editorial boards of several international journals, Associate Editor of the *Journal of Reproductive Biotechnology and Fertility*, and Co-Editor-in-Chief of *Andrologia*.

Reference

1. Sies H (1985) Oxidative stress: introductory remarks. In: Oxidative stress. pp 1–8

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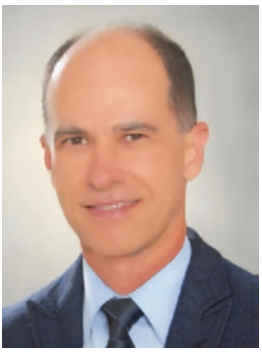

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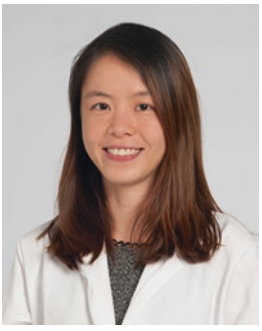

Siew May Wang, MD Dr. Wang received her medical degree from the University of Glasgow, United Kingdom, in 2012, graduating with honors. She worked as a medical doctor in Singapore before moving to the United States in 2016. Siew May is a student researcher at the American Center for Reproductive Medicine at Cleveland Clinic. Her research interests are in the area of oxidative stress, sperm DNA damage, and male infertility.

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Chapter 1
Overview and Sources of Reactive Oxygen Species (ROS) in the Reproductive System

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1.1 Introduction

Reactive oxygen species (ROS) are highly reactive molecules that are generated from oxygen metabolism. They can be free radicals or non-radicals. Free radicals are molecules that contain at least one unpaired valence electron at their outer shell, making them highly reactive and short lived [1]. Among all the ROS, superoxide anion ($\bullet O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\bullet OH$) are the most known examples. Reactive nitrogen species (RNS), is the subclass of ROS that contain nitrogen compound [2]. Both ROS and RNS, when present in physiological amount, have important roles in normal cellular functions such as fighting against infection, regulating different intercellular signaling pathways and facilitating normal maturation and fertilization in reproductive systems [1, 3–7]. However, when

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ROS present in high concentration, overwhelming the antioxidant defense system, oxidative stress results, and this may lead to cellular dysfunction via lipid peroxidation, protein and DNA damages [8]. Due to such damaging effect on the cells, OS is related to many pathological conditions including infertility [3, 9].

This chapter introduces concepts of free radicals, redox reactions, and then focuses on ROS, RNS and their sources at cellular levels, particularly in both male and female reproductive systems as well as covering a brief overview of oxidative stress.

1.1.1 Free Radicals

Free radicals are molecules that contain at least one unpaired valence electron, making them highly reactive and short-lived [1]. Among all the free radicals, hydroxyl radical (•OH), and superoxide anion ($\bullet O_2^-$) are the most known examples. They derive from molecular oxygen under reducing conditions; however, because of their reactivity, these free radicals can have deleterious effect on normal cellular functions when present in high concentrations [1]. Other examples of free radicals are hydroxyl radical (•OH), superoxide anion ($\bullet O_2^-$), nitric oxide (•NO), lipid peroxyl ($\bullet LOO^-$) and thiyl (•RS) [9].

1.1.1.1 Superoxide Anion ($O_2\bullet^-$)

Univalent reduction of oxygen will result in the formation of superoxide anion ($O_2\bullet^-$) [1]. This reaction can happen in the mitochondrial electron transport chain (ETC) without the involvement of any enzymatic activity by the action of oxidation-reduction reactive intermediates such semi-ubiquinone [10]. On the other hand, enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and xanthine oxidase (XO) can also mediate this reaction. High amounts of superoxide anions are usually released from neutrophils and macrophages by the action of different isoforms of NADPH oxidase. These superoxide anions can then be converted into non-radical species, hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), enzymatically with oxidative enzymes such as superoxide dismutase (SOD) or nonenzymatically [4].

$$e^- + O_2 \rightarrow O_2 \bullet^-$$

$$\xrightarrow{\text{SOD}}$$

$$2O_2 \bullet^- + 2H^+ \rightarrow O_2 + H_2O_2$$

1.1.1.2 Hydroxyl Radical (•OH)

The interaction between hydrogen peroxide (H_2O_2) and reduced transition metals such as copper and iron results in Fenton reaction, which leads to the formation of hydroxyl radical (•OH). In addition, the exposure of water molecules to ionizing radiation can also result in the production of hydroxyl radical [1, 11, 12].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$$

1.1.2 Oxidation-Reduction (Redox) Reactions

Redox reactions or oxidation–reduction reactions are reactions that involve transfer of electrons between two chemicals. The number of electrons accepted, lost or shared is known as the oxidation number. When the oxidation number of any chemical increases, it is said that this chemical is oxidized and the process is known as oxidation. In contrast, when the oxidation number is decreased, the chemical is reduced and the process is known as reduction C [12, 13]. Iron rust is a good example of a redox reaction. As shown below, iron (Fe) loses electrons to oxygen. Hence, the oxidation number of iron increases while the oxidation number of oxygen decreases. In other words, iron is oxidized while oxygen is reduced at the same time. This is what happens when an iron nail rusts [13].

$$4Fe^{2+} + O_2 \rightarrow 4Fe^{3+} + 2O^{2-}$$

1.1.2.1 Haber-Weiss Reaction

Haber-Weiss reaction is another example of a redox reaction. In Haber-Weiss reaction, superoxide anion interacts with hydrogen peroxide in the presence of a metal ion (as a catalyst) to produce a hydroxyl radical [13]. The net reaction can be broken down into two reactions. The first step involves the reduction of ferric to ferrous ion; followed by the second step, the Fenton reaction. Along with the Fenton reaction, these two reactions are important in generating the highly reactive hydroxyl radicals [14, 15].

$$\text{Reaction1} : Fe^{3+} + O_2 \bullet^- \rightarrow Fe^{2+} + O_2$$

$$\text{Reaction2} : Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$$

$$\text{Net Reaction} : O_2 \bullet^- + H_2O_2 \rightarrow \bullet OH + OH^- + O_2$$

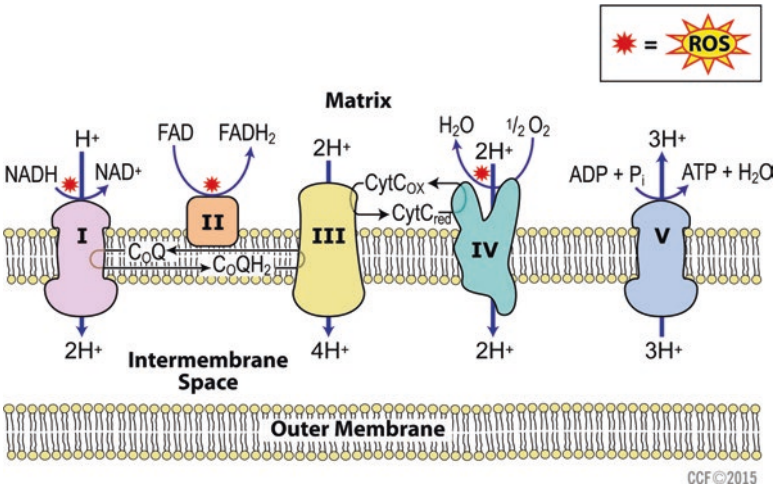

Fig. 1.1 Generation of ROS via electron transport chain

1.1.2.2 Electron Transport Chain (ETC)

Electrons passage through a couple of specialized enzymes known as ETC in the mitochondria is important in cellular energy (adenosine triphosphate, ATP) production. It involves a series of redox reactions. Electrons first enter the chain after the reduction of NADH and $FADH_2$, both of which act as electron carriers. Cytochrome oxidase, acts as the final acceptor of these electrons, enhances the tetravalent reduction of oxygen into water through a process known as oxidative phosphorylation [16]. During this process, a small amounts of oxygen (2–5%) are univalently reduced into free radicals [17]. Ubisemiquinone, a part of ETC, is considered the main site of electron leak leading to the generation of superoxide anions (Fig. 1.1) [4].

1.1.3 Reactive Oxygen Species (ROS)

The term reactive oxygen species (ROS) includes the reduced form of oxygen and their reaction products with other molecules. Some but not all ROS are free radicals [1]. Superoxide anion and hydroxyl radical are examples of radical ROS, whereas hydrogen peroxide and hypochlorite ion are non-radical ROS (Table 1.1). ROS usually have high reactivity due to the extra-unpaired electron at their outer shells and their half-lives are in the order of milliseconds [10, 18, 19]. The hydroxyl radical is the most damaging radical because it cannot be scavenged easily in our bodies [1, 20].

Intracellular ROS can be produced by stimulation of tightly regulated NADPH intracellular oxidases and peroxidases or as an alternate product by other enzymes

Table 1.1 Radical and non-radical reactive oxygen species (ROS)

ROS	
Free radicals	Non-radicals
Hydroxyl radical (•OH)	Hydrogen peroxide (H_2O_2)
Superoxide anion ($•O_2^-$)	Singlet oxygen ($^1O_2$)
Lipid peroxyl ($•LOO^-$)	Ozone (O_3)
Thiyl (•RS)	Lipid peroxide (LOOH)
	Peroxynitrite ($ONOO^-$)

such as cytochrome P450 and nitric oxide synthase. Additionally, ROS can be produced by the leakage of electrons from the ETC (Fig. 1.1), which is regarded as the main ROS source in most cells and xanthine oxidase [4, 21, 22].

The univalent reduction of oxygen in the mitochondria generates superoxide anion, which can be further reduced by the action of mitochondrial superoxide dismutase into hydrogen peroxide [21]. As previously mentioned, ubisemiquinone, a component of mitochondrial respiratory chain, is the most common site for this univalent reduction of oxygen. While small amounts of reduced oxygen exist in all functional cells, large quantities of superoxide anion and its derivatives are generated by neutrophils and activated macrophages via the NADPH oxidase pathway [4].

Another ROS production pathway involves xanthine oxidase (XO). The conversion of hypoxanthine into xanthine by XO and xanthine into uric acid yields superoxide radicals [23]. But only a small amount of ROS is released by the activity of XO under normal circumstances. However, increased degradation of ATP as a result of metabolic stress activates XO leading to increased production of superoxide anion [23]. As a matter of fact, XO has been implicated as a major contributor to ROS levels in certain pathological conditions involving ischemia and reperfusion [24].

Inflammation and infection also play a significant role in generating ROS. For instance, a high production of ROS has been noted in endothelial cells induced by tumor necrosis factor (TNF) [25], lymphocytes can produce ROS via the 5-lipoxygenase (5-LO) pathway possibly with CD 40 ligation [26], and cyclooxygenase-1 generates ROS after stimulated by different chemicals including TNFα, interleukin-1, bacterial lipopolysaccharide, and tumor promoter 4-O-tetradecanoylphorbol-13-acetate (TPA).

Interestingly, oxidation of dopamine can also lead to ROS production and this has been shown to be related to neurodegenerative diseases like Parkinson's disease [4].

1.1.3.1 Physiological Roles

ROS are formed during normal cellular metabolism. They are involved in many physiological processes, including the activation of the immune system. For instance, phagocytes activated as part of the immune system release hydrogen peroxide to kill the pathogens. Superoxide anion is also known to have a role in fighting

Table 1.2 Radical and non-radical reactive nitrogen species (RNS)

RNS	
Free radicals	Non-radicals
Nitric oxide ($\bullet NO^-$)	Nitrogen dioxide: NO_2
Nitrous acid (HNO_2^-)	Dinitrogen tetraoxide: N_2O_4

against infections [1, 27]. On the other hand, ROS released by non-phagocytic cells, including fibroblast, endothelial cells and cardiac myocytes is involved in regulating different intercellular signaling pathways. These cells contain various NADPH oxidase isoforms [4, 5, 28, 29]. Furthermore, physiological amount of ROS is required in spermatozoa capacitation, hyper-activation, acrosome reaction and sperm-oocyte fusion [30].

1.1.4 Reactive Nitrogen Species (RNS)

Reactive Nitrogen species (RNS) are special forms of ROS that contain nitrogen [2]. Similar to ROS, RNS can also include radicals such as nitric oxide (NO) and nitrous acid (HNO_2) and non-radicals such as nitrogen dioxide (NO2) and dinitrogen tetraoxide (N_2O_4) (Table 1.2). Nitric oxide synthase (NOS) can convert L-arginine and oxygen to NO and L-citrulline in the presence of multiple cofactors, including calcium, NADPH, flavin mononucleotide (FMN), calmodulin and flavin adenine dinucleotide (FAD) [31]. Furthermore, different forms of RNS including nitrosonium cation (NO^+), nitroxyl anion (NO^-) or peroxynitrite ($ONOO^-$) can be produced as a result of the interactions of NO with various chemicals. Although NO is not reactive enough to cause DNA damage, NO can react with superoxide anion to produce peroxynitrite ($ONOO^-$) [32].

1.1.4.1 Physiological Roles

RNS are involved in many physiological mechanisms and processes including production of several hormones and regulation of inflammatory response by inhibiting platelets aggregation and preventing the margination of neutrophils to endothelial cells [33, 34].

NO can alter the heme moiety of guanlate cyclase resulting in alteration in the structure of the enzyme leading to the activation of the enzyme. The activated form of guanylate cyclase results in the production of cGMP, which controls the activity of phosphodiesterases, protein kinases and ion channels. These modulations are required for many physiological processes including the control of the smooth muscle tone and suppressing platelet adhesion [35].

1.1.5 Sources of ROS in Male Reproductive System

1.1.5.1 Generation of ROS

Superoxide anion is considered to be the most abundant ROS in human sperm and it is the precursor of other ROS types. Accepting another electron to form hydrogen peroxide and reacts with other radicals such as nitric oxide to form peroxynitrate can further reduce superoxide anion. Finally, it can also react with itself by dismutation, a reaction that is catalyzed by SOD to produce hydrogen peroxide. In the presence of catalase and a transition metal, hydrogen peroxide is converted to the hydroxyl radical, one of the strongest oxidants in nature [9].

The generation of such ROS occurs via two methods: (1) in the cell membranes, using NADPH oxidases, and (2) in the mitochondria, using NADH oxido-reductase.

NADPH Oxidases

NADPH oxidase enzymes are located in the cell membranes of the spermatozoa [9]. NADPH oxidases are a group of enzymes that catalyses the conversion of molecular bivalent oxygen to superoxide. The substrates for these reactions are NADPH molecules generated by the hexose monophosphate shunt. NOX 5 gene was found to be expressed in NADPH oxidase in the spermatozoa, located both in the flagella/neck region and the acromsome and hence, may involve in this process [36].

Electron Leakage in the Mitochondria

ROS are generated by sperm mitochondria under normal cellular respiration. Spermatozoa are rich in mitochondria because they constantly require ATP for motility [37]. Electron leakage from cytochromes in the ETC sources the monovalent reduction of oxygen to the superoxide anion. There is a link between ROS production and NADH oxido-reductases activity in the inner and outer mitochondrial membranes within respiring spermatozoa. NADH oxido-reductase (ubiquinone oxido reductase) catalyses electrons transfer from NADH to coenzyme Q10 in the ETC. Electron leakage occurs during reverse electron transfer, which acts as a source of electrons for the reduction of oxygen to the superoxide anion.

Hyperoxic conditions also increase ROS production [38]. When sperm respiration increases due to an increased need for ATP, the concentration of molecular oxygen in the matrix of the mitochondria increases thus leading to increased superoxide production as well. However, it is interesting to note that ROS production also increases under hypoxic conditions as ROS producing sites in mitochondria have a higher affinity for oxygen than the cytochrome oxidases under such conditions [39].

1.1.5.2 Sources of ROS in Seminal Plasma

Immature/Abnormal Spermatozoa

A marked difference has been noted in the quantity of ROS produced by spermatozoa at different stage of maturation, suggesting that the level of maturation affects ROS production [40].

Immature spermatozoa are characterized by retaining excessive residual cytoplasm (ERC) around their mid-piece, which is normally expelled during spermiogenesis. ERC contains high levels of glucose-6-phosphate dehydrogenase (G6PDH), a cytosolic enzyme that utilizes the hexose monophosphate shunt to produce abnormally high levels of NADPH. Excessive NADPH results in a greater production of superoxide anions by NADPH oxidases [41]. A positive correlation of high ROS levels with excessive cytoplasmic retention in spermatozoa is documented [42].

Furthermore, abnormal morphological sperm produce more ROS than their normal counterparts [43]. In one study, a negative correlation was observed between the production of ROS and the number of healthy sperm with normal or borderline morphology. In the same study, a positive correlation of was seen between ROS production and the number of sperm with amorphous heads, tail defects, excess cytoplasm and abnormal midpiece [44].

Leukocytes

Activated leukocytes (peroxidase positive) produce large quantities of ROS and it has been shown that leukocytes are the predominant source of ROS in raw human semen. ROS produced by leukocytes is believed to account for toxicity against human sperm.

Peroxidase positive leukocytes are primarily macrophages and polymorphonuclear (PMN) leukocytes originating from the prostate and seminal vesicles [40]. Such leukocytes are often found in the seminal fluid. In one study in which seminal oxidative stress (OS) was artificially-induced, removal of leukocytes by "Dyanabeads" reduced ROS levels significantly [45].

Although it is generally accepted that an association exists between the number of leukocytes in the seminal fluid and excessive ROS production, OS can still result in the absence of leukocytospermia. Of note, one study reported that ROS production from PMN up to a concentration of 0.5×10^6/ml is negligible [46].

On the contrary, when PMN leukocytes are activated by stimuli like infection and/or inflammation, a discharge of ROS ensues [47]. For instance, neutrophils that contain myeloperoxidase use superoxide anions to oxidize chloride ions. This results in the production of hypochlorous acid (HOCL), which acts as an anti-bacterial agent but is also a kind of ROS. Moreover, leukocytes increase the production of NADPH by the hexose monophosphate shunt, thus increasing the activity of NADPH oxidases that ultimately increase the production of superoxide anion.

As a matter of fact, a strong association exists between the presence of male accessory gland infections (MAGI) and seminal ROS levels. Prostatitis, prostato-vesiculitis and prostato-vesiculo-epididymitis are the commonest MAGI; the most prevalent being prostatitis. The immune response to MAGI results in an increased number of leukocytes in the male genital tract, which subsequently leads to ROS production [9].

Studies have also linked bacterial prostatitis and the autoimmune response to prostate antigens with excessive ROS production [48]. Although it is generally accepted that granulocytes in the prostatic fluid are a source of ROS, semen of individuals suffering from chronic prostatitis that do not contain leukocytes may also have elevated ROS levels, suggesting that another source of ROS exist in the prostate gland.

The degree of OS is increased when the prostatitis is associated with seminal vesiculitis. Along the same lines, when compared to prostato-vesiculitis and prostatitis, a higher number of white blood cells and ROS levels are observed in prostato-vesiculo-epididymitis [49].

Varicocele

Varicocele is the dilatation of veins in pampiniform plexus around the spermatic cord. Studies have associated varicocele with many abnormal sperm parameters, including DNA damage. The latter has been linked to increased levels of ROS and OS. The higher the varicocele grade, the higher the ROS production [30].

1.2 Conclusion

Despite the strong evidence indicating that animal sperm make ROS, the ability of human sperm to produce significant amounts of ROS is surrounded by controversy. Notwithstanding, some evidence that human sperm produce ROS exists but experiments should ensure that sperm preparations are free of leukocytes, since leukocytes are prolific sources of ROS.

1.2.1 Sources of ROS in Female Reproductive System

While the role of ROS has been extensively investigated in the male reproductive system, the literature is relatively poor in studies addressing the role of ROS in the female counterpart. Nonetheless, ROS have been shown to be associated with pathologies of the female reproductive system, including preeclampsia, polycystic ovary syndrome, recurrent pregnancy loss and endometriosis [3].

In the female reproductive system, ROS are mainly found in the cells of ovarian follicles, the fallopian tube, the endometrium and the peritoneum.

1.2.2 Steroidogenesis and Ovulation

Completion of meiosis I during oocyte maturation is induced by an increase in ROS and is inhibited by an increase in antioxidants. The contrary occurs during meiosis II, thus indicating that ROS play an important role during the pre-ovulatory states [50]. Besides, steroidogenesis is also linked to increased ROS production in the ovaries by utilizing the cytochrome P450 pathway [51].

An earlier study in the rat suggested that ROS levels in corpus luteum increased during the regression phase [52]. Newer discoveries shed light to the relationship between different hormones and ROS production in the luteal phase. In the growing follicle, ROS induces apoptosis whereas FSH and reduced glutathione (GSH) are anti-apoptotic. FSH stimulates estrogen production within the granulosa cells, which in turn increases catalase that inhibits apoptosis [53].

On the contrary, excessive production of LH increases ROS production. The LH surge in the ovulatory is followed by a post-surge inflammatory phase. If LH secretion is in excess, the post-surge inflammatory precursors may increase thus causing OS-induced damage due to the overly produced ROS [21, 54].

After ovulation, the corpus luteum produces progesterone. It was found that both copper/zinc-superoxide dismutase (Cu/Zn-SOD) and progesterone levels increase in the early-mid luteal phase but decline in the regression phase. Lipid peroxide opposes Cu/Zn-SOD and progesterone in both phases [21, 54]. On the contrary, manganese-superoxide dismutase (Mn-SOD) is not affected by progesterone levels and is independent in its action, whereby its levels are sustained to protect the luteal cells from OS-induced inflammation [21].

1.2.3 Graffian Follicle

The Graffian follicle has been studied as a potential source of ROS because it contains macrophages, neutrophils, granulosa cells and the oocyte [21].

The oocyte has shown to be a source of ROS by different mechanisms. First, oxidative phosphorylation within the oocyte utilizes O_2 to produce energy (ATP). Second, the XO system eliminates toxic wastes in the form of uric acid, but at the expense of generating ROS. Lastly, the NADPH oxidase system whereby the oxidative burst produces HOCl to kill microbes. All these systems are prominent sources of ROS in the oocyte [21].

Aging is defined by the gradual loss of organ and tissue functions. The ageing of oocytes was linked with ROS production that may increase oocyte damage [20].

This hypothesis has led researchers to speculate that ROS might be related to the occurrence of congenital anomalies in neonates seen originated form older women [21]. It has been observed that oocytes from women at advanced reproductive age carry dysfunctions at the mitochondrial DNA level and chromosomal aneuploidy The marker that has been utilized to assess mitochondrial DNA (mtDNA) damage is an oxidized derivative of the deoxyguanosine base, namely oxodeoxyguanosine (8-OHdG) [54].

Menstrual blood is one pivotal way females lose iron (Fe) As discussed earlier, Fe potentiates oxidative damage—and its build-up in the body poses a higher risk on menopausal women to OS-related diseases [21]. Menopause also causes decreased production of estrogen along with its protective properties against oxidative damage of the endometrium [55].

1.2.4 Granulosa Cells and Cumulus Mass Cells

The granulosa cells are one of the main sources of ROS in the graffian follicle. Undifferentiated granulosa cells give rise to the cumulus oophorus, which anchors the oocyte and keeps it in place. SOD is the main antioxidant present in these cells to counteract the ROS production [20, 56]. OS in the granulosa cells and cumulus oophorus may damage the cell contents, including DNA [54]. There has been shown that a positive correlation between 8-OHdG levels in the granulosa cells and poor oocyte quality [57, 58] but the exact mechanisms of ROS production in the granulosa cells and cumulus mass remain unclear.

1.2.5 Follicular Fluid

ROS and antioxidants are found in the follicular fluid (FF), which levels have been linked with oocyte quality [50]. Since FF is produced by both granulosa cells and theca cells interna [59], it is likely these elements are the sources of ROS and antioxidants. The most abundant antioxidants in the follicular fluid are non-enzymatic, including Vitamin C, Vitamin E, glutathione (GSH), hypotaurine and taurine [50].

The FF has high metabolic activity and contains steroid hormones, growth factors, leukocytes, cytokines and granulosa cells, all of which are known to increase ROS production [50].

ROS levels have been studied in the FF of women trying to conceive via assisted reproductive technology (ART). Total Antioxidant Capacity (TAC) were lower in those who fail to conceive [60]. In another study, TAC levels were shown to be higher in FF aspirates that yielded oocytes that successfully fertilized via ART [61].

1.2.6 Fallopian Tube

Nitric oxide (NO) synthase is present in human tubal cells, which catalyzes the formation of NO, the most common source of reactive nitrogen species (RNS). NO is a potent vasodilator, and its presence in the tubal cells promotes fallopian tube contractions. These contractions aid to move the oocyte as well as spermatozoa [62]. Increased NO levels protect the fallopian tubes from microbes, but such levels are also toxic to spermatozoa. Conversely, decreased NO levels is associated with impaired tube contractions, with negative effects on both spermatozoa and ovum [54].

As mentioned earlier, NO is formed from O_2 and L-arginine, and it further reacts with superoxide anion to form peroxynitrite. Peroxyinitrite induces lipid peroxidation and nitration of tyrosine molecules on enzymes essential for signal transduction.

There are essentially three types of NOS: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). eNOS has vasodilator effects during pregnancy and its production is stimulated by LH and hCG. iNOS is mainly associated with pathological conditions as it is found within macrophages that await stimulation by cytokines [3]. High levels of NOS over stimulate the tyrosine-dependent enzymes and cause cytotoxicity. On the other hand, low levels of NOS due to absence of L-arginine is associated with increased superoxide anion levels, thus causing OS. NO absence has been shown to be a key factor in disruption of the vascular system that leads to infertile states [54].

Notably, the fallopian tubes are rich in antioxidant enzymes [3, 20, 21, 50, 54, 63–69]. The effect of ROS on sperm capacitation can be blocked by these antioxidants. Alternative sources of physiological ROS, possibly generated by the cumulus cells might promote capacitation and prepare the sperm to fertilize.

1.2.7 Peritoneal Cavity

The main sources of ROS in the peritoneal cavity are macrophages, endometrial cells, red blood cells and menstrual-reflux debris [65]. Idiopathic infertile women were shown to have elevated levels of peritoneal leukocytes, up to 95 % of which are macrophages. Increased levels of malonaldehyde, a stable end-product of lipid peroxidation, was found in patients with idiopathic female infertility [21, 39, 65, 66, 70, 71]. Furthermore, the presence of higher level of ROS and lower level of antioxidants in the peritoneum has been suggested to cause endometriosis, a common cause of female infertility [63].

1.2.8 Endometrium

ROS levels in the physiological range generated within endometrial cells ultimately increase in prostaglandin F 2alpha ($PGF_{2\alpha}$), which in turn plays a role in luteal regression [72]. Moreover, withdrawal of estrogen and progesterone causes a

decrease in SOD levels and a subsequent increase in ROS levels, which promotes NF-KB transcription factor to express cyclo-oxygenase-2 (COX-2) enzyme. COX-2 will catalyze the synthesis of PGF2a that contributes to the process of endometrial shedding [73].

1.3 Conclusion

Although there is strong evidence indicating that ROS are generated by several cellular elements and systems in the female reproductive tract, the role of ROS remains to be further elucidated.

1.3.1 Oxidative Stress (OS)

As highly reactive molecules, ROS readily react with a wide range of biological molecules, including unsaturated fatty acids, sulphydryl proteins and nucleic acids [8]. In combating the ROS, there is antioxidant defense mechanism in our body, which are enzymes or non-enzymes that are able to neutralize these reactive molecules. The loss of the required balance between the production of ROS and the elimination of these radicals by the antioxidant defense system leads to a condition known as oxidative stress (OS). This can be caused by either high ROS production or decreased antioxidants or even both of them together. As discussed earlier, ROS are formed during the normal cellular metabolism. However, when ROS levels increase beyond the normal capacity of antioxidant defense, OS occurs and can result in cellular dysfunction or cell death through different mechanisms including lipid peroxidation, nucleic acid and protein damages directly or indirectly [8]. Apoptosis is defined as a unique form of programmed cell death, which plays a significant role in controlling the growth and homeostasis of different organisms [4]. An association between increased ROS levels and apoptosis is also well documented [74, 75].

The extent of these damages due to OS depends on several factors, which include the extent of anti-oxidation in the environment and lipid saturation. As unsaturated fatty acids contain unconjugated double bonds adjacent to the methylene groups, this makes the methylene carbon-hydrogen bonds weaker and thus more susceptible to peroxidative damage [76]. Other factors like the presence of metal binding proteins (MBP), including transferrin and albumin [77, 78] also seem to play a role; these molecules help to bind iron that are present in the cell, which would otherwise potentiate the oxidative damage.

Due to its damaging effect on the cells, OS is related to many pathological conditions and diseases including cancer, ischemia and infertility, as well as non-pathological process including aging [3]. The negative effects of OS to the male and female reproductive systems can be found in Chap. 4.

References

1. Nunes-Silva A, Freitas-Lima L (2014) The association between physical exercise and Reactive Oxygen Species (ROS) production. J Sports Med Doping Stud 4(152):2161–0673.1000152
2. Doshi SB et al (2012) Role of reactive nitrogen species in male infertility. Reprod Biol Endocrinol 10(109):10.1186
3. Agarwal A, Gupta S, Sharma RK (2005) Role of oxidative stress in female reproduction. Reprod Biol Endocrinol 3:28
4. Droge W (2002) Free radicals in the physiological control of cell function. Physiol Rev 82(1):47–95
5. Keisari Y, Braun L, Flescher E (1983) The oxidative burst and related phenomena in mouse macrophages elicited by different sterile inflammatory stimuli. Immunobiology 165(1):78–89
6. Wu G et al (2004) Glutathione metabolism and its implications for health. J Nutr 134(3):489–492
7. Agarwal A et al (2014) Effect of oxidative stress on male reproduction. World J Mens Health 32(1):1–17
8. Martindale JL, Holbrook NJ (2002) Cellular response to oxidative stress: signaling for suicide and survival. J Cell Physiol 192(1):1–15
9. Agarwal A et al (2014) Mechanisms of oligozoospermia: an oxidative stress perspective. Syst Biol Reprod Med 60(4):206–216
10. Boveris A, Cadenas E, Stoppani A (1976) Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. Biochem J 156:435–444
11. Deby C, Goutier R (1990) New perspectives on the biochemistry of superoxide anion and the efficiency of superoxide dismutases. Biochem Pharmacol 39(3):399–405
12. Fridovich I (1978) The biology of oxygen radicals. Science 201(4359):875–880
13. Sen CK (2001) Antioxidant and redox regulation of cellular signaling: introduction. Med Sci Sports Exerc 33(3):368–370
14. Haber F, Weiss J (1932) Über die katalyse des hydroperoxydes. Naturwissenschaften 20(51):948–950
15. Kehrer JP (2000) The Haber–Weiss reaction and mechanisms of toxicity. Toxicology 149(1):43–50
16. Fromm HJ, Hargrove MS (2012) Electron transport and oxidative phosphorylation. In: Essentials of biochemistry. Springer, New York. pp 223–238
17. Schneider CD, Oliveira AR (2004) Oxygen free radicals and exercise: mechanisms of synthesis and adaptation to the physical training. Rev Bras Med Esporte 10(4):308–313
18. Boveris A, Chance B (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem J 134:707–716
19. Cadenas E et al (1977) Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome c reductase from beef-heart mitochondria. Arch Biochem Biophys 180(2):248–257
20. Fujii J, Iuchi Y, Okada F (2005) Fundamental roles of reactive oxygen species and protective mechanisms in the female reproductive system. Reprod Biol Endocrinol 3(1):43
21. Agarwal A, Allamaneni SS (2004) Role of free radicals in female reproductive diseases and assisted reproduction. Reprod Biomed Online 9(3):338–347
22. Al Ghouleh I et al (2011) Oxidases and peroxidases in cardiovascular and lung disease: new concepts in reactive oxygen species signaling. Free Radic Biol Med 51(7):1271–1288
23. Cantu-Medellin N, Kelley EE (2013) Xanthine oxidoreductase-catalyzed reactive species generation: a process in critical need of reevaluation. Redox Biol 1(1):353–358
24. Mittal M et al (2014) Reactive oxygen species in inflammation and tissue injury. Antioxid Redox Signal 20(7):1126–1167
25. Chen X et al (2008) Role of reactive oxygen species in tumor necrosis factor-alpha induced endothelial dysfunction. Curr Hypertens Rev 4(4):245–255
26. Ha YJ, Seul HJ, Lee JR (2011) Ligation of CD40 receptor in human B lymphocytes triggers the 5-lipoxygenase pathway to produce reactive oxygen species and activate p38 MAPK. Exp Mol Med 43(2):101–110

27. Frey RS, Ushio–Fukai M, Malik AB (2009) NADPH oxidase-dependent signaling in endothelial cells: role in physiology and pathophysiology. Antioxid Redox Signal 11(4):791–810
28. Lander HM et al (1997) A molecular redox switch on p21ras structural basis for the nitric oxide-p21ras interaction. J Biol Chem 272(7):4323–4326
29. Nathan CF, Root R (1977) Hydrogen peroxide release from mouse peritoneal macrophages: dependence on sequential activation and triggering. J Exp Med 146(6):1648–1662
30. Agarwal A, Hamada A, Esteves SC (2012) Insight into oxidative stress in varicocele-associated male infertility: part 1. Nat Rev Urol 9(12):678–690
31. Perry JM, Marletta MA (1998) Effects of transition metals on nitric oxide synthase catalysis. Proc Natl Acad Sci 95(19):11101–11106
32. Pacher P, Beckman JS, Liaudet L (2007) Nitric oxide and peroxynitrite in health and disease. Physiol Rev 87(1):315–424
33. Olas B, Wachowicz B (2007) Role of reactive nitrogen species in blood platelet functions. Platelets 18(8):555–565
34. Wang L et al (2012) Specific role of neutrophil inducible nitric oxide synthase in murine sepsis-induced lung injury in vivo. Shock 37(5):539–547
35. Rosselli M, Keller R, Dubey RK (1998) Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. Hum Reprod Update 4(1):3–24
36. Musset B et al (2012) NOX5 in human spermatozoa expression, function, and regulation. J Biol Chem 287(12):9376–9388
37. Griveau JF, Le Lannou D (1997) Reactive oxygen species and human spermatozoa: physiology and pathology. Int J Androl 20(2):61–69
38. Turrens JF (2003) Mitochondrial formation of reactive oxygen species. J Physiol 552(2): 335–344
39. Attaran M et al (2000) The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. Int J Fertil Womens Med 45(5):314–320
40. Gil-Guzman E et al (2001) Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation. Hum Reprod 16(9):1922–1930
41. Rengan AK et al (2012) An investigation of excess residual cytoplasm in human spermatozoa and its distinction from the cytoplasmic droplet. Reprod Biol Endocrinol 10:92
42. Gomez E et al (1996) Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. J Androl 17:276–287
43. Said TM et al (2005) Impact of sperm morphology on DNA damage caused by oxidative stress induced by β-nicotinamide adenine dinucleotide phosphate. Fertil Steril 83(1):95–103
44. Aziz N et al (2004) Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index. Fertil Steril 81(2):349–354
45. Ford WC, Whittington K, Williams AC (1997) Reactive oxygen species in human sperm suspensions: production by leukocytes and the generation of NADPH to protect sperm against their effects. Int J Androl 20(Suppl 3):44–49
46. Oborna I et al (2009) Reactive oxygen species in human semen in relation to leukocyte contamination. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 153(1):53–57
47. Lavranos G et al (2012) Investigating ROS sources in male infertility: a common end for numerous pathways. Reprod Toxicol 34(3):298–307
48. Shang Y et al (2014) The effect of chronic bacterial prostatitis on semen quality in adult men: a meta-analysis of case-control studies. Sci Rep 4:7233
49. Dinesh V, Shamsi M, Dada R (2012) Supraphysiological free radical levels and their pathogenesis in male infertility. Reprod Sys Sexual Disorders 1(114):2
50. Behrman HR et al (2001) Oxidative stress and the ovary. J Soc Gynecol Investig 8(1 Suppl): S40–S42
51. Aitken RJ, Buckingham D, Harkiss D (1993) Use of a xanthine oxidase free radical generating system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa. J Reprod Fertil 97(2):441–450
52. Sugino N et al (1993) Changes in activities of superoxide dismutase and lipid peroxide in corpus luteum during pregnancy in rats. J Reprod Fertil 97(2):347–351

53. Devine PJ, Perreault SD, Luderer U (2012) Roles of reactive oxygen species and antioxidants in ovarian toxicity. Biol Reprod 86(2):27
54. Agarwal A et al (2012) The effects of oxidative stress on female reproduction: a review. Reprod Biol Endocrinol 10:49
55. Catt JW, Henman M (2000) Toxic effects of oxygen on human embryo development. Hum Reprod 15(Suppl 2):199–206
56. Burton GJ, Jauniaux E (2011) Oxidative stress. Best Pract Res Clin Obstet Gynaecol 25(3):287–299
57. Tamura H et al (2008) Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. J Pineal Res 44(3):280–287
58. Seino T et al (2002) Eight-hydroxy-2′-deoxyguanosine in granulosa cells is correlated with the quality of oocytes and embryos in an in vitro fertilization-embryo transfer program. Fertil Steril 77(6):1184–1190
59. Ruder EH, Hartman TJ, Goldman MB (2009) Impact of oxidative stress on female fertility. Curr Opin Obstet Gynecol 21(3):219
60. Pasqualotto EB et al (2004) Effect of oxidative stress in follicular fluid on the outcome of assisted reproductive procedures. Fertil Steril 81(4):973–976
61. Bedaiwy MA et al (2012) Effect of follicular fluid oxidative stress parameters on intracytoplasmic sperm injection outcome. Gynecol Endocrinol 28(1):51–55
62. Ekerhovd E, Norström A (2004) Involvement of a nitric oxide-cyclic guanosine monophosphate pathway in control of fallopian tube contractility. Gynecol Endocrinol 19(5):239–246
63. Agarwal A, Durairajanayagam D, du Plessis SS (2014) Utility of antioxidants during assisted reproductive techniques: an evidence based review. Reprod Biol Endocrinol 12:112
64. Agarwal A, Saleh RA, Bedaiwy MA (2003) Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril 79(4):829–843
65. Das S et al (2006) Reactive oxygen species level in follicular fluid—embryo quality marker in IVF? Hum Reprod 21(9):2403–2407
66. Oyawoye O et al (2003) Antioxidants and reactive oxygen species in follicular fluid of women undergoing IVF: relationship to outcome. Hum Reprod 18(11):2270–2274
67. Salmon AB, Richardson A, Pérez VI (2010) Update on the oxidative stress theory of aging: does oxidative stress play a role in aging or healthy aging? Free Radic Biol Med 48(5):642–655
68. Shkolnik K et al (2011) Reactive oxygen species are indispensable in ovulation. Proc Natl Acad Sci 108(4):1462–1467
69. Sugino N et al (1996) Changes in activity of superoxide dismutase in the human endometrium throughout the menstrual cycle and in early pregnancy. Hum Reprod 11(5):1073–1078
70. Paszkowski T et al (1995) Selenium dependent glutathione peroxidase activity in human follicular fluid. Clin Chim Acta 236(2):173–180
71. Jozwik M et al (1999) Oxidative stress markers in preovulatory follicular fluid in humans. Mol Hum Reprod 5(5):409–413
72. Sugino N et al (2004) Withdrawal of ovarian steroids stimulates prostaglandin F2alpha production through nuclear factor-kappaB activation via oxygen radicals in human endometrial stromal cells: potential relevance to menstruation. J Reprod Dev 50(2):215–225
73. Wu B et al (2014) ROS are critical for endometrial breakdown via NF-κB–COX-2 signaling in a female mouse menstrual-like model. Endocrinology 155(9):3638–3648
74. Willie A (1980) Cell death: the significance of apoptosis. Int Rev Cytol 68:251–306
75. Slater AF et al (1995) Signalling mechanisms and oxidative stress in apoptosis. Toxicol Lett 82:149–153
76. Wathes DC, Abayasekara DRE, Aitken RJ (2007) Polyunsaturated fatty acids in male and female reproduction. Biol Reprod 77(2):190–201
77. Quinlan GJ, Martin GS, Evans TW (2005) Albumin: biochemical properties and therapeutic potential. Hepatology 41(6):1211–1219
78. Van Campenhout A et al (2005) Cell-mediated LDL oxidation: the impact of transition metals and transferrin. Biochem Biophys Res Commun 338(3):1617–1624

Chapter 2
Methods to Measure Reactive Oxygen Species (ROS) and Total Antioxidant Capacity (TAC) in the Reproductive System

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2.1 Introduction

ROS production in the male reproductive tract has become a real concern because of their potential toxic effects on quality and function of sperm [1–3]. ROS is produced by abnormal, immature, morphologically abnormal spermatozoa and contaminated white blood cells especially the polymorphonuclear granulocytes in the seminal ejaculates [4–7]. Examples of ROS include the superoxide anion ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), the extremely reactive hydroxyl radical ($\bullet OH$) and the peroxyl radical (HO_2^-) [8]. Reactive nitrogen species are often considered to be a subclass of ROS. It includes nitric oxide (NO), nitrous oxide (N_2O), peroxynitrite (NO_3^-), nitroxyl anion (HNO), and peroxynitrous acid (HNO_3) [5–7, 9]. High levels of ROS have a detrimental effect on sperm concentration [10–16], motility [10, 12, 14, 16], abnormal sperm morphology [12, 17–21] as well as increase DNA damage [22–24], apoptosis [25] and result in sperm dysfunction [26–30]. Increased presence of ROS with progressive depletion of antioxidant reserves results in oxidative stress, which is strongly correlated, with the etiology of male infertility [28, 31–33].

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There are two methods by which ROS can be measured; direct and the indirect methods. The direct methods measure the ROS directly whereas indirect methods measure their oxidized products. Direct assays include chemiluminescence, nitroblue tetrazolium test (NBT), cytochrome *c* reduction, flow cytometry, electron spin resonance, and xylenol orange-based assay. Indirect methods include measurement by myeloperoxidase test, measurement of redox potential, lipid peroxidation levels, levels of chemokines, antioxidants, and antioxidant enzymes measuring levels of DNA damage and proteomic alterations (Table 2.1).

2.1.1 Types of Semen Sample Used to Detect ROS

Various types of semen samples can be used to detect ROS levels, some of which include the unprocessed seminal ejaculate, the processed semen sample by swim up and by density gradient centrifugation [20, 42]. Seminal ejaculate comprises of not only spermatozoa but also all other secretions from prostate and seminal vesicles and other accessory glands and cellular components such as round cells, leukocytes and epithelial cells. Levels of ROS are reflective of the de novo status of the ROS in the sample. In a simple wash and resuspend sample, the seminal plasma is removed but the leukocytes, round cells and debris remain in the sample. The sperm prepared by swim-up separates the actively motile sperm from the non-motile sperm and the debris. Similarly, in the density gradient separation, the spermatozoa are separated on the basis of their densities, which results in the separation of actively motile and morphologically normal sperm. The density gradient technique is used to measure ROS levels in both mature and immature spermatozoa [20, 42].

2.1.2 Measurement of ROS

2.1.2.1 Nitroblue Tetrazolium Test

Nitroblue Tetrazolium or the NBT test is based on the generation of ROS by sperm and leukocytes by using the compound Nitroblue Tetrazolium. NBT is a yellow water-soluble nitro-substituted aromatic tetrazolium compound that reacts with cellular superoxide ions to form formazan derivative that can be monitored spectrophotometrically [72]. This test is based on the principle that when heterogeneous samples such as seminal ejaculate are stained with NBT; it results in the formation of colored formazan due to reduction of NBT. This has been shown to correlate with impaired sperm function [72]. NBT is an electron acceptor that becomes reduced in the presence of free oxygen radicals to form a blue-black compound, formazan [73]. The spermatozoa containing this formazan can be also be stained histochemically and scored under microscope.

The method involves preparation of NBT solution by adding phosphate- buffered saline (PBS) with NBT powder. This is then used to stain the whole ejaculate, i.e., leukocytes and abnormal spermatozoa. The tubes are centrifuged and the pellet

Table 2.1 Direct and indirect assays measuring ROS

Method	Type	Principle	Advantage	Reference
Cytochrome c reduction test	Direct	The reduction of ferricytochrome c to ferrocytochrome c is used to detect superoxide formation	Gold standard for measuring extracellular superoxide anions	[34]
Electron spin resonance (ESR) Electron paramagnetic resonance (EPR)	Direct	The magnetic properties of unpaired electrons in free radicals enable them to absorb electromagnetic radiation on application of external magnetic field and this then generate absorption spectra utilizing the energy of electron spin state, which is measured by ESR spectrophotometers Provides direct detection of the "instantaneous" presence of free radical species in a sample Plays a major role in the assessment of most of the oxidants characterized by very short half-life (nanoseconds to microseconds) usually by using stabilizing molecules called spin-traps/probes	This is used to measure oxidative stress on proteins and lipids Simple, high sensitivity and specificity Detects free radicals and paramagnetic molecules. The magnetic field-based EPR detection enables nondestructive (in vitro) and noninvasive (in vivo) measurements of biological samples EPR spectroscopy, coupled with the use of paramagnetic probes, is a potential technique for accurate and precise determination of ROS concentrations in a variety of biological samples	[5, 25, 34, 35] [36, 37]
Xylenol orange based assay	Direct	This uses automated analyzer. The ROS in semen oxidizes ferrous to ferric ion and this forms a colored complex with xylenol orange in an acidic medium, the color intensity of which can be measured spectrophotometrically. Results are expressed in μmol H_2O_2 equiv./L	It is rapid, easy, stable, inexpensive, reliable and sensitive	[38]
Aromatic traps	Direct	Used to measure ROS produced in vivo. Salicylates and phenylalanine are used which reacts with free radicals to form more stable products	Used to measure ROS in cardiovascular and cerebrovascular systems	[35]
ROS measurement by chemiluminescence	Direct	Measures real time production of ROS. Uses two probes—luminol and Lucigenin. Luminol measures Global ROS levels both extracellular and intracellular (supreroxide anion, hydrogen peroxide, hydroxyl radical) Lucigenin is specific for superoxide anion and hydroxyl radical	Chemiluminescence is robust, sensitive and specific method	[39–45]

(continued)

Table 2.1 (continued)

Method	Type	Principle	Advantage	Reference
Flow cytometry	Direct	ROS measurement of hydrogen peroxide and superoxide anion by flow cytometry. Dihydroethidium measures intracellular superoxide anion and dichlorofluoroscein diacetate for intracellular hydrogen peroxide	Requires very low amounts of spermatozoa, high specificity gofer intracellular ROS in spermatozoa	[46–48]
Endtz test	Indirect	ROS is mainly generated by leukocytes. The myeloperoxidase is used to stain polymorphonuclear granulocytes. But does not provide any information regarding ROS generation by spermatozoa	Indirect indicator of excessive ROS generation by leukocytes in semen	[4, 38, 49]
Redox potential GSH/GSSG	Indirect	Balance of reduced glutathione and its oxidized form (GSSG) gives an indication of ROS levels in vivo. GSH/GSSG levels are measured biochemically or using high performance liquid chromatography	Can be used to measure oxidative stress in-vitro and in-vivo	[7, 20, 50]
Total antioxidant capacity	Indirect	Measures total antioxidants in seminal plasma	Rapid colorimeter method	[51, 52]
Thiobarbituric acid assay (TBARS)	Indirect	Measures lipid peroxidation. Detects malondialdehyde (MDA-TBA) adduct by colorimetry or fluoroscopy	Simple but non specific	[53, 54]
Isoprostane	Indirect	Liquid chromatography-tandem mass spectrometry	Specific, stable compound	[55]
HNE-HIS Adduct ELISA	Indirect	ELISA	Rapid, helps in quantification	[56–58]
DNA damage	Indirect	Measures single and double stranded DNA fragmentation by sperm chromatin structure assay, TUNEL assay, sperm chromatin dispersion assay or comet assay	Measure single or double strand DNA breaks, robust, sensitive method (SCSA and TUNEL)	[6, 59–68]
Oxidation reduction potential	Indirect	Measures the redox balance in a given biological system. It measures all known and unknown oxidants and antioxidants in a given sample	High sensitivity, specificity and accuracy. Can be measured both in seminal ejaculates and in seminal plasma (both fresh and frozen)	[69–71]

formed at the bottom is used to make smears. The smears are air dried and using the Wright stain, the slides are stained again and scored under microscope [73]. Leukocytes are scored as: no detectable formazan (−), scattered or few formazan granules (+), intermediate density (++), and cells filled with formazan (+++). Spermatozoa are scored as follows: formazan occupying 50 % or less of the cytoplasm (+) and more than 50 % of cytoplasm (++) [73]. NBT reaction reflects the ROS generating activity in the cytoplasm of cells, and therefore it can help determine the cellular origin of ROS in semen [74].

The cells stained with NBT are NBT positive cells and there exists a relation between the NBT+ cells and the levels of ROS in the same suspension [73]. This test helps in identifying the source of ROS whether it's sperm or the infiltrating leukocytes in the semen. It is important to distinguish between the sources of ROS, as the clinical implications of infiltrating leukocytes are different from the pathological conditions in which sperm are themselves the source of ROS [75]. The advantages of this method include that it is readily available, inexpensive and has high sensitivity. It provides information about the differential contribution of leukocytes and abnormal spermatozoa in the production of ROS i.e., the cellular origin of ROS in the sample [73]. The major limitation of this test is that presence of other cellular reductases may also reduce NBT. Furthermore, changes in the cellular content of various oxido-reductases may also alter the rates of NBT reduction [74].

2.1.2.2 Chemiluminescence Assay

Chemiluminescence is one of the most commonly employed methods used to detect ROS in semen sample [44, 75, 76]. The reaction causes emission of light, which is measured with a luminometer. The two major types of luminometer include the photon counting luminometer and the direct current luminometer. The photon counting measures the individual photon whereas the direct counting measures the current passing through a luminometer. These are measured as photons per minute or relative light units, respectively [77].

Luminometers are also classified as single tube luminometer, which can measure only single sample, or multiple tube luminometer, which can measure multiple samples at any time, and this is the one used in research laboratories. The third one is the plate luminometer, which utilizes a 96 well plate to read multiple samples at a time [77].

The basic principle of chemiluminescence is the measurement of emitted light due to a chemical reaction occurring between chemical reagents and the ROS generated. The following equation shows two reactants A and B in presence of an excited intermediate [◊] resulting in emission of light. The decay of this excited state [◊] to a lower energy level causes light emission.

$$[A] + [B] \rightarrow [\Diamond] \rightarrow [Products] + \text{light}$$

There are two major types of probes used in chemiluminescence which include Luminol and Lucigenin (Table 2.2).

Table 2.2 Major types of probes for measuring ROS by chemiluminescence

Luminol	Lucigenin
1. It works through one electron oxidation	1. This works through one electron reduction
2. Measures both intracellular as well as extracellular ROS	2. It measures only the extracellular ROS
3. Hydrogen peroxide radical and oxygen radical are involved	3. It involves measuring the superoxide anion

Fig. 2.1 Autolumat 953 Plus Luminometer used in the measurement of ROS by chemiluminescence assay. Multiple tubes can be loaded simultaneously for measuring ROS. The luminometer can be connected with a computer and monitor

The reagents used are the stock luminol probe (100 mm), the working luminol (5 mm) and the dimethylsulfoxide (DMSO) solution [78]. The procedure is performed in an indirect light. A luminometer is attached to a computer (Fig. 2.1). A total of 11 tubes are used that include 3 blank tubes which contain only the PBS, 3 negative controls which contains PBS + luminol (working solution), 2 tubes which has the patient sample and + luminol, 3 positive control which contains PBS+ hydrogen peroxide (50 μL) + luminol (Fig. 2.2). The tubes are loaded in to the luminometer (Berthold, Autolumat Plus LB 953) and a real time [lot of the ROS levels produced in each sample are visualized on the computer monitor (Fig. 2.3) and the results can be visualized and printed in an excel sheet [78].

The factors affecting chemiluminescence reactions include sample volume, time of analysis, viscosity of the sample, concentration of reactants, reagent injection, temperature control, human error, background luminescence. The major advantages include that it is high specificity and sensitivity and can measure both intracellular as well as extracellular ROS [41]. The major disadvantages are: (1) it cannot measure multiple markers simultaneously and (2) the level of ROS decline with the time after ejaculation due to short half-life of ROS. Factors affecting ROS measurement are: (1) luminometer calibration; (2) sensitivity and the dynamic range as well as

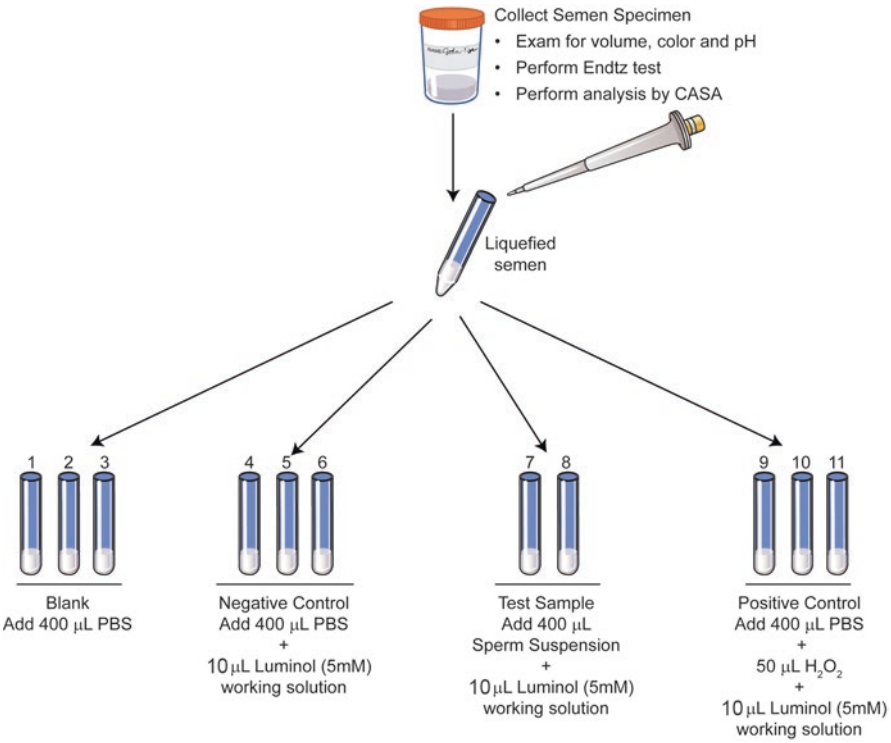

Fig. 2.2 Preparing the tubes for ROS measurement. A total of 11 tubes are labeled from S1 to S11: Blank, negative control, test sample and positive control. Luminol is added to all tubes except the blank. Hydrogen peroxide is added only to the positive control

the units used; (3) concentration and the type of probe used; (4) concentration and the volume of semen used, and (5) temperature of the instrument at the time of measurement. Semen age, viscosity of the sample, repeated centrifugation, use of media containing albumin that can generate spurious signals; spikes and sensitivity of luminol to pH changes are other variables that influence ROS production. ROS is an independent factor of male factor infertility [26]. We have reported different cutoff values for ROS in processed semen samples [4, 6, 17] and seminal ejaculates [17, 21, 24, 43].

We have recently revised the reference range of ROS in seminal ejaculates [43]. ROS levels >102 RLU/s/10^6 sperm are considered abnormal. At this cutoff ROS sensitivity is 76 % with a positive predictive value of 82.1 %. When the controls were strictly comprised of individuals who had established pregnancy, the cutoff was slightly lower at <93 RLU/s/×10^6 sperm. The sensitivity increased to 93.8 % indicating that the test can differentiate subjects that are fertile from those that are not. Levels of ROS >102 RLU/s/×10^6 sperm must be considered pathological.

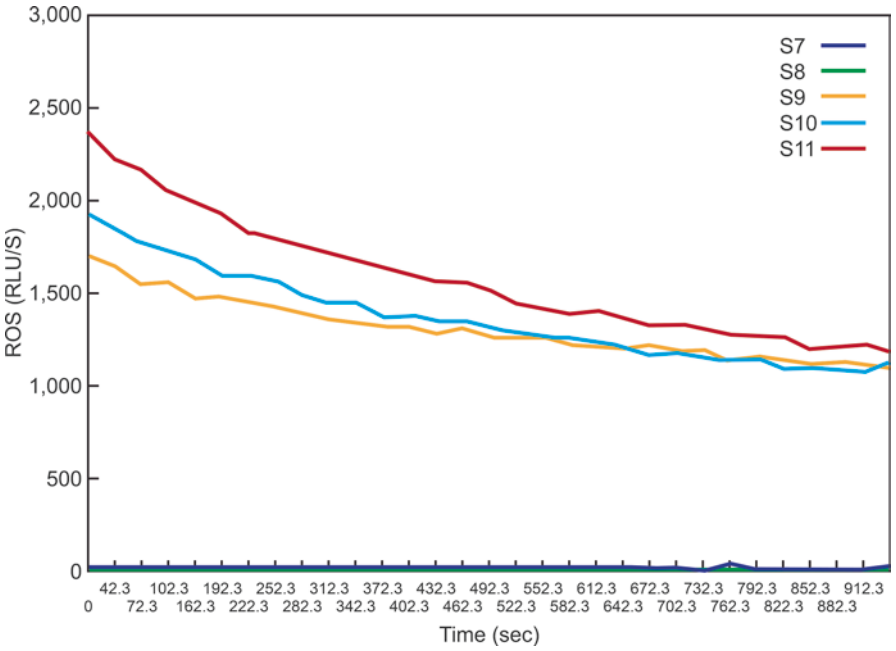

Fig. 2.3 A typical graph showing the ROS levels in the 11 tubes (S1–S11). As seen here, only the positive controls have significantly higher levels of ROS. Those producing low levels (Tubes S1–S8) of ROS are seen very close to the *X* axis

The major diagnostic application of chemiluminescence is that it provides independent assessment of the quality of the ejaculate and this is extremely important in patients with unexplained infertility as these patients demonstrate high levels of ROS despite conventional semen parameters within normal ranges [20, 77, 79–81]. The reproducibility of ROS by chemiluminescence assay makes the test sensitive and reliable in measuring ROS levels. ROS levels in the mature spermatozoa may have both diagnostic and prognostic importance as elevated ROS levels in mature spermatozoa may reflect oxidative stress in semen samples that will be used for ART purpose and may also be used to predict the fertilizing potential of the spermatozoa. This can be accomplished by characterizing the semen samples that are used in ART based on the established reference values of ROS.

The intracellular levels of ROS can be measured by flow cytometry using dihydrofluorescein diacetate (DCFH) to detect intracellular hydrogen peroxide radicals. This dye is oxidized to the highly fluorescent derivative dichlorofluorescein (DCF), which is detected by the use of flow cytometer [46–48, 82]. A counterstain dye for nucleic acid (propidium iodide) is used to exclude the apoptopic spermatozoa [83]. Dihydroethidium (DE) can be used to detect intracellular levels of superoxide anions [46, 48, 83]. The results are interpreted as percentage of fluorescent spermatozoa [83].

2.1.3 Measurement of DNA Fragmentation

Reduced fertility, embryo development, increased rates of miscarriages has been reported in cases of higher sperm DNA damage [84–86]. Several etiological factors such as cigarette smoking, irradiation, chemotherapy, leukocytospermia, varicocele, cancer, elevated levels of ROS, abnormalities during chromatin packaging and advancing age have demonstrated compromised sperm DNA quality [31, 83, 87–91].

Oxidative stress is responsible for single strand breaks in DNA [20, 91]. Furthermore, apoptosis can also occur as a result of increased oxidative stress and result in DNA fragmentation. Several studies show that infertile men have high number of sperm with single or double stranded fragmentation [59, 92–94].

Several tests have been introduced to measure the sperm DNA damage [64, 88, 95–101]. The methodological approaches by which sperm DNA damage is investigated in these tests are varied. Some tests measure abnormalities in sperm chromatin whereas others measure direct DNA strand fragmentation. Among such test the most commonly used are the sperm chromatin structure assay (SCSA) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) [87, 97, 99, 102, 103]. These are briefly described below:

2.1.3.1 Sperm Chromatin Structure Assay (SCSA)

Sperm Chromatin Structure Assay (SCSA) detects damaged sperm DNA using flow cytometry of acridine orange stained sperm. It is based on the susceptibility of DNA breaks to acid denaturation. Low pH treatment opens the DNA strands at the sites of breaks. Staining by acridine orange is highly precise and repeatable and comparable between fresh and frozen samples. The DNA damage is induced by exposing to denaturing conditions. This utilizes the metachromatic properties of acridine orange to distinguish single stranded/red fluorescence and double stranded/native DNA/green fluorescence [59, 60, 83]. The DNA fragmentation index (DFI) is the ratio of percentage of sperms showing red fluorescence/total fluorescence (red + green) [48, 59].

The SCSA also measures sperm with high DNA stainability (%HDS) which is related to the nuclear histones retained in immature sperm and shown to be predictive of pregnancy failure [60]. The current clinical threshold 25 % DFI that categorized patient into a statistical probability of the following: (a) longer time to natural pregnancy, (b) low odds of IUI pregnancy, (c) more miscarriages, or (d) no pregnancy [60]. The test is precise, repeatable with acceptable DNA fragmentation that has a threshold of placing a man at risk of infertility.

2.1.3.2 Terminal deoxynucleotidyl Transferees dUTP Nick End Labeling (TUNEL) Assay

TUNEL assay utilizes a template-independent DNA polymerase called terminal deoxynucleotidyl transferase (TdT) that non-preferentially adds deoxyribonucleotides to 3′ hydroxyl (OH) single- and double-stranded DNA. Deoxyuridine

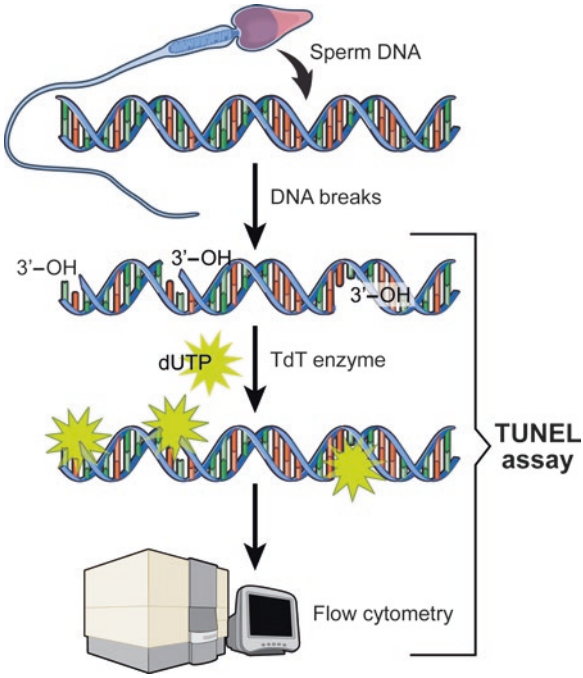

Fig. 2.4 Schematic of the DNA staining by the TUNEL assay

Fig. 2.5 Set up of the bench top flow cytometer

triphosphate (dUTP) is the substrate that is added by the TdT enzyme to the free 3′-OH break-ends of DNA (Fig. 2.4) [96, 99].

The more DNA strand breaks sites that are present, the more labels are incorporated within a cell. This identifies the in situ DNA breaks. Here, the 3′ hydroxyl free ends are labeled using a fluorescent label which on passing through a flow cytometer generates fluorescence, which is directly proportional to the number of strand breaks [96, 99]. Various protocols are used to detect DNA damage via TUNEL assay like fluorescein isothiocyanate labeled dUTP system and Apoptosis detection kit. DNA fragmentation can be measured by both a simple bench top flow cytometer (Fig. 2.5). This test is highly sensitive and specific [96, 99]. It measures a definite end point and is considered to provide better prediction regarding the potential of embryo implantation [86, 102].

Many factors involved in the processing, fixation and permeabilization of the specimen adversely affect the clinical implication of TUNEL assay [104]. The difficulty in the permeablization of sperm chromatin due to its highly dense compaction and tightly packed protamines plays a major role in reduced sensitivity preventing TdT from directly interacting with DNA strand breaks [105]. Unlike SCSA, the cutoff values of sperm DNA fragmentation for TUNEL have not been clearly established [61]. Studies have reported DNA fragmentation measured by TUNEL assay to range from 12 to 36.5 % at which no pregnancies were reported [106, 107]. A cutoff value of >19.2 % and 16.8 % has been recently shown to have >90 % specificity [61]. The specificities can be further increased by including only men with established pregnancies as controls. The high specificity and positive predictive value is important particularly in cases of idiopathic and unexplained infertility.

2.1.3.3 Epifluorescence Using Acridine Orange Dye

Acridine orange is a nucleic acid specific, fluorescent, cationic dye. It interacts with DNA by intercalation and by electrostatic interaction with RNA or single stranded DNA [96]. Fluorescence microscopy is used along with acridine orange dye. There is exposure to acid, which denatures DNA with single- or double stranded breaks. The dye acridine orange binds to DNA. The double stranded DNA fluoresces green and single stranded DNA gives red color. In addition the sperm suspension after staining with the Apo-direct kit for the TUNEL assay can also be visualized for DNA fragmentation by the fluorescence microscope (Fig. 2.6). Sperm stained green indicate sperm with DNA fragmentation [96]. This method uses fluorescence, which is relatively rapid, simple and inexpensive. The major disadvantage of this technique is the heterogeneous staining and color fading of the slides. Also, the presence of indistinct colors ranging from red to green interferes with the results [108].

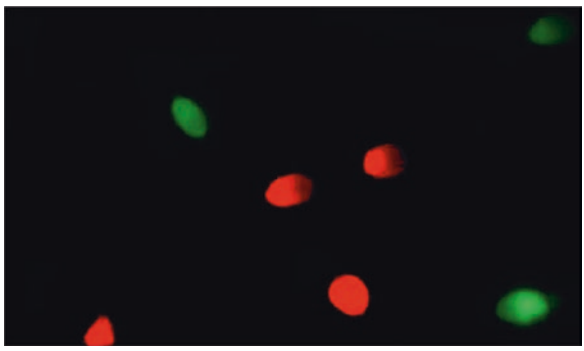

Fig. 2.6 Fluorescent staining showing intact (*red*) spermatozoa and spermatozoa with DNA fragmentation (*green*)

2.1.3.4 Comet Assay

This is a single gel electrophoresis method, which basically measures the breaks in DNA [66]. Electrophoresis is used to mobilize DNA fragments that are produced from nucleoids after being depleted of proteins. It is based on the general concept that DNA fragments resulting from pre-existing DNA breaks have different mobility in the electrophoretic field depending on the relative size of the fragment. This generates morphological differences between nuclei containing fragmented DNA when examined under fluorescent microscopy. The resulting image represents a "comet" that consists of a head and a tail chromatin in the direction of the anode. Larger the size of the comet, higher is the level of DNA fragmentation [109, 110]. Thus, sperm with more DNA breaks shows intense comet tail [111]. At the end of electrophoresis, all the broken strands of DNA migrate towards the anode and this forms a comet tail, which can be used to assess the DNA damage using the fluorescence microscope or cytometer [64]. The comet's tail length and fluorescent intensity is directly proportional to the degree of DNA fragmentation [65].

The assay can be performed in both neutral as well as alkaline environments. In neutral buffer, double stranded DNA damage is measured while in alkaline environment DNA can be denatured, both single– (SS) and double stranded (DS) DNA damage can be measured due to unwinding of the DNA strands [112]. Under the influence of electric field, there is separation of broken DNA strands (SS and DS) [65, 113–116]. After separation, the broken DNA fragments migrate towards the tail forming comet tail and the intact DNA remains confined to the head forming the comet's head [117]. The major limitation of the comet assay for its routine use in fertility labs is (1) slide processing is time consuming and (2) it requires electrophoresis equipment and fluorescence microscopy.

2.1.3.5 Sperm Chromatin Dispersion (SCD) Assay

Sperm chromatin dispersion (SCD) assay uses the Halosperm kit to differentiate between non-fragmented spermatozoa from the fragmented spermatozoa [64]. This test is used in laboratories with no access to flow cytometry. It can be visualized using bright field or fluorescence microscopy. It is based on a controlled species-specific DNA denaturation to produce single-stranded DNA stretches from any DNA breaks, coupled with controlled DNA depletion [67, 101, 118, 119]. The process involves (1) integration of sperm sample into an inert agarose microgel on pretreated slide, (2) controlled acid denaturation of DNA, (3) controlled protein depletion. Normal sperm produce halos of dispersed chromatin around a dense core. In fragmented DNA, no halos of dispersed chromatin are produced [120, 121]. The halos can further be classified according to their morphology and the results can be expressed for each patient against the established cutoff criteria (Fig. 2.7). The results show a strong correlation when compared with indirect assessments of DNA damage such as the SCSA or comet assay [64, 122]. The advantages and disadvantages of the sperm DNA fragmentation assays are shown in Table 2.3.

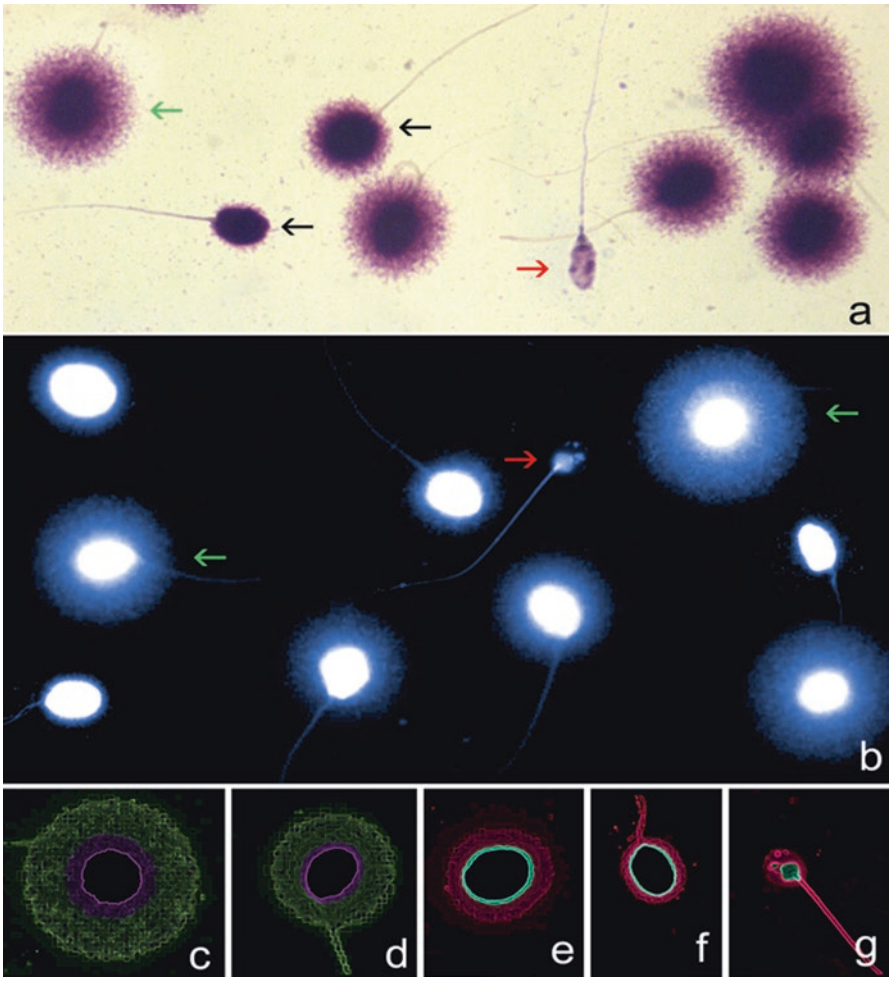

Fig. 2.7 Assessment of sperm DNA fragmentation using the sperm chromatin dispersion (SCD) test. Nucleoids from human spermatozoa obtained with the improved SCD procedure (Halosperm, Halotech DNA, SL, Madrid, Spain) under (**a**) bright field microscopy and Wright's stain (**b**) under fluorescence microscopy and DAPI staining. *Green arrows* target spermatozoa containing a normal DNA molecule. *Red arrows* target a highly fragmented spermatozoon (degraded sperm). (**c–g**) Electronic filtered images showing a series of nucleoids with different levels of sperm DNA damage. Nucleoids with highlighted core delineation in *green* correspond to (**c**) large (**d**) and medium halos of dispersed chromatin representing a normal DNA molecule. Nucleoids in *red* are spermatozoa containing fragmented DNA and are represented by (**e**) small or (**f**) no halos of dispersed chromatin and (**g**) degraded spermatozoa. Bright-field and fluorescence microphotographs were obtained using a motorized fluorescence microscope controlled with software for automatic scanning and image digitization (Leica Microsystems, Barcelona, Spain). The microscope was equipped with a Leica EL6000 metal halide fluorescence light source and Plan-Fluotar 60× objectives with three independent filter blocks (DAPI-5060B; FITC- 3540B and TRITC-A; Semrock, Rechestern NY, USA). A charge coupled device (Leica DFC350 FX, Leica Microsystems, Barcelona, Spain) was used for image capture (Courtesy of Prof. Jaime Gosálvez, Madrid, Spain)

Table 2.3 Assays measuring DNA fragmentation

Assay	Advantage	Disadvantage	Reference
SCSA	Established clinical thresholds, robust and sensitive assay; uses metachromatic acridine orange staining and flow cytometry; requires only 10,000 cells; can be done in fresh or frozen samples	Not available in commercial kits, calculations are complex, acid induced denaturation. Not performed in routine andrology labs	[59, 60]
TUNEL	High sensitivity and specificity. Can be done on fresh or frozen samples. Associated with fertility and available in commercial kits. Measures definite end point	Thresholds not standardized, not specific to oxidative damage, need for special equipment (flow cytometer or fluorescence microscope). Results affected by fixation and permeabilization of sperm. Although can be measured by fluorescence microscopy, results are subjective and prone to inaccuracy due to heterogenous nature of staining and instability of the stain	[61, 86, 96, 99, 104–107]
Comet	Requires small number of cells; high sensitivity, measures breaks in DNA; correlates with seminal parameters. Assay can be performed in neutral and in alkaline environment	Time and labor intensive, not specific to oxidative damage, requires special imaging software, lacks correlation with fertility. Requires electrophoresis and fluroscence microscopy. Slide processing is time consuming	[63, 64, 66, 111]
SCD	Differentiates fragmented from non-fragmented spermatozoa. Does not require flow Cytometry. Can be visualized both by bright field and fluorescence microscopy Strong correlation with SCSA or Comet assay	Interobserver subjectivity to categorize the halos is a limitation of SCD	[64, 67, 68, 101, 118, 119, 122]

SCSA = Sperm chromatin structure assay, *TUNEL* = Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), *SCD* = Sperm chromatin dispersion

2.1.4 Other Methods for Measuring ROS

In addition to the more popular methods of measuring DNA damage described above there are other less common techniques reported in literature. These are shown in Tables 2.4 and 2.5.

Table 2.4 Different methods to measure total antioxidants

Technique	Principle	Advantage	Disadvantage	Reference
TEAC	Inhibition method	Standard by other assays		[123]
ORAC	Inhibition method	High specificity, responds to numerous antioxidants, gross differentiation of aqueous and lipid soluble antioxidants	Time consuming	[124]
FRAP	Reduction of $Fe^3 \rightarrow Fe^2$	Simple, inexpensive	Does not measure SH-group containing radicals	[125]
Enhanced Chemiluminescence	Chemiluminescence	Accurate	Cumbersome, expensive instrumentation, time consuming, signal reagent might reduce in intensity	[126]
Colorimetric	Colorimetric analysis	Less time consuming, relatively inexpensive, convenient, can be used as an in office test	Significantly expensive reagents	[51, 52]
ROS-TAC Score[a]	Chemiluminescence	Better predictor compared to ROS and TAC alone	Requires statistical modeling	[6, 127]
ORP[a] using the RedoxSYS Diagnostic System	Galvanostat based mechanism	Prognostic marker (cORP), easy, less time consuming, requires less expertise, can be used for frozen specimens	Affected by viscosity of the sample	[69–71, 128]

TAEC = Trolox equivalent antioxidant capacity, *ORAC* = Oxidation radical absorbance capacity, *FRAP* = Ferric reducing ability assay, *ORP* = Oxidation reduction potential

[a] These techniques do not directly measure ROS or TAC but rather predict the OS status in the sample

2.1.4.1 Measurement of Total Antioxidant Capacity

The total antioxidant capacity (TAC) is a parameter that can be measured by evaluating the reducing ability of various antioxidants present in semen against an oxidative reagent such as hydrogen peroxide, and measuring the effect on the substrate [51, 52]. The reaction can be measured with a spectrophotometer, colorimeter, depending on

Table 2.5 Various techniques to measure lipid peroxidation

Technique	Principle	Advantage	Disadvantage	Reference
Thiobarbituric acid assay (TBARS)	MDA-TBA adduct detection by colorimetry or fluoroscopy	Simple but non specific	Rigorous controls are required	[53, 54]
Isoprostane	EIA/Liquid chromatography-tandem mass spectrometry	Specificity, stable compound	Labor intensive and expensive cost of equipment	[55]
HNE-His Adduct ELISA	ELISA	Rapid, helps in quantification	Chances of cross reactivity	[56–58, 129]

MDA = malondialdehyde, *TBA* = Thiobarbituric acid, *HNE-His* = hydroxynonenal histidine

the substrate. Most techniques employed to estimate TAC measure the low molecular weight, chain breaking antioxidants and do not include the contribution of antioxidant enzymes (glutathione group of enzymes, catalase, and superoxide dismutase) and metal binding proteins.

Colorimetric Analysis

The antioxidant assay is based on the principle that aqueous and lipid antioxidants in the seminal plasma specimens inhibit the oxidation of the 2, 20-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) to $ABTS^{+}$. Under the reaction conditions used, the antioxidants in the seminal plasma cause suppression of the absorbance at 750 nm to a degree that is proportional to their concentration. A stable blue–green color is formed [49, 50]. The capacity of the antioxidants present in the sample to prevent ABTS oxidation is compared with that of standard Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analogue. Results are reported as micromoles of Trolox equivalent. Clear seminal plasma is used for the assay.

Ten microliters of metmyoglobin and 150 μL of chromogen are added to all standard/sample wells. The reaction is initiated by adding 40 μL of hydrogen peroxide as quickly as possible. The plate is incubated for 5 min at room temperature on a horizontal shaker and absorbance monitored at 750 nm using a microplate reader. The total antioxidant concentration of each sample can be calculated using the equation obtained from the linear regression of the standard curve by substituting the average absorbance values for each sample into the equation:

$$\text{Antioxidant}\left(\mu\text{M}\right)=\frac{\text{Unknown average absorbance}-\text{Y}\quad\text{intercept}}{\text{Slope}}\times\text{dilution}\times 1000$$

Men with oxidative stress have reduced levels of total antioxidants in their seminal plasma.

Some of the other methods measuring antioxidants are illustrated in Table 2.4.

Oxygen Radical Absorbance

Oxygen radical absorbance capacity (ORAC) is a technique that works on utilizing the unique property of phycoerthrins. It works on the sample principle as Trolox equivalent antioxidant capacity (TEAC) [123, 124].

Ferric Reducing Ability Assay

Ferric Reducing Ability Assay (FRAP) is a simple and automated test working on the principle of reduction of ferric to ferrous ions. Antioxidants present in the seminal plasma reduce ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex to Fe^{2+}-TPTZ and form a blue colored complex. Comparing the changes in absorbance occurring at 593 nm gives an estimate of the antioxidant capacity [125].

2.1.4.2 ROS-TAC Score

It is a novel parameter derived from the ROS concentration and seminal TAC values called ROS-TAC score. The ROS and TAC values from controls are used to create a scale of these two variables using controls as reference point. Both values are normalized for distribution after converting ROS to the log of ROS + 1. Both log ROS + 1 and TAC are standardized to Z scores so that both will have the same variability. Standardized scores are calculated by subtracting the mean value of the controls from the individuals observed values and dividing by the standard deviation of the control population.

The standardized values are analyzed with principal component analysis, which provides linear combinations or weighted sums that account for most variability among correlated variables [6]. The ROS-TAC scores are novel measures of oxidative stress and superior than the individual ROS and TAC alone as it is capable of discriminating fertile from infertile men [6, 49, 127]. Infertile men with male factor or idiopathic diagnoses were reported to show low ROS-TAC scores. Infertile men with higher ROS-TAC scores were able to initiate pregnancies compared to those who had low scores and had failed pregnancy [6].

2.1.4.3 Measurement of Redox Potential

Redox potential or oxidation- reduction potential (ORP) is a novel measure of oxidative stress or a redox balance in a given biological system. It has been shown to correlate with illness or injury that is related to a state of oxidative stress [69, 70, 130, 131]. Unlike measuring a single marker of oxidative stress, the Redox system allows the assessment of all known and unknown oxidants and antioxidants in a given sample. It is based on the electrical conductance relative to an internal reference standard according to Nernst equation

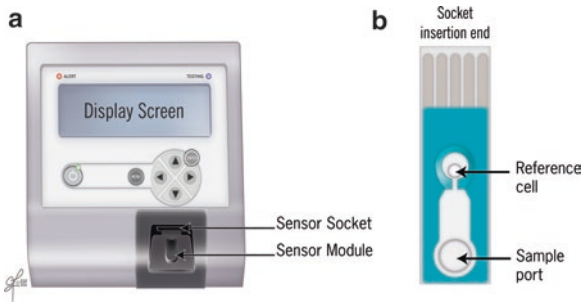

Fig. 2.8 Measurement of oxidation reduction potential (ORP) in semen sample using (**a**) MiOXSYS analyzer and (**b**) Sensor. A 30 μL sample is loaded on the sensor sample port and the sensor is inserted in the sensor socket for ORP measurement

$$E\left(ORP\right)=E_0-RT/nF$$

Where E is the Redox potential or ORP, E_0 is the standard potential of a redox system measured with respect to hydrogen electron, which is arbitrarily assigned an E_0 of 0 V, R is the gas constant, T is the absolute temperature measured in degrees Kelvin and F is Faraday's constant [69, 128]. It has been measured in whole blood, plasma, urine, cerebrospinal fluid, saliva, tears and amniotic fluid [70, 130, 131]. ORP values higher than the established reference values are indicative of oxidative stress [132]. More recently ORP values have been reported in fresh and frozen semen and seminal plasma samples [71]. It involves the RedoxSYS or MiOXSYS Analyzer and the sensor strip which has an application port where the sample is loaded (Fig. 2.8).

It measures two parameters. The first parameter, static ORP or sORP is snapshot of the current oxidative stress in the sample that may be induced due to infection, inflammation or disease. The second parameter is capacity ORP or cORP, and this parameter gives us an estimate of the antioxidants reserves present. The sperm concentration in the sample is calculated and the sORP and cORP results are normalized and expressed as sORP/10^6 sperm or cORP/10^6 sperm. Initial results have shown high sensitivity, specificity and accuracy of ORP reflecting its potential application as a diagnostic tool in oxidative stress related male infertility [133].

2.1.5 Measurement of Lipid Peroxidation

Polyunsaturated fatty acids (PUFAs) containing cellular macromolecules are particularly susceptible to ROS. Lipid peroxidation is characterized by breakdown of these PUFAs into lipid peroxides due to oxidative stress [55]. Lipid peroxides are unstable indicators which on decomposition form more complex and reactive compounds such as 4-hydroxynonenal (4-HNE), acrolein (ACR) and malondialdehyde (MDA).

Compared to free radicals, these aldehydic products are relatively stable and are able to move freely and react with molecules like DNA, proteins and lipids. These

products not only modify proteins and alter their function but are also considered as cytotoxic second messengers of oxidative stress. This property of these aldehydic fragments makes them highly utilized in biomedical research [56, 57]. Measurement of the end products of lipid peroxidation is a widely accepted marker of oxidative stress. Some of the common methods to measure lipid peroxidation are described below:

2.1.5.1 HNE-His Adduct ELISA/HNE Adduct Competitive ELISA

HNE-His Adduct ELISA functions on the principle of enzyme immunoassay and has been developed for quantification and rapid detection of HNE-His protein adducts. 4-HNE can react with lysine, histidine or cysteine residues in protein to form adducts. A 96-well titer ELISA plate is taken and protein samples (10 µg/mL) or BSA standards 0–200 µg/mL are adsorbed onto it. The HNE-protein adducts present in the standard or sample are probed with a primary antibody, (anti-HNE-His antibody). This is followed by addition of a HRP conjugated secondary antibody. Standard curves prepared from predetermined HNE-BSA standards are used to compare and quantify the HNE-protein adduct content in an unknown sample. Using a primary wavelength of 450 nm, the absorbance of each well is read on a microplate reader. Results are expressed as pmol/mg [58].

2.1.5.2 Isprostane (IsoP) Method

Another important biomarker of lipid peroxidation is 8-isoprostane (IsoP) measured as ng/mL [129]. It is a specific end product belonging to family of eicosanoids derived from nonenzymatic peroxidation of polyunsaturated fatty acids [34]. The advantages of using the IsoP marker are: it is not produced by enzymatic pathways like cyclooxygenase and lipoxygenase pathways of arachidonic acid, it is stable and it can be quantified in seminal plasma.

2.1.5.3 TBARS Assay

Malonaldehyde (MDA) is a reactive compound formed during lipid peroxidation [134]. The thiobarbituric acid (TBA) assay is one of the commonly used tests to assess changes in MDA. The assay detects TBA-reactive substances (TBARS) via spectrophotometry, high performance liquid chromatography (HPLC), colorimetry or spectrofluorescence [53]. MDA and TBA are reacted together to form the MDA-TBA adduct. This is carried out in acidic conditions and under a high temperature (90–100 °C). This adduct formation is measured colorimetrically at 530–540 nm or fluorometrically at an excitation wavelength of 530 nm and an emission wavelength of 550 nm [54]. Lipid peroxidation in sperm is expressed as nmol MDA 10^{-7} sperm [135–137]. The advantages and disadvantages of various lipid peroxidation methods are shown in Table 2.5.

2.1.6 Measurement of ROS-Induced Post-Translational Modifications

Reactive oxygen species can modify proteins resulting in altered functions such as activation or inhibition of transcription factors, signal transducers and enzymes [100, 138, 139]. These result in alterations in structural and functional integrity of specific proteins. Most of the oxidants will react with several amino acids to yield multiple products. Only a few stable and specific products those are characteristic of a selected oxidant that may be used as a signature for any selected type of ROS. Three principal type of post-translational modification of proteins are induced by ROS, namely, S-glutathionylation (GSS-R), nitrotyrosine modifications (Nitro-Y) and carbonylation. Carbonylation, nitration and thionylation of proteins are regarded as the most common post-translational modifications leading to dysfunction of proteins [139, 140].

Nitrotyrosine is formed by the reaction of peroxynitrite or donors of NO• with tyrosine residues. It can be produced by the sperm cell by the reaction of superoxide and NO•. The nitrotyrosine protein modification can result in alteration of protein function or structure. Higher amounts of nitrotyrosine were found in patients with impaired motility (athenozoospermia) or spermatic duct cord blood in varicocele patients [141–143]. Reactive carbonyls are produced by direct protein oxidation (oxidation on Trp, Lys, Arg, Pro, and Thr) reaction with low- or high-molecular weight dicarbonyls (modifications of Lys, Arg, and Cys) generated during lipid peroxidation and glycoxidation, and oxidative degradation of Amadori products.

Similarly, for detection of quantification of carbonyl modification of spermatozoa proteins, BSA standards or protein samples (10 μg/mL) are adsorbed onto a 96-well plate for 2 h at 37 °C. The protein carbonyls present in the sample or standard are derivatized to DNP hydrazone and probed with an anti-DNP antibody, followed by an HRP conjugated secondary antibody. The protein carbonyl content in unknown sample is determined by comparing with a standard curve prepared from a predetermined reduced and oxidized BSA standard.

Introduction of carbonyl group to protein involves oxidative modification of specific amino acids namely proline, arginine, lysine and threonine, to aldehyde or ketone and their eventual cleavage. Protein carbonylation is irreversible and leads to structural and functional alterations in the protein and the carbonylated proteins are generally destined to proteosomal breakdown [144].

Products of oxidation such as protein carbonyls are useful for detection and in estimation of ROS levels in a semen sample [145]. Protein carbonyls are chemically stable, more reliable and a frequently used marker for protein oxidation [146]. ELISA is commonly used to quantify the levels of protein carbonyl content in the seminal plasma by colorimetric analysis [147].

Both nitrosylation and carbonylation can be measured by ELISA. ELISA is a commonly used technique to detect and quantify a specific protein in a complex mixture. The analysis is done by immobilizing proteins in microplate wells using specific antibodies. The specific antibodies are chemically linked to biological enzymes.

The activities of these enzymes produce a measurable signal when they are mixed with solutions containing appropriate substances [148]. The end product is a stable color that can be measured colorimetrically or fluorometrically if fluorophore-labeled antibodies are used for signal generation, especially in multiplex arrays.

Competitive ELISA kit can be used for nitrotyrosine quantitation. In this assay, the unknown protein nitrotyrosine sample or nitrated BSA standards is first added to a nitrated BSA preabsorbed EIA plate. After a brief incubation, an anti-nitrotyrosine antibody is added, followed by an HRP conjugated secondary antibody. The protein nitrotyrosine content in unknown sample is determined by comparing with a standard curve prepared from predetermined nitrated BSA standard.

2.1.7 Measurement of ROS-Induced Protein Alterations–Proteomic Analysis

Proteomics and bioinformatics tools can be utilized to understand alterations in proteins as a result of exposure of spermatozoa to reactive oxygen species or oxidative stress. It also helps in demonstrating that how post-translational modifications, such as phosphorylation, proteolytic cleavages, glycosylation and mutations, bring about changes in the physiological functions of the spermatozoa. Global change occurs in proteomic profile of human spermatozoa and seminal plasma under oxidative stress conditions [149–151].

Differential regulation of protein expression in infertile patients has been reported with variations in ROS level as evidenced by global proteomic profiling [28]. Both spermatozoa and seminal plasma proteome influence fertilization and implantation in infertile men with various levels of ROS or oxidative stress [28, 150–152]. Most commonly employed techniques to understand sperm specific proteins include 2D polyacrylamide gel electrophoresis (2D-PAGE), differential in gel electrophoresis (DIGE) and Liquid Chromatography-Mass spectrometry or LC-MS/MS. Global proteomic analysis involves analysis of pooled or individual test samples (either spermatozoa or seminal plasma from semen samples of infertile men exhibiting oxidative stress).

Exposure to different levels of ROS has shown that in the seminal proteome, proteins involved in biomolecule metabolism, protein folding and protein degradation are differentially modulated infertile patient group exposed to low, ROS, medium ROS and high ROS in comparison to fertile controls. In the sperm proteome, differentially expressed proteins with distinct reproductive functions have been demonstrated only in men with low, medium or high ROS levels [28]. Similarly in the seminal plasma proteome, pathways involved in post-translational modification of proteins, protein folding (heat shock proteins, molecular chaperones) and developmental disorder are overexpressed in the high ROS group compared with fertile control group [152].

2.1.7.1 Validation of Proteins of Interest

Proteins of interest that are modified by ROS and identified by proteomic and bioinformatic analysis can be further validated in order to be categorized as a potential biomarker of ROS in spermatozoa or seminal plasma. Potential biomarker candidates of interest can be validated by Western blot analysis utilizing specific antibodies to proteins of interest, or by ELISA and Immunochemistry.

Western Blot Analysis

Western blot analysis is a commonly used technique that helps in quantification of proteins and also in identification of a target protein from a complex mixture of proteins extracted from a cell. It involves the separation of proteins using gel electrophoresis on the basis of their 3-D structure or denatured proteins by the length of the polypeptide. The gel is carefully transferred onto a membrane (PVDF), thus producing a band for each protein. After washing and blocking, it is incubated with primary antibodies specific to the protein of interest. Following incubation with a secondary antibody, the unbound antibodies are washed off. Bound antibodies are detected by chemiluminescence. Proteins are quantified by comparing it to a standard protein (Fig. 2.9) [153–155].

Immunochemistry

Immunocytochemistry is a technique employed on spermatozoa to localize antigen expression. It works on the principle of epitope-antibody interactions. Positive staining patterns are visualized using a molecular label, which can be fluorescent or

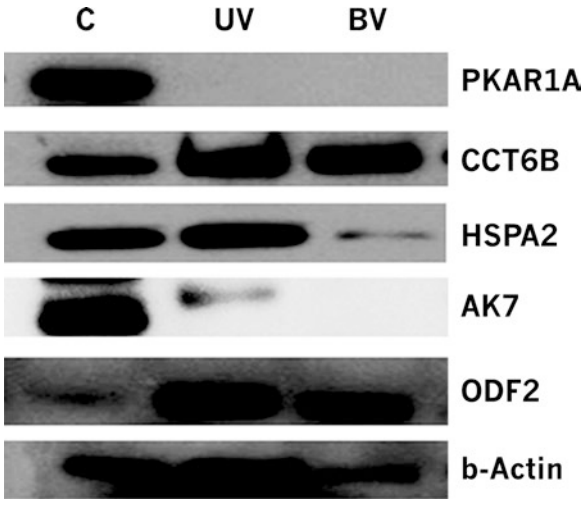

Fig. 2.9 Validation of 5 proteins by western blot analysis in control (fertile men) and infertile men with unilateral varicocele (UV) or bilateral varicocele (BV)

chromogenic. Briefly, an aliquot of 10×10^6spermatozoa is centrifuged initially at 500 g for 5 min. It is then fixed for 15 min in 2 % paraformaldehyde. The fixed cells are then washed in 0.1 M phosphate buffered saline. The cells are then resuspended in 0.1 M glycine/PBS, and they are then transferred to poly-L-lysine coated coverslips to settle overnight. The spermatozoa are permeabilized using Triton X-100-PBS (0.2 %). This is carried out for 10 min at room temperature and is followed by a wash in PBS.

By mixing 3 % BSA solution (900 mg PBS, 30 mg BSA and 100 μl goat serum) for 30 min, non-specific antibodies are inhibited. The cells are then rinsed in PBS and treated with the primary antibody of interest, diluted in PBS overnight at a temperature of 4 °C in a humidified chamber. The cells are washed in PBS and treated with the secondary antibody in PBS for 1 h at 37 °C. They are then re-washed in PBS. The cells are mounted on frosted slides and images are captured on a fluorescent microscope at 530 nm and positive staining patterns are identified [153]. The localization of the protein can be in the acrosome, neck, mid piece or the tail [156].

Proteins of interest can also be identified by ELISA utilizing the antibodies to proteins of interest.

ELISA

Protein of interest can also be validated by ELISA. The proteins (antigen) is directly or indirectly immobilized and coated onto the surface of the wells of the microplate. All unsaturated surface binding sites are then covered by adding blocking antibodies. Antigen specific antibodies are then added and incubated. These antibodies bind to the antigens. A signal is generated by the primary or secondary tag present on the specific antibody when the antibody binds to the antigen. This signal is then detected and quantification of the protein is done by measuring the signal [148, 157].

2.2 Conclusion

Oxidative stress is recognized as an important factor in male infertility. Accurate assessment is therefore critical in the laboratory evaluation of male infertility attributed to oxidative stress. In this chapter we have described various techniques that can be used to measure reactive oxygen species or their end-products using simple techniques such as nitroblue testing to ROS measurement by chemiluminescence assay, measuring antioxidants or end products of oxidative stress such as lipid peroxidation, DNA damage or protein modifications. We have highlighted the value of the newer tools such as proteomics in identifying oxidatively modified proteins and validating these proteins. The ultimate goal is to identify potential markers of oxidative stress to assist in elucidating the underlying mechanism of oxidative stress related sperm dysfunction that ultimately results in male infertility and assists the clinicians in the management of these patients.

References

1. Kessopoulou E et al (1992) Origin of reactive oxygen species in human semen: spermatozoa or leucocytes? J Reprod Fertil 94(2):463–470
2. Aitken J, Fisher H (1994) Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. Bioessays 16(4):259–267
3. Saleh RA, Agarwal A (2002) Oxidative stress and male infertility: from research bench to clinical practice. J Androl 23(6):737–752
4. Shekarriz M et al (1995) Incidence and level of seminal reactive oxygen species in normal men. Urology 45(1):103–107
5. Sharma RK, Agarwal A (1996) Role of reactive oxygen species in male infertility. Urology 48(6):835–850
6. Sharma RK et al (1999) The reactive oxygen species-total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. Hum Reprod 14(11):2801–2807
7. Sikka SC (2001) Relative impact of oxidative stress on male reproductive function. Curr Med Chem 8(7):851–862
8. Agarwal A et al (2014) Effect of oxidative stress on male reproduction. World J Mens Health 32(1):1–17
9. Doshi SB et al (2012) Role of reactive nitrogen species in male infertility. Reprod Biol Endocrinol 10:109
10. Agarwal A et al (2014) Mechanisms of oligozoospermia: an oxidative stress perspective. Syst Biol Reprod Med 60(4):206–216
11. Attaran M et al (2000) The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. Int J Fertil Womens Med 45(5):314–320
12. Aziz N et al (2004) Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index. Fertil Steril 81(2):349–354
13. Agarwal A et al (2003) Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril 79(4):829–843
14. Ochsendorf FR et al (1994) Chemiluminescence in semen of infertile men. Andrologia 26(5):289–293
15. Pasqualotto FF et al (2000) Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation. Fertil Steril 73(3): 459–464
16. Athayde KS et al (2007) Development of normal reference values for seminal reactive oxygen species and their correlation with leukocytes and semen parameters in a fertile population. J Androl 28(4):613–620
17. Agarwal A et al (1994) Relationship of sperm parameters with levels of reactive oxygen species in semen specimens. J Urol 152(1):107–110
18. Zini A et al (1993) Reactive oxygen species in semen of infertile patients: levels of superoxide dismutase- and catalase-like activities in seminal plasma and spermatozoa. Int J Androl 16(3):183–188
19. Gomez E et al (1996) Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. J Androl 17(3):276–287
20. Gil-Guzman E et al (2001) Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation. Hum Reprod 16(9):1922–1930
21. Agarwal A et al (2014) Relationship amongst teratozoospermia, seminal oxidative stress and male infertility. Reprod Biol Endocrinol 12:45
22. de Lamirande E, Gagnon C (1992) Reactive oxygen species and human spermatozoa. I. Effects on the motility of intact spermatozoa and on sperm axonemes. J Androl 13(5):368–378
23. Kemal DN et al (2000) Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa. Fertil Steril 74(6):1200–1207

24. Desai N et al (2009) Physiologic and pathologic levels of reactive oxygen species in neat semen of infertile men. Fertil Steril 92(5):1626–1631
25. Agarwal A, Said TM (2005) Oxidative stress, DNA damage and apoptosis in male infertility: a clinical approach. BJU Int 95(4):503–507
26. Agarwal A et al (2006) Reactive oxygen species as an independent marker of male factor infertility. Fertil Steril 86(4):878–885
27. Koppers AJ et al (2008) Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. J Clin Endocrinol Metab 93(8):3199–3207
28. Ayaz A et al (2015) Impact of precise modulation of reactive oxygen species levels on spermatozoa proteins in infertile men. Clin Proteomics 12(1):4
29. Cassina A et al (2015) Defective human sperm cells are associated with mitochondrial dysfunction and oxidant production. Biol Reprod 93(5):119
30. Treulen F et al (2015) Mitochondrial permeability transition increases reactive oxygen species production and induces DNA fragmentation in human spermatozoa. Hum Reprod 30(4):767–776
31. Tremellen K (2008) Oxidative stress and male infertility – a clinical perspective. Hum Reprod Update 14(3):243–258
32. Hamada A et al (2013) Insight into oxidative stress in varicocele-associated male infertility: part 2. Nat Rev Urol 10(1):26–37
33. Hamada A et al (2012) Unexplained male infertility: diagnosis and management. Int Braz J Urol 38(5):576–594
34. Agarwal A et al (2005) Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. Indian J Exp Biol 43(11):963–974
35. Buettner GR (1987) Spin trapping: ESR parameters of spin adducts. Free Radic Biol Med 3(4):259–303
36. Mrakic-Sposta S et al (2012) Assessment of a standardized ROS production profile in humans by electron paramagnetic resonance. Oxid Med Cell Longev 2012:973927
37. Mrakic-Sposta S et al (2014) A quantitative method to monitor reactive oxygen species production by electron paramagnetic resonance in physiological and pathological conditions. Oxid Med Cell Longev 2014:306179
38. Deepinder F, Agarwal A (2009) Chapter 39: Determination of seminal oxidants (reactive oxygen species). In: Lipshultz LI, Howards S, Neiderberger CS. (eds) Infertility in the male, 4th edn. Cambridge University Press, Cambridge, p 618–632
39. Agarwal A et al (2008) Chapter 10: Oxidative stress measurement in patients with male or female factor infertility. In: Popov I, Lewin G. Handbook of chemiluminescent methods in oxidative stress assessment, Transworld Research Network, p 195–218
40. Kobayashi H et al (2001) Quality control of reactive oxygen species measurement by luminol-dependent chemiluminescence assay. J Androl 22(4):568–574
41. Agarwal A et al (2004) Chemiluminescence technique for measuring reactive oxygen species. Reprod Biomed Online 9(4):466–468
42. Benjamin D et al (2012) Methods for the detection of ROS in human sperm samples. Studies on men's health and fertility. Humana, New York, pp 257–274
43. Agarwal A et al (2015) Reference values of reactive oxygen species in seminal ejaculates using chemiluminescence assay. J Assist Reprod Genet 32(12):1721–1729
44. Aitken RJ et al (1992) Reactive oxygen species and human spermatozoa: analysis of the cellular mechanisms involved in luminol- and lucigenin-dependent chemiluminescence. J Cell Physiol 151(3):466–477
45. Kashou AH et al (2013) Assessment of oxidative stress in sperm and semen. Methods Mol Biol 927:351–361
46. Guthrie HD, Welch GR (2006) Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. J Anim Sci 84(8):2089–2100
47. De Iuliis GN et al (2006) Definitive evidence for the nonmitochondrial production of superoxide anion by human spermatozoa. J Clin Endocrinol Metab 91(5):1968–1975

48. Mahfouz R et al (2009) Evaluation of chemiluminescence and flow cytometry as tools in assessing production of hydrogen peroxide and superoxide anion in human spermatozoa. Fertil Steril 92(2):819–827
49. Sharma RK et al (2001) Relationship between seminal white blood cell counts and oxidative stress in men treated at an infertility clinic. J Androl 22(4):575–583
50. Jones DP (2002) Redox potential of GSH/GSSG couple: assay and biological significance. Methods Enzymol 348:93–112
51. Miller NJ et al (1993) A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin Sci (Lond) 84(4):407–412
52. Mahfouz R et al (2009) Diagnostic value of the total antioxidant capacity (TAC) in human seminal plasma. Fertil Steril 91(3):805–811
53. Chirico S et al (1993) Lipid peroxidation in hyperlipidaemic patients. A study of plasma using an HPLC-based thiobarbituric acid test. Free Radic Res Commun 19(1):51–57
54. Grotto D et al (2007) Rapid quantification of malondialdehyde in plasma by high performance liquid chromatography-visible detection. J Pharm Biomed Anal 43(2):619–624
55. Halliwell B, Chirico S (1993) Lipid peroxidation: its mechanism, measurement, and significance. Am J Clin Nutr 57(5 Suppl):715S–724S, discussion 724S–725S
56. Zarkovic N (2003) 4-hydroxynonenal as a bioactive marker of pathophysiological processes. Mol Aspects Med 24(4-5):281–291
57. Spickett CM (2013) The lipid peroxidation product 4-hydroxy-2-nonenal: advances in chemistry and analysis. Redox Biol 1:145–152
58. Weber D et al (2013) Measurement of HNE-protein adducts in human plasma and serum by ELISA-Comparison of two primary antibodies. Redox Biol 1:226–233
59. Evenson DP et al (1999) Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod 14(4):1039–1049
60. Evenson DP (2013) Sperm chromatin structure assay (SCSA$^{(R)}$). Methods Mol Biol 927:147–164
61. Sharma RK et al (2010) TUNEL as a test for sperm DNA damage in the evaluation of male infertility. Urology 76(6):1380–1386
62. Aitken RJ et al (2013) The source and significance of DNA damage in human spermatozoa; a commentary on diagnostic strategies and straw man fallacies. Mol Hum Reprod 19(8):475–485
63. Simon L et al (2011) Sperm DNA damage measured by the alkaline Comet assay as an independent predictor of male infertility and in vitro fertilization success. Fertil Steril 95(2):652–657
64. Ribas-Maynou J et al (2013) Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay. Andrology 1(5):715–722
65. Simon L, Carrell DT (2013) Sperm DNA damage measured by comet assay. Methods Mol Biol 927:137–146
66. McKelvey-Martin VJ et al (1997) Two potential clinical applications of the alkaline single-cell gel electrophoresis assay: (1). Human bladder washings and transitional cell carcinoma of the bladder; and (2). Human sperm and male infertility. Mutat Res 375(2):93–104
67. Fernandez JL et al (2003) The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. J Androl 24(1):59–66
68. Fernandez JL et al (2005) Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. Fertil Steril 84(4):833–842
69. Shapiro HM (1972) Redox balance in the body: an approach to quantitation. J Surg Res 13(3):138–152
70. Rael LT et al (2007) Oxidation-reduction potential and paraoxonase-arylesterase activity in trauma patients. Biochem Biophys Res Commun 361(2):561–565
71. Agarwal A et al (2015) Establishing the oxidation-reduction potential in semen and seminal plasma. Fertil Steril 104(3), e146

72. Baehner RL et al (1976) The biochemical basis of nitroblue tetrazolium reduction in normal human and chronic granulomatous disease polymorphonuclear leukocytes. Blood 48(2):309–313
73. Esfandiari N et al (2003) Utility of the nitroblue tetrazolium reduction test for assessment of reactive oxygen species production by seminal leukocytes and spermatozoa. J Androl 24(6):862–870
74. Fridovich I (1997) Superoxide anion radical (O2-.), superoxide dismutases, and related matters. J Biol Chem 272(30):18515–18517
75. Agarwal A et al (2008) Clinical relevance of oxidative stress in male factor infertility: an update. Am J Reprod Immunol 59(1):2–11
76. Aitken RJ et al (2004) Shedding light on chemiluminescence: the application of chemiluminescence in diagnostic andrology. J Androl 25(4):455–465
77. Agarwal A et al (2014) Characterizing semen parameters and their association with reactive oxygen species in infertile men. Reprod Biol Endocrinol 12:33
78. Agarwal et al (2016) Reactive oxygen species (ROS) measurement. In: Agarwal A, Gupta S, Sharma R (eds) Andrological evaluation of male infertility: a laboratory guide. Springer Publishing, New York, pp 155–164. Chapter 21
79. Aitken RJ, Clarkson JS (1987) Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. J Reprod Fertil 81(2):459–469
80. Aitken R et al (1991) Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. Am J Obstet Gynecol 164(2):542–551
81. Ollero M et al (2001) Characterization of subsets of human spermatozoa at different stages of maturation: implications in the diagnosis and treatment of male infertility. Hum Reprod 16(9):1912–1921
82. Aziz N et al (2010) Comparison of chemiluminescence and flow cytometry in the estimation of reactive oxygen and nitrogen species in human semen. Fertil Steril 94(7):2604–2608
83. Agarwal A et al (2009) Markers of oxidative stress and sperm chromatin integrity. Methods Mol Biol 590:377–402
84. Mammoto A et al (1996) Reactive oxygen species block sperm-egg fusion via oxidation of sperm sulfhydryl proteins in mice. Biol Reprod 55(5):1063–1068
85. Seli E et al (2004) Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. Fertil Steril 82(2):378–383
86. Zini A et al (2008) Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: systematic review and meta-analysis. Hum Reprod 23(12):2663–2668
87. Schulte RT et al (2010) Sperm DNA damage in male infertility: etiologies, assays, and outcomes. J Assist Reprod Genet 27(1):3–12
88. Alshahrani S et al (2014) Infertile men older than 40 years are at higher risk of sperm DNA damage. Reprod Biol Endocrinol 12:103
89. Mahfouz R et al (2010) Semen characteristics and sperm DNA fragmentation in infertile men with low and high levels of seminal reactive oxygen species. Fertil Steril 94(6):2141–2146
90. Said TM et al (2005) Novel association between sperm deformity index and oxidative stress-induced DNA damage in infertile male patients. Asian J Androl 7(2):121–126
91. De Iuliis GN et al (2009) Mobile phone radiation induces reactive oxygen species production and DNA damage in human spermatozoa in vitro. PLoS One 4(7), e6446
92. Hoshi K et al (1996) The relationship between acridine orange fluorescence of sperm nuclei and the fertilizing ability of human sperm. Fertil Steril 66(4):634–639
93. Gopalkrishnan K et al (1999) Use of acridine orange to evaluate chromatin integrity of human spermatozoa in different groups of infertile men. Andrologia 31(5):277–282
94. Larson KL et al (2000) Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. Hum Reprod 15(8):1717–1722
95. Palermo GD et al (2014) Perspectives on the assessment of human sperm chromatin integrity. Fertil Steril 102(6):1508–1517

96. Sharma R, Agarwal A (2011) Chapter 14: Laboratory evaluation of sperm chromatin: TUNEL assay. In: Zini A, Agarwal A (eds) Sperm chromatin: biological and clinical applications in male infertility and assisted reproduction, Springer Science+Business Media, LLC, p 201–215
97. Samplaski MK et al (2010) New generation of diagnostic tests for infertility: review of specialized semen tests. Int J Urol 17(10):839–847
98. Sharma R et al (2013) Sperm DNA fragmentation analysis using the TUNEL assay. Methods Mol Biol 927:121–136
99. Sharma R et al (2016) Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using bench top flow cytometer for evaluation of sperm DNA fragmentation in fertility laboratories: protocol, reference values, and quality control. J Assist Reprod Genet 33(2):291–300
100. Esteves SC et al (2015) Diagnostic accuracy of sperm DNA degradation index (DDSi) as a potential noninvasive biomarker to identify men with varicocele-associated infertility. Int Urol Nephrol 47(9):1471–1477
101. Gosalvez J et al (2014) Characterisation of a subpopulation of sperm with massive nuclear damage, as recognised with the sperm chromatin dispersion test. Andrologia 46(6):602–609
102. Zini A, Sigman M (2009) Are tests of sperm DNA damage clinically useful? Pros and cons. J Androl 30(3):219–229
103. Feijo CM, Esteves SC (2014) Diagnostic accuracy of sperm chromatin dispersion test to evaluate sperm deoxyribonucleic acid damage in men with unexplained infertility. Fertil Steril 101(1):58–63.e3
104. Muratori M et al (2010) Small variations in crucial steps of TUNEL assay coupled to flow cytometry greatly affect measures of sperm DNA fragmentation. J Androl 31(4):336–345
105. Lewis SE et al (2013) The impact of sperm DNA damage in assisted conception and beyond: recent advances in diagnosis and treatment. Reprod Biomed Online 27(4):325–337
106. Henkel R et al (2003) DNA fragmentation of spermatozoa and assisted reproduction technology. Reprod Biomed Online 7(4):477–484
107. Henkel R et al (2004) Influence of deoxyribonucleic acid damage on fertilization and pregnancy. Fertil Steril 81(4):965–972
108. Shamsi MB et al (2011) Sperm DNA integrity assays: diagnostic and prognostic challenges and implications in management of infertility. J Assist Reprod Genet 28(11):1073–1085
109. Baumgartner A et al (2009) The comet assay in male reproductive toxicology. Cell Biol Toxicol 25(1):81–98
110. Tice RR et al (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 35(3):206–221
111. Hughes CM et al (1996) A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. Mol Hum Reprod 2(8):613–619
112. Tarozzi N et al (2007) Clinical relevance of sperm DNA damage in assisted reproduction. Reprod Biomed Online 14(6):746–757
113. Cortes-Gutierrez EI et al (2014) Interpreting sperm DNA damage in a diverse range of mammalian sperm by means of the two-tailed comet assay. Front Genet 5:404
114. Fernandez JL et al (2001) DBD-fish on neutral comets: simultaneous analysis of DNA single- and double-strand breaks in individual cells. Exp Cell Res 270(1):102–109
115. Rivero MT et al (2003) Patterns of DNA migration in two-dimensional single-cell gel electrophoresis analyzed by DNA breakage detection-fluorescence in situ hybridization. Environ Mol Mutagen 42(3):223–227
116. Zee YP et al (2009) Evidence that single-stranded DNA breaks are a normal feature of koala sperm chromatin, while double-stranded DNA breaks are indicative of DNA damage. Reproduction 138(2):267–278
117. Klaude M et al (1996) The comet assay: mechanisms and technical considerations. Mutat Res 363(2):89–96

118. Fernandez JL et al (2000) DNA breakage detection-FISH (DBD-FISH) in human spermatozoa: technical variants evidence different structural features. Mutat Res 453(1):77–82
119. Muriel L et al (2004) Structure of human sperm DNA and background damage, analysed by in situ enzymatic treatment and digital image analysis. Mol Hum Reprod 10(3):203–209
120. Ankem MK et al (2002) Novel assay for determining DNA organization in human spermatozoa: implications for male factor infertility. Urology 59(4):575–578
121. Agarwal A, Said TM (2012) Sperm chromatin assessment. Textbook of assisted reproductive techniques. Fourth edn: volume 1: Laboratory perspectives. p 75
122. Chohan KR et al (2006) Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. J Androl 27(1):53–59
123. Rice-Evans C, Miller NJ (1994) Total antioxidant status in plasma and body fluids. Methods Enzymol 234:279–293
124. Cao G et al (1993) Oxygen-radical absorbance capacity assay for antioxidants. Free Radic Biol Med 14(3):303–311
125. Benzie IF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 239(1):70–76
126. Said TM et al (2003) Enhanced chemiluminescence assay vs colorimetric assay for measurement of the total antioxidant capacity of human seminal plasma. J Androl 24(5):676–680
127. Pasqualotto FF et al (2008) Semen quality and oxidative stress scores in fertile and infertile patients with varicocele. Fertil Steril 89(3):602–607
128. Bar-Or R et al (2012) Method and apparatus for measuring oxidation-reduction potential. Google patents
129. Khosrowbeygi A, Zarghami N (2007) Levels of oxidative stress biomarkers in seminal plasma and their relationship with seminal parameters. BMC Clin Pathol 7:6
130. Rael LT et al (2009) Plasma oxidation-reduction potential and protein oxidation in traumatic brain injury. J Neurotrauma 26(8):1203–1211
131. Rael LT et al (2009) Injury severity and serum amyloid A correlate with plasma oxidation-reduction potential in multi-trauma patients: a retrospective analysis. Scand J Trauma Resusc Emerg Med 17:57
132. Kohen R, Nyska A (2002) Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicol Pathol 30(6):620–650
133. Agarwal A et al (2017) Diagnostic application of oxidation-reduction potential assay for measurement of oxidative stress: clinical utility in male factor infertility. Reprod Biomed Online 34(1):48–57
134. Benedetti S et al (2012) Differences in blood and semen oxidative status in fertile and infertile men, and their relationship with sperm quality. Reprod Biomed Online 25(3):300–306
135. Fatma BA et al (2009) Sperm quality improvement after date seed oil in vitro supplementation in spontaneous and induced oxidative stress. Asian J Androl 11(3):393–398
136. Potts RJ et al (2000) Seminal plasma reduces exogenous oxidative damage to human sperm, determined by the measurement of DNA strand breaks and lipid peroxidation. Mutat Res 447(2):249–256
137. Bell M et al (1992) Time course of hydrogen peroxide induced changes in the lipid peroxidation of human sperm membranes. Adv Contracept Deliv Syst 8(1-2):144–150
138. Agarwal A et al (2015) Major protein alterations in spermatozoa from infertile men with unilateral varicocele. Reprod Biol Endocrinol 13:8
139. Radi R (2004) Nitric oxide, oxidants, and protein tyrosine nitration. Proc Natl Acad Sci U S A 101(12):4003–4008
140. Dalle-Donne I et al (2006) Biomarkers of oxidative damage in human disease. Clin Chem 52(4):601–623
141. Herrero MB et al (2001) Tyrosine nitration in human spermatozoa: a physiological function of peroxynitrite, the reaction product of nitric oxide and superoxide. Mol Hum Reprod 7(10):913–921
142. Morielli T, O'Flaherty C (2015) Oxidative stress impairs function and increases redox protein modifications in human spermatozoa. Reproduction 149(1):113–123

143. Romeo C et al (2003) Preliminary report on nitric oxide-mediated oxidative damage in adolescent varicocele. Hum Reprod 18(1):26–29
144. Bollineni RC et al (2014) Carbonylated plasma proteins as potential biomarkers of obesity induced type 2 diabetes mellitus. J Proteome Res 13(11):5081–5093
145. Dalle-Donne I et al (2003) Protein carbonylation in human diseases. Trends Mol Med 9(4):169–176
146. Stadtman ER, Berlett BS (1991) Fenton chemistry. Amino acid oxidation. J Biol Chem 266(26):17201–17211
147. El-Taieb MA et al (2009) Oxidative stress and epididymal sperm transport, motility and morphological defects. Eur J Obstet Gynecol Reprod Biol 144(Suppl 1):S199–S203
148. Engvall E, Perlmann P (1971) Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8(9):871–874
149. Hamada A et al (2013) Two-dimensional differential in-gel electrophoresis-based proteomics of male gametes in relation to oxidative stress. Fertil Steril 99(5):1216–1226, e2
150. Sharma R et al (2013) Proteomic analysis of seminal fluid from men exhibiting oxidative stress. Reprod Biol Endocrinol 11:85
151. Sharma R et al (2013) Proteomic analysis of human spermatozoa proteins with oxidative stress. Reprod Biol Endocrinol 11:48
152. Agarwal A et al (2015) Comparative proteomic network signatures in seminal plasma of infertile men as a function of reactive oxygen species. Clin Proteomics 12(1):23
153. Mathews ST et al (2009) Imaging systems for westerns: chemiluminescence vs. infrared detection. Methods Mol Biol 536:499–513
154. Edmondson DG, Dent SY (2001) Identification of protein interactions by far western analysis. Curr Protoc Protein Sci. Chapter 19: p. Unit 19 7
155. Agarwal A et al (2015) Proteomic signatures of infertile men with clinical varicocele and their validation studies reveal mitochondrial dysfunction leading to infertility. Asian J Androl 18(2):282–291
156. Salvolini E et al (2013) Involvement of sperm plasma membrane and cytoskeletal proteins in human male infertility. Fertil Steril 99(3):697–704
157. Nakane PK, Pierce GB Jr (1967) Enzyme-labeled antibodies for the light and electron microscopic localization of tissue antigens. J Cell Biol 33(2):307–318

Chapter 3
Physiological Roles of Reactive Oxygen Species (ROS) in the Reproductive System

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3.1 Introduction

Reactive oxygen species (ROS) are highly reactive oxidizing agents which play certain physiological and pathological roles in the human body [1]. These substances are normally present within the cells. ROS are mostly free radicals. Free radicals have been labeled as molecular entities containing at least one unpaired electron which gives rise to a highly reactive group of compounds [2]. Moderate concentrations of ROS are vital in maintaining a number of physiologic processes in both male and female reproductive systems. ROS can be acquired through two different classes of sources, either endogenous (cellular) or exogenous. Unlike endogenous ROS, exogenous sources such as ultraviolet light, chemotherapeutic agents and inflammatory cytokines do not play a major role in generating ROS in the female reproductive system [3]. Endogenous sources, however, are the most prominent birthplace from which ROS are derived. The major intracellular sources of ROS include the electron

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transport chain in the mitochondria, endoplasmic reticulum as well as the peroxisomes [4]. Conversely, other extra-mitochondrial ROS sources exist through numerous enzymatic pathways. Examples include NADPH oxidase, xanthine oxidase, lipoxygenases, cyclooxygenases and the cytochrome P450 systems [5].

Examples of ROS include molecules such as superoxide ($O2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), peroxyl ($ROO^{\bullet}$) and hydrogen peroxide (H_2O_2). There are also some other types of ROS-derived molecules that might not be as naturally prevalent. For instance, hypochlorous acid (HOCL), a well-known component of bleach and bactericidal agents is regarded as one of these less commonly acknowledged ROS members. The ROS family mentioned in this chapter also includes Reactive Nitrogen Species (RNS) [6]. Regarding RNS, nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$) and peroxynitric acid ($ONOO^{-}$) constitute the majority of RNS.

Under normal circumstances, a balance between the concentrations of oxidants and antioxidants is maintained. In case of an overproduction of ROS, or in cases where the body is unable to eliminate excess ROS, oxidative stress (OS) occurs, leading to cell and tissue damage. ROS may inflict damage to DNA, proteins and cell membranes [1]. The pathogenic role played by ROS will be discussed in Chapter 6Under physiologic conditions, cells exhibit defensive mechanisms against an increased concentration of ROS. Antioxidants are scavenging molecules that convert ROS to H_2O to prevent the ROS elevation and the resultant OS. They are divided into enzymatic and non-enzymatic antioxidants [7, 8]. Enzymatic antioxidants, known as natural antioxidants, neutralize excess ROS and prevent it from interfering with cellular or membrane structures. Enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GSR) [4]. Non-enzymatic antioxidants, also known as synthetic antioxidants, are essentially dietary supplements. Dietary intake of antioxidant vitamins and minerals such as vitamin C, vitamin E, selenium, zinc, taurine, hypotaurine, glutathione, beta carotene and carotene greatly influences the body's complex antioxidant system [7–9].

Vitamin C is a chain breaking antioxidant that is responsible for halting the propagation of peroxidative processes. Vitamin C plays also a role in recycling oxidized vitamin E and glutathione [10]. Taurine, hypotaurine and transferrin protect the embryo from oxidative stress and are mainly found in tubal and follicular fluid [11]. Glutathione is present in the oocyte and in tubal fluid; it contains sulfur chemical groups and has a role in enhancing the development of the zygote from the 2-cell cluster all the way to the morula or the blastocyst stage [11].

The physiological role of ROS has been first examined by Aitken et al. [12] who suggested that ROS were positively involved in the normal sperm physiology. The authors' observations indicated that physiological levels of ROS are crucial for the completion of most, if not all of the maturational stages that spermatozoa undergo, maximizing their fertilizing potential. On the other hand, spermatozoa are particularly susceptible to lipid peroxidation because of the relatively elevated levels of polyunsaturated fatty acids present in their plasma membrane. Furthermore, spermatozoa lack adequate amounts of cellular repair mechanisms and antioxidant defense systems. The reason behind this is the unusually low cytoplasmic volume, which happens after spermatozoa extrude ample volumes of their cytoplasm during maturation [13].

ROS also play a role in various physiological processes in the female reproductive system, including follicular development, ovarian steroidogenesis, ovulation, formation of the corpus luteum, luteolysis, germ cell function, maintenance of pregnancy and parturition.

This chapter will focus on the physiological role of ROS in both male and female reproductive systems.

3.1.1 Physiological Roles of ROS in Male Reproductive System

3.1.1.1 Spermatogenesis and Sperm Maturation

ROS are involved in the physiology of the sperm maturational stages, particularly during epididymal transit. At this stage, spermatozoa go through large-scale changes, including remodeling of the plasma membrane and nuclear material, installment of the signal transduction and enzymatic machinery essential to the completion of capacitation and hyperactivation [13]. Inoperable at this stage of maturation, some surface proteins are released while others, mainly novel proteins, get strongly attached and rearranged onto the plasma membrane [14–16].

Unlike somatic cells, histone packing is not sufficient to maintain the biochemical integrity of the genetic material stored in spermatozoa. The reason is spermatozoa are deficient in the preventative and DNA repair systems that are usually found in other somatic cells [17]. Therefore, additional forms of stability are required to ensure that nuclear genome contained within a spermatozoon's chromatin entanglement does not get damaged during the migration from the caput to epididymis cauda. This unique form of stability is achieved through the oxidation of the thiol groups present in protamines, which are small nuclear proteins that eventually replace histones in the process of spermiogenesis, producing particularly strong disulfide bonds between cysteine residues. The formation of disulfide bonds occurs in the epididymis cauda, where spermatozoa are stored prior to ejaculation [18]. As a matter of fact, regulatory redox mechanisms that produce ROS have shown to be implicated in the proper chromatin packing of multiple mammalian species, including humans [18]. Approximately 98.5 % of the cysteine residues present in human spermatozoa are found in the oxidized form. ROS contribute to the generation of the disulfide bonds by acting as oxidizing agents providing oxidative power [19]. Mature spermatozoa located in the epididymis cauda were found to produce ROS as indicated by an extemporaneous luminol-peroxidase signal [20].

Moreover, the contribution of ROS, particulary peroxides, in the formation of the protective mitochondrial covering, known as the ''mitochondrial capsule", has been confirmed. If this capsule is not formed correctly, a marked impairment of cellular function, predominantly relating to power generation, will be noticed [21]. Peroxides exert an oxidizing influence on an enzyme known as phospholipid hyperoxide glutathione peroxidase (PHGPx). The product of this oxidation reacts with a protein thiol group, forming a selenedisulfide bond.

3.1.1.2 Sperm Capacitation

Capacitation has been recognized as one of the crucial sperm maturation events taking place inside the female genital tract. During capacitation, spermatozoa acquire a status of hyperactive motility, also known as hyperactivation, which is accompanied by an increase in the cellular responsiveness to chemotactic signalling [22]. It normally takes place as a post-ejaculatory phase, optimizing the spermatozoa's fertilizing capacity. This process ensures that only viable and mature sperm reach the oocyte. The molecular background of capacitation includes cellular changes on a large scale, namely, an efflux of cholesterol, influxes of HCO_3^- and $Ca^{2+,}$ ROS generation, increase in cAMP, pH, protein phosphorylation (Ser/Thr and Tyr) and membrane hyperpolarization [23, 24]. Currently, the most frequently used in vitro method to distinguish between capacitated and non-capacitated sperm is by assessing the spermatozoa's ability to undergo the acrosome reaction (AR). It has been found that the AR can be induced by Ca^{2+} ionophore A23187, progesterone, zona pellucida (ZP), and lysophosphatodiylcholine (LPC) [25].

Hyperactivation is generally regarded as a marker of capacitation. Hyperactive spermatozoa demonstrate a unique pattern of motility characterized by an overall non-linear motility. They also experience a considerable lateral displacement of the head together with elevated amplitude, asymmetric flagellar movements. This type of motility provides spermatozoa with the sufficient propulsive force necessary to penetrate the cumulus cells and zona pellucida surrounding the oocyte. Although hyperactivation is essential for fertilization, it is not as important for the AR. Hyperactive spermatozoa are unlikely to exhibit substantial stagnation in the female oviductal epithelium, thus facilitating progressive movement. There have been studies suggesting that progesterone secreted at the zona pellucida of an oocyte might initiate the biochemical cascade of hyperactivation [26]. Nevertheless, the biochemical background regarding hyperactivation is yet to be fully elucidated. Despite of that, some notable cellular breakthroughs have been reported. These include: a rise in cAMP activity and pH [26], fluxes of HCO_3^- [23], an elevation in the concentration levels of intracellular calcium ($Ca^{2+)}$ [27, 28] and increased production of ROS [29].

In order to illustrate the physiological role of ROS more clearly, spermatozoa were incubated with minimal levels of H_2O_2. This eventually stimulated the initiation of capacitation, thereby increasing the overall rate of sperm-oocyte fusion [30]. This experiment relied on sperm hyperactivated motility and the readiness to undergo the acrosome reaction in response to exposure to the A23187 calcium ionophore [31]. Likewise, extensive research has been carried out to identify the physiological role of ROS during capacitation. The presence of ROS plays an essential role in amplifying the numbers of P-Tyr proteins, mainly those with a molecular weight of around 100 kDa [32, 33].

It is generally believed that H_2O_2 is central to the process of capacitation, whereas O_2^- mainly acts as a regulatory molecule for hyperactivation [34]. On the contrary, physiological levels of hydrogen peroxide seems to have a minor impact on acrosome reaction [34, 35]. Superoxide anion has also similar effects, albeit to a slighter extent compared to H_2O_2 [29, 36].

Nitric oxide (NO), which is a type of RNS, has been found to enhance capacitation specifically in the female reproductive system. When catalase, which converts hydrogen peroxide to hydroxide radical is added to a sample where sufficient NO is available, capacitation is promptly prevented, thus indicating that the NO-induced effects are achieved through complex mechanisms involving hydrogen peroxide. Further proof is that cAMP, a nucleotide greatly involved in the biochemical framework of capacitation, gets severely depleted whenever ROS scavengers or nitric oxide synthase (NOS) inhibitors are present [37].

As for hyperactivation, studies report that O_2^- performs the most fundamental role in regulating hyperactive motility, but the effect of H_2O_2 varies depending upon the concentration used. When ROS were generated via an array of distinctively different methods, including the administration of fetal cord serum (FCS), fetal cord serum ultrafiltrate (FCSu), and xanthine + xanthine oxidase (X + XO), results supporting the role of superoxide were obtained [28]. $\bullet O_2^-$ has been found to be purposely helpful in vivo, because hyperactivation was swiftly inhibited in the presence of SOD mixed with FCS or even FCSu [38]. As the case is with capacitation, the completion of hyperactivation cannot take place without phosphorylation of some certain proteins. A prime example of these is the A-kinase anchoring protein, otherwise known as AKAP. This protein acts to bind the protein kinase A to the fibrous sheath of a spermatozoon [39].

The protein kinase A (PKA)-dependent pathway is thought to regulate the process of capacitation. The process is initiated by influxes of calcium and bicarbonate ions, which enter the cell due to the inactivation of a unique type of plasma membrane channel, known as ATP-dependent Ca^{2+} regulatory channel (PMCA) [37]. The entire ROS dependent capacitation cascade is sensitive to high pH [40]. One study showed that removing HCO_3^- from an incubating medium blocks ROS generation. The increased levels of calcium ions, together with ROS activate the downstream amplifier molecule adenylate cyclase (AC), which acts on ATP converting it to the nucleotide cyclic adenosine monophosphate (cAMP). It is worth noting that O_2^- is specifically implicated in activating AC by oxidizing a thiol group. The raised cAMP levels promote PKA activity that triggers Ser/Thr phosphorylation, which is linked to both the activation of protein tyrosine kinases (PTKs) and inhibition of protein tyrosine phosphatases (PTPs). PKA activation is also associated with the stimulation of NADPH oxidase, an essential ROS-generating enzyme. PTKs phosphorylate tyrosine residues located in the fibrous sheath of spermatozoa. Where superoxide is hypothesized to directly promote tyrosine phosphorylation, H_2O_2 seems to work indirectly by activating PTKs and inhibiting PTPs [37].

3.1.1.3 Acrosome Reaction

The acrosome reaction (AR) is characterized by the release of proteolytic enzymes, mainly acrosin and hyaluronase, and modification in the anterior part of the head, which facilitate sperm movement through the cumulus oophorus and subsequent sperm-oocyte fusion. This process takes place immediately after capacitation [9, 23].

In vivo, the AR starts when the ZP3 ligand on the oocyte binds to the sperm plasma membrane, perhaps with some progesterone assistance [41]. ROS were shown to increase sperm affinity to ZP3 ligand, possibly through the increased expression of complementary receptors on sperm plasma membrane, including phosphorylation of three specific plasma membrane proteins on the sperm head, fertilin beta, spermadhesin family, and P47 [42].

In vitro, AR can be initiated by sperm incubation with recombinant zona pellucida glycoprotein 3 (ZP3), progesterone or ROS, but reports are mixed. In one study involving both normozoospermic and oligozoospermic individuals, low to moderate levels of free radicals (above 17.46 counts/viable spermatozoa) were found to be associated with mature spermatozoa. On the contrary, high levels of ROS in the seminal plasma were associated with low numbers of viable mature spermatozoa. Whereas no clear relationship between the level of free radicals and the action of the acrosomal enzymes, particular acrosin, has been found by some investigators [43], others have reported that adding ROS to incubation media activates the AR [44, 45]. Furthermore, O_2^-, H_2O_2 and even NO were able to exert positive influences on the AR, provided they were present in low concentrations [46–49]. Hydrogen peroxide seems to have a positive effect on AR whereas adding catalase has an opposite effect.

Collectively, these results suggest that ROS are implicated in the AR in vitro, but the exact mechanisms involved have not been fully elucidated [30, 35, 44]. Since both capacitation and acrosome involve tyrosine phosphorylation of certain proteins, such as those with molecular weights of 70, 76, 81 and 105 kDa, it has been suggested that there must be a common chemical pathway regulating both capacitation and acrosome reaction [47].

As far as the cellular mechanisms of AR are concerned, an influx of Ca^{2+} ions (coming from the acrosomal calcium store and extracellular compartment) into the cytosolic compartment is thought to initiate the overall process [50]. It has been speculated that ROS generated through enzymatic action might induce this influx of calcium ions. A possible pathway might involve NADPH, which is present at the plasma membrane level, converting normal diatomic oxygen to the highly reactive superoxide radical [51]. The next step in the AR would be the cleavage of phosphatidylinositol-4, 5-biphosphate (PIP2) forming diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 activates actin-severing proteins, which then break actin threads that are located in the intermembrane space between the plasma and acrosomal membranes, causing the fusion of the two membranes. The acrosomal material is exocytotically released afterwards. Subsequently, DAG galvanizes a special type of protein kinase, known as protein kinase C (PKC) which causes an additional influx of calcium through the plasma membrane Ca^{2+} ATPase channel. DAG also activates (via phosphorylation) a unique type of fatty acid hydrolase referred to phospholipase 2 (PLA2), which cleaves the second carbon of the triacylglycerol molecule liberating a membrane fatty acid. This aims to increase the plasma membrane fluidity in preparation of successful sperm-oocyte fusion [52].

3.1.1.4 Sperm-oocyte Fusion

After AR, sperm-oocyte fusion takes place. This step involves plasma membrane fluidity that is dependent upon a high polyunsaturated fatty acid content and activity of phospholipase A2. It has been suggested that O_2^- generated by sperm may act as a membrane phospholipase. As a result, there is an increase in the levels of lysophosphatidylcholine—a fusogenic type of lysophospholipid kinked with the AR [53]. Others have demonstrated that superoxide anion can activate phospholipase A2 (PLA2), which in turn promotes the release of plasma membrane lysolipids similar to the process of AR itself [54, 55].

The association between increased levels of ROS and sperm-oocyte fusion has been confirmed by Aitken et al. [52, 56]. A pronounced increase in sperm-oocyte fusion was observed in spermatozoa exhibiting major tyrosine phosphorylation, which suggests that the events accompanying capacitation and AR are related to the ones involved in sperm-oocyte fusion. ROS, typically H_2O_2 and O_2^-, have been shown to increase fertilization rates [30, 50, 52]. Notably, addition of SOD or catalase is associated with a marked reduction in fusogenicity.

Likewise the activation of phospholipase A2, de-esterification of membrane phospholipids is also important for increasing membrane fluidity [57]. Phospholipase A2 activity is significantly reduced by dephosphorylation. ROS are proposed to inhibit PTPases, thus enhancing PLA2 activity [58]. A more direct optimization mechanism might be the fact that ROS activate PKC via the action of protein tyrosine kinase. PKC is known to phosphorylate phospholipases, which subsequently triggers an augmented PLA2 activity [41, 59, 60].

An additional contribution by ROS is chemotaxis during the migration of spermatozoa towards the oocyte in the female genital tract, as a response to the secretion of progesterone by the cumulus oophorus cells. Chemotaxis is not just confined to the presence of progesterone, but could also be initiated by follicular fluid. Sanchez et al. carried out an experimental study aimed to determine the role of ROS in mediating chemotaxis. Specimens were divided into four treatment conditions: (1) spermatozoa in BWW medium (as a control), (2) spermatozoa where 0.1 ROS-producing 0.1 mM phorbol 12-myristate 13-acetate (PMA) was added, (3) spermatozoa where H_2O_2-scaveging catalase was applied, and (iv) a final preparation with both catalase and PMA. The outcome measures were capacitation status, ROS-production, and most importantly, chemotaxis level. All three parameters were examined at time zero and after 3 h of incubation under conditions facilitating the occurrence of capacitation [61].

At time zero, there was no remarkable change in the chemotaxis of the cultures under PMA treatment or PMA and catalase treatment. However, the treatment containing catalase revealed a significant fall in the level of chemotactic spermatozoa. At the end of the 3-h incubation period, spermatozoa treated with PMA were shown to have become less responsive to the chemoattractant compared to the other groups. The same culture was significantly more sluggish in responding compared to time zero. Adding catalase seemed to have reversed that effect, with respect to the results

at time zero. These experimental results suggest that there must be some sort of physiological gateway of ROS at which they assist chemotaxis. Beyond that point, ROS result in oxidative stress which in turns cause the downfall of chemotaxis.

3.2 Conclusion

ROS are not strictly harmful to male reproductive health. On the contrary, ROS are currently regarded as essential for the proper maturation and formation of healthy sperm, provided ROS are within so-called physiological ranges (Fig. 3.1). The proposed mechanism of action for ROS-related physiology is that ROS mediate the oxidation-reduction of thiol-groups, which controls the activation of AC, cAMP, and many other biochemical intermediates further downstream. ROS have been involved in strengthening DNA integrity by enhancing chromatin stability and formation of the mitochondrial capsule by facilitating the creating of disulfide bonds. Hydrogen peroxide has been considered the major type of ROS to sperm physiology, including capacitation and AR, whereas superoxide anion is primarily involved in hyperactivation. At present, not all mechanisms in the ROS-mediated sperm maturation have been elucidated.

3.2.1 Physiological Roles of ROS in the Female Reproductive System

3.2.1.1 Follicular Development

During the follicular phase of the menstrual cycle, a number of primordial follicles undergo development and maturation, and this process often results in one or more dominant mature follicles. These dominant follicles are the only ones destined for ovulation; other follicles that start to develop during the follicular phase will eventually undergo follicular atresia. Follicular atresia is influenced by three factors: loss of sensitivity to gonadotropic hormones (mainly FSH), loss of steroidogenic function and lastly mediation of ROS [2, 62].

Follicular angiogenesis is induced by hypoxia of the granulosa cells; a vital process for follicular growth and development [63]. ROS act as signal transducers or intracellular messengers of this angiogenic response [64]. The source of ROS in the follicular fluid is currently unknown, but it has been speculated that ROS might be generated by the oocyte itself, granulosa cells as well as endothelial and thecal cells. The theca interna is richly vascularized, thus providing a gateway for the passage of factors from the circulation into the follicular fluid [65]. Furthermore, follicular fluid is known to contain cytokines, neutrophils and macrophages, all prominent ROS sources [66]. The ovary initiates defensive mechanisms for detoxification and protection against ROS. These include enzymes such as catalase and superoxide

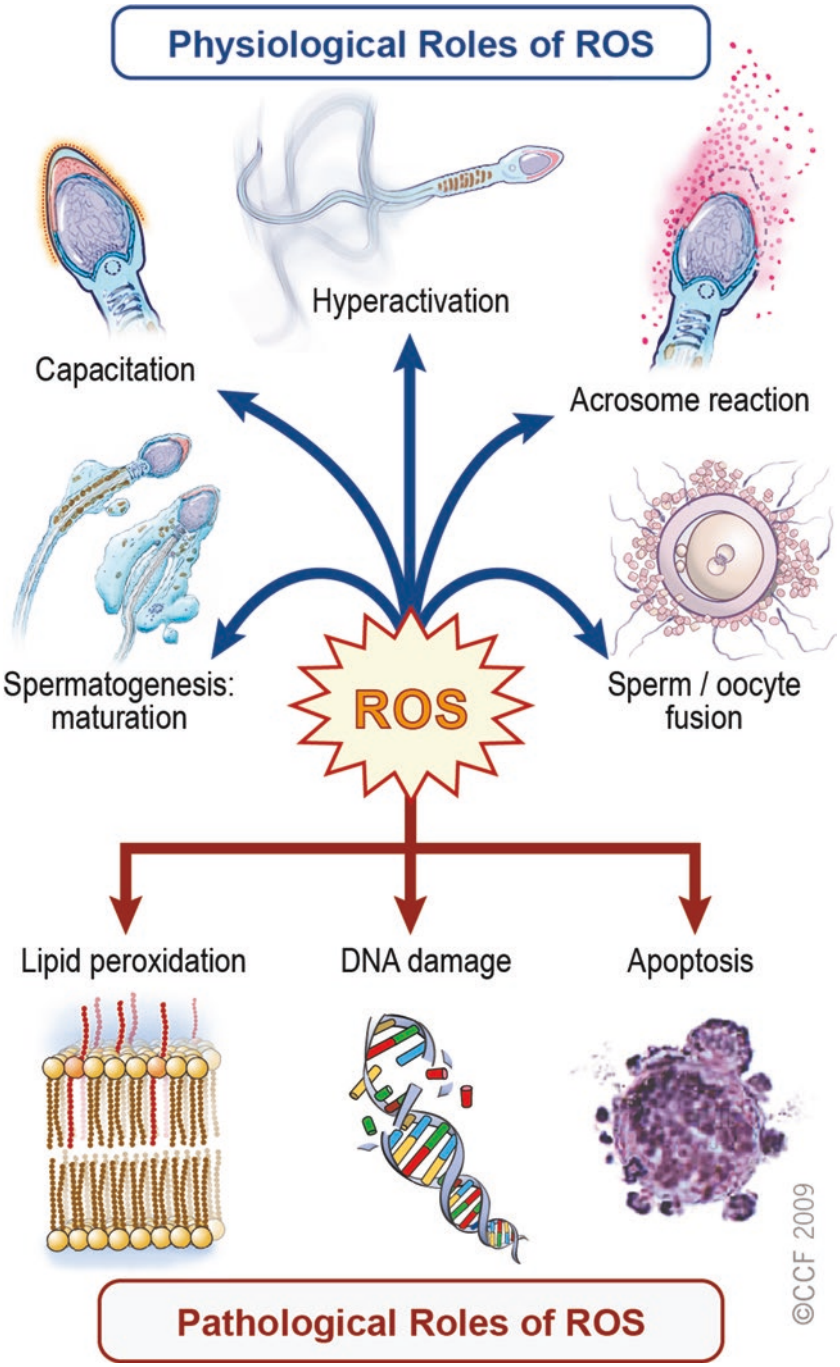

Fig. 3.1 Physiological roles of ROS include maturation, capacitation, hyperactivation, the acrosome reaction and sperm-oocyte fusion. Pathological roles of ROS include lipid peroxidation, DNA damage and apoptosis

dismutase, antioxidants such as vitamins C and E [67], carotenoid lutein [68] and the peroxidase cofactor reduced glutathione [69]. It has been suggested that a correlation between poor oocyte quality and abnormally low amounts of ROS in follicular fluid exists [66]. The free oxygen radical and related metabolites play essential roles in the cellular metabolism, prompting the breakdown of follicular walls for ovulation to occur [66].

3.2.1.2 Ovarian Steroidogenesis

Steroid hormones greatly influence the production of ROS in the uterus [70]. In the presence of estradiol, uterine weight increases along with a 200-fold increase in peroxidase activity [71]. This increase is largely due eosinophils present in the leukocyte infiltrate, which provide a substantial amount of peroxidase [2]. The function of peroxidase is to ensure to the uterus an antibacterial environment [72]. Peroxidase also regulates the levels of estrogen through a negative feedback mechanism [73].

The apical plasma membrane of the endometrial epithelium hosts NADPH oxidase, which is responsible for dismutation of superoxide anion. This, in turn, results in the production of hydrogen peroxide which is the major source of peroxidases [74]. Hydrogen peroxide plays a role in generating prostaglandins through enzymatic mechanisms, catalyzed by cyclooxygenase [75], as well as non-enzymatic pathways [76].

Arachidonic acid is an unsaturated fatty acid that serves as the main precursor of a family of lipid compounds called eicosanoids. Eicosanoids possess hormone-like properties and provide a very wide range of other compounds, such as prostaglandins. Once oxygen is added to arachidonic acid, cyclooxygenases produce different prostaglandins, including $PGF_{2\alpha,}$ which play essential roles in chemotaxis, implantation as well as degradation of the corpus luteum [2]. Phospholipase A2 is a calcium-dependent enzyme, which acts on the phospholipids in the membrane to produce arachidonic acid. Phospholipase A2 activity can be enhanced by lipid peroxidation and inhibited by antioxidants [77, 78].

3.2.1.3 Ovulation

Ovulation is frequently compared to an acute inflammation given it includes vascular swelling, a buildup of immune cells and the immediate production of prostaglandins [2]. Superoxide levels increase when ovulation takes place. Conversely, LH causes a temporary increase in SOD activity during ovulation [67]. Leukocytes located around pre-ovulatory follicles are a potential source of ROS during ovulation [79]. Interestingly, ovulation is significantly impaired in the presence of SOD [80]. It has been hypothesized that free radicals cause membrane damage which initiate the production of prostaglandins through the cyclooxygenase system by activation of phospholipases. These prostaglandins are essential for ovulation to occur.

Oxygen free radicals induce OS in the ovarian follicles, which elicit apoptosis of granulosa cells and instigates ovulation, whereas ROS scavengers have an inhibitory effect [81, 82]. Indomethacin inhibits cyclooxygenase activity, thus also inhibiting ovulation [2]. A rise in LH levels activates polymorphonuclear (PMN) cells, which leads to an increase in ROS production in the ovary. PMN cells exhibit LH receptors; these can facilitate oxygen production as soon as they are activated [83]. Oxygen plays various physiologic roles during ovulation.

3.2.1.4 Corpus Luteum

Formation

Following ovulation, granulosa and thecal cells differentiate to give rise to the corpus luteum. The corpus luteum is a temporary endocrine gland responsible for progesterone and estradiol secretion [70]. LH exhibits luteotropic action in the maintenance of a high level of vitamin E in the corpus luteum. On the contrary, LH has been shown to cause a remarkable drop in the level of ascorbic acid in the corpus luteum [84]. Due to the presence of high levels of antioxidant vitamins, it has been suggested that ROS might have functional significance in the corpus luteum [2].

An association between ROS and progesterone has also been suggested. Progesterone seems to have the ability to modulate ROS generation. Low ROS levels exert luteotropic effects and maintain the corpus luteum. During the formation of the corpus luteum, the level of antioxidants increases and ROS activity decreases [1].

Luteolysis

If pregnancy does not occur, the corpus luteum undergoes luteolysis [70]. This process involves two changes: halting the progesterone secretion which is also referred to as "functional luteolysis", and modifications in the structure of the corpus luteum as degeneration of cells takes place [85]. In addition, a decrease in the activity of SOD occurs coupled with an increase in ROS; this serves to exert luteolytic effects on the CL [1]. This instant increase in the level of ROS triggers the expression of cyclooxygenase-2 (COX-2), which leads to activation of nuclear factor-kappa B (NF-kB) and a successive stimulation of $PGF_{2\alpha}$ synthesis [86]. $PGF_{2\alpha}$ prompts luteal cells as well as phagocytic leukocytes to produce superoxide anion [87, 88].

Action of $PGF_{2\alpha}$ inhibits the formation of cAMP by LH [85]. Depletion of ascorbic acid and lipid peroxidation occur within 2 and 4 h after treatment with $PGF_{2\alpha}$, but interestingly enough, both returned to their normal physiological concentrations after 24 h. Since ascorbate plays a role in synthesis of collagen, ascorbic acid depletion might be correlated with structural luteolysis [89]. $PGF_{2\alpha}$ boosts production of hydrogen peroxide while simultaneously decreases the level of progesterone in rat luteal tissue [90]. It is worth noting that the action of $PGF_{2\alpha}$ applies to most species, but primates are an exception. The factors responsible for triggering luteolysis in

humans are currently unknown, even though a number of studies proposed that hydrogen peroxide generation performs some sort of luteolytic function [2].

During luteolysis, a decrease in luteal blood flow occurs and this initiates activation of the xanthine-xanthine oxidase system, a system responsible augmenting the production of ROS and causing tissue damage [91]. This system generates superoxide anion that is not very reactive compared to other radicals. However, it can still inhibit LH stimulation of progesterone secretion induced by cAMP as well as cAMP synthesis, which appear to be due to G-protein uncoupling mechanisms [92, 93]. One of the other functions of the superoxide anion is its ability to activate phospholipase A2 which plays a role in production of arachidonic acid [54].

Hydrogen peroxide is vital in regression of the corpus luteum. It instantly inhibits LH-dependent cAMP and progesterone production and is responsible for depletion of ATP within the cells. This breakdown of ATP is primarily the result of peroxide-induced DNA damage [93]. Translocation of cholesterol across the outer mitochondrial membrane is a rate-limiting step in which it is metabolized into progesterone. Hydrogen peroxide inhibits this step and therefore blocks steroidogenesis [94]. The effect of increased lipid peroxidation by hydrogen peroxide has a physiological role because its subproducts can enhance phospholipase A2 activity thus leading to an increased production of prostaglandins [76, 95]. This suggests that H_2O_2 might actually be part of a positive feedback mechanism related to ROS induction and synthesis of $PGF_{2\alpha}$ [70].

The origin of hydrogen peroxide in the ovary remains elusive. Since H_2O_2 is critical in luteolysis, its suppression must play a role in prevention of regression of the corpus luteum, and this is required for the maintenance of early pregnancy [2]. For example, adenosine expresses progonadotropic actions in ovarian cells [96] and this can inhibit the production of ROS by neutrophils when exposed to catalysts like the colony-stimulating factor [97]. In addition, the ovary produces transforming growth factor-β which has the ability to deactivate macrophages and diminish the respiratory burst [98]. Research has shown that α-interferon can antagonize the effect of γ-interferon, which usually activates the respiratory burst in macrophages [99].

3.2.1.5 Maintenance of Pregnancy

Despite the fact that ROS are considered waste products of metabolic oxidations, small concentrations might in fact participate in physiological signaling pathways in the embryo [100]. ROS play a role in signal transduction and have the ability to exert vasoactive effects on the placenta [101]. Decidual macrophages are involved in the regulation of apoptosis during the process of implantation. This is necessary for invasion of the developing embryo during pregnancy [102]. When exposed to bacterial lipopolysaccharides, decidual macrophages produce superoxide anion and TNF-α -an inflammatory cytokine- and these actions are thought to play a role in protecting the fetus against intrauterine infections [103]. Recent studies have shown that oxidative stress and hypoxia trigger normal placental apoptosis of the trophoblast. These conditions are vital for growth and development of the embryo and placenta [5]. Conversely, a high level of oxidative stress has disastrous effects on pregnancy. During the early stages of placental development, a high level of

oxidative stress has been linked to miscarriage, intrauterine growth restriction, pre-eclampsia and embryo resorption [101]. Early human pregnancy failure has been associated with defective placental antioxidant systems [104].

3.2.1.6 Parturition

In conditions of high metabolic demand, ROS levels are known to increase remarkably in humans [1]. During parturition, neutrophils and monocytes invade uterine tissues as part of an inflammatory event which takes place in humans and animals [105]. When an increased expression of cytokines in the myometrium and cervix develops during labor, migration of leukocytes to the site of inflammation is actively enhanced [106]. These leukocytes are responsible for producing superoxide anion by combining hypoxanthine with xanthine oxidase, which triggers a rise in intracellular calcium thus leading to myometrial contraction [107]. H_2O_2 acts as a contractile agent on smooth muscles, participating in a number of mechanisms including the opening of Ca^{2+} release channels as well as the reuptake of Ca^{2+} in the sarcoplasmic reticulum, and modulating myofibrilar Ca^{2+} sensitivity [108]. Therefore, the process of labor can be thought of as a positive feedback system involving ROS and mediators of inflammation (prostaglandins and cytokines) [109, 110].

Before parturition can take place, serum concentrations of progesterone are reduced and levels of estrogen are elevated. Estrogen boosts ROS generation through pro-inflammatory actions, bringing about metabolic and enzymatic changes. Together, ROS and estrogen enhance myometrial contractions and uterine involution, which is essentially the shrinkage of the uterus after childbirth [110]. During the last phase of gestation, a tremendous increase in the enzymatic antioxidant reserve takes place in order to prepare for life in an oxygen-rich environment [111].

3.3 Conclusion

ROS participate in development of follicles, ovarian steroidogenesis and ovulation, breakdown of the corpus luteum and maintenance of pregnancy. Because their actions are transient, experimental tests remain complex and challenging. Future perspectives would include setting a more accurate cut-off value differentiating physiologic from pathologic levels of ROS in different organisms, and how this can affect reproductive health and fertility.

References

1. Rizzo A et al (2012) Roles of reactive oxygen species in female reproduction. Reprod Domest Anim 47(2):344–352
2. Riley JC, Behrman HR (1991) Oxygen radicals and reactive oxygen species in reproduction. Proc Soc Exp Biol Med 198(3):781–791

3. Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. Nature 408(6809):239–247
4. Agarwal A, Gupta S, Sharma RK (2005) Role of oxidative stress in female reproduction. Reprod Biol Endocrinol 3:28
5. Al-Gubory KH, Fowler PA, Garrel C (2010) The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. Int J Biochem Cell Biol 42(10):1634–1650
6. Halliwell B, Cross CE (1994) Oxygen-derived species: their relation to human disease and environmental stress. Environ Health Perspect 102(Suppl 10):5–12
7. Pierce JD, Cackler AB, Arnett MG (2004) Why should you care about free radicals? RN 67(1):38–42, quiz 43
8. Van Langendonckt A, Casanas-Roux F, Donnez J (2002) Oxidative stress and peritoneal endometriosis. Fertil Steril 77(5):861–870
9. Agarwal A, Saleh RA, Bedaiwy MA (2003) Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril 79(4):829–843
10. Chan AC (1993) Partners in defense, vitamin E and vitamin C. Can J Physiol Pharmacol 71(9):725–731
11. Guerin P, El Mouatassim S, Menezo Y (2001) Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum Reprod Update 7(2):175–189
12. Aitken RJ, Clarkson JS, Fishel S (1989) Generation of reactive oxygen species, lipid peroxidation, and human sperm function. Biol Reprod 41(1):183–197
13. Kovalski N, De Lamirande E, Gagnon C (1992) Reactive oxygen species generated by human neutrophils inhibit sperm motility: protective effect of seminal plasma and scavengers. Fertil Steril 58(4):809–816
14. Gil-Guzman E et al (2001) Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation. Hum Reprod 16(9):1922–1930
15. Ford WC (2004) Regulation of sperm function by reactive oxygen species. Hum Reprod Update 10(5):387–399
16. Vernet P, Aitken RJ, Drevet JR (2004) Antioxidant strategies in the epididymis. Mol Cell Endocrinol 216(1–2):31–39
17. Aitken RJ et al (1998) Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. Biol Reprod 59(5):1037–1046
18. Saowaros W, Panyim S (1979) The formation of disulfide bonds in human protamines during sperm maturation. Experientia 35(2):191–192
19. Rousseaux J, Rousseaux-Prevost R (1995) Molecular localization of free thiols in human sperm chromatin. Biol Reprod 52(5):1066–1072
20. Aitken RJ et al (2004) Redox activity associated with the maturation and capacitation of mammalian spermatozoa. Free Radic Biol Med 36(8):994–1010
21. Roveri A et al (2001) PHGPx and spermatogenesis. Biofactors 14(1–4):213–222
22. Eisenbach M (1999) Mammalian sperm chemotaxis and its association with capacitation. Dev Genet 25(2):87–94
23. de Lamirande E, Leclerc P, Gagnon C (1997) Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Mol Hum Reprod 3(3):175–194
24. Visconti PE et al (2002) Novel signaling pathways involved in sperm acquisition of fertilizing capacity. J Reprod Immunol 53(1–2):133–150
25. Baker MA, Aitken RJ (2004) The importance of redox regulated pathways in sperm cell biology. Mol Cell Endocrinol 216(1–2):47–54
26. O'Flaherty C, de Lamirande E, Gagnon C (2006) Positive role of reactive oxygen species in mammalian sperm capacitation: triggering and modulation of phosphorylation events. Free Radic Biol Med 41(4):528–540
27. Aitken RJ (1999) The Amoroso Lecture. The human spermatozoon – a cell in crisis? J Reprod Fertil 115(1):1–7
28. Suarez SS (2008) Control of hyperactivation in sperm. Hum Reprod Update 14(6):647–657

29. de Lamirande E, Gagnon C (1995) Capacitation-associated production of superoxide anion by human spermatozoa. Free Radic Biol Med 18(3):487–495
30. Aitken RJ et al (1995) Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. J Cell Sci 108(Pt 5):2017–2025
31. Griveau JF, Renard P, Le Lannou D (1994) An in vitro promoting role for hydrogen peroxide in human sperm capacitation. Int J Androl 17(6):300–307
32. Leclerc P, de Lamirande E, Gagnon C (1997) Regulation of protein-tyrosine phosphorylation and human sperm capacitation by reactive oxygen derivatives. Free Radic Biol Med 22(4):643–656
33. Dona G et al (2011) Endogenous reactive oxygen species content and modulation of tyrosine phosphorylation during sperm capacitation. Int J Androl 34(5 Pt 1):411–419
34. de Lamirande E et al (1997) Reactive oxygen species and sperm physiology. Rev Reprod 2(1):48–54
35. Griveau J, Renard P, LANNOU D (1995) Superoxide anion production by human spermatozoa as a part of the ionophore-induced acrosome reaction process. Int J Androl 18(2):67–74
36. de Lamirande E, Gagnon C (1995) Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects. Hum Reprod 10(suppl 1):15–21
37. Zini A, De Lamirande E, Gagnon C (1995) Low levels of nitric oxide promote human sperm capacitation in vitro. J Androl 16(5):424–431
38. Yeoman RR, Jones WD, Rizk BM (1998) Evidence for nitric oxide regulation of hamster sperm hyperactivation. J Androl 19(1):58–64
39. Leclerc P, de Lamirande E, Gagnon C (1996) Cyclic adenosine 3',5' monophosphate-dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. Biol Reprod 55(3):684–692
40. Aitken RJ et al (1998) On the cellular mechanisms by which the bicarbonate ion mediates the extragenomic action of progesterone on human spermatozoa. Biol Reprod 58(1):186–196
41. Breitbart H, Spungin B (1997) The biochemistry of the acrosome reaction. Mol Hum Reprod 3(3):195–202
42. Breitbart H, Naor Z (1999) Protein kinases in mammalian sperm capacitation and the acrosome reaction. Rev Reprod 4(3):151–159
43. Ichikawa T et al (1999) Reactive oxygen species influence the acrosome reaction but not acrosin activity in human spermatozoa. Int J Androl 22(1):37–42
44. de Lamirande E, Gagnon C (1993) A positive role for the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. Int J Androl 16(1):21–25
45. Boerke A et al (2008) Capacitation-dependent reorganization of microdomains in the apical sperm head plasma membrane: functional relationship with zona binding and the zona-induced acrosome reaction. Theriogenology 70(8):1188–1196
46. Herrero MB, de Lamirande E, Gagnon C (2003) Nitric oxide is a signaling molecule in spermatozoa. Curr Pharm Des 9(5):419–425
47. de Lamirande E et al (1998) Involvement of reactive oxygen species in human sperm arcosome reaction induced by A23187, lysophosphatidylcholine, and biological fluid ultrafiltrates. J Androl 19(5):585–594
48. Leclerc P, De Lamirande E, Gagnon C (1998) Interaction between Ca2, cyclic 3, 5 adenosine monophosphate, the superoxide anion, and tyrosine phosphorylation pathways in the regulation of human sperm capacitation. J Androl 19:434–443
49. O'Flaherty CM, Beorlegui NB, Beconi MT (1999) Reactive oxygen species requirements for bovine sperm capacitation and acrosome reaction. Theriogenology 52(2):289–301
50. Aitken RJ et al (1996) The extragenomic action of progesterone on human spermatozoa is influenced by redox regulated changes in tyrosine phosphorylation during capacitation. Mol Cell Endocrinol 117(1):83–93
51. Aitken RJ, Buckingham DW, West KM (1992) Reactive oxygen species and human spermatozoa: analysis of the cellular mechanisms involved in luminol- and lucigenin-dependent chemiluminescence. J Cell Physiol 151(3):466–477

52. Aitken RJ (1997) Molecular mechanisms regulating human sperm function. Mol Hum Reprod 3(3):169–173
53. Ohzu E, Yanagimachi R (1982) Acceleration of acrosome reaction in hamster spermatozoa by lysolecithin. J Exp Zool 224(2):259–263
54. Sawada M, CARLSON JC (1991) Rapid plasma membrane changes in superoxide radical formation, fluidity, and phospholipase A2 activity in the corpus luteum of the rat during induction of luteolysis*. Endocrinology 128(6):2992–2998
55. Wu X, Sawada M, Carlson J (1992) Stimulation of phospholipase A2 by xanthine oxidase in the rat corpus luteum. Biol Reprod 47(6):1053–1058
56. Griveau JF, Le Lannou D (1997) Reactive oxygen species and human spermatozoa: physiology and pathology. Int J Androl 20(2):61–69
57. Griveau JF et al (1995) Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. J Reprod Fertil 103(1):17–26
58. Goldman R, Ferber E, Zort U (1992) Reactive oxygen species are involved in the activation of cellular phospholipase A2. FEBS Lett 309(2):190–192
59. Zor U et al (1993) Reactive oxygen species mediate phorbol ester-regulated tyrosine phosphorylation and phospholipase A2 activation: potentiation by vanadate. Biochem J 295(Pt 3):879–888
60. Xu J et al (2003) Oxidant-mediated AA release from astrocytes involves cPLA(2) and iPLA(2). Free Radic Biol Med 34(12):1531–1543
61. Sanchez R et al (2010) Human sperm chemotaxis depends on critical levels of reactive oxygen species. Fertil Steril 93(1):150–153
62. Margolin Y, Aten RF, Behrman HR (1990) Antigonadotropic and antisteroidogenic actions of peroxide in rat granulosa cells. Endocrinology 127(1):245–250
63. Tropea A et al (2006) Regulation of vascular endothelial growth factor synthesis and release by human luteal cells in vitro. J Clin Endocrinol Metab 91(6):2303–2309
64. Basini G et al (2004) Effect of reduced oxygen tension on reactive oxygen species production and activity of antioxidant enzymes in swine granulosa cells. Biofactors 20(2):61–69
65. Tarin JJ (1996) Potential effects of age-associated oxidative stress on mammalian oocytes/embryos. Mol Hum Reprod 2(10):717–724
66. Attaran M et al (2000) The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. Int J Fertil Womens Med 45(5):314–320
67. Laloraya M, Laloraya MM (1988) Changes in the levels of superoxide anion radical and superoxide dismutase during the estrous cycle of Rattusnorvegicus and induction of superoxide dismutase in rat ovary by lutropin. Biochem Biophys Res Commun 157(1):146–153
68. Chew B, Holpuch D, O'fallon J (1984) Vitamin A and β-carotene in bovine and porcine plasma, liver, corpora lutea, and follicular fluid. J Dairy Sci 67(6):1316–1322
69. Mattison D et al (1982) Ontogeny of ovarian glutathione and sensitivity to primordial oocyte destruction by cyclophosphamide. Pediatr Pharmacol 3(1):49–55
70. de Lamirande E, Gagnon C (1994) Reactive oxygen species (ROS) and reproduction. In: D. Armstrong (ed.), Free radicals in diagnostic medicine. Springer. pp 185–197
71. Lyttle CR, DeSombre ER (1977) Uterine peroxidase as a marker for estrogen action. Proc Natl Acad Sci 74(8):3162–3166
72. Klebanoff S, Smith D (1970) Peroxidase-mediated antimicrobial activity of rat uterine fluid. Gynecol Obstet Invest 1(1):21–30
73. Klebanoff SJ (1965) Inactivation of estrogen by rat uterine preparations. Endocrinology 76:301–311
74. Ishikawa Y, Hirai K, Ogawa K (1984) Cytochemical localization of hydrogen peroxide production in the rat uterus. J Histochem Cytochem 32(6):674–676
75. Hemler ME, Cook HW, Lands WE (1979) Prostaglandin biosynthesis can be triggered by lipid peroxides. Arch Biochem Biophys 193(2):340–345
76. Morrow JD et al (1990) A series of prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. Proc Natl Acad Sci U S A 87(23):9383–9387

77. Phan SH et al (1989) Xanthine oxidase activity in rat pulmonary artery endothelial cells and its alteration by activated neutrophils. Am J Pathol 134(6):1201–1211
78. Robison TW, Sevanian A, Forman HJ (1990) Inhibition of arachidonic acid release by nordihydroguaiaretic acid and its antioxidant action in rat alveolar macrophages and Chinese hamster lung fibroblasts. Toxicol Appl Pharmacol 105(1):113–122
79. Brannstrom M, Mayrhofer G, Robertson SA (1993) Localization of leukocyte subsets in the rat ovary during the periovulatory period. Biol Reprod 48(2):277–286
80. Miyazaki T et al (1991) Effect of inhibition of oxygen free radical on ovulation and progesterone production by the in-vitro perfused rabbit ovary. J Reprod Fertil 91(1):207–212
81. Kodaman PH, Behrman HR (2001) Endocrine-regulated and protein kinase C-dependent generation of superoxide by rat preovulatory follicles. Endocrinology 142(2):687–693
82. Shkolnik K et al (2011) Reactive oxygen species are indispensable in ovulation. Proc Natl Acad Sci U S A 108(4):1462–1467
83. Shirai F et al (2002) Human peripheral blood polymorphonuclear leukocytes at the ovulatory period are in an activated state. Mol Cell Endocrinol 196(1–2):21–28
84. Parlow AF (1972) Influence of differences in the persistence of luteinizing hormones in blood on their potency in the ovarian ascorbic acid depletion bioassay. Endocrinology 91(4):1109–1112
85. Carlson JC, Wu XM, Sawada M (1993) Oxygen radicals and the control of ovarian corpus luteum function. Free Radic Biol Med 14(1):79–84
86. Sugino N et al (2002) Nuclear factor-κB is required for tumor necrosis factor-α-induced manganese superoxide dismutase expression in human endometrial stromal cells. J Clin Endocrinol Metabol 87(8):3845–3850
87. Sawada M, Carlson JC (1994) Studies on the mechanism controlling generation of superoxide radical in luteinized rat ovaries during regression. Endocrinology 135(4):1645–1650
88. Tanaka M et al (2000) Participation of reactive oxygen species in PGF2alpha-induced apoptosis in rat luteal cells. J Reprod Fertil 120(2):239–245
89. Endo T et al (1993) Hydrogen peroxide evokes antisteroidogenic and antigonadotropic actions in human granulosa luteal cells. J Clin Endocrinol Metab 76(2):337–342
90. Riley JC, Behrman HR (1991) In vivo generation of hydrogen peroxide in the rat corpus luteum during luteolysis. Endocrinology 128(4):1749–1753
91. Sugino N et al (1993) Effects of ovarian ischemia-reperfusion on luteal function in pregnant rats. Biol Reprod 49(2):354–358
92. Gatzuli E, Aten RF, Behrman HR (1991) Inhibition of gonadotropin action and progesterone synthesis by xanthine oxidase in rat luteal cells. Endocrinology 128(5):2253–2258
93. Behrman HR, Preston SL (1989) Luteolytic actions of peroxide in rat ovarian cells. Endocrinology 124(6):2895–2900
94. Behrman HR, Aten RF (1991) Evidence that hydrogen peroxide blocks hormone-sensitive cholesterol transport into mitochondria of rat luteal cells. Endocrinology 128(6):2958–2966
95. Sevanian A et al (1988) Lipid peroxidation and phospholipase A2 activity in liposomes composed of unsaturated phospholipids: a structural basis for enzyme activation. Biochim Biophys Acta 961(3):316–327
96. Soodak LK, Musicki B, Behrman HR (1988) Selective amplification of luteinizing hormone by adenosine in rat luteal cells. Endocrinology 122(3):847–854
97. Nathan CF (1989) Respiratory burst in adherent human neutrophils: triggering by colony-stimulating factors CSF-GM and CSF-G. Blood 73(1):301–306
98. Tsunawaki S et al (1988) Deactivation of macrophages by transforming growth factor-beta. Nature 334(6179):260–262
99. Yoshida R, Murray HW, Nathan CF (1988) Agonist and antagonist effects of interferon alpha and beta on activation of human macrophages. Two classes of interferon gamma receptors and blockade of the high-affinity sites by interferon alpha or beta. J Exp Med 167(3):1171–1185
100. Covarrubias L et al (2008) Function of reactive oxygen species during animal development: passive or active? Dev Biol 320(1):1–11

101. Myatt L, Cui X (2004) Oxidative stress in the placenta. Histochem Cell Biol 122(4):369–382
102. Abrahams VM et al (2004) Macrophages and apoptotic cell clearance during pregnancy. Am J Reprod Immunol 51(4):275–282
103. Singh U et al (2005) Immunological properties of human decidual macrophages – a possible role in intrauterine immunity. Reproduction 129(5):631–637
104. Liu AX et al (2006) Proteomic analysis on the alteration of protein expression in the placental villous tissue of early pregnancy loss. Biol Reprod 75(3):414–420
105. Golightly E, Jabbour HN, Norman JE (2011) Endocrine immune interactions in human parturition. Mol Cell Endocrinol 335(1):52–59
106. Tornblom SA et al (2005) Non-infected preterm parturition is related to increased concentrations of IL-6, IL-8 and MCP-1 in human cervix. Reprod Biol Endocrinol 3:39
107. Masumoto N et al (1990) Superoxide anion increases intracellular free calcium in human myometrial cells. J Biol Chem 265(36):22533–22536
108. Appiah I et al (2009) Hydrogen peroxide affects contractile activity and anti-oxidant enzymes in rat uterus. Br J Pharmacol 158(8):1932–1941
109. Conner EM, Grisham MB (1996) Inflammation, free radicals, and antioxidants. Nutrition 12(4):274–277
110. Jenkin G, Young IR (2004) Mechanisms responsible for parturition; the use of experimental models. Anim Reprod Sci 82–83:567–581
111. O'Donovan DJ, Fernandes CJ (2004) Free radicals and diseases in premature infants. Antioxid Redox Signal 6(1):169–176

Chapter 4
Negative Effects of Oxidative Stress (OS) on Reproductive System at Cellular Level

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4.1 Introduction

There are multiple physiological processes occuring at the cellular as well as at the molecular level in the reproductive system. They are important in maintaining the homeostatic balance between various ions; and such balance is essential for preserving the quality of both the sperm and oocyte. Disturbance in this may lead to infertility and oxidative stress (OS) is believed to be the major culprit [1].

Reactive oxygen species (ROS) are generated as byproducts of oxygen metabolism. They usually contain at least one unpaired electron and play important physiological roles. Antioxidants, on the other hand, help in combating ROS by converting these highly reactive metabolites into water and molecular oxygen. The state when there is an excess in ROS related to antioxidants results in OS [2].

OS has a negative impact on several male physiological parameters and processes such as motility, morphology and concentration at the cellular level [1, 3]. It can cause cellular damage [4], DNA damage, and other deleterious effects [2] on both male and female reproductive systems. Similarly, high ROS levels in the various biological windows such as follicular fluid, peritoneal fluid and hydrosalpingeal fluid have all been reported to have adverse impacts on fertility. These microenvironments are essential in creating an ideal environment for nurturing oocytes and the resultant development of competent and mature oocytes that are necessary for suc-

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cessful reproductive outcomes. Alterations in these fluid windows can lead to abnormal reproductive processes and affect the follicle quality [5].

This chapter discusses the negative effects of OS on male and female reproductive systems and fertility, focuses particularly on the effects at the cellular level.

4.1.1 Negative Effects of Oxidative Stress in Male Reproductive System

4.1.1.1 Sperm Parameters

Sperm Concentration

One of the most common semen abnormalities is that of low concentration ($<10 \times 10^6$ sperm/mL), which is known as oligozoospermia, and it is a main contributor to male infertility [6]. The reasons behind oligozoospermia are numerous; which include inefficient spermatogenesis, smoking, fever and use of recreational drugs. Oligozoospermia can also result from testicular trauma, chromosomal disorders and hormonal imbalances [7]. Recent studies have shown a strong correlation between ROS levels and the sperm count [6, 8–10]; where oligozoospermic men produce higher levels of ROS [9]. The exact mechanism of oligozoospermia is not well understood even though it has been suggested [11–13] that chromosomal abnormalities may play a role [9, 11–13]. Another relationship seen with increasing levels of ROS is an increase in apoptosis of mature spermatozoa, resulting in oligozoospermia [14, 15]. As apoptosis continues, DNA damage caused by ROS accelerates apoptosis of the germs cell and leads to a further decline in sperm count [5].

Sperm Motility

Acquisition of motility is critical for spermatozoa to swim towards the oocyte and fertilize in the female reproductive tract after insemination. The sperm motility is generated by axoneme, a highly organized structure that composed of more than 250 proteins. It occurs after the proteins undergo tyrosine phosphorylation, a crucial modification process in the regulation of flagella motility [16].

OS is one of the most common factors known to affect sperm motility. It can occur due to many reasons such as a defect in the mitochondrial ultrastructure, which was found to be related to decreased sperm motility in oligozoospermia. Alteration in the expression of mitochondrial proteins was also found to cause an impairment of sperm parameters as seen in asthenozoospermic men (motility <40 %), as this can disrupt the phosphorylation of proteins responsible for motility [17].

Increased ROS levels can also lead to lipid peroxidation (LPO), which results in oxidation of polyunsaturated fatty acids in the plasma membrane and the formation of malondialdehyde (MDA), a reactive aldehyde [18]. In asthenozoospermic and

oligoasthenozoospermic men, significantly high levels of MDA were observed and the motility impairment was attributed to changes in membrane fluidity brought by oxidation [19–22]. It has been shown that MDA can lead to activation of the caspase cascade and externalization of phosphatidylserine, leading to apoptosis [22]. Furthermore, the addition of lipid aldehydes such as 4-hydroxynonenal (4-HNE) and acrolein, the simplest unsaturated aldehyde, causes a dose-dependent loss of both total and progressive motility (Fig. 4.1) [23]. A dose-dependent decline in motility was reported in spermatozoa treated with hydrogen peroxide. These effects clearly show that oxidative stress has an impact on the sperm motility [24].

Sperm Morphology

Normal range of spermatozoa morphology values along with other semen parameters have been lowered in the past few decades [25]. The average value for normal sperm morphology is about 4 % [26]. In a study of men with good sperm motility and concentration but poor morphology, it suggested that the sperm morphology best reflects its fertilization potential.

Most of the morphological abnormalities have been reported to be caused by genetic and chromosomal abnormalities such as aneuploidy of the sex chromosomes [27]. However, OS can also cause morphological defect in the spermatozoa, impairing its fertilizing ability [28]. A study showed that increased proportion of spermatozoa with mid-piece defects had increased production of ROS [29]. These mid-piece defects have been negatively correlated with in-vitro fertilization rates [30]. The presence of excessive cytoplasm has been speculated as the cause of such ROS production [31].

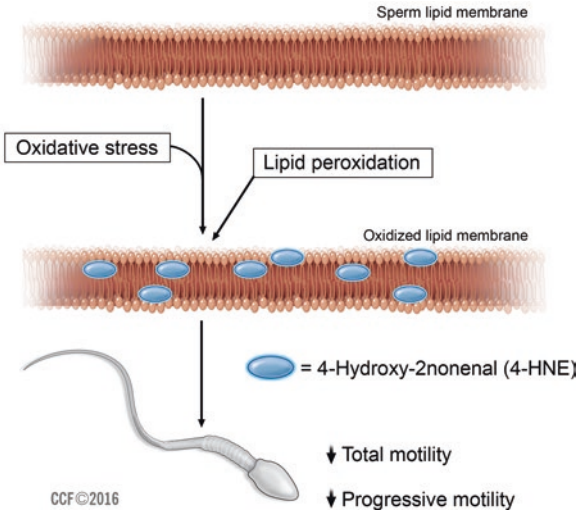

Fig. 4.1 The effect of lipid peroxidation of 4-HNE on sperm motility

Furthermore, Agarwal et al. showed that men with normal white blood cell levels and normal spermatozoa parameters but manifesting teratozoospermia (abnormal sperm morphology) displayed increased production of ROS [28]. This further suggests abnormal morphology of spermatozoa contributes to infertility by increasing ROS production.

Apart from mid-piece defects, it has also been shown that OS can cause amorphous head defect in the spermatozoa [32].

Although these studies have highlighted the correlations between spermatozoa morphological defects and increased ROS levels, the exact mechanism is not fully understood and more research is warranted [28, 32, 33].

4.1.1.2 Lipid Peroxidation

Lipid peroxidation (LPO) is a consequence of OS, which leads to the oxidation of membrane lipid components. This process impairs the fluidity of the cell membrane, inactivates membrane-enzymes and increases its permeability for non-specific ions [34].

Spermatozoa are highly susceptible to OS due the presence of high amounts of polyunsaturated fatty acids in their cell membranes that weakens the methyl carbon-hydrogen bond [35]. Interestingly, Huszar et al. reported a positive relationship between creatine kinase activity (which involves in reflecting the degree of cytoplasmic extrusion) and the rate of LPO (as measured by MDA formation), indicating that creatine kinase could be used as a biomarker of OS [36].

4.1.1.3 Mitochondrial Membrane Potential (MMP)

Like all cells, spermatozoa contain mitochondria for various normal physiological processes. But unlike other cells, spermatozoa mitochondria are physically separated in a subcellular compartment known as the mid-piece, which indicates how crucial and specific its functionality is [37]. The membrane potential of the mitochondria is strictly regulated in order for it to function properly [35]. Any disruption in highly regulated function could cause the disruption and breakdown of the organelle and cell as a whole.

One of the factors that can alter mitochondrial potential is OS, which ultimately leads to the disruption in mitochondrial homeostasis. It does so by affecting the mitochondrial membrane potential (MMP), which could depict a negative correlation with functional spermatozoa parameters, such as motility and fertilization ability. Electrophilic aldehydes that are generated from OS-induced LPO, enter the mitochondria, inhibit the electron transport chain, results in the leakage of large amounts of ROS. The ROS then attack the membrane of the mitochondria and affect its integrity by altering the MMP [22].

MMP can also be affected by spermatozoa released from the germinal epithelium carrying surplus residual cytoplasm as a result of impaired spermatogenesis.

This retention of residual cytoplasm has been positively correlated with increased ROS generation and has been suggested to be driven by the cytosolic enzyme glucose-6-phosphate dehydrogenase [38, 39]. In the event of elevated ROS levels, lipid metabolism and redox reaction responsible genes are also affected [40]. In a study on testicular inflammatory disease, it was reported that the MMP showed marked reduction as a result of the testicular inflammation [41]. Rapid loss of adenosine tri-phosphate (ATP) due to LPO also causes spermatozoa morphological defects, and a decrease in viability ultimately decreasing the motility [42, 43]. In contrast, no effect of MMP on spermatozoa motility has been reported, which further supports that metabolic pathways and processes involved in motility are not fully understood yet [44].

4.1.1.4 Protein Phosphorylation

The tyrosine phosphorylation of a protein is of high importance, not only because it plays a role in the activation of specific proteins but also its role in flagellar motility in the spermatozoa of many species [16, 45]. For example, the spermatozoa axoneme, as previously mentioned, is activated by phosphorylation [45–49]. Several other proteins such as hexokinase and AKAP82, which were found to be phosphorylated have also been suggested to be involved in the activation of sperm motility [50]. The phosphorylation of these proteins usually involves cAMP-dependent protein kinases [46, 47].

It has been suggested that elevated ROS levels may lead to a decrease in axonemal protein phosphorylation and thus, reduce membrane fluidity [51, 52]. Interestingly, RNS, a subclass or ROS cause a toxic effect when they interact with superoxide anions to form peroxynitrite (Fig. 4.2) [53, 54]. Peroxynitrite and its breakdown products can induce peroxidative damage and nitrosation of trysoine molecules responsible for signal transductions [55, 56]. Furthermore, it affects the kinases and phosphatases used in capacitation, thereby disrupting tyrosine phosphorylation and lowering its fertilizing potential [57]. Spermatozoa of infertile patients fail to undergo tyrosine phosphorylation, and this could suggest that OS has a role in this impairment, as it is one of the leading causes of infertility [58].

4.1.1.5 Acrosome Reaction

Prior to binding and penetration of the oocyte zona pellucida, the spermatozoa must be able to undergo acrosome reaction; a process by which proteolytic enzymes are released from the acrosome in order to facilitate zona pellucida binding and sperm surface remodeling in preparation for sperm-oocyte fusion [59]. Controlled ROS levels are required for triggering the acrosome reaction as well as promoting the binding ability of human spermatozoa with the zona pellucida [44]. However, excessive amounts of ROS impair membrane dependent processes such as acrosome reaction and sperm-oocyte fusion [60, 61].

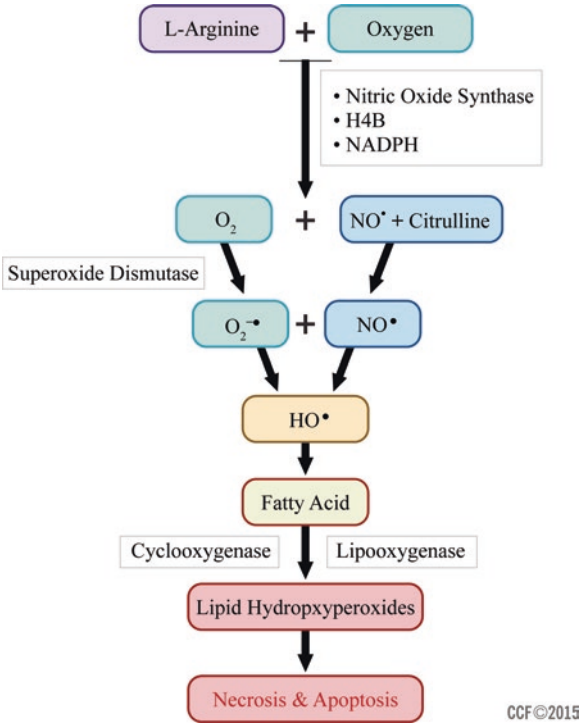

Fig. 4.2 Illustration of involvement of NO synthase in forming RNS and ROS, leading to the formation of lipid hydroperoxides, which could result in necrosis and apoptosis

4.1.1.6 DNA Damage

A healthy and intact spermatozoa DNA is required for fertilization. Any alteration in DNA structure could alter or lead to loss of function of proteins [62]. An example would be alteration and deletion in mitochondrial DNA which negatively influence cellular homeostasis and leads to a decrease in spermatozoa functionality [44]. Spermatozoa and seminal plasma with low antioxidant enzyme levels caused by homeostatic imbalances have been associated with impaired spermatozoa function and DNA integrity. This usually occurs when a cell is under OS induced by ROS or RNS [23, 63].

High concentrations of NO have been found to induce detrimental effects on mitochondrial and nuclear DNA via deamination, oxidation and nitration [64–66]. Moreover, elevated levels of aneuploidy have been positively correlated with increased levels of spermatozoa DNA damage, potentially resulting in genetic abnormalities [67]. Another cause of DNA damage results from LPO induced by OS, in which the electrophilic aldehydes induce the nuclear DNA damage [22]. The type of DNA damage could be in the DNA structure as a whole or on proteins at the molecular level. This occurs in the form of base modifications, deletions, cross-links or even frame shifts [5]. In addition, RNS can also cause chromosomal rearrangements and microdeletions [68, 69]. Also, it has been observed that single

stranded breaks in the DNA of spermatozoa are a result of OS, while double stranded breaks are also a result of 4-HNE exposure, a major product of LPO [70].

Although spermatozoa possess the first base excision repair (BER) enzyme for DNA damage repair, the lack of the second enzyme of the process, oxoguanine glycosylase (OGG1), is a reason for their susceptibility to OS as this damage can only be repaired by the oocyte after fertilization [71]. The intrinsic apoptotic pathway of spermatozoa does not seem to have an apparent increase in DNA fragmentation because the spermatozoa mitochondria, which are a source of ROS generation, and the nucleus, are in two different physically separated subcellular compartments [22]. As stated earlier, the integrity and correct DNA modifications are required for proper fertilization to occur [62]. Sperm DNA damage has been associated negatively with live-birth rates after IVF [72].

4.1.1.7 Sperm-oocyte Fusion

For sperm-oocyte fusion to occur, a spermatozoon must first penetrate the cervical mucous membrane in the female reproductive tract to be able to fuse with an oocyte. The capacity of spermatozoa to fuse with an oocyte is related to their ability to penetrate the cervical mucous membrane [73]. Following penetration, the spermatozoon must be able to undergo capacitation to further activate the cell. This process is triggered only when a spermatozoon detects the ionic and environmental changes in the female reproductive tract [16].

Like many reproductive physiological processes, capacitation requires a low and controlled level of ROS for sperm-oocyte interaction by increasing the membrane fluidity and fusion rates [74–76]. Physiological levels of NO, hydrogen peroxide and superoxide anions are important in the regulation of tyrosine phosphorylation that is associated with capacitation [38, 61, 77]. An elevated ROS levels in the genitourinary tract is associated with poor sperm-oocyte fusion. Disturbances such as OS-induced DNA damage, in either spermatozoa or oocyte integrity, will have an impact on their functions that will ultimately affect the chances of fusion and thus fertilization [78, 79]. OS not only damages the DNA but also causes LPO, resulting in a poor sperm-oocyte fusion and adhesion [19–21].

4.1.1.8 Fertilizing Capacity

Sperm require low (physiological) amounts of ROS for proper fertilizing ability to occur during capacitation [24]. This occurs when ROS triggers the activation of adenylate cyclase, increases intracellular calcium levels and phosphorylating protein kinases rendering them active [47, 80–82]. In a study using in vitro fertilization (IVF) as a biological end point, hyperactivation was found to be the most critical single characteristic of sperm movement that predicts the fertilizing potential of human spermatozoa [83, 84].

Elevated ROS levels have been associated with abnormal spermatozoa development and fertilizing capacity [74]. Henkel et al. examined IVF and ICSI outcomes in patients with ROS induced spermatozoa DNA damage and showed that the women had low pregnancy rate [85]. RNS have also been implicated in poor spermatozoa function and fertilizing ability [86–88]. Another study showed that spermatozoa with a deficiency in superoxide dismutase 1 (SOD1) caused a decrease in fertilizing ability during IVF procedures. This was attributed to increased LPO, decreased ATP levels and malfunctioning of tyrosine phosphorylation [89].

Furthermore, a study conducted by Yeung et al. proposed that ROS could have another positive influence on fertilization capacity [90]. However, another study on patients with oligozoospermia undergoing ICSI concluded that patients with spermatozoa concentration below 10×10^6 sperm/mL showed significantly lower fertilization rates [91]. Interestingly enough, some studies suggested the possibility that excessive antioxidant levels could restrain spermatozoa fertilization capacity by lowering ROS levels below the physiological levels [92–94]. Despite the known negative effects of ROS, some studies have proven otherwise, that ROS may not have any negative impact on IVF and intracytoplasmic injection (ICSI) outcome [90, 95]. Establishing an ROS cut-off value for fertilization rates and pregnancy outcomes is still controversial because there is still nonconformity with the study design and protocols used for ROS measurement [96].

4.1.1.9 Apoptosis

Apoptosis is defined as programmed cell death, which is essential in an organism's development [97]. Apoptosis is an important phenomenon for the male germ line in early development as well as in adulthood [22]; it regulates the germ cell-Sertoli cell ratio and eliminates damaged germ cells from the seminiferous tubules in response to stimuli [22].

Any disruption in spermatogenesis will cause the germ cells to default to apoptosis, namely the Fas/FasL apoptotic cascade [98]. Sertoli cells regulate the induction of apoptosis and remove about 50% of the cells during meiosis I of spermatogenesis by marking them with Fas type apoptotic markers for phagocytosis and elimination [99–101]. However, defective or abortive apoptosis can cause cells that are destined to be eliminated to escape apoptosis and get released in the ejaculate, leading to abnormalities in the embryo after fertilization [102].

Elevated levels of RNS damage in the mitochondrial membrane induce cytochrome-c release and activate the caspase cascade for initiation of apoptosis [68, 69]. A study observed that increased levels of H_2O_2 in mature spermatozoa had an increase in the percentage of apoptotic spermatozoa; however, there was no significant increase in the percentage of dead sperm [103, 104]. Another study by Mahfouz et al. showed that addition of H_2O_2 to mature spermatozoa decreased both apoptotic and dead cells [105]. Although apoptosis is sometimes required and useful for physiological functions, OS has an impact on spermatogenesis and transduction pathways of apoptosis. As this process is not fully understood, more

studies need to be conducted to further explain the mechanism of action and the pathways involved.

4.1.2 Negative Effects of Oxidative Stress in Female Reproductive System

4.1.2.1 Follicular Fluid

Follicular fluid provides an optimal microenvironment for the maturation of the oocyte before fertilization and can influence the IVF outcome parameters such as fertilization, embryo cleavage, and pregnancy rates [106]. Follicular fluid originates from the transfer of the blood plasma components, which crosses the follicular-blood barrier and from secretory contributions by granulosa and theca cells. The fluid contains steroid hormones, growth factors, leukocytes, cytokines and macrophages. All of these factors can lead to elevated ROS production [107, 108].

Adequate level of ROS is important in fertilization and implantation. Oxidative stress, on the other hand, influences the oocyte and embryo quality and thus the fertilization rate [109]. However, the existing literature regarding the impact of ROS on reproductive outcomes is controversial. A study done by Jozwik et al. reported that the concentration of oxidative stress markers such as conjugated dienes, lipid hydroperoxides and thiobarbituric acid-reactive substances in follicular fluid did not reflect the reproductive potential of oocytes [110]. In contrast, Attaran et al. reported higher levels of ROS in women who became pregnant with IVF [107], whereas Pasqualotto et al. reported elevated follicular fluid LPO in women who became pregnant versus those who did not [111].

High levels of ROS or low levels of antioxidants can affect oocyte maturation. SOD, has a crucial role in protecting the oocyte during their maturation phase by converting superoxide anions to less harmful substances like hydrogen and water [112]. Higher total antioxidant capacity (TAC) level increases fertilization potential in women undergoing IVF [113]. Deficiency of selenium-dependent glutathione peroxidase levels has also been reported to result in fertilization failure [114]. This is in agreement with studies conducted on genetically manipulated mice where a decrease in the litter size and number of litters per month was seen with deficiency of superoxide dismutase (SOD) level [115, 116]. Persistently elevated ROS level can ultimately lead to toxic effects on the embryos and impairs cleavage patterns of the embryos [117]. Borowiecka et al. evaluated the levels of lipid and protein peroxidation markers in the follicular fluid of patients undergoing IVF. Oxidative stress markers were examined in pregnant women versus those that were not. A two-fold increase in levels of protein carbonyl was reported in non-pregnant women. The study demonstrates that elevated levels of lipid and protein peroxidation levels in the fluid might have a negative impact on IVF outcome. Their findings support the idea that increased level of oxidative stress markers in follicular fluid is an important predictor of fertility outcomes [118].

Bedaiwy et al. reported higher follicular fluid TAC, fluid ROS-TAC scores and lower follicular fluid ROS levels were associated with pregnancy after ICSI [119]. Follicular fluid NO concentrations have been negatively associated with embryo quality and rate of cleavage [120]. Although ROS may serve important physiological roles within the ovary, the cyclical production of these damaging agents over years may lead to a higher cumulative risk of ovarian pathology that would probably be exacerbated under conditions of reduced antioxidant status [121].

Das et al. examined the effect of ROS on follicular fluid of women undergoing IVF and related these levels to embryo formation and quality. A favorable effect of ROS on percentage of embryo formation with ROS levels of up to 100 counted photons per second (cps) in grade II and grade III oocytes have been reported and embryo formation declines at ROS levels higher than 100cps. An overall negative correlation was reported between higher follicular fluid ROS and embryo quality [122].

Basini et al. reported that oxidative stress did not affect swine follicular growth although concentrations of hydrogen peroxide, hydroperoxides were significantly reduced during follicle growth. In addition, these authors reported significantly reduced concentrations of all enzymatic and nonenzymatic antioxidants levels with the exception of catalase during follicle development, suggesting that other factors may be involved in ROS detoxification during follicle development [123]. Similarly, during follicle maturation, the theca interna cells play an important role in protecting the developing oocyte from ROS by acting as blood-follicular barrier [124].

Antioxidants block the resumption of meiosis, whereas the generation of ROS initiates oocyte maturation in the follicle. Similarly, other investigators have shown that high levels of SOD activity in the follicular fluid are associated with a smaller success rate of IVF that are characteristic of women of advancing age [125, 126]. A positive correlation between the antioxidant capacity of the follicular fluid and that are successfully fertilized or augmented steroidogenesis has been reported [113, 127]. Apoptosis of granulosa cells can induce OS, and negatively affect the follicle development and result in poor oocyte quality in the presence of endometriosis [128].

A recent investigation examining oxidative stress and poor oocyte quality among women undergoing IVF and embryo transfer indicated that concentration of 8-OHdG (a measure of DNA oxidation) was significantly greater in the follicular fluid with a high rate (≥30%) of degenerative oocytes [129]. Shaeer et al. measured the OS markers such as lipid peroxidation (MDA), nitrite/nitrate ratio (NO_2/NO_3 ratio), reduced glutathione (GSH), reduced glutathione/oxidized glutathione ratio (GSH/GSSG ratio) by high performance liquid chromatography (HPLC) in follicular fluid. The study demonstrates that follicular fluid OS is aggravated in obesity and has a negative impact on pregnancy outcome of ICSI cycles [130].

Lower follicular fluid concentration of β-carotene with associated lower IVF success rates has been reported among smokers [131], suggesting that follicular loss of β-carotene occurs in response to tobacco-related oxidative stress. Both active and passive smoking may require increased antioxidant requirements [132, 133].

In obese and infertile women, presence of oxidized low-density lipoprotein (oxLDL) and enzyme antioxidants in the follicles (intrafollicular) may be a

contributory factor in reproductive disorders [134]. These investigators measured the concentrations of oxLDL and the activities of superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase in the serum and follicular fluid of obese and non-obese women. A thousand-fold lower level of oxLDL was seen in the follicular fluid compared to the levels in the serum. Higher levels were seen in the catalase activity and not in the SOD levels. Elevated levels of oxLDL in the follicular fluid of obese women were associated with higher catalase activity indicating excessive oxidative stress [134].

An association between adverse effects of OS on oocyte meiotic spindle (MS) assembly have been demonstrated in mouse oocytes [135]. These results corroborate the findings that non-visualization of MS is fairly correlated with higher follicular levels of ROS. Women with PCOS were reported to have a number of oocytes with meiotic spindle visualization and higher mean ROS levels in follicular fluid. This indicates a poorer oocyte and thus embryo quality in these women, which may be attributed to an excessive generation of ROS and LPO, and to an impaired antioxidant defense mechanism in follicular fluid of PCOS women. In contrast, women with endometriosis were observed to have a low follicular fluid oxidative stress status and good meiotic spindle imaging results, suggesting a possible role of endometrial receptivity rather than oocyte quality accounting for low pregnancy rates in these women [136].

In the existing literature, some studies suggest optimum level of follicular fluid ROS to be a marker for predicting success in IVF cycles [137], others have demonstrated a toxic effect of high ROS levels in follicular fluid on oocytes and lower pregnancy rates [113, 138]. Recent studies also indicate high follicular fluid ROS levels tend to decrease the fertilization potential of oocytes; furthermore, a higher percentage of good quality embryos were observed corresponding to ROS levels <100 cps [139].

4.1.2.2 Peritoneal Fluid

Peritoneal environment and associated peritoneal fluid have a complex role in OS induced female infertility. Reactive oxygen species (ROS) are produced by peritoneal fluid mononuclear leucocytes in endometriosis patients [140]. Production of ROS is known to increase after activation of immune cells, especially polymorphonuclear leukocytes and macrophages. Consequently, ROS are likely to be important mediators in the etiopathogenesis of endometriosis [140, 141].

Murphy et al. reported increased concentrations of oxidized low density lipoproteins in the peritoneal fluid of women in whom the disease was developing [142, 143]. Oxidative modification of these molecules involves peroxidation of the lipid component, which leads to release of aldehydes, such as MDA, and reaction with lysine residues of proteins [144]. It has been hypothesized that endometriosis is associated with ROS in the peritoneal and tubal fluid, adversely affecting sperm motility and function [145], and increasing the growth and adhesion of endometrial cells in the peritoneal cavity [142, 146]. Sperm motility has been negatively correlated with

peritoneal fluid volume [147, 148], suggesting the possibility that increased fluid volume may be a causative factor in endometriosis-associated infertility. Zeller et al. observed a significant increase in ROS production by resting peritoneal macrophages in peritoneal fluid samples collected during mid-luteal phase of menstruation in women with endometriosis compared with fertile controls [140]. It is hypothesized that peritoneal fluid diffuses into the fallopian tubes where it may cause damage to sperm, as they are known to be sensitive to OS [149]. Peritoneal fluid of women with idiopathic infertility demonstrated a higher concentration of ROS compared with fertile controls, but these levels were not significantly different from women with endometriosis [148].

In the presence of macrophages, cytokines are released in the peritoneal fluid due to inflammation. Cytokines such as interleukin (IL) 1,2,5,8 and 10 and tumor necrosis factor (TNF) may also be implicated in infertile patients with endometriosis [141]. Significantly lower levels of vitamin E have been reported in the peritoneal fluid of women with endometriosis, as a result of the increased OS [150]. OS is also known to be the contributing factor in the formation of intraperitoneal adhesions; one of the key factors implicated in infertility associated with endometriosis [151].

Peritoneal fluid of women with endometriosis-induced infertility has been found to have elevated ROS and/or poor antioxidant defense mechanisms [152–154]. While some studies failed to observe increased OS in the peritoneal fluid of patients with endometriosis [148, 155, 156], others have reported increased levels of OS markers in women with infertility [142, 150, 157–159]. High concentrations of MDA, pro-inflammatory cytokines (IL-6, TNF-alpha, and IL-beta), angiogenic factors (IL-8 and VEGF), monocyte chemo-attractant protein-1 [160], and ox-LDL [161] have all been reported in the peritoneal fluid of infertile patients. Pro-inflammatory and chemotactic cytokines play a central role in the recruitment and activation of phagocytic cells, which are the main producers of both ROS and RNS [160]. The elevated levels of ROS have detrimental effects on gametes. Mansour et al. demonstrated that oocytes incubated with peritoneal fluid of women with endometriosis exhibited increased DNA damage [162]. A concurrent study by the same group showed that even spermatozoa co-incubated with peritoneal fluid of women with endometriosis exhibited increased DNA fragmentation [162, 163].

Patients with endometriosis tend to have lower pregnancy rates than those without the disease. In addition to the toxic effect on spermatozoa, low oocyte and embryo quality may be mediated by ROS and this may contribute to the subfertility experienced by patients with endometriosis [164]. Intraperitoneal administration of melatonin, a potent scavenger of free radicals, has been shown to cause regression of endometriotic lesions in rat endometriosis explant models [165–167] by neutralizing OS [168, 169].

4.1.2.3 Hydrosalpingeal Fluid

Hydrosalpingeal fluid (HSF) is fluid that is produced in an inflamed fallopian tube, either unilaterally, or bilaterally [170]. Hydrosalpinx is caused by occlusion of the fimbriated end of the fallopian tube near the ovary, and retention of HSF in the site,

followed by swelling and distension triggers the loss of its anatomical shape and its functionality. Hydrosalpinx represents a post-inflammatory state of OS. It exhibits elevated production of ROS and MDA levels and low total antioxidant capacity [171]. The tubal fluid originating from the hydrosalpinx has been shown in a murine experimental model to have embryotoxic properties. The development of blastocysts was inversely correlated to the percentage of hydrosalpingeal fluid added to the murine two-cell embryo culture when compared with controls without HSF. These correlated positively with the production of ROS [171]. Several possible mechanisms for the embryotoxic properties of the hydrosalpingeal fluid have been suggested, including presence of microorganisms, endotoxins, cytokines, OS and lack of nutrients [172].

Bedaiwy et al. hypothesized that blastocyst maturation is dependent on low levels of ROS in hydrosalpingeal fluid, and increased concentration of the above mentioned parameters (except LPO, which did not show any significant change) are indicative of improvement in blastocyst growth [171]. Several research studies have examined the mechanisms by which the HSF exerts embryotoxicity. Some of the proposed mechanisms for hydrosalpingeal embryotoxicity include oxidative stress, toxins in the fluid, altered endometrial receptivity or the embryos are washed away. Chan et al. reported the mechanistic pathways by which hydrosalpingeal fluid exerts toxicity using the rat embryo model. They investigated the impact of hydrosalpinx fluid on rat embryos by assessing the levels of 8-iso prostaglandin f2-alpha levels and protein content of mouse embryos. They did not find a correlation between higher levels of OS markers and morphologic abnormalities in the embryos [173]. Bedaiwy et al. aspirated hydrosalpingeal fluid from 11 infertile women with confirmed hydrosalpinges. ROS and enzymatic TAC (both measured by chemiluminescence), and lipid peroxidation (measured by TBARS) were quantified. 2-cell mouse embryos were incubated with 25, 50 or 75 % hydrosalpingeal fluid and observed for blastocyst development. Blastocyst development was inversely correlated with concentration of hydrosalpingeal fluid. It was demonstrated in embryos cultured in 75 % hydrosalpingeal fluid compared to control embryos. Lipid peroxidation was not significantly related to blastocyst development, and TAC was detectable in only 18 % of samples, although TAC was at a level unlikely to affect blastocyst development. Blastocyst development was positively correlated with ROS concentration, but overall low ROS concentration was suggestive of healthy endosalpinx rather than a concentration potentially deleterious to the developing embryos [171].

Embryotoxicity is normally associated with the presence of hydrosalpingeal fluid that is not necessarily caused by an excess of ROS. The excess ROS levels detected in hydrosalpingeal fluid may originate as byproducts from other natural physiological processes such as cellular respiration. Higher levels of ROS may also be generated by the inflammatory response resulting from chronic salpingitis [174]. The definite reasons for embryotoxicity associated with hydrosalpinx are not completely identified and the recommendations are to surgically remove hydrosalpinx before IVF.

4.1.2.4 Oocyte Quality and DNA Oxidation

A mature competent oocyte is required to achieve fertilization. In order to accomplish this, the oocyte needs to be nurtured in an optimal microenvironment. Among several factors attributing to detrimental influences of cellular damage to the oocyte and the cellular response to protect it, the mechanism of ROS induced damage is important (Fig. 4.3). 8-Oxo-2′-deoxyguanosine (8-oxo-dG) is one of the products of DNA oxidation, and any increase in its level is indicative of OS [175]. A correlation was noted between oocyte quality and the level of 8-OHdG in granulosa cells in patients undergoing IVF-ET and ICSI. A negative relationship was reported between fertilization rates and OS from granulosa cell resulting in increased level of 8-OHdG in both cumulus and granulosa cells. Similarly, a negative correlation was seen between 8-OHdG and production of good embryos [176]. Higher levels of 8-OHdG have been reported in patients with endometriosis when compared with other categories of infertility such as tubal factor, male factor and unknown. Bedaiway et al. conducted a study to find the correlation between the early embryonic development and the level of ROS in culture media on day 1 after insemination. High level of ROS associated with a low fertilization were reported in day 1, and low blastocyst rates on day 3, and lower pregnancy rates on day 5 day and high embryonic fragmentation rates with ICSI but not with IVF [120]. Addition of vitamins E and C, especially vitamin C was found to improve blastocyst development [177]. In another study, LPO levels were examined and elevated ROS level in follicular fluid did not result in oocyte fertilization supporting the hypothesis that higher LPO levels and lower level of TAC result in reduced fertilization rate due to reduced availability of antioxidants [113].

Guanine is more prone to oxidative damage. Oxidation of guanine leads to the formation of 7, 8-dihydro-8-oxoguanine (8-oxo-G), which is one of the major oxidative DNA adducts. It is known that 8-oxo-G is considered as a biomarker for OS in DNA [178]. In a study done by de Carvalho et al., [179] the histological levels of hydroxyl-2-deoxyguanosine were examined in biopsy specimens from healthy biopsy specimens including cervix, tubes, uterus, peritoneum, and topic endometrium in secretory phase and compared with benign gynecological biopsy tissues including hydrosalpinges, leiomyoma, adenomyosis and tubal cysts. Oxidative DNA damage was confirmed in these benign gynecologic disorders by the presence of high DNA damage as demonstrated by immunohistochemical staining of 8-hydroxy-2-deoxyguanosine. These authors concluded that hydrosalpinges, leiomyoma, and adenomyosis exhibit the highest amounts of oxidative DNA damage in the pelvic cavity [179].

Oocyte quality has been assessed by visualization of meiotic spindle and ROS levels. Aspirated follicular fluid from mature oocytes was examined in study patients with endometriosis, PCOS and tubal infertility. Following ovarian stimulation (long stimulation protocol), higher rates of meiotic spindle visualization were seen in mature oocytes and these were associated with lower ROS levels [180]. Oocytes with higher rates of spindle visualization yielded good quality (grade 1) embryos within each subgroup of patients. Disorganization of the meiotic spindles could result in chromosomal dispersion, failure of normal fertilization, chromosomal

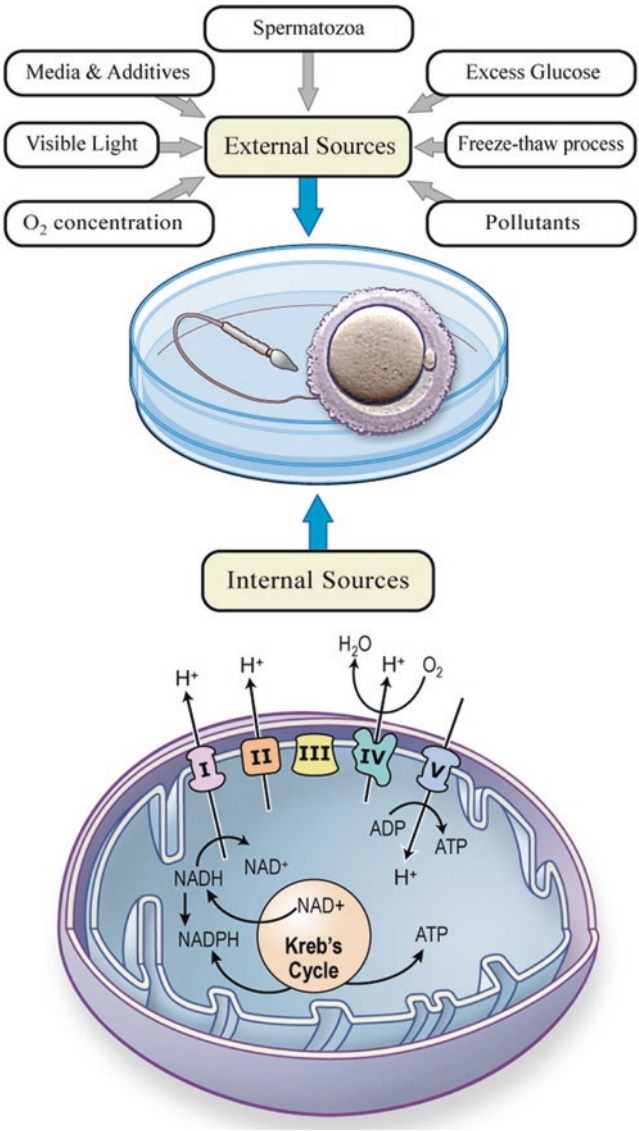

Fig. 4.3 Illustration of different internal and external factors that could have detrimental effects on sperm and oocytes

disjunction and karyotypic abnormalities in the embryo. Most studies on microtubular damage of oocytes have documented the sensitivity of the MII spindle to OS induced by cryopreservation. This increased buildup of ROS results in depolymerization of the microtubules, which are more prone to OS compared with variations in the chromosomal alignment. This may explain the poor oocyte quality and low

fertilization and poor pregnancy outcomes both in vitro and in patients with endometriosis. These changes were validated using hydrogen peroxide as an exogenous inducer of ROS and also with the addition of 200 ng/mL of TNF-α. The effects of TNF-alpha inhibitors on microtubular structure and chromosomal alignments aimed at reducing alterations provoked by the cytokine and ROS should be examined cautiously. This is important when studying the potential benefits of combining such inhibitors with antioxidant(s) in an effort to enhance oocyte quality [181, 182].

4.2 Conclusion

ROS are important factors for a variety of mechanisms occurring in the body. In this chapter, the negative impacts of OS on male and female reproductive systems at cellular level are elaborated. Elevated levels of ROS and their effects in spermatozoa, follicular fluid, peritoneal fluid, hydrosalpingeal fluid and oocyte and embryo were discussed. Imbalance between ROS and antioxidants results in OS, which lead to damage of lipids, proteins and DNA. The mechanisms involved in such damage along with their possible consequences at cellular level have all been discussed. There are still areas regarding the influence of OS on male and female reproductive systems at cellular level that remain unclear and hence further studies are warranted.

References

1. Aitken RJ et al (2014) Oxidative stress and male reproductive health. Asian J Androl 16(1):31–38
2. Agarwal A et al (2006) The role of free radicals and antioxidants in reproduction. Curr Opin Obstet Gynecol 18(3):325–332
3. Agarwal A et al (2014) Mechanisms of oligozoospermia: an oxidative stress perspective. Syst Biol Reprod Med 60(4):206–216
4. Sharma RK et al (1999) The reactive oxygen species-total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. Hum Reprod 14(11):2801–2807
5. Agarwal A et al (2003) Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril 79(4):829–843
6. Milardi D et al (2012) Male fertility and reduction in semen parameters: a single tertiary-care center experience. Int J Endocrinol 2012:649149
7. McLachlan RI (2013) Approach to the patient with oligozoospermia. J Clin Endocrinol Metab 98(3):873–880
8. Guz J et al (2013) Comparison of oxidative stress/DNA damage in semen and blood of fertile and infertile men. PLoS One 8(7), e68490
9. Kullisaar T et al (2013) Increased levels of hydrogen peroxide and nitric oxide in male partners of infertile couples. Andrology 1(6):850–858
10. Mahanta R et al (2012) Association of oxidative stress biomarkers and antioxidant enzymatic activity in male infertility of North-East India. J Obstet Gynaecol India 62(5):546–550
11. Montjean D et al (2012) Sperm transcriptome profiling in oligozoospermia. J Assist Reprod Genet 29(1):3–10
12. Pylyp LY et al (2013) Chromosomal abnormalities in patients with oligozoospermia and non-obstructive azoospermia. J Assist Reprod Genet 30(5):729–732

13. Zhang ZB et al (2012) Male infertility in Northeast China: a cytogenetic study of 135 patients with non-obstructive azoospermia and severe oligozoospermia. J Assist Reprod Genet 29(1):83–87
14. Gandini L et al (2000) Study of apoptotic DNA fragmentation in human spermatozoa. Hum Reprod 15(4):830–839
15. Agarwal A, Sekhon LH (2011) Oxidative stress and antioxidants for idiopathic oligoasthenoteratospermia: is it justified? Indian J Urol 27(1):74–85
16. Inaba K (2003) Molecular architecture of the sperm flagella: molecules for motility and signaling. Zoolog Sci 20(9):1043–1056
17. Amaral A et al (2007) The expression of polymerase gamma and mitochondrial transcription factor A and the regulation of mitochondrial DNA content in mature human sperm. Hum Reprod 22(6):1585–1596
18. Pryor WA, Stanley JP (1975) Letter: a suggested mechanism for the production of malonaldehyde during the autoxidation of polyunsaturated fatty acids. Nonenzymatic production of prostaglandin endoperoxides during autoxidation. J Org Chem 40(24):3615–3617
19. Potts RJ et al (2000) Seminal plasma reduces exogenous oxidative damage to human sperm, determined by the measurement of DNA strand breaks and lipid peroxidation. Mutat Res 447(2):249–256
20. Aitken RJ (1995) Free radicals, lipid peroxidation and sperm function. Reprod Fertil Dev 7(4):659–668
21. Jedrzejczak P et al (2005) Consequences of semen inflammation and lipid peroxidation on fertilization capacity of spermatozoa in in vitro conditions. Int J Androl 28(5):275–283
22. Aitken RJ, Baker MA (2013) Causes and consequences of apoptosis in spermatozoa; contributions to infertility and impacts on development. Int J Dev Biol 57(2–4):265–272
23. Aitken RJ, Curry BJ (2011) Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. Antioxid Redox Signal 14(3):367–381
24. Morielli T, O'Flaherty C (2015) Oxidative stress impairs function and increases redox protein modifications in human spermatozoa. Reproduction 149(1):113–123
25. Carlsen E et al (1992) Evidence for decreasing quality of semen during past 50 years. BMJ 305(6854):609–613
26. Metzger et al (2010) International association of diabetes and pregnancy study groups recommendations on the diagnosis and classification of hyperglycemia in pregnancy. Diabetes Care 33(3):676–682
27. Yeung CH et al (2009) Coiled sperm from infertile patients: characteristics, associated factors and biological implication. Hum Reprod 24(6):1288–1295
28. Agarwal A, Tvrda E, Sharma R (2014) Relationship amongst teratozoospermia, seminal oxidative stress and male infertility. Reprod Biol Endocrinol 12:45
29. Aziz N et al (1996) The sperm deformity index: a reliable predictor of the outcome of oocyte fertilization in vitro. Fertil Steril 66(6):1000–1008
30. Lim CC et al (1998) Human sperm morphology and in vitro fertilization: sperm tail defects are prognostic for fertilization failure. Andrologia 30(1):43–47
31. Ollero M et al (2001) Characterization of subsets of human spermatozoa at different stages of maturation: implications in the diagnosis and treatment of male infertility. Hum Reprod 16(9):1912–1921
32. Aziz N et al (2004) Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index. Fertil Steril 81(2):349–354
33. Pasqualotto FF et al (2008) Poor semen quality and ROS-TAC scores in patients with idiopathic infertility. Urol Int 81(3):263–270
34. Wong-Ekkabut J et al (2007) Effect of lipid peroxidation on the properties of lipid bilayers: a molecular dynamics study. Biophys J 93(12):4225–4236
35. Koppers AJ et al (2010) Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa. Free Radic Biol Med 48(1):112–119

36. Huszar G, Vigue L (1994) Correlation between the rate of lipid peroxidation and cellular maturity as measured by creatine kinase activity in human spermatozoa. J Androl 15(1):71–77
37. Aitken RJ et al (2007) Proteomic changes in mammalian spermatozoa during epididymal maturation. Asian J Androl 9(4):554–564
38. Aitken RJ (1999) The Amoroso Lecture. The human spermatozoon – a cell in crisis? J Reprod Fertil 115(1):1–7
39. Huszar G, Vigue L (1993) Incomplete development of human spermatozoa is associated with increased creatine phosphokinase concentration and abnormal head morphology. Mol Reprod Dev 34(3):292–298
40. Li YC et al (2006) An oligonucleotide microarray study on gene expression profile in mouse testis of experimental cryptorchidism. Front Biosci 11:2465–2482
41. Allen JA et al (2004) Bacterial endotoxin lipopolysaccharide and reactive oxygen species inhibit Leydig cell steroidogenesis via perturbation of mitochondria. Endocrine 25(3):265–275
42. Gharagozloo P, Aitken RJ (2011) The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. Hum Reprod 26(7):1628–1640
43. Bansal AK, Bilaspuri GS (2010) Impacts of oxidative stress and antioxidants on semen functions. Vet Med Int 2010:1–10
44. Amaral A et al (2013) Mitochondria functionality and sperm quality. Reproduction 146(5):R163–R174
45. Visconti PE et al (1995) Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development 121(4):1139–1150
46. Hayashi H et al (1987) Involvement of tyrosine protein kinase in the initiation of flagellar movement in rainbow trout spermatozoa. J Biol Chem 262(34):16692–16698
47. Visconti PE et al (1995) Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. Development 121(4):1129–1137
48. Dey CS, Brokaw CJ (1991) Activation of Ciona sperm motility: phosphorylation of dynein polypeptides and effects of a tyrosine kinase inhibitor. J Cell Sci 100(Pt 4):815–824
49. Si Y, Okuno M (1999) Role of tyrosine phosphorylation of flagellar proteins in hamster sperm hyperactivation. Biol Reprod 61(1):240–246
50. Carrera A, Gerton GL, Moss SB (1994) The major fibrous sheath polypeptide of mouse sperm: structural and functional similarities to the A-kinase anchoring proteins. Dev Biol 165(1):272–284
51. de Lamirande E, Gagnon C (1992) Reactive oxygen species and human spermatozoa. I. Effects on the motility of intact spermatozoa and on sperm axonemes. J Androl 13(5):368–378
52. de Lamirande E, Gagnon C (1992) Reactive oxygen species and human spermatozoa. II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. J Androl 13(5):379–386
53. Sikka SC (2001) Relative impact of oxidative stress on male reproductive function. Curr Med Chem 8(7):851–862
54. Sikka SC et al (1995) Role of oxidative stress and antioxidants in male infertility. J Androl 16(6):464–468
55. Rosselli M et al (1998) Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. Hum Reprod Update 4(1):3–24
56. Archer S (1993) Measurement of nitric oxide in biological models. FASEB J 7(2):349–360
57. de Lamirande E, O'Flaherty C (2008) Sperm activation: role of reactive oxygen species and kinases. Biochim Biophys Acta 1784(1):106–115
58. Buffone MG et al (2005) Capacitation-associated protein tyrosine phosphorylation and membrane fluidity changes are impaired in the spermatozoa of asthenozoospermic patients. Reproduction 129(6):697–705
59. Aitken RJ (2006) Sperm function tests and fertility. Int J Androl 29(1):69–75, discussion 105–108

60. Sawyer DE et al (2003) Quantitative analysis of gene-specific DNA damage in human spermatozoa. Mutat Res 529(1–2):21–34
61. de Lamirande E et al (2009) Control of superoxide and nitric oxide formation during human sperm capacitation. Free Radic Biol Med 46(10):1420–1427
62. Talwar P, Hayatnagarkar S (2015) Sperm function test. J Hum Reprod Sci 8(2):61–69
63. Gong S et al (2012) Low amounts and high thiol oxidation of peroxiredoxins in spermatozoa from infertile men. J Androl 33(6):1342–1351
64. Lee NP, Cheng CY (2008) Nitric oxide and cyclic nucleotides: their roles in junction dynamics and spermatogenesis. Adv Exp Med Biol 636:172–185
65. Lee NP, Cheng CY (2004) Nitric oxide/nitric oxide synthase, spermatogenesis, and tight junction dynamics. Biol Reprod 70(2):267–276
66. Amiri I et al (2007) Nitric oxide level in seminal plasma and its relation with sperm DNA damages. Iran Biomed J 11(4):259–264
67. Enciso M et al (2013) Increased numbers of DNA-damaged spermatozoa in samples presenting an elevated rate of numerical chromosome abnormalities. Hum Reprod 28(6):1707–1715
68. Makker K et al (2009) Oxidative stress & male infertility. Indian J Med Res 129(4):357–367
69. Koppenol WH et al (1992) Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. Chem Res Toxicol 5(6):834–842
70. Badouard C et al (2008) Determination of new types of DNA lesions in human sperm. Zygote 16(1):9–13
71. Smith TB et al (2013) The presence of a truncated base excision repair pathway in human spermatozoa that is mediated by OGG1. J Cell Sci 126(Pt 6):1488–1497
72. Simon L et al (2013) Sperm DNA damage has a negative association with live-birth rates after IVF. Reprod Biomed Online 26(1):68–78
73. Aitken RJ et al (1992) Sperm penetration into a hyaluronic acid polymer as a means of monitoring functional competence. J Androl 13(1):44–54
74. Ko EY et al (2014) Male infertility testing: reactive oxygen species and antioxidant capacity. Fertil Steril 102(6):1518–1527
75. Calamera J et al (2003) Superoxide dismutase content and fatty acid composition in subsets of human spermatozoa from normozoospermic, asthenozoospermic, and polyzoospermic semen samples. Mol Reprod Dev 66(4):422–430
76. Khosrowbeygi A, Zarghami N (2007) Fatty acid composition of human spermatozoa and seminal plasma levels of oxidative stress biomarkers in subfertile males. Prostaglandins Leukot Essent Fatty Acids 77(2):117–121
77. Aitken RJ et al (1998) A novel signal transduction cascade in capacitating human spermatozoa characterised by a redox-regulated, cAMP-mediated induction of tyrosine phosphorylation. J Cell Sci 111(Pt 5):645–656
78. Rajesh KT et al (2002) Oxidative stress associated DNA damage in testis of mice: induction of abnormal sperms and effects on fertility. Mutat Res 513(1–2):103–111
79. Aitken RJ, Koppers AJ (2011) Apoptosis and DNA damage in human spermatozoa. Asian J Androl 13(1):36–42
80. O'Flaherty C et al (2006) Positive role of reactive oxygen species in mammalian sperm capacitation: triggering and modulation of phosphorylation events. Free Radic Biol Med 41(4):528–540
81. Leclerc P et al (1996) Cyclic adenosine 3',5' monophosphate-dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. Biol Reprod 55(3):684–692
82. de Lamirande E, O'Flaherty C (2012) Sperm capacitation as an oxidative event. In: Agarwal A, Aitken RJ, Alvarez JG (eds) Studies on men's health and fertility, Applied basic research and clinical practice. Springer + Business Media, LCC, Humana Press, New York, pp 57–94
83. Sukcharoen N et al (1995) Predicting the fertilizing potential of human sperm suspensions in vitro: importance of sperm morphology and leukocyte contamination. Fertil Steril 63(6):1293–1300

84. Sukcharoen N et al (1995) Definition of the optimal criteria for identifying hyperactivated human spermatozoa at 25 Hz using in-vitro fertilization as a functional end-point. Hum Reprod 10(11):2928–2937
85. Henkel R et al (2003) DNA fragmentation of spermatozoa and assisted reproduction technology. Reprod Biomed Online 7(4):477–484
86. Baker MA, Aitken RJ (2005) Reactive oxygen species in spermatozoa: methods for monitoring and significance for the origins of genetic disease and infertility. Reprod Biol Endocrinol 3:67
87. Cornwell TL et al (1994) Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. Am J Physiol 267(5 Pt 1):C1405–C1413
88. Zhang H, Zheng RL (1996) Possible role of nitric oxide on fertile and asthenozoospermic infertile human sperm functions. Free Radic Res 25(4):347–354
89. Tsunoda S et al (2012) Impaired fertilizing ability of superoxide dismutase 1-deficient mouse sperm during in vitro fertilization. Biol Reprod 87(5):121
90. Yeung CH et al (1996) Production of reactive oxygen species by and hydrogen peroxide scavenging activity of spermatozoa in an IVF program. J Assist Reprod Genet 13(6):495–500
91. Arikan II et al (2012) ICSI cycle outcomes in oligozoospermia. Clin Exp Obstet Gynecol 39(3):280–282
92. Menezo YJ et al (2007) Antioxidants to reduce sperm DNA fragmentation: an unexpected adverse effect. Reprod Biomed Online 14(4):418–421
93. Bjelakovic G et al (2015) Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. Sao Paulo Med J 133(2):164–165
94. Verma A, Kanwar KC (1998) Human sperm motility and lipid peroxidation in different ascorbic acid concentrations: an in vitro analysis. Andrologia 30(6):325–329
95. Hammadeh ME et al (2008) Reactive oxygen species, total antioxidant concentration of seminal plasma and their effect on sperm parameters and outcome of IVF/ICSI patients. Arch Gynecol Obstet 277(6):515–526
96. Agarwal A et al (2005) Correlation of reactive oxygen species levels with the fertilization rate after in vitro fertilization: a qualified meta-analysis. Fertil Steril 84(1):228–231
97. Vaux DL, Flavell RA (2000) Apoptosis genes and autoimmunity. Curr Opin Immunol 12(6):719–724
98. Lin YC et al (2010) FasL gene-deficient mice display a limited disruption in spermatogenesis and inhibition of mono-(2-ethylhexyl) phthalate-induced germ cell apoptosis. Toxicol Sci 114(2):335–345
99. Billig H et al (1996) Gonadal cell apoptosis: hormone-regulated cell demise. Hum Reprod Update 2(2):103–117
100. Pentikainen V et al (1999) Fas regulates germ cell apoptosis in the human testis in vitro. Am J Physiol 276(2 Pt 1):E310–E316
101. Sakkas D et al (1999) Origin of DNA damage in ejaculated human spermatozoa. Rev Reprod 4(1):31–37
102. Celik-Ozenci C, Tasatargil A (2013) Role of poly(ADP-ribose) polymerases in male reproduction. Spermatogenesis 3(2), e24194
103. Kumar PG et al (1991) Superoxide radical level and superoxide dismutase activity changes in maturing mammalian spermatozoa. Andrologia 23(2):171–175
104. Said TM et al (2004) Human sperm superoxide anion generation and correlation with semen quality in patients with male infertility. Fertil Steril 82(4):871–877
105. Mahfouz RZ et al (2010) Sperm viability, apoptosis, and intracellular reactive oxygen species levels in human spermatozoa before and after induction of oxidative stress. Fertil Steril 93(3):814–821
106. Keisari Y et al (1983) The oxidative burst and related phenomena in mouse macrophages elicited by different sterile inflammatory stimuli. Immunobiology 165(1):78–89
107. Attaran M et al (2000) The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. Int J Fertil Womens Med 45(5):314–320
108. Agarwal A et al (2004) Chemiluminescence technique for measuring reactive oxygen species. Reprod Biomed Online 9(4):466–468

109. Agarwal A et al (2005) Oxidative stress and its implications in female infertility – a clinician's perspective. Reprod Biomed Online 11(5):641–650
110. Jozwik M et al (1999) Oxidative stress markers in preovulatory follicular fluid in humans. Mol Hum Reprod 5(5):409–413
111. Pasqualotto EB et al (2004) Effect of oxidative stress in follicular fluid on the outcome of assisted reproductive procedures. Fertil Steril 81(4):973–976
112. Bedaiwy MA et al (2002) Prediction of endometriosis with serum and peritoneal fluid markers: a prospective controlled trial. Hum Reprod 17(2):426–431
113. Oyawoye O et al (2003) Antioxidants and reactive oxygen species in follicular fluid of women undergoing IVF: relationship to outcome. Hum Reprod 18(11):2270–2274
114. Paszkowski T et al (1995) Selenium dependent glutathione peroxidase activity in human follicular fluid. Clin Chim Acta 236(2):173–180
115. Ho YS et al (1998) The nature of antioxidant defense mechanisms: a lesson from transgenic studies. Environ Health Perspect 106(Suppl 5):1219–1228
116. Matzuk MM et al (1998) Ovarian function in superoxide dismutase 1 and 2 knockout mice. Endocrinology 139(9):4008–4011
117. De Iuliis GN et al (2006) Definitive evidence for the nonmitochondrial production of superoxide anion by human spermatozoa. J Clin Endocrinol Metab 91(5):1968–1975
118. Borowiecka M et al (2012) Oxidative stress markers in follicular fluid of women undergoing in vitro fertilization and embryo transfer. Syst Biol Reprod Med 58(6):301–305
119. Bedaiwy MA et al (2012) Effect of follicular fluid oxidative stress parameters on intracytoplasmic sperm injection outcome. Gynecol Endocrinol 28(1):51–55
120. Bedaiwy MA et al (2004) Differential growth of human embryos in vitro: role of reactive oxygen species. Fertil Steril 82(3):593–600
121. Behrman HR et al (2001) Oxidative stress and the ovary. J Soc Gynecol Investig 8(1 suppl):S40–S42
122. Das S et al (2006) Reactive oxygen species level in follicular fluid – embryo quality marker in IVF? Hum Reprod 21(9):2403–2407
123. Basini G et al (2008) Reactive oxygen species and anti-oxidant defences in swine follicular fluids. Reprod Fertil Dev 20(2):269–274
124. Shiotani M et al (1991) Immunohistochemical localization of superoxide dismutase in the human ovary. Hum Reprod 6(10):1349–1353
125. Sabatini L et al (1999) Superoxide dismutase activity in human follicular fluid after controlled ovarian hyperstimulation in women undergoing in vitro fertilization. Fertil Steril 72(6):1027–1034
126. Carbone MC et al (2003) Antioxidant enzymatic defences in human follicular fluid: characterization and age-dependent changes. Mol Hum Reprod 9(11):639–643
127. Appasamy M et al (2008) Evaluation of the relationship between follicular fluid oxidative stress, ovarian hormones, and response to gonadotropin stimulation. Fertil Steril 89(4):912–921
128. Nakahara K et al (1998) Ovarian fecundity in patients with endometriosis can be estimated by the incidence of apoptotic bodies. Fertil Steril 69(5):931–935
129. Tamura H et al (2008) Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. J Pineal Res 44(3):280–287
130. Shaeer EK, Soliman SA, El-Ghareeb AA-b (2014) Obesity and follicular fluid oxidative stress: relationship to ICSI outcome. Middle East Fertil Soc J 19(2):139–143
131. Tiboni GM et al (2004) Influence of cigarette smoking on vitamin E, vitamin A, beta-carotene and lycopene concentrations in human pre-ovulatory follicular fluid. Int J Immunopathol Pharmacol 17(3):389–393
132. Alberg A (2002) The influence of cigarette smoking on circulating concentrations of antioxidant micronutrients. Toxicology 180(2):121–137
133. Dietrich M et al (2003) Smoking and exposure to environmental tobacco smoke decrease some plasma antioxidants and increase gamma-tocopherol in vivo after adjustment for dietary antioxidant intakes. Am J Clin Nutr 77(1):160–166

134. Bausenwein J et al (2010) Elevated levels of oxidized low-density lipoprotein and of catalase activity in follicular fluid of obese women. Mol Hum Reprod 16(2):117–124
135. Zuelke KA et al (1997) Glutathione oxidation is associated with altered microtubule function and disrupted fertilization in mature hamster oocytes. Biol Reprod 57(6):1413–1419
136. Rajani S et al (2012) Assessment of oocyte quality in polycystic ovarian syndrome and endometriosis by spindle imaging and reactive oxygen species levels in follicular fluid and its relationship with IVF-ET outcome. J Hum Reprod Sci 5(2):187–193
137. Deepinder F, Agarwal A (2009) Determination of seminal oxidants. In: Howards S, Lipshultz L, Niederbeger C (eds) Infertility in the male, 4th edn. Cambridge University Press, Cambridge, pp 618–632. Chapter 39
138. Baka S, Malamitsi-Puchner A (2006) Novel follicular fluid factors influencing oocyte developmental potential in IVF: a review. Reprod Biomed Online 12(4):500–506
139. Miller NJ et al (1993) A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin Sci (Lond) 84(4):407–412
140. Zeller JM et al (1987) Enhancement of human monocyte and peritoneal macrophage chemiluminescence activities in women with endometriosis. Am J Reprod Immunol Microbiol 13(3):78–82
141. Bedaiwy MA, Falcone T (2003) Peritoneal fluid environment in endometriosis. Clinicopathological implications. Minerva Ginecol 55(4):333–345
142. Murphy AA et al (1998) Endometriosis: a disease of oxidative stress? Semin Reprod Endocrinol 16(4):263–273
143. Murphy AA et al (1998) Macrophage scavenger receptor(s) and oxidatively modified proteins in endometriosis. Fertil Steril 69(6):1085–1091
144. Fernandez JL et al (2005) Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. Fertil Steril 84(4):833–842
145. Curtis P et al (1993) Adverse effects on sperm movement characteristics in women with minimal and mild endometriosis. Br J Obstet Gynaecol 100(2):165–169
146. Portz DM et al (1991) Oxygen free radicals and pelvic adhesion formation: I. Blocking oxygen free radical toxicity to prevent adhesion formation in an endometriosis model. Int J Fertil 36(1):39–42
147. van Furth R et al (1979) Characteristics of human mononuclear phagocytes. Blood 54(2):485–500
148. Wang Y et al (1997) Importance of reactive oxygen species in the peritoneal fluid of women with endometriosis or idiopathic infertility. Fertil Steril 68(5):826–830
149. Storey BT (1997) Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. Mol Hum Reprod 3(3):203–213
150. Mier-Cabrera J et al (2008) Effect of vitamins C and E supplementation on peripheral oxidative stress markers and pregnancy rate in women with endometriosis. Int J Gynaecol Obstet 100(3):252–256
151. Kyama CM et al (2008) Endometrial and peritoneal expression of aromatase, cytokines, and adhesion factors in women with endometriosis. Fertil Steril 89(2):301–310
152. Polak G et al (2013) Increased levels of oxidative stress markers in the peritoneal fluid of women with endometriosis. Eur J Obstet Gynecol Reprod Biol 168(2):187–190
153. Polak G et al (2010) Peritoneal fluid iron levels in women with endometriosis. Ginekol Pol 81(1):20–23
154. Ngo C et al (2009) Reactive oxygen species controls endometriosis progression. Am J Pathol 175(1):225–234
155. Ho HN et al (1997) Total antioxidant status and nitric oxide do not increase in peritoneal fluids from women with endometriosis. Hum Reprod 12(12):2810–2815
156. Polak G et al (2001) Total antioxidant status of peritoneal fluid in infertile women. Eur J Obstet Gynecol Reprod Biol 94(2):261–263
157. Jackson LW et al (2005) Oxidative stress and endometriosis. Hum Reprod 20(7):2014–2020
158. Szczepanska M et al (2003) Oxidative stress may be a piece in the endometriosis puzzle. Fertil Steril 79(6):1288–1293

159. Sharma I et al (2010) Role of 8-iso-prostaglandin F2alpha and 25-hydroxycholesterol in the pathophysiology of endometriosis. Fertil Steril 94(1):63–70
160. Aitken RJ et al (2004) Shedding light on chemiluminescence: the application of chemiluminescence in diagnostic andrology. J Androl 25(4):455–465
161. Rong R et al (2002) Induction of monocyte chemotactic protein-1 in peritoneal mesothelial and endometrial cells by oxidized low-density lipoprotein and peritoneal fluid from women with endometriosis. Fertil Steril 78(4):843–848
162. Mansour G et al (2009) The impact of peritoneal fluid from healthy women and from women with endometriosis on sperm DNA and its relationship to the sperm deformity index. Fertil Steril 92(1):61–67
163. Mansour G et al (2009) L-carnitine supplementation reduces oocyte cytoskeleton damage and embryo apoptosis induced by incubation in peritoneal fluid from patients with endometriosis. Fertil Steril 91(5 Suppl):2079–2086
164. Augoulea A et al (2009) The role of the oxidative-stress in the endometriosis-related infertility. Gynecol Endocrinol 25(2):75–81
165. Ozcelik B et al (2003) Effect of melatonin in the prevention of post-operative adhesion formation in a rat uterine horn adhesion model. Hum Reprod 18(8):1703–1706
166. Guney M et al (2008) Regression of endometrial explants in a rat model of endometriosis treated with melatonin. Fertil Steril 89(4):934–942
167. Paul S et al (2008) Role of melatonin in regulating matrix metalloproteinase-9 via tissue inhibitors of metalloproteinase-1 during protection against endometriosis. J Pineal Res 44(4):439–449
168. Hoshi K et al (1996) The relationship between acridine orange fluorescence of sperm nuclei and the fertilizing ability of human sperm. Fertil Steril 66(4):634–639
169. Gopalkrishnan K et al (1999) Use of acridine orange to evaluate chromatin integrity of human spermatozoa in different groups of infertile men. Andrologia 31(5):277–282
170. Doshi SB et al (2012) Role of reactive nitrogen species in male infertility. Reprod Biol Endocrinol 10(109):10.1186
171. Bedaiwy MA et al (2002) Relationship between oxidative stress and embryotoxicity of hydrosalpingeal fluid. Hum Reprod 17(3):601–604
172. Strandell A, Lindhard A (2002) Why does hydrosalpinx reduce fertility? The importance of hydrosalpinx fluid. Hum Reprod 17(5):1141–1145
173. Chan LY et al (2003) A study of teratogenicity of hydrosalpinx fluid using a whole rat embryo culture model. Hum Reprod 18(5):955–958
174. Kubo A et al (1987) Oxygen radical generation by polymorphonuclear leucocytes of beige mice. Clin Exp Immunol 70(3):658–663
175. de Souza-Pinto NC et al (2001) Repair of 8-oxodeoxyguanosine lesions in mitochondrial DNA depends on the oxoguanine dna glycosylase (OGG1) gene and 8-oxoguanine accumulates in the mitochondrial dna of OGG1-defective mice. Cancer Res 61(14):5378–5381
176. Seino T et al (2002) Eight-hydroxy-2′-deoxyguanosine in granulosa cells is correlated with the quality of oocytes and embryos in an in vitro fertilization-embryo transfer program. Fertil Steril 77(6):1184–1190
177. Wang X et al (2002) Vitamin C and vitamin E supplementation reduce oxidative stress-induced embryo toxicity and improve the blastocyst development rate. Fertil Steril 78(6):1272–1277
178. Kim JE et al (2004) 8-Oxoguanine induces intramolecular DNA damage but free 8-oxoguanine protects intermolecular DNA from oxidative stress. FEBS Lett 556(1–3):104–110
179. de Carvalho LF et al (2013) Mapping histological levels of 8-hydroxy-2′-deoxyguanosine in female reproductive organs. J Mol Histol 44(1):111–116
180. Chao HT et al (2005) Repeated ovarian stimulations induce oxidative damage and mitochondrial DNA mutations in mouse ovaries. Ann N Y Acad Sci 1042:148–156
181. Choi WJ et al (2007) Oxidative stress and tumor necrosis factor-alpha-induced alterations in metaphase II mouse oocyte spindle structure. Fertil Steril 88(4 Suppl):1220–1231
182. Yang HW et al (1998) Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. Hum Reprod 13(4):998–1002

Chapter 5
Extrinsic Factors Inducing Oxidative Stress (OS) in Male and Female Reproductive Systems

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5.1 Introduction

Reactive oxygen species (ROS) are free radical and non-free radical oxidizing agents that play a normal physiological role in the body at low physiological amount [1]. Biological reactions and mitochondrial processes generate regular amounts of ROS. In the female reproductive system, ROS plays an essential role in signal transduction pathways and metabolic pathways in processes such as follicular development, oocyte maturation, ovulation, implantation, steroidogenesis, and maintenance of pregnancy. In the male reproductive system, ROS are critical in spermatozoa capacitation, hyperactivation, acrosome reaction and sperm-oocyte fusion [2, 3].

When ROS increases beyond the physiological levels, overwhelming opposing antioxidant forces, oxidative stress (OS) results. When this occurs, ROS can lead to lipid peroxidation (LPO), DNA damage, and OS-induced apoptosis and autophagy, all of which are harmful to the cells especially in the highly susceptible gametes [2].

There are many internal and external sources of ROS. This chapter will focus only on the extrinsic factors. The two main subcategories include lifestyle and

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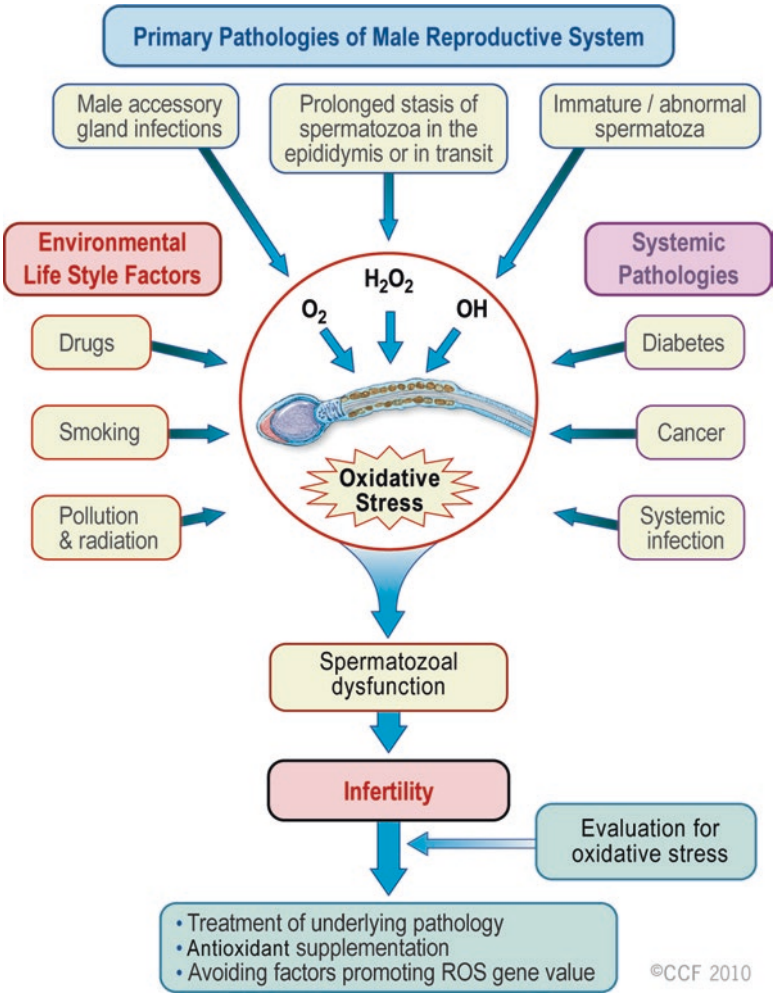

Fig. 5.1 Factors that affect sperm function

environment. Under lifestyle factors, we discuss the effects of habits, occupation, and general health. Under environment factors, we cover pollution, radiation, and altitude. We will review the impact of these factors on ROS levels and OS in more detail.

The significance of this review is in its relevance to our daily lives. We all lead a different individual lifestyle and many of our lifestyle factors and exposures to different environmental conditions may impact our body's reproductive system (Fig. 5.1). The main goal of this chapter is to provide an insight into the various extrinsic factors and the mechanisms by which they induce OS.

5.2 Lifestyle Factors

5.2.1 Habits

5.2.1.1 Smoking

Cigarette smoking (CS) causes a multitude of adverse corporeal health effects. It can lead to cardiovascular diseases, respiratory diseases, and cancer. Unbeknownst to many, smoking has a significantly negative impact on the reproductive health of both males and females [4]. Smoking is, however, one of the most clinically relevant and manageable OS-inducing extrinsic factors.

CS contains over 4000 chemicals, many of which are toxic, carcinogenic and mutagenic [4, 5]. Some of these chemicals include polycyclic aromatic hydrocarbons [such as benzo(a)pyrene, BaP], aromatic amines, and heavy metals (i.e. Cd, Cr, Pb, Ni) [6, 7]. Smoking affects the body primarily via two mechanisms: inducing abnormally high ROS levels and/or weakening antioxidant defenses, ultimately leading to OS and cellular damage [5, 6, 8–10].

Increased ROS Mechanisms

In two similar studies by Gannon et al. [8, 9], CS exposure and its effect on stimulating the autophagy pathway and dysregulating mitochondrial processes in mice were explored. Autophagy pathway is described as the breakdown of damaged organelles or macromolecules by phagosome-lysosome degradation [8]. They demonstrated that CS was involved in follicular damage, OS induction, autophagy pathway activation and downregulation of superoxide dismutase 2 (SOD2)'s expression, an important antioxidant enzyme in the mitochondria, as well as possibly altering mitochondrial functions. They found three main mechanisms by which CS exposure induces OS; (1) by increasing the activity of heat shock protein 25, (2) activating cytochrome P450 enzymes by CS-containing chemical aryl hydrocarbon receptor (AhR) agonists like BaP, and (3) by increasing leukocytes production. Furthermore, progenitor cells in smokers also displayed a greater amounts of ROS production [9]. According to Jennings et al., CS interferes with the redox potential of thiols, and the high nitrogen dioxide (NO_2) content in CS induces the production of sulfur-free radicals, influencing disulfide bridge formation, and leads to oxidative damage [11].

CS contains approximately 10^{17} oxidant molecules/puff of smoke, of which a significant portion, 10^{14}, are ROS. CS occurs in two phases: gas and tar (particulate). A few studies show that CS gas and tar particles are pro-oxidants that increase LPO of cellular membranes [12, 13]. In the gas phase, short-lived ROS, such as superoxide anion and nitrous oxide, exist and react to form the highly reactive peroxynitrite radical. In the tar phase, long-lived hydroquinones, involved in redox cycle processes, form superoxide radicals and hydrogen peroxides. Auto-oxidation of hydroquinone and

catechol, for example, generates hydrogen peroxide (H_2O_2), which is iron (Fe) catalyzed to highly reactive hydroxyl radicals via Fenton reaction. CS can also stimulate iron release from ferritin and activate the autophagy pathway, leading to the generation of ROS [6]. These hydroquinones and peroxides cause oxidative DNA and cellular damage.

In a study comparing the levels of lipoperoxides (LP) and nitrous oxide (NO) in smokers and non-smokers, smokers were found to have a significant greater amounts of LP and NO in the plasma and LP in the erythrocytes compared to non-smokers [14]. Another related study showed an increase in malondialdehyde (MDA), a chemical product of the lipid peroxidation of polyunsaturated fatty acids. Regardless of gender, MDA levels were higher in smokers as compared to non-smokers [15]. These studies further elucidate the generation of ROS in CS.

Mechanisms of Decreased Antioxidant levels

CS exposure decreases SOD2 activity in the mitochondria of granulosa cells in mice and that progenitor cells had decreased plasma antioxidant concentrations [8]. Glutathione-S-transferases (GST) are part of the glutathione enzyme family, important antioxidant biomolecules. Mai et al. reported that CS causes a decrease in GST [6]. These lead to OS as both GST and SOD2 are important in counterbalancing the overpowering pro-oxidants.

Smoking also reduces intracellular and extracellular antioxidant concentrations of ascorbate, beta-carotene and alpha-tocopherol, a form of vitamin E [16]. Acute CS exposure shows a decrease in glutathione peroxidase levels (GSH-Px) but surprisingly, chronic exposure increases glutathione antioxidant activity. This increase, however, is not significant enough to relieve the OS induced [17].

CS is shown to exhaust plasma antioxidants both *in vitro* and *in vivo*. Keeping dietary intake as a control variable among active, passive, and nonsmokers, active smokers were found to have a lower antioxidant capacity (i.e. lower plasma ascorbic acid levels). Both side-stream (second-hand or passive smoke) and mainstream (active or inhaled) smoke decreases antioxidant defenses, particularly in beta-carotene levels [12, 18]. In another study comparing groups of smokers and non-smokers, SOD, catalase, glutathione peroxidase activities were measured in erythrocytes, while vitamin C, E, and beta-carotene levels were monitored in the plasma. Smokers displayed a significant decrease in SOD, catalase and GSH-Px activities, as well as in vitamin C, E, and beta-carotene levels relative to non-smokers [14]. Such compromised antioxidant system may result in OS.

In Hulea et al.'s study, they focused on the effect of age on oxidant and antioxidant levels in smokers and non-smokers. The authors reported that the activity of SOD and GSH-Px was elevated in smokers from the age of 18–45 and diminished in smokers from the age of 46–80. This study reveals important information, particularly for males. It demonstrates that during the ages of 18–45, the antioxidant defenses may be working efficiently, leading to higher activities of SOD and GSH-Px, but as the smokers past the age of 45, the defenses are weakened and the plasma/tissue are under a state of continuous OS [19].

According to the Second National Health and Nutrition Examination Survey, smokers are less likely to consume as many fruits/vegetables as non-smokers and consequently, do not obtain satisfactory levels of these essential vitamins and antioxidants [12]. Furthermore, studies show that dietary antioxidants supplements provide only limited protection to smokers [20, 21]. Thus, over time, the beneficial effect of these supplements is diminished and smokers display declining antioxidant defenses.

Studies have commented on the reverse-effects of smoking cessation on ROS and OS levels. One study measured oxidative damage via values of known pro-oxidant inducers and antioxidant capacity. It explicated that after 1 year of quitting smoking, reformed non-smokers yield values of pro-oxidants and antioxidants that are not significantly different from original non-smokers. This data potentially suggests a promising future health and reproductive capacity in reformed non-smokers [14].

Overall, the association between cigarette smoking and OS is likely exist. Smoking is harmful to the male and female reproductive systems as it interferes with vital processes via OS. CS either attenuates antioxidant defenses or stimulates production of abnormally high amounts of ROS, yielding oxidative damage. Quitting smoking seems to be the most viable option to restore the body's OS and ROS to homeostatic levels.

5.2.1.2 Alcohol

Alcohol consumption is common around the world. Unfortunately, this lifestyle factor causes unfavorable effects on the health of male and female reproductive systems [22]. Studies have shown that hormone levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone are decreased in alcohol consumers. Additionally, sperm parameters are significantly reduced in alcoholics [23, 24]. A study carried out by Maneesh et al. reported that OS was correlated with an increase in lipid peroxidation byproducts and a reduction in antioxidants, and possibly led to a reduction in testosterone level [25].

One way in which alcohol induces OS in male and female reproductive systems is through the production of ROS. ROS is generated in response to alcohol metabolism by the microsomal ethanol-oxidizing system (MEOS). Ethanol (EtOH) metabolism also leads to the formation of NADH; hence, augments the electron transport chain (ETC), which increases oxygen consumption and ROS formation [26–28]. Furthermore, the overproduction of ROS also decreases the activity of the antioxidant superoxide dismutase (SOD) and lowers glutathione (GSH) levels [29].

The OS induced by EtOH metabolism may activate the oxidation steps of the Maillard reaction, which increases the production of advanced glycation end products (AGE). The accumulation of AGE is considered to be toxic [30]. When AGE bind to its receptor (RAGE), it causes an inflammatory state via the transcription factor NF-kappa B activation and subsequently by cytokine expression [31–36]. Alcohol also speeds up the process of OS by increasing apoptosis, altering cell structures and damaging tissue; as well as, stimulates the production of free radicals

from damage to mitochondria in addition to the already weakened antioxidant defense system caused by alcohol consumption [37–39].

Studies show that the production of AGE can be prevented by supplementations derived from vitamin B and/or vitamins A, C and E [40–42]. Unfortunately, most alcohol abusers suffer from diet deficiencies including protective antioxidants [43]. It is agreed among most experts that it is best to avoid more than two drinks a day to prevent the adverse effects of alcohol abuse.

5.3 General Health

5.3.1 Diet/Undernutrition

The United Nations Food and Agriculture Organization approximated 805 million of the 7.3 billion people in the world as chronically undernourished in 2012–2014. The majority of these malnourished individuals reside in underdeveloped and developing countries [44]. Proper management of diet is essential in maintaining the physiological processes and preventing the development of OS and hence the subsequent pathologies.

There are two main types of undernutrition: (1) protein-energy malnutrition, and (2) micronutrient deficiency. Protein malnutrition is considered more detrimental because proteins are essential components of normal physiology. The two kinds of protein-undernutrition are known as marasmus and kwashiorkor. Marasmus is characterized by rapid weight loss and decrease in total energy intake of any of the fundamental macromolecules, while Kwashiorkor's primary onset is in young children usually between the ages of 1–3 and is differentially characterized by edema [44, 45].

Akinola et al., studied the effects of severe malnutrition on OS in rats. They compared rats that were normal (control), marasmus, kwashiorkor and marasmic-kwashiorkor, a more severe combination. The measured SOD activity was much lower in rats with marasmic-kwashiorkor compared to the control group and catalase activity was much greater compared to all other groups. This shows that the antioxidant system has been altered, indicating a potential advent of OS. Because antioxidants are interdependent, if one or few antioxidant enzymes are affected, the whole system could be compromised, multiplying the negative effects [45].

Most of the other studies reported similar results; they established the compromised antioxidant defenses due to poor diet and nutrition. An animal study exhibited that pregnant female Wistar rats that were fed half of their normal diet, ended in producing offspring with decreased SOD activity and a subsequent increase in superoxide anion concentration. This *in vivo* study suggests that intrauterine undernutrition could lead to OS [46]. Another study induced protein malnutrition in maternal rats, the offspring of which had either hypoactive or hyperactive antioxidant enzyme activity in their pancreatic islets (such as SOD). In this case, SOD activity was increased without co-activation of catalase and glutathione peroxidase. This lack of

co-activation could lead to increase in hydrogen peroxide formation. These results demonstrate that early modifications in antioxidant capacity can engender OS later on in life [47].

Additional studies in rats have demonstrated the diminished antioxidant defenses due to poor diet. Maternal protein malnutrition increased xanthine oxidase (XO) expression and LPO in the islets of 15 month-male offspring. Manganese-SOD (Mn-SOD), Copper/Zinc-SOD (Cu/Zn-SOD), and heme-oxygenase-1 (HO-1) activities were reduced [48]. Poor dietary antioxidants intake further weakens the defense system, leaving the body vulnerable to OS [49]. In babies born small for their gestational age via maternal malnourishment, an increase in OS and DNA damage has been reported [50].

One study revealed that plasma concentrations of malondialdehyde-modified low-density lipoproteins (MDA-LDL) were higher in individuals with low body mass index (BMI). Because MDA acts as a biomarker of oxidative damage and LPO in the body, higher MDA indicates greater levels of ROS [50, 51].

In summary, adequate nutrition is required for normal physiological functions. An increase in ROS production, evidenced by high levels of MDA was reported [51]. However, when the body is malnourished, an impaired antioxidant defenses is the root cause of OS.

5.3.2 Obesity/Overnutrition

Obesity also plays a significant role in inducing OS in the reproductive system. Obesity reduces the levels of circulating testosterone and increases estradiol levels. Furthermore, obesity can cause infertility by decreasing sperm concentrations and increasing DNA damage [52–54]. Usually, diet goes hand-in-hand with obesity, especially a high-energy diet (HED) with high amounts of fats and carbohydrates. HED can contribute to OS in the testicular environment [55].

Metabolic syndrome (MS) is a condition that is closely linked to obesity. There are several mechanisms by which MS causes infertility: (1) the excess adipose tissue results in the conversion of testosterone to estrogen; consequently, leading to the development of secondary hypogonadism through the inhibition of the hypothalamic-pituitary-gonadal axis [56], (2) in severely obese subjects, there is an accumulation of suprapubic and inner thigh fat which can lead to increase in scrotal temperature [57], and (3) MS is also associated with systemic pro-inflammatory states and thus increased OS in both males and females [58].

Even though the rate of beta-oxidation increases directly with HED [59], testicular mitochondria may not be capable of oxidizing all the fatty acids at the same rate as the consumption; causing an energy overload and leading to mitochondrial stress. The resultant mitochondrial stress disturbs the regular functions of the testicular ETC. As mitochondria are the major producers of ROS, mitochondrial stress predisposes mitochondria to trigger a cascade of oxidative damage to the testicular environment [55]. A recent study reported that there was a decoupling in the activities

of the mitochondrial complexes I and III in the testis of rats that were on HED [55]. These decoupling of activities of both complexes reverses the electron flow, disrupting the ETC functions and lowering the levels of adenosine triphosphate (ATP) production. This disturbance leads to the deficiency of energy production and ROS overproduction. What makes these events even more germane is the fact that mitochondria are essential for proper sperm functions [60]. HED-induced OS is accompanied by decreased and damaged mitochondrial DNA (mtDNA) [55]; this is crucial since mtDNA codes for proteins that are essential for oxidative phosphorylation, and also because mtDNA is very susceptible to OS. Mutations intensify the effect of impaired ETC leading to more ROS production [55]. This also applies to the female reproductive system, where an accumulation in intracellular fat can disturb mitochondrial functions and potentially damage the mitochondria; hence, disturbing the ETC leading to a deficit in ATP production. This is crucial since the mitochondria in oocytes are important for normal embryonic metabolism [61].

Another mechanism as to how HED induces OS was found in the same study, where HED affects the testicular antioxidant defense system by limiting the testicular antioxidant capacity, and decreasing the expression of ROS-detoxifying enzymes peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α) and sirtuin 3 (SIRT 3) [55]. PGC-1α regulates OS regulatory pathways to activate ROS-detoxifying enzymes in stress conditions, and this is all facilitated by the action of SIRT 3 [62].

Generally, obesity induces OS due to the fact that adipose tissue releases pro-inflammatory cytokines, which promote leukocytes production of ROS [63]. The OS causes lipid peroxidation, DNA damage and ineffective sperm-oocyte interaction [64, 65]. Furthermore, the eating habits associated with obesity such as HED induces the release of ROS from over-stressed mitochondria and impedes ROS-detoxifying pathways, leading up to OS that damages mtDNA and henceforth sperm parameter and function. These adverse effects also, to a degree, affect the female reproductive system, where mitochondrial damage is vital to oocytes instead.

5.3.3 Psychological Stress

Stress is a major part of every person's life, and is stimulated by various sources that differ from one person to another. There are studies that demonstrate mental stress causes OS, as it is associated with lower antioxidant levels such as GSH and SOD, and higher levels of pro-oxidants. Overall, stress is linked to increase in ROS in the seminal plasma and impaired sperm quality, specifically in males. Similarly, stress can trigger OS in the female reproductive system, impairing oocyte development [66, 67].

Other studies show that stress and depression are associated with infertility since they are thought to reduce testosterone and LH levels in the blood; disturbing gonadal functions and leading to reduced spermatogenesis and sperm parameters [68, 69]. However, it is still unclear whether stress and depression are the causes of low testosterone levels or vice versa, despite the link between the two [70].

5.3.4 Medications

Medications have profound effects on our body, operating through a vast network of various signaling pathways. There are many proposed mechanistic effects of medicines on ROS and OS. Bactericidal antibiotics can overproduce ROS and lead to mitochondrial dysfunction in mammal cells. Conversely, studies on bacteriostatic agents, like tetracycline, indicate no significant increase in ROS production [71].

Anticancer drugs, like doxorubicin (DOX) and cisplatin, display rather elaborate mechanisms for ROS production. Cisplatin and DOX are both known to diminish antioxidant reserves [72]. DOX has a particular affinity for the mitochondria and thus, accumulates there. Ordinary DOX, or quinone DOX, in the presence of NADH, converts to semiquinone DOX via one-electron reduction. Semiquinone DOX, in turn, reacts with O_2 to form superoxide radicals and reproduce quinone DOX [73]. Further processing of the abundant superoxide radical can lead to formation of hydrogen peroxide and other free radicals. DOX also causes accumulation of intracellular iron, which generates harmful hydroxyl radicals via Fenton reaction. Thus, DOX-induced propagation of the superoxide radical and regeneration of quinone DOX can greatly enhance OS [74].

Non-steroidal anti-inflammatory drugs (NSAIDs) can also induce LPO and mitochondrial damage. Sulindac sulfide, a specific NSAID, increase levels of ROS biomarkers [75]. Acetaminophen in mice decreased catalase and GSH activity, allowing physiological levels of H_2O_2 and hydroperoxides to become more potent and caused cellular damage. This is evidenced by reported damage of mice hepatocytes in the study [76, 77]. A recent study reported that co-intake of DOX and taxane induces OS, demonstrating that co-administration of multiple drugs could possibly have greater detrimental effects [78].

Although many of the studies indicate significant effects of on causing OS, further research is required on distinct classes of these drugs in order to create a more complete picture in term of their underlying mechanism in inducing OS.

5.4 Environment

5.4.1 Environmental Pollution

5.4.1.1 General Pollutants

Environmental pollutants are prevalent, on both lands and seas. Many of the toxic substances that we are exposed to originate from human activities and a few from natural disasters [79]. Examples of human sources of pollutants include industrial discharges and products, mining, petroleum refining, smelting, plastic manufacturing, and cosmetics [79, 80]. Specific pollutants include nitric oxide, sulfur dioxide, carbon tetrachloride, ozone, wood dust, particulate matter (PM), volatile organic compounds (VOC), bisphenol A (BPA), xenoestrogens, and phthalates [79, 81–83].

Xenoestrogens are steroid-mimetic chemicals that target the mitochondria, inciting DNA damage and organelle dysfunction. One review correlated this mitochondrial damage to increases in ROS levels [79]. BPA, a chemical found in plastics, is reported to increase OS. Exposure to BPA throughout embryonic and neonatal stages in mice also showed increases in tissue peroxidation, but strikingly also displayed increases in antioxidant enzyme activity [84]. In a study with mature male mice, BPA diminished the antioxidant protective enzymes and LPO remained high [85]. This suggests that exposure to BPA possibly triggered adaptive mechanisms in some mice, leading to a greater antioxidant expression [84, 86]. A third study focused on the DJ-1 gene, which is involved in oxidative protection and ROS elimination, and its relation to BPA exposure. As BPA concentrations rose, DJ-1 activity lessened and cells began to die. This indicates that BPA triggers hydroxyl radical production, overwhelming DJ-1 and other protective mechanisms [87].

Carbon tetrachloride, or CCl_4, found in ambient air, refrigerants, oil solvents, and aerosols, is a substance that binds to lipids and proteins and induces peroxidation by reacting with oxygen to form CCl_4-related peroxy free radical (CCl_3OO) [88]. VOC, from fuel burning, solvents, paints, and glues, form hydroxyl radicals and increase ROS levels [89, 90]. These ROS incite LPO and diminish GSH activity [81, 91]. Ozone initiates OS in the cell via ozonide and hydrogen peroxide radical formations [89]. Concentrated ambient particles (CAP) doubled ROS levels from steady-state conditions in rat lung and heart, by Fenton reactions or by altering redox mechanisms. CAP, like BPA, increases SOD activity, indicating adaptive functioning [86].

When mice were exposed to mono-(2-ethylhexyl) and di-(2-methylhexyl) phthalates, chemicals found in plastics, antioxidant mechanisms were inhibited and follicle growth was stunted. After providing antioxidant supplements, follicle growth returned [92]. Another study found that di-(2-methylhexyl) phthalates induced accumulation of superoxide radicals due to diminished SOD1 expression [93]. These studies surmise that phthalates overproduce ROS.

Particulates (PM), including wood dust and secondary particles like sulfur and nitrous oxides, also induce OS. Wood dust, primarily affecting carpenters, has ROS-stimulatory effects and shows reduction of glutathione function [94]; it is also reported to increase DNA damage and chromosomal aberrations.

Sulfur dioxide (SO_2), originates from fossil fuel combustion, can also damage DNA. One proposed mechanism is the conversion of SO_2 derivatives, sulfite and bisulfite, into peroxyl radicals after reacting with O_2. These radicals proliferate LPO [83]. Benzene similarly induced oxidative DNA damage. Using OS biomarkers, researchers showed that PM increased MDA levels, a byproduct of peroxidation, suggesting oxidative damage to lipids/proteins in the plasma [95].

Exposure to heavy metals such as lead (Pb), cadmium (Cd), zinc (Zn), chromium (Cr), and iron (Fe) can lead to metal toxicity and OS. Cadmium displaces zinc, preventing zinc from scavenging free radicals. In a similar study, cadmium in cigarettes, reduced the bioavailability of selenium, a vital element in the antioxidant mechanism [12]. Iron toxicity generates free radicals. Similarly, lead is able to replace vital bivalent cations, increasing ROS and decreasing antioxidant forces. When chromium (III) is oxidized in excess atmospheric oxygen to chromium (VI),

it becomes a stronger oxidizing agent and soluble/mobile in water, reacting with thiols to produce high levels of ROS [96].

Overall, many of these pollutants seem to either systemically upregulate ROS production or downregulate antioxidant protective mechanisms, engendering OS. However, future studies are required in order to determine the actual effect of elevated OS on physiological functions.

5.4.1.2 Pesticides

Pesticides are frequently used worldwide for the protection of crops. Ironically, there are some pesticides that have been found to be harmful, specifically to the reproductive systems of both men and women. Studies have shown that pesticides such as lindane, methoxychlorate and dioxin-TCDD have been linked to testicular OS in rats and mice [97–99]. However, carbendazim, a broad-spectrum fungicide, has been reported to have harmful effects in rodents as well as humans, altering sperm parameters, testicular weight and seminiferous tubule diameters [100, 101]. One of the studies done on rats showed that carbendazim was capable of inducing OS, causing harmful effects on the male reproductive system. Damaged steroidogenic enzymes and antioxidant defenses can engender OS. Carbendazim also increases hydrogen peroxide radicals and lipid peroxidation in Leydig cells [102].

Regarding organophosphate pesticides, studies have shown decreased activity and levels of antioxidant enzymes in combination with increased lipid peroxidation [103]. The extent of damage caused by organophosphate pesticides or compounds depends on the amount and length of exposure. A decrease in GSH with increased ROS generation is what triggers the OS. Chlorpyrifos is an example of an organophosphate pesticide that can lead to lipid peroxidation and DNA strand breaks induced by ROS [104].

5.4.1.3 Radiation

Cell-Phone Radiation

Most people are aware of the potentially harmful effects of solar radiation, but radiation from a mobile device, such as cell-phone, can also increase ROS. In males, almost exclusively, studies have linked cell-phone radiation or radio-frequency electromagnetic radiation (RF-EMR) to OS.

One study on male Wistar rats exposed to EMR displayed decreases in glutathione and SOD activity and increases in catalase activity and MDA levels [105]. In another study of human spermatozoa, RF-EMR incited mitochondrial ROS generation and increased DNA damage. Researchers believed that the radiation caused electron seepage and subsequently led to ROS production [106]. Long term mobile phone radiation-exposure exhibited significant decreases in antioxidant capacity and increases in NADH oxidase activity, possibly stimulating ROS [107].

In summary, cell-phone radiation shows a fairly strong correlation with ROS production and the onset of OS in males. This prompts understanding of the effect of RF-EMR on female reproductive cells/organs and OS, but this idea has currently not been explored.

5.4.1.4 High Altitude

Studies have indicated that high altitude environments, areas containing lower amounts of molecular oxygen, may cause changes in ROS levels in the body, leading to OS [108].

One study in rats demonstrated that sporadic exposure to 4000 m altitude increased LPO in both the slow and fast twitch muscle fibers. Over a period of four weeks, LPO did not vary significantly, but protein oxidation levels remained high [109]. Five-day exposure to 7576 m altitudes in rats, showed marked increase in LPO as well [110]. A similar study comparing people in Peru residing at sea level (control) and at 4300 m areas, reported OS with acute and chronic residence at high-altitudes [111].

Conversely, the following studies did not indicate significant presence of OS. Sheep were exposed to high-altitude environments and developed complications in their corpus luteum. They were later provided vitamin supplements to study possible OS formation. The supplements proved ineffective and reported levels of OS biomarkers were low, labeling hypoxia, and not OS, as the main contributor to luteal dysfunction in the sheep [112]. Humans exposed to 4500 m altitude strikingly displayed increased antioxidant activities (rise of glutathione and SOD activity), indicating adaptive functioning.

Based on these varying results, the effect of high altitude on OS remains elusive. Additional research in animal exposure to high altitudes and corresponding studies in human populations living in high-altitude or relocating from low to high altitude areas could be insightful.

References

1. Tremellen K (2008) Oxidative stress and male infertility – a clinical perspective. Hum Reprod Update 14(3):243–258
2. Agarwal A et al (2014) Mechanisms of oligozoospermia: an oxidative stress perspective. Syst Biol Reprod Med 60(4):206–216
3. Rizzo A et al (2012) Roles of reactive oxygen species in female reproduction. Reprod Domest Anim 47(2):344–352
4. Centers for Disease Control and Prevention (US), National Center for Chronic Disease Prevention and Health Promotion (US), Office on Smoking and Health (US) (2010) How tobacco smoke causes disease: The biology and behavioral basis for smoking-attributable disease: a report of the surgeon general
5. Valavanidis A, Vlachogianni T, Fiotakis K (2009) Tobacco smoke: involvement of reactive oxygen species and stable free radicals in mechanisms of oxidative damage, carcinogenesis

and synergistic effects with other respirable particles. Int J Environ Res Public Health 6(2):445–462
6. Mai Z et al (2014) The effects of cigarette smoke extract on ovulation, oocyte morphology and ovarian gene expression in mice. PLoS One 9(4), e95945
7. Ashraf MW (2012) Levels of heavy metals in popular cigarette brands and exposure to these metals via smoking. Scientific World Journal 2012
8. Gannon AM, Stämpfli MR, Foster WG (2013) Cigarette smoke exposure elicits increased autophagy and dysregulation of mitochondrial dynamics in murine granulosa cells. Biol Reprod 88(3):63
9. Gannon AM, Stämpfli MR, Foster WG (2011) Cigarette smoke exposure leads to follicle loss via an alternative ovarian cell death pathway in a mouse model. Toxicol Sci 2011:kfr279
10. Augood C, Duckitt K, Templeton A (1998) Smoking and female infertility: a systematic review and meta-analysis. Hum Reprod 13(6):1532–1539
11. Jennings PC et al (2011) Increased zona pellucida thickness and meiotic spindle disruption in oocytes from cigarette smoking mice. Human Reprod. p deq393
12. Preston A (1990) Cigarette smoking-nutritional implications. Prog Food Nutr Sci 15(4):183–217
13. Van der Vaart H et al (2004) Acute effects of cigarette smoke on inflammation and oxidative stress: a review. Thorax 59(8):713–721
14. Zhou J et al (2000) Effects of cigarette smoking and smoking cessation on plasma constituents and enzyme activities related to oxidative stress. Biomed Environ Sci 13(1):44–55
15. Bridges A et al (1993) Age, sex, cigarette smoking and indices of free radical activity in healthy humans. Eur J Med 2(4):205–208
16. Ghanem ENB et al (2015) The α-tocopherol form of vitamin e reverses age-associated susceptibility to Streptococcus pneumoniae lung infection by modulating pulmonary neutrophil recruitment. J Immunol 194(3):1090–1099
17. Aoshiba K, Nagai A (2003) Oxidative stress, cell death, and other damage to alveolar epithelial cells induced by cigarette smoke. Tob Induc Dis 1(3):1–8
18. Dietrich M et al (2003) Smoking and exposure to environmental tobacco smoke decrease some plasma antioxidants and increase gamma-tocopherol in vivo after adjustment for dietary antioxidant intakes. Am J Clin Nutr 77(1):160–166
19. Hulea S et al (1994) Cigarette smoking causes biochemical changes in blood that are suggestive of oxidative stress: a case-control study. J Environ Pathol Toxicol Oncol 14(3–4):173–180
20. Priéme H et al (1997) No effect of supplementation with vitamin E, ascorbic acid, or coenzyme Q10 on oxidative DNA damage estimated by 8-oxo-7, 8-dihydro-2'-deoxyguanosine excretion in smokers. Am J Clin Nutr 65(2):503–507
21. Charles HH et al (1996) Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. N Engl J Med 334(18):1145–1149
22. Boyden TW, Pamenter RW (1983) Effects of ethanol on the male hypothalamic-pituitary-gonadal axis. Endocr Rev 4(4):389–395
23. Muthusami KR, Chinnaswamy P (2005) Effect of chronic alcoholism on male fertility hormones and semen quality. Fertil Steril 84(4):919–924
24. Goverde HJ et al (1995) Semen quality and frequency of smoking and alcohol consumption – an explorative study. Int J Fertil Menopausal Stud 40(3):135–138
25. Maneesh M et al (2006) Alcohol abuse-duration dependent decrease in plasma testosterone and antioxidants in males. Indian J Physiol Pharmacol 50(3):291–296
26. Agarwal A, Prabakaran SA (2005) Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. Indian J Exp Biol 43(11):963–974
27. Lieber CS (2004) The discovery of the microsomal ethanol oxidizing system and its physiologic and pathologic role. Drug Metab Rev 36(3–4):511–529
28. Dahchour A et al (2005) Production of reactive oxygen species following acute ethanol or acetaldehyde and its reduction by acamprosate in chronically alcoholized rats. Eur J Pharmacol 520(1–3):51–58

29. Kovacic P (2005) Unifying mechanism for addiction and toxicity of abused drugs with application to dopamine and glutamate mediators: electron transfer and reactive oxygen species. Med Hypotheses 65(1):90–96
30. Spiteller G (2008) Peroxyl radicals are essential reagents in the oxidation steps of the Maillard reaction leading to generation of advanced glycation end products. Ann N Y Acad Sci 1126:128–133
31. Yan SD et al (1994) Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. J Biol Chem 269(13):9889–9897
32. Bierhaus A et al (1998) AGEs and their interaction with AGE-receptors in vascular disease and diabetes mellitus. I. The AGE concept. Cardiovasc Res 37(3):586–600
33. Kislinger T et al (1999) N(epsilon)-(carboxymethyl)lysine adducts of proteins are ligands for receptor for advanced glycation end products that activate cell signaling pathways and modulate gene expression. J Biol Chem 274(44):31740–31749
34. Basta G et al (2002) Advanced glycation end products activate endothelium through signal-transduction receptor RAGE: a mechanism for amplification of inflammatory responses. Circulation 105(7):816–822
35. Haslbeck KM et al (2007) Activation of the RAGE pathway: a general mechanism in the pathogenesis of polyneuropathies? Neurol Res 29(1):103–110
36. Kovacic P, Somanathan R (2011) Cell signaling and receptors in toxicity of advanced glycation end products (AGEs): alpha-dicarbonyls, radicals, oxidative stress and antioxidants. J Recept Signal Transduct Res 31(5):332–339
37. Sun AY et al (2001) Ethanol and oxidative stress. Alcohol Clin Exp Res 25(5 Suppl ISBRA):237S–243S
38. Alikhani M et al (2007) Advanced glycation end products induce apoptosis in fibroblasts through activation of ROS, MAP kinases, and the FOXO1 transcription factor. Am J Physiol Cell Physiol 292(2):C850–C856
39. Chen BH, Jiang DY, Tang LS (2006) Advanced glycation end-products induce apoptosis involving the signaling pathways of oxidative stress in bovine retinal pericytes. Life Sci 79(11):1040–1048
40. Khalifah RG, Baynes JW, Hudson BG (1999) Amadorins: novel post-Amadori inhibitors of advanced glycation reactions. Biochem Biophys Res Commun 257(2):251–258
41. Stracke H et al (2001) Efficacy of benfotiamine versus thiamine on function and glycation products of peripheral nerves in diabetic rats. Exp Clin Endocrinol Diabetes 109(6):330–336
42. Miyata T, Kurokawa K, van Ypersele de Strihou C (2000) Relevance of oxidative and carbonyl stress to long-term uremic complications. Kidney Int Suppl 76:S120–S125
43. Koch OR et al (2004) Oxidative stress and antioxidant defenses in ethanol-induced cell injury. Mol Aspects Med 25(1–2):191–198
44. (2015) World hunger and poverty facts and statistics. Accessed 1 July 2015 from http://www.worldhunger.org/articles/Learn/world hunger facts 2002.htm
45. Akinola F, Oguntibeju O, Alabi O (2010) Effects of severe malnutrition on oxidative stress in Wistar rats. Sci Res Essays 5(10):1145–1149
46. Franco Mdo C et al (2002) Enhanced oxidative stress as a potential mechanism underlying the programming of hypertension in utero. J Cardiovasc Pharmacol 40(4):501–509
47. Theys N et al (2009) Early low protein diet aggravates unbalance between antioxidant enzymes leading to islet dysfunction. PLoS One 4(7), e6110
48. Tarry-Adkins JL et al (2010) Poor maternal nutrition leads to alterations in oxidative stress, antioxidant defense capacity, and markers of fibrosis in rat islets: potential underlying mechanisms for development of the diabetic phenotype in later life. FASEB J 24(8):2762–2771
49. Sies H, Stahl W, Sevanian A (2005) Nutritional, dietary and postprandial oxidative stress. J Nutr 135(5):969–972
50. Gupta P et al (2004) Oxidative stress in term small for gestational age neonates born to undernourished mothers: a case control study. BMC Pediatr 4:14
51. Higashi Y et al (2003) Low body mass index is a risk factor for impaired endothelium-dependent vasodilation in humans: role of nitric oxide and oxidative stress. J Am Coll Cardiol 42(2):256–263

52. Nguyen RH et al (2007) Men's body mass index and infertility. Hum Reprod 22(9):2488–2493
53. Koloszar S et al (2005) Effect of body weight on sperm concentration in normozoospermic males. Arch Androl 51(4):299–304
54. Kort HI et al (2006) Impact of body mass index values on sperm quantity and quality. J Androl 27(3):450–452
55. Rato L et al (2014) Pre-diabetes alters testicular PGC1-alpha/SIRT3 axis modulating mitochondrial bioenergetics and oxidative stress. Biochim Biophys Acta 1837(3):335–344
56. Fejes I et al (2006) Effect of body weight on testosterone/estradiol ratio in oligozoospermic patients. Arch Androl 52(2):97–102
57. Dandona P et al (2005) Metabolic syndrome: a comprehensive perspective based on interactions between obesity, diabetes, and inflammation. Circulation 111(11):1448–1454
58. Davi G, Falco A (2005) Oxidant stress, inflammation and atherogenesis. Lupus 14(9):760–764
59. Chanseaume E et al (2007) Chronological approach of diet-induced alterations in muscle mitochondrial functions in rats. Obesity (Silver Spring) 15(1):50–59
60. Bajpai M, Gupta G, Setty BS (1998) Changes in carbohydrate metabolism of testicular germ cells during meiosis in the rat. Eur J Endocrinol 138(3):322–327
61. Bloomer RJ, Fisher-Wellman KH (2009) Systemic oxidative stress is increased to a greater degree in young, obese women following consumption of a high fat meal. Oxid Med Cell Longev 2(1):19–25
62. Kong X et al (2010) Sirtuin 3, a new target of PGC-1alpha, plays an important role in the suppression of ROS and mitochondrial biogenesis. PLoS One 5(7), e11707
63. Singer G, Granger DN (2007) Inflammatory responses underlying the microvascular dysfunction associated with obesity and insulin resistance. Microcirculation 14(4–5):375–387
64. Kodama H et al (1997) Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. Fertil Steril 68(3):519–524
65. Twigg J et al (1998) Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. Hum Reprod 13(6):1429–1436
66. Eskiocak S et al (2005) Association between mental stress & some antioxidant enzymes of seminal plasma. Indian J Med Res 122(6):491–496
67. Eskiocak S et al (2006) Effect of psychological stress on the L-arginine-nitric oxide pathway and semen quality. Braz J Med Biol Res 39(5):581–588
68. Gollenberg AL et al (2010) Semen quality in fertile men in relation to psychosocial stress. Fertil Steril 93(4):1104–1111
69. Schweiger U et al (1999) Testosterone, gonadotropin, and cortisol secretion in male patients with major depression. Psychosom Med 61(3):292–296
70. Hall E, Burt VK (2012) Male fertility: psychiatric considerations. Fertil Steril 97(2):434–439
71. Kalghatgi S et al (2013) Bactericidal antibiotics induce mitochondrial dysfunction and oxidative damage in Mammalian cells. Sci Transl Med 5(192):192ra85
72. Santos NA et al (2007) Cisplatin-induced nephrotoxicity is associated with oxidative stress, redox state unbalance, impairment of energetic metabolism and apoptosis in rat kidney mitochondria. Arch Toxicol 81(7):495–504
73. Chen Y et al (2007) Collateral damage in cancer chemotherapy: oxidative stress in nontargeted tissues. Mol Interv 7(3):147–156
74. Deavall DG et al (2012) Drug-induced oxidative stress and toxicity. J Toxicol 2012:645460
75. Adachi M et al (2007) Nonsteroidal anti-inflammatory drugs and oxidative stress in cancer cells. Histol Histopathol 22(4):437–442
76. Lores Arnaiz S et al (1995) Oxidative stress by acute acetaminophen administration in mouse liver. Free Radic Biol Med 19(3):303–310
77. Reid AB et al (2005) Mechanisms of acetaminophen-induced hepatotoxicity: role of oxidative stress and mitochondrial permeability transition in freshly isolated mouse hepatocytes. J Pharmacol Exp Ther 312(2):509–516
78. Pieniazek A et al (2013) Oxidative stress induced in rat liver by anticancer drugs doxorubicin, paclitaxel and docetaxel. Adv Med Sci 58(1):104–111

79. Al-Gubory KH (2014) Environmental pollutants and lifestyle factors induce oxidative stress and poor prenatal development. Reprod Biomed Online 29(1):17–31
80. Miller MR, Shaw CA, Langrish JP (2012) From particles to patients: oxidative stress and the cardiovascular effects of air pollution. Future Cardiol 8(4):577–602
81. Simeonova R et al (2014) Some in vitro/in vivo chemically-induced experimental models of liver oxidative stress in rats. Biomed Res Int 2014:706302
82. Farahat SA, Ibrahim YH, Abdel-Latif MN (2010) Genotoxicity and oxidative stress due to exposure to wood dust among carpenters. Egypt J Occup Med 34(1):83–95
83. Meng Z et al (2004) DNA damaging effects of sulfur dioxide derivatives in cells from various organs of mice. Mutagenesis 19(6):465–468
84. Kabuto H, Amakawa M, Shishibori T (2004) Exposure to bisphenol A during embryonic/fetal life and infancy increases oxidative injury and causes underdevelopment of the brain and testis in mice. Life Sci 74(24):2931–2940
85. Chitra KC, Latchoumycandane C, Mathur PP (2003) Induction of oxidative stress by bisphenol A in the epididymal sperm of rats. Toxicology 185(1–2):119–127
86. Gurgueira SA et al (2002) Rapid increases in the steady-state concentration of reactive oxygen species in the lungs and heart after particulate air pollution inhalation. Environ Health Perspect 110(8):749–755
87. Ooe H et al (2005) Induction of reactive oxygen species by bisphenol A and abrogation of bisphenol A-induced cell injury by DJ-1. Toxicol Sci 88(1):114–126
88. Boll M et al (2001) Mechanism of carbon tetrachloride-induced hepatotoxicity. Hepatocellular damage by reactive carbon tetrachloride metabolites. Z Naturforsch C 56(7–8):649–659
89. Lodovici M, Bigagli E (2011) Oxidative stress and air pollution exposure. J Toxicol 2011:487074
90. Kjellstrom T et al (2006) Air and water pollution: burden and strategies for control. In: Jamison DT et al (eds) Disease control priorities in developing countries. World Bank, The International Bank for Reconstruction and Development/The World Bank Group, Washington, DC
91. Ranjbar A et al (2014) Propofol attenuates toxic oxidative stress by CCl4 in liver mitochondria and blood in rat. Iran J Pharm Res 13(1):253–262
92. Kay VR, Chambers C, Foster WG (2013) Reproductive and developmental effects of phthalate diesters in females. Crit Rev Toxicol 43(3):200–219
93. Wang W et al (2012) Mono-(2-ethylhexyl) phthalate induces oxidative stress and inhibits growth of mouse ovarian antral follicles. Biol Reprod 87(6):152
94. Aitken J, Fisher H (1994) Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. Bioessays 16(4):259–267
95. Sorensen M et al (2003) Linking exposure to environmental pollutants with biological effects. Mutat Res 544(2–3):255–271
96. Jaishankar M et al (2014) Toxicity, mechanism and health effects of some heavy metals. Interdiscip Toxicol 7(2):60–72
97. Chitra KC et al (2001) Effect of lindane on antioxidant enzymes in epididymis and epididymal sperm of adult rats. Asian J Androl 3(3):205–208
98. Latchoumycandane C, Chitra KC, Mathur PP (2003) 2,3,7,8-tetrachlorodibenzo- p-dioxin (TCDD) induces oxidative stress in the epididymis and epididymal sperm of adult rats. Arch Toxicol 77(5):280–284
99. Latchoumycandane C, Mathur PP (2002) Induction of oxidative stress in the rat testis after short-term exposure to the organochlorine pesticide methoxychlor. Arch Toxicol 76(12):692–698
100. Carter SD, Hess RA, Laskey JW (1987) The fungicide methyl 2-benzimidazole carbamate causes infertility in male Sprague-Dawley rats. Biol Reprod 37(3):709–717
101. Akbarsha MA et al (2001) Spermatotoxic effect of carbendazim. Indian J Exp Biol 39(9):921–924
102. Rajeswary S et al (2007) Modulation of antioxidant defense system by the environmental fungicide carbendazim in Leydig cells of rats. Reprod Toxicol 24(3–4):371–380

103. Julka D, Pal R, Gill KD (1992) Neurotoxicity of dichlorvos: effect on antioxidant defense system in the rat central nervous system. Exp Mol Pathol 56(2):144–152
104. Kovacic P, Pozos RS (2006) Cell signaling (mechanism and reproductive toxicity): redox chains, radicals, electrons, relays, conduit, electrochemistry, and other medical implications. Birth Defects Res C Embryo Today 78(4):333–344
105. Kesari KK, Kumar S, Behari J (2011) Effects of radiofrequency electromagnetic wave exposure from cellular phones on the reproductive pattern in male Wistar rats. Appl Biochem Biotechnol 164(4):546–559
106. De Iuliis GN et al (2009) Mobile phone radiation induces reactive oxygen species production and DNA damage in human spermatozoa in vitro. PLoS One 4(7), e6446
107. Desai NR, Kesari KK, Agarwal A (2009) Pathophysiology of cell phone radiation: oxidative stress and carcinogenesis with focus on male reproductive system. Reprod Biol Endocrinol 7:114
108. Radak Z et al (2014) The effects of high-altitude exposure on reactive oxygen and nitrogen species. In: Laher I (ed) Systems biology of free radicals and antioxidants. Springer, Berlin, pp 407–416
109. Bakonyi T, Radak Z (2004) High altitude and free radicals. J Sports Sci Med 3(2):64–69
110. Kumar D et al (1999) Biochemical and immunological changes on oral glutamate feeding in male albino rats. Int J Biometeorol 42(4):201–204
111. Jefferson JA et al (2004) Increased oxidative stress following acute and chronic high altitude exposure. High Alt Med Biol 5(1):61–69
112. Parraguez VH et al (2013) Fertility in a high-altitude environment is compromised by luteal dysfunction: the relative roles of hypoxia and oxidative stress. Reprod Biol Endocrinol 11:24

Chapter 6
Pathological Roles of Oxidative Stress (OS) in Diseases Related to Female Reproductive System

Sajal Gupta, Gulfam Ahmad, My Tran, Ghada Al Hayaza, and Zeina Kayali

6.1 Introduction

Nowadays, approximately 14 % of women in reproductive age have difficulties to conceive in the United States [1]. Several strategies have been utilized to overcome infertility, including assisted reproductive technology (ART). Although *in vitro* fertilization (IVF) is still the most commonly used technique, the number of ART cycles involving intracytoplasmic sperm injection (ICSI) has markedly increased [2].

It has been recognized that environmental and lifestyle factors modulate the couple's fertility status through a series of known and unknown mechanisms, but oxidative stress (OS) seems to play a major intermediary. Interestingly, the ovaries, the testes, and the uterus are among those organs with the highest amount of mitochondria in the human body. On the one hand, this is needed because of the high requirement of energy production via ATP. On the other hand, it makes these organs highly susceptible to elevated levels of reactive oxygen species (ROS). An increase in ROS production can be mainly due to mitochondrial dysfunction. OS and endoplasmic reticulum stress (ER stress) can cause cell death or cell dysfunction. ER stress is involved in protein misfolding, whereas OS is involved in several physiological pathways. ROS in optimal levels are important in the ovaries to modulate luteal and

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granulosa cells function. However, elevated OS is associated with ovarian aging. Furthermore, high levels of ROS in the peritoneal cavity and fallopian tubes makes the latter inappropriate environments for oocytes, spermatozoa and embryos, thus affecting fertilization and implantation [3]. It has been suggested that excessive OS in the second trimester pregnancy is associated with fetal growth restriction [4, 5]. Despite the aforementioned association between OS and female infertility, further research is needed to elucidate its clinical implications [6].

In this chapter, the current knowledge about the role of excessive levels of ROS in the female reproductive system is discussed.

6.2 Polycystic Ovarian Syndrome (PCOS)

Polycystic ovarian syndrome (PCOS) is one of the most common endocrine abnormalities, affecting 18 % of women of reproductive age. PCOS is characterized by hyperandrogenism and ovulatory dysfunction [3]. The clinical presentation includes menstrual and skin disorders, central adiposity, insulin resistance and endothelial dysfunction. Increased levels of ROS seem to aggravate this condition [3, 7, 8].

Patients with PCOS exhibit mitochondrial dysfunction that leads to high ROS production and low oxygen consumption and glutathione (GSH) levels. The decreased antioxidant capacity further aggravates oxidative stress (OS), thus creating a state of inflammation. In hyperglycemic patients, the mononuclear cells produce ROS that is followed by an increase in transcription factors such as TNF-α and NF-κβ. This results in insulin resistance and further increases OS [9]. Lifestyle modifications have been postulated to be critical to reduce OS in PCOS patients, particularly those exhibiting hyperglycemia [3].

PCOS is considered as a spectrum of metabolic and endocrine abnormalities. Infertility treatment in such patients remains a challenge, even with the available options such as IVF and embryo transfer (ET) [10]. High miscarriage and low conception rates have been reported in obese PCOS patients [11]. Incompetent oocytes, higher intra-follicular androgen levels and low fertilization rates have also been observed in PCOS patients undergoing ART [12].

Since oocyte quality is critical to embryo development, it has been suggested that oocyte spindle imaging could be used to determine oocyte quality [13–16]. Oocytes with a visible spindle in Polscope microscopy seem to develop better than counterparts with no or less visible spindle [16–18]. In one study involving PCOS patients subjected to IVF, Chattopadhayay et al. explored the effect of OS in the follicular fluid (FF) on oocytes' meiotic spindle (MS) formation to predict the embryo quality. It was found that the OS markers were much higher while MS formation was much lower in PCOS women than women with tubal infertility; and poorer quality embryos were found in the same group even though not statistically significant. On the other hand, Rajini et al. examined MS in women with endometriosis, PCOS and tubal blockage; and they found that MS was observed the lowest in the PCOS group. Also within each group, oocytes with spindle visualization produced a higher number of Grade 1 embryos and lower ROS levels in FF [19]. These study suggested

that high ROS and low number of oocytes with visualized may contribute to the etiopathogenesis of infertility in women with PCOS.

The follicular fluid contains neutrophils and macrophages, which produces ROS and cytokines. Despite being needed at low levels for oocyte and embryo competence, high amounts of ROS has shown to exert toxicity [15]. It has been suggested that good quality embryos were obtained in environments containing <100 cps (counted photons per second) ROS [20].

In the second trimester of both normal and complicated pregnancies OS can be associated with fetal growth restriction and retardation [4, 5]. In PCOS women advanced glycation end products (AGEs) and advanced oxidation protein products (AOPP), which are the markers of OS, are elevated. Advanced oxidation protein products are increased in women with PCOS [21].

AGEs enhance the oxidative state by transcription factors, which increase ROS levels and deplete antioxidants [22]. Offspring of women diagnosed with PCOS may show growth disorders, either large or small for gestational age. Cytokines production is stimulated by AGEs that may cause disruption in placentation [23].

The association between hyperandrogenism seen in PCOS women and OS has also been reported. Excessive OS was correlated with insulin resistance, high blood pressure and BMI [24].

Excessive OS can be due to the increased number of leukocytes, increased expression of p47 (phox) gene and increased plasma thiobarbituric acid-reactive substances. Furthermore, the antioxidants glutathione and haptoglobin are decreased in females diagnosed with PCOS [22].

The first line of defense against ROS is superoxide dismutase (SOD), which catalyzes the conversion of superoxide to hydrogen peroxide (H_2O_2). For normal cell function, a balance between superoxide and hydrogen peroxide is needed, thus it is essential that optimal levels of SOD are achieved in the FF. However, H_2O_2 should be removed as soon as possible since it reacts with superoxide radicals resulting in highly reactive hydroxyl radicals. Accumulation of hydrogen peroxide has shown to negatively affect both cAMP dependent and non-cAMP dependent steroidogenesis, thus inhibiting progesterone secretion and gonadotropin action [25]. On the other hand, there was an obvious decrease in SOD serum levels in women with PCOS compared to controls. These authors also noted that levels of SOD in the serum were higher than those in the FF [25]. As mentioned earlier, PCOS women have increased levels of ROS due to insulin resistance [3] and excessive free fatty acids [25], which might explain the increased utilization of SOD in order to neutralize ROS.

Weight loss, lifestyle modification and increased physical activity have been shown effective in improving fertility in obese PCOS patients. Recently, the Dietary Approach to Stop Hypertension (DASH) was proposed to help in the treatment of PCOS. This diet contains low-glycemic index and low-energy dense diet, and was reported to benefit patients with type 2 diabetes and gestational diabetes. The DASH diet increases plasma total antioxidant capacity and GSH levels, which may help in the control of OS levels and the increased lipid levels [26–28]. Moreover, adherence to DASH diet has been effective to decrease body fat and insulin resistance, which ultimately result in decreased testosterone levels.

6.3 Pregnancy Loss

Spontaneous abortion is defined by an unexpected pregnancy loss before 20 weeks of gestation [3]. Its risk increases with advanced maternal age. For instance, the incidence of spontaneous abortion is 8.9 % in women between 20–24 years of age whereas it is 74.7 % in those of 45 years and older [29]. On the other hand, recurrent pregnancy loss (RPL) occurs only in 1–3 % of pregnancies and is characterized by three or more consecutive miscarriages [3]. In 50 % of the cases, RPL origin is unknown etiology, but some studies showed that OS might be contributory [3, 30]. Interestingly, decreased levels of vitamin E, vitamin A, β-carotene, glutathione peroxidase (GPx), SOD and catalase, and increased levels of GSH are produced to compensate for the increased ROS levels as reported in patients with RPL [3, 31, 32]. This may be associated with abnormal placentation during the first trimester that result in syncytiotrophoblast deterioration, thus contributing to RPL [33].

In different pathological conditions unfolded or misfolded proteins accumulate in the ER leading to what is known as endoplasmic reticulum stress. ROS affects endoplasmic function by disturbing the calcium homeostasis. A study conducted by Gao et al. in 2012 suggested ER stress induced by OS as a possible contributory factor for unexplained miscarriage. Decidual cells were used to estimate the levels of proteins and unfolded protein response (UPR). These cells treated with hydrogen peroxide (H_2O_2) had elevated levels of glucose regulated protein 78 (GRP 78) and ubiquitinated proteins [34]. Normal levels of valosin-containing proteins (VCP) are needed for proper cell function. Extreme OS can affect the activity of UPR by reducing VCP in decidual cells thus causing cell damage, inhibition of cell growth, and apoptosis. This accumulation activates a cascade called UPR. The cascade leads to inhibition of translation and increases the number of chaperones and folding enzymes; it also enhances ER associated degradation of misfolded proteins (ERAD). This cascade stimulates PERK and IRE1/X-box binding protein-1 activating transcription factor 6 (ATF6). It has been found that decidual cells from patients with early pregnancy loss (EPL) have low levels of ER chaperones and GRP 78. GPR78 acts by inhibiting UPR, thus protecting against cell apoptosis and tissue damage in non-stressed cells. UPR inhibition is achieved by binding to PERK, IRE1 and ATF6, which are then kept as inactive forms. ER chaperones and GPR 78 can be used as markers of ER stress.

These aforementioned findings indicate that H_2O_2 causes an increase in the levels of both ubiquitin and GPR 78 in the decidual cells. Caspase-4 fragments, which are used to determine ER stress, are found in high levels in the decidual cells exposed to H_2O_2, further indicating that caspase is activated in these cells. In conclusion, OS can stimulate UPR and ER stress in decidual cells leading to apoptosis and cell damage. High levels of misfolded or unfolded proteins are associated with ER stress, making this pathophysiology pathway an attractive explanation to early pregnancy loss [34].

Women with RPL exhibit higher levels of uterine natural killer cells (uNK) than normal pregnancies [35], albeit his association is not unequivocal [36]. The density

of uNK cells is directly associated with the density of endometrial vessels throughout the luteal phase. It is also directly associated to the differentiation of endometrial arterioles in the vascular smooth muscle cell (VSMC). VSMC allows pre-implantation blood flow by encircling placental spiral arteries. Higher levels of uNK cells in women with RPL may cause an overly developed spiral arteries in association to VSMC, which will lead to inappropriate blood flow to the fetal-placental unit [37]. Disorganized blood flow to the feto-placental unit may lead to pregnancy loss. OS markers were found to be higher in women with higher miscarriage rates in comparison to gestational aged matched control women [38]. Understanding the connection between the different immune responses and RPL along with the understanding of OS markers might lead to a greater success in the treatment of RPL [39–42].

In a study conducted by Talukdar et al., the effects of coenzyme Q10 on Th1 (T helper cell type 1)/Th2(T helper cell type 2) in women with unexplained RPL were evaluated [43]. A key step to a successful pregnancy is the shift from Th1 to Th2 response. It has been suggested that high levels of ROS and Th1-type immunity are associated with RPL. The authors found that the levels of co-enzyme Q10 were lower in patients with RPL than controls. Elevated Th1-type immune response (IFN-c-producing CD3+CD4+) and decreased Th2-type lymphocytes (IL-4-producing CD3+CD4+) were also noticed in the affected patients with RPL. The study also showed that RPL is stimulated by excessive ROS production, which saturated the capacity of antioxidants and stimulated OS and inflammation. Coenzyme Q10 regulates the cell membrane as well as acts as an antioxidant. It is located in the inner mitochondrial membrane and functions as both an electron carrier and lipophilic substance. CoQ10 supplementation is suggested to improve the chances of women with RPL achieve full term pregnancies [43].

6.4 Preeclampsia

Preeclampsia (PE) is a serious disease characterized by hypertension and proteinuria [44]. It usually manifests during the 20th week of gestation or later. The main pathophysiology mechanisms seem to involve focal vasospasm and movement of fluid from the intravascular to extravascular space. The imbalance between vasoconstrictors and vasodilators are speculated to underlie vasospasm, which affect blood perfusion to some organs. Oxidative stress has also been implicated in preeclampsia pathophysiology. Inadequate spiral artery conversion causes the generation of OS and leads to decreased placental perfusion associated with a low-level ischemic-reperfusion injury [3]. Humoral factors are then secreted into the maternal systemic circulation due to vascular dysfunction, which aggravates organ injury [45]. Trophoblastic and endothelial cells are also capable of generating ROS and the production is increased by the ischemic-reperfusion injury [46]. This oxidative stress state is associated with the secretion of anti-angiogenic factors as well as cytokines, which stimulates the adhesion of leukocytes and platelets to the endothelium [45]. OS enhances nitration of p38 MAPK activity reducing its catalytic function.

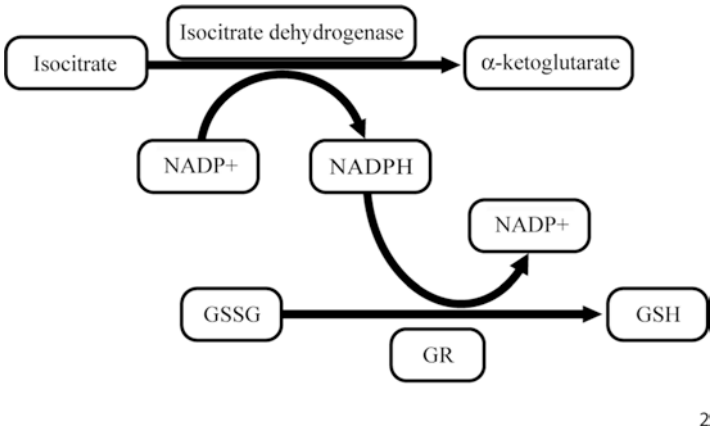

Fig. 6.1 Glutahtione (GSH) and redox regulation in the oocytes

The above processes are thought to be associated with the fetal growth restriction noted in preeclampsia [47].

Added to this, high levels of circulating H_2O_2 are associated with decreased nitric oxide (NO), a vasodilator, thus explaining hypertension. Other sources of ROS include neutrophils, monocytes, and CD4+ T cells. Neutrophils increase superoxide anion levels, which is accompanied by decreased NO levels leading to endothelial cell damage [44, 48, 49].

Endothelial dysfunction might add to the understanding of preeclampsia. When trophoblastic cells invade the maternal decidua, the spiral arteries of the decidua undergo important changes making them high-capacity low-resistance blood vessels. Impaired invasion of fetal trophoblast is one of the characteristics of PE. The decreased remodeling capacity in PE leads to diminished blood flow to the placenta. Hypertension is a compensatory mechanism to overcome the decreased blood flow.

The balance between antioxidants and oxidants, including NO, is disturbed in PE thus causing a state of OS as already mentioned. Impaired redox reactions induce cellular apoptosis (Fig. 6.1) [44, 49, 50].

NO is a vasodilator that mediates function of endothelial cells by regulating the vascular tone, platelet aggregation, leukocyte adhesion, and smooth muscle development [51]. Anion superoxide ($\cdot O_2^-$) is another important free radical in PE. Reperfusion causes oxidative damage after ischemia via conversion of xanthine dehydrogenase (XD) to xanthine oxidase (XO). Also, the metabolism in an ischemic tissue involves ATP breakdown to hypoxanthine (HX). Xanthine and hypoxanthine can be converted to uric acid by XO. XO can also convert oxygen to $^{\bullet}O^-$ and H_2O_2 [44]. This scenario becomes more complicated in the placenta where iron needs to be restricted from passing to the syncytiotrophoblast and is stored in the tissue until its concentration increases in the maternal circulation. Metals including iron produce pro-oxidants named hydroxyl radicals ($^{\bullet}OH$) by Fenton reaction (Fig. 6.2) [52].

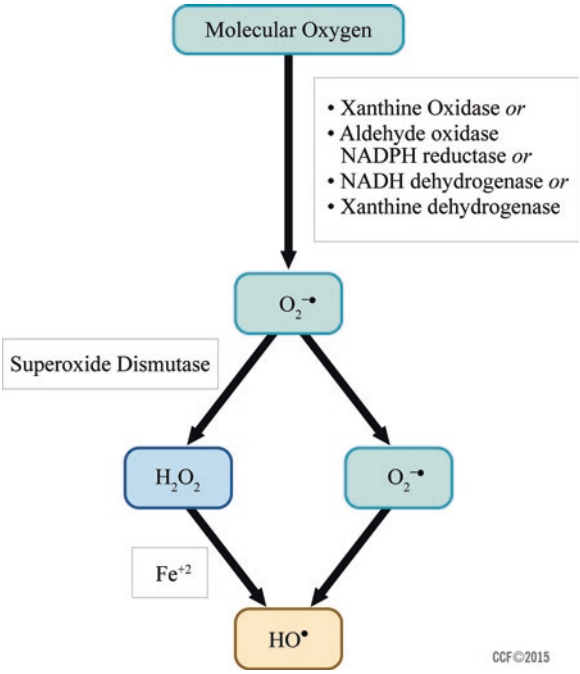

Fig. 6.2 Fenton reaction. Molecular oxygen can be metabolized by different enzymes to form superoxide anion (O^{-}), which then undergo dismutation forming hydrogen peroxide (H_2O_2). In the presence of metal (e.g. Fe^{2+}), Fenton reaction occurs leading to the formation of hydroxyl radical

A different source of superoxide is NADPH oxidases. It has been shown that the NADPH oxidase NOX1, triggered by the angiotensin II cascade, is highly expressed in PE placentas and causes inflammation. Lastly, mitochondria are equally essential during pregnancy to maintain the metabolic function of the placenta. But under pathological conditions it turns out to be another source of superoxide. Mitochondrial dysfunction has a potent effect on both fetal and placental growth and function. Noticeably, oxidative stress in PE has a role in the development of endothelial dysfunction [44].

In pregnancy, NO production is stimulated by NO synthase (NOS), which has three different isoforms, namely, neural (nNOS), endothelial (eNOS), and inducible NOS (iNOS). In the vascular endothelium eNOS is expressed to maintain NO synthesis and the vascular tone, but also inhibits the inflammatory state caused by leukocytes and platelets adhesion [45]. Elevated levels of ROS appear to suppress eNOS [53]. Moreover, NO is scavenged by ROS forming peroxynitrite ($ONOO^{-}$). This peroxynitrite impedes different vascular signaling pathways. Also, it oxidizes DNA, proteins, and lipids. The increase in both ROS and $ONOO^{-}$ are believed to be the cause of the reduction in the availability of NO and a possible cause of endothelial dysfunction. This can be a the mechanistic basis of the underlying etiology of PE [45]. eNOS is important at the cellular level to maintain the vascular function in pregnancy. High levels of free radicals impair eNOS function leading to vascular dysfunction, which characterizes PE.

The treatment strategy for patients with preeclampsia remains experimental and there is no well-known intervention for this disease. Although enzymatic antioxidants might be effective in preventing the development of preeclampsia, studies with the use of vitamins C and E have not yield conclusive results [54].

6.5 Menopause

Maternal age is directly related to oocyte quality. Age related reproductive dysfunctions, including menopause, are associated with decreased oocyte quality, increased mitochondrial DNA (mtDNA) damage, and chromosomal aneuploidy [55, 56]. Typically, as women age her body fat, blood lipids, and iron stores increase [3]. Hormonal changes in menopausal women include a markedly elevation of pituitary gonadotropins and decrease in the levels of anti-Mullerian hormone [57]. The increase in LH and FSH can increase estrogen biosynthesis in the corpus luteum. In post-menopausal women, estrogen exert a concentration dependent either pro-oxidant or anti-oxidant roles [58]. Estrogen deficiency prevents its antioxidant protective effect. Although estrogen helps in the prevention of bone turnover by inhibiting osteoclast formation and function, in menopausal women macrophages produce TNF-alpha increasing osteoblast and osteoclast formation that results in bone turnover [59].

It has been suggested that oxidative stress is exacerbated in menopausal women partially due with impaired antioxidant reserve [59]. A healthy diet and antioxidant supplementation may improve antioxidant levels of post-menopausal women.

6.6 Endometriosis

Endometriosis is a chronic, benign, inflammatory disease characterized by the growth of ectopic endometrial glands and stroma, often implanted in the ovaries and the cul-de-sac, but that can also be found in the lungs, urinary tract and the central nervous system [3, 60, 61]. Patients presenting with endometriosis can be asymptomatic or have a diverse range of symptoms as well as present with infertility.

Infertility and chronic pelvic pain are the two most prevalent clinical symptoms of endometriosis, however, dysmenorrhea, dyspareunia and bladder/bowel symptoms can also be manifested [62]. It is an estrogen-dependent disease that is classified into three types: peritoneal, ovarian and rectovaginal septum endometriosis (deep endometriosis). Depending on the location of the ectopic endometrial tissue in the abdomen, each is a distinct type of disease and differs from the other in the pathogenesis and presentation. Lesions of peritoneal endometriosis appear in the peritoneum whereas those of ovarian endometriosis present in the ovaries. Deep endometriosis is considered the most chronic and invasive form of endometriosis since infiltration of endometriotic tissue is required [63]. Many studies have shown that endometriosis affects 10 % of reproductive aged women, greater than 33 % of

women with chronic pelvic pain and 5-50% of women with infertility [60, 64–66]. The gynecological disorder of endometriosis is common affecting almost seven million of US women [67].

Although many studies report high prevalence of endometriosis, its pathogenesis remains unclear and it is therefore considered a multifactorial disease, which could result from a genetic predisposition. Among the known factors of endometriosis; coelomic metaplasia, metastatic spread, retrograde menstruation and altered immunity are the leading theories behind the causation of endometriosis [62]. The coelomic metaplasia theory, proposed in the 1960s explaining endometriosis at distant sites, is based upon the theory of presence of endometrial stem cells in the peritoneum. These stem cells undergo metaplasia to develop lesions of endometriosis [68]. Also, the presence of endometrial implants in the pelvic cavity could be explained by the theory claiming that menstrual tissue is transported from the uterus to the pelvis through lymphatics and veins [62]. On the other hand, retrograde menstruation is the most accepted theory behind the etiology of endometriosis, clearly explaining the presence of endometrial tissue in the peritoneal cavity as a result of its backflow through the fallopian tubes [3, 62]. Ectopic endometrial tissue then develops its new blood supply and proliferates in the pelvic/peritoneal cavity. Higher volume of refluxed menstrual blood has been reported in women with endometriosis than in control [69]. Nevertheless, retrograde menstruation cannot be considered as a definitive cause of endometriosis because it has been seen that the occurrence of retrograde menstruation is indistinguishable in women with and without endometriosis [62]. Furthermore, women with deficient cell mediated immunity have a higher chance of developing endometriosis as a result of retrograde menstruation. In these women, leukocytes are not capable of differentiating the ectopic endometrial fragments from the reflux therefore failing to clear it out. The cytotoxicity of natural killer cells against endometrial cells was found to be significantly lower in patients with endometriosis compared with controls explaining their contribution to the development of the disease [70]. The other important cause linked to endometriosis is oxidative stress as discussed below.

6.6.1 Biomarkers of Oxidative Stress (OS) in Endometriosis

Oxidative stress (OS) also plays a significant role in the development and progression of endometriosis. Patients suffering from abundant retrograde menstruation and defective immune response are more prone to iron accumulation in the peritoneal cavity as a consequence of the increase number of erythrocytes in the reflux. An abundance of iron decreases the capacity of ferritin to store it, leading to its accumulation. This in turn leads to the production of free radicals, which act as a catalyst in the Fenton reaction [71].

During normal cellular metabolism, reactive oxygen species (ROS) are generated and antioxidants present in the body prevent their toxicity. However, an imbalance between the generation of ROS and antioxidants can lead to oxidative stress, a factor contributing to the pathogenesis of endometriosis. Pathological levels of

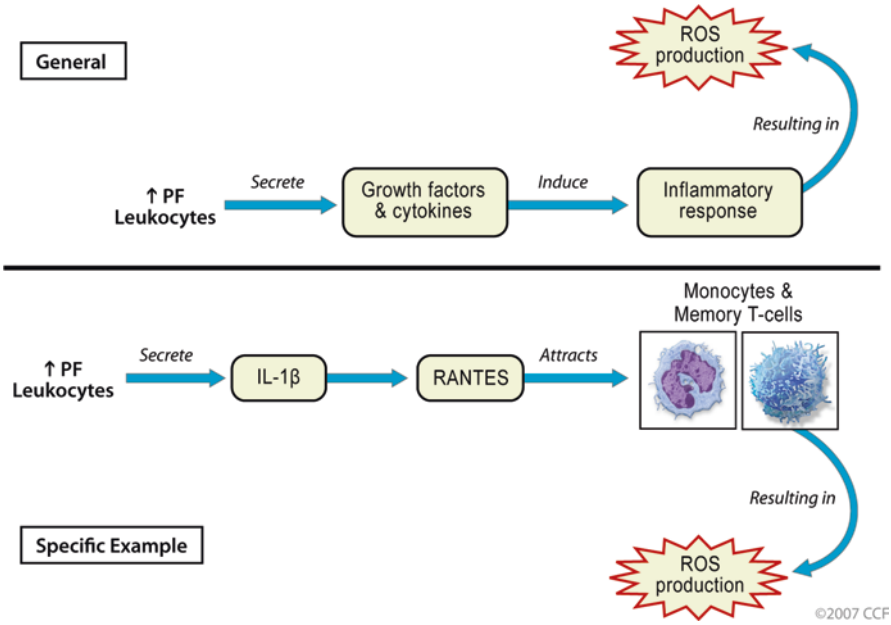

Fig. 6.3 Productions of ROS in the peritoneal environment. Increase in peritoneal fluid, leukocytes lead to release of growth factors and cytokines (including regulated on activation, normal T-cell expressed and secreted (RANTES)); which then induce an inflammatory response (by activating different cells e.g. monocytes, T-cells), resulting in ROS production

ROS have been reviewed by Defrere et al. and Agarwal et al. and were found to contribute to the regulation of Nuclear Factor-κβ (NF-κβ) -a transcriptional factor involved in the progression of the disease. NF-κβ stimulates the release of pro-inflammatory cytokines like IL-6, TNF-α, and IL-β, which helps in the recruitment of phagocytes (Fig. 6.3). It is an established fact that phagocytes are the major contributors in the production of ROS [3, 71]. The link between high levels of ROS and NF-kB has been reviewed by Gupta et al. [72].

Furthermore, higher levels of 8-iso-prostaglandin F2- alpha (8-iso-PGF2-alpha) were identified in the urine and peritoneal fluid of women with endometriosis. This study, conducted by Sharma et al., found that an increase in levels of 8-iso-PGF2-alpha resulted in lipid peroxidation and thus, oxidative stress. The measurement of 8-iso-PGF2-alpha can be used as a biomarker for oxidative stress in patients with endometriosis [73].

6.6.2 Proteomics and Oxidative Stress in Endometriosis

The role of proteomics has been explored to provide a linkage between oxidative stress and endometriosis [74]. Prieto et al. measured the levels of superoxide dismutase (SOD), an antioxidant enzyme, in the follicular fluid of women with endometriosis,

and found a decrease in SOD leading to oxidative stress [75]. Higher levels of Afamin, a vitamin E binding protein, were detected in the peritoneal fluid of patients with endometriosis, which caused a decrease in the scavenging role of antioxidants leading to oxidative stress [76]. While this study indicated an increase in a protein contributing to oxidative stress in patients with endometriosis, another study noted a decrease in hemopexin, a protein responsible for inhibiting the production of reactive oxygen species [77]. These two findings show a clear correlation between oxidative stress and endometriosis. Heme binding protein was also found to be lower than normal further underlining the relevance of oxidative stress in relation to endometriosis [63]. Thioredoxin binding protein (TBP2) plays a role in the regulation of thioredoxin (TRX), an antioxidant responsible for cell proliferation and apoptosis. TBP2 stimulates apoptosis in cases of increased levels of ROS. Decreased levels of TRX and TBP2 were noted in a study that measured their levels in the peritoneal fluid of women with endometriosis. The following results marked a decrease in antioxidant activity as well as cell apoptosis explaining the development of oxidative stress related endometriosis [78].

Heat shock proteins (HSP) are chaperones stimulating cell proliferation by inhibiting apoptosis. They are found in low concentration under normal physiological states, but accumulate in conditions where cells undergo physiological stress [3]. Elevated levels of HSP70, a member of the HSP family, were measured and found to lead to increased proliferation of ectopic endometrial cells present outside the pelvic cavities [79]. In a systematic review, it has been suggested that these oxidants released as a result of an increase in HSP70 influence the progression of ectopic endometrial cells by stimulating pro-inflammatory cytokines [3]. Regardless of its increase in the aforementioned study, the role HSP contributes to oxidative stress related endometriosis is still unclear [74]. Contradictory reports in the literature have shown that levels of HSP90, another member of the HSP family, were evaluated in ectopic endometrial tissue and showed an increase in one study [80] but a decrease in another [81].

6.6.3 Metabolomics and Oxidative Stress in Endometriosis

Metabolomics is the study of metabolites in relation to the etiology and pathophysiology of disease. Proton Nuclear Magnetic Resonance Spectroscopy Based Targeted Metabolite Profiling (NMR) was used to measure the levels of OS biomarkers and detect metabolite imbalances in patients with endometriosis to further comprehend the causation of their disease. Among the 135 women recruited for the study, 55 % were diagnosed with endometriosis and comprised the study group whereas 45 % with tubal factor infertility were used as controls. The inclusion criteria strictly allowed only those who did not receive any medical or hormonal treatment for the past three months to participate in the study. The exclusion criteria was any history of chocolate cysts being removed, pelvic tuberculosis and gynecological surgeries like abdominal or lower pelvic surgeries. Patients were asked to present fasting and in their early follicular phase, and venous blood was drawn and the following parameters were measured in the serum: levels of reactive oxygen species (ROS),

total antioxidant capacity (TAC), lipid peroxidation (LPO), superoxide dismutase (SOD), glutathione (GSH), and catalase. Metabolic profiling using NMR was then performed on 26 samples from the study group and 24 from the controls. An increase in ROS and LPO was noted while a decrease in the antioxidant levels of SOD, catalase and GSH were observed in the serum of the study group in comparison to the controls. A decline in the levels of GSH contributes to the biosynthesis of ophthalamate, also releasing 2-hydroxybutyrate as a by-product. Metabolic profiling displayed elevated levels of both opthalamate and 2-hydroxyvutyrate indicating oxidative stress in patients with endometriosis. Advanced oxidation protein products (AOPP), albumin aggregations impaired by OS, were also measured in the study [67]. The presence of ROS in the plasma induces oxidation of amino acids through their reaction with chlorinated oxidants finally forming AOPP [82]. These findings determine the contribution of oxidative stress to the pathophysiology of endometriosis. This study also provides a more comprehensive assessment of free radicals as well as their end products such as AOPP and how it translates into changing metabolic profile and its relationship with OS.

6.6.4 Treatment of Oxidative Stress Associated with Endometriosis

Being an estrogen dependent disease, it has been suggested that endometriosis could be approached using gonadotropin-releasing hormone agonists (GnRHa) as well as contraceptive pills, both of which generate an estrogen-deficient state. Consequently, growth of ectopic endometrial tissue is impeded and symptoms of endometriosis are relieved [61]. Furthermore, treating infertile women with endometriosis using GnRHa has proved to improve embryo implantation and to increase pregnancy rates [83]. Aromatase inhibitors have also shown to alleviate symptoms of endometriosis by hindering the activity of Aromatase P450, an enzyme responsible for the biosynthesis of estrogen in the ovaries [60]. Aromatase activity is significantly higher in patients with endometriosis in comparison to those without the condition (Fig. 6.4). Increased estrogen levels caused by aromatase P450 stimulate prostaglandin E2 further activating aromatase and progressing the development of ectopic endometrial deposits [84].

Oxidative stress, which is triggered by chronic inflammation in patients with endometriosis, also contributes to the progression of endometriosis. The increase in ROS production affects the quality of the oocytes of these patients as well as implantation explaining infertility in women with endometriosis. In a study conducted by Tamura et al., GnRHa was administered to twenty-three infertile women diagnosed with endometriosis to test its effect on oxidative stress related endometriosis and infertility. The ultralong group, consisting of eleven patients, received three courses of gonadotropin releasing hormone agonist (GnRHa), followed by a standard controlled ovarian hyperstimulation. The control group on the other hand, which consisted of the twelve patients, received one course of GnRHa in the mid luteal phase,

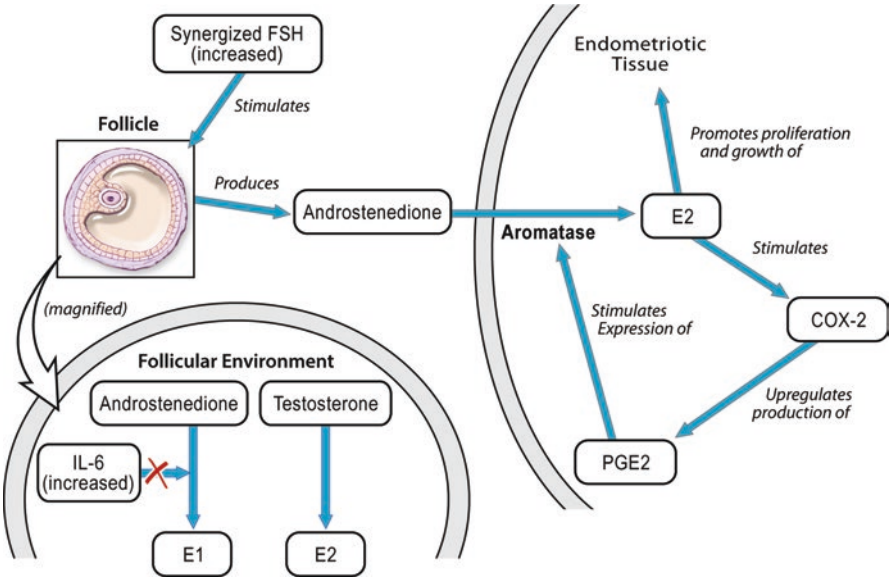

Fig. 6.4 Role of aromatase in a persistent endometriotic state

followed by a standard controlled ovarian hyperstimulation as well. During oocyte retrieval, follicular fluid aspirated from each woman of the two groups was analyzed. Fertilization, implantation and pregnancy rates were compared between the ultralong and control groups as well. Oxidative stress markers, antioxidants and the numbers of matured follicles and retrieved oocytes were also measured and compared. Implantation rates in the ultralong group (21.4 %) as well as pregnancy rates (27.3 %) were significantly higher than those in the control group (8.3 and 8.3 % respectively). Levels of Tumor necrosis factor (TNF) α, a key oxidative stress marker in patients with endometriosis, showed a remarkable decrease in the ultralong group (5.8 ± 3.2 pg/ml) in comparison to the control group (10.6 ± 3.2 pg/ml). Melatonin, an antioxidant hormone released from the pineal gland, notably increased in the follicular fluid of the ultralong group (139.2 ± 45.7 pg/ml) when comparing it to the control group (85.6 ± 27.4 pg/ml). The study strongly indicates that the administration of GnRHa alleviates the negative impacts of oxidative stress markers in infertile women with endometriosis, leading to an increase their fertility and pregnancy outcomes [83]. Desferoxamine (DFO), an iron chelator could also be used as an effective treatment for oxidative stress related endometriosis. Iron chelators decrease the detrimental effects of oxidative stress caused by iron overload in the peritoneal cavities as a result of retrograde menstruation in patients with endometriosis. In these women, DFO must be administered directly into the peritoneal cavity using intrapelvic implants because of the heavy menstrual cycles these women suffer from. This ensures that DFO will absorb the iron overload present in the peritoneal cavity exclusively, and not further decrease the total body iron, which has already been reduced as a result of the heavy menstrual cycles in these women [71].

An antioxidant rich diet has also shown to play a role in endometriosis regression [3]. A study conducted by Mier-Cabrera et al. showed the correlation between administering vitamin E and C to patients with endometriosis and its effect on certain OS markers. After 4 months of daily supplementation with bars containing vitamins E and C, the levels of malondialdehyde (MDA) and lipid hydroperoxides in the plasma and peritoneal fluids of the study group decreased significantly in comparison to the control group. However, no improvement in pregnancy rates was reported, suggesting that despite the positive impact of antioxidants on OS biomarkers in these women, no definitive treatment has been standardized [85]. Future adequate powered randomized controlled trials are needed to study the alleviation of OS in endometriosis as well as pregnancy outcomes. Treatment needs to be individualized for patients targeted towards the type of endometriosis that each woman is suffering from.

6.7 Gestational Diabetes Mellitus (GDM)

Gestational diabetes mellitus (GDM) is a common pregnancy complication characterized by a state of carbohydrate intolerance. Due to the hyperglycemic situation these women suffer from, the pancreatic beta cells have to release more insulin to compensate, sustaining high insulin levels and developing insulin resistance [86, 87]. The prevalence of GDM has been increasing, especially in women who are obese and in those of advanced age [88, 89]. During routine screening, 2–13 % of pregnant women are diagnosed with GDM [90]. GDM is a risk factor for developing maternal conditions such as gestational hypertension, preeclampsia and cardiovascular diseases [91, 92]. These women are also prone to developing type 2 diabetes mellitus later in life. A study conducted by Teede et al. demonstrated that 26-70 % of GDM women developed type 2 diabetes mellitus 10–15 years after delivery [89]. GDM is also associated with adverse fetal and neonatal outcomes such as macrosomia, shoulder dystocia and development of obesity later in life [90, 91].

The pathophysiology explaining insulin resistance and changes in glucose tolerance in these women is still unclear; however OS was found to play a crucial role in the development of GDM [93, 94]. A study conducted by Zhu et al. reported elevated levels of plasma C-reactive proteins (CRP) in patients with GDM compared to controls. An increase in this inflammatory biomarker was found to be correlated to a decrease in the efficacy of the glutathione antioxidant system as well as an increase in lipid peroxidation [88]. On the other hand, red blood cells (RBC) possess antioxidant characteristics under normal conditions, therefore contributing to OS when the RBCs develop defects. For example, it has been noted that increased levels of physiologically modified RBCs result in lack of scavenging activity in patients with GDM, further underlining the relationship between oxidative state and the development of the disease [88, 95].

Oxidants and antioxidants are produced abundantly in the placenta and the GDM placenta was found to have increased levels of OS markers such as xanthine oxidase

and malondialdehyde and decreased capacity to scavenge the free radicals [94, 96]. Furthermore, elevated levels of placental 8-isoprostane have been documented in patients with GDM compared to controls. An elevation in this lipid peroxidation marker additionally correlates OS to GDM [94].

It has been suggested that GDM develops as a result of increased intake of heme-iron supplementation, a common prescription among pregnant women [97, 98]. It was reported that the risk of developing GDM increases by 51 % with every 1 mg intake of heme-iron [99]. OS related GDM induced by heme-iron intake was found to increase lipid peroxidation, DNA damage and enhance insulin resistance [100]. Contradictory findings have been reported concerning the presence of OS biomarkers in GDM patients caused by the induction of the oxidative stress as a result of excess iron [100].

An accumulation of catalytic iron inducing OS, caused by compromised fetuses explains the variability in the results. It has been reported that enzymes such as peroxidases can be used to diminish the detrimental effects of ROS in GDM patients. Furthermore, the DASH diet has shown to decrease ROS production and treat insulin resistance in GDM [101]. Those studies demonstrate that women with GDM have decreased ability to counterbalance the oxidative state they suffer from, which is reflected by their insulin resistance. Nevertheless, further research is needed to determine effective therapeutic interventions to reduce OS, alleviate insulin resistance and treat GDM in these women.

6.8 Unexplained Infertility

A couple is diagnosed with unexplained infertility when all other reasons of infertility such as ovulatory disorders and hormonal imbalances as well as the male factor have been ruled out. Hence, it is considered an exclusion diagnosis and is more prevalent in females with advanced age [102]. Among the couples who consult for infertility treatment, approximately 15 % are diagnosed with unexplained infertility [103]. The pathophysiology behind unexplained infertility is still unclear, however OS can possibly contribute to this condition [104]. The imbalance between ROS and antioxidant levels in the peritoneal fluid of these patients is mainly due to defects in their scavenging systems. Decreased antioxidant activity of both glutathione and vitamin E could very likely be the basis of OS related unexplained infertility [105].

Furthermore, homocysteine, an amino acid triggering OS and apoptosis [106], was found to accumulate in cases of folate deficiency. Folate, a B vitamin, plays a crucial role in female reproduction [107]. Polymorphisms in folate metabolism pathways have been investigated to determine their role in the etiology of unexplained infertility. It has been reported that women with unexplained infertility have polymorphisms in the 5,10-methylenetetrahydrofolate reductase (MTHFR) 677C/T gene [108]. This gene is required for the conversion of homocysteine to methionine through the folate methylation cycle, a process involved in the methylation of DNA,

lipids and proteins [109]. Defects in this gene lead to reduced and unstable enzymatic activity resulting in hyperhomocysteinemia [110, 111]. This condition induces ROS production by inhibiting antioxidant enzymes such as glutathione peroxidase and SOD leading to elevated levels of peroxyl and superoxide radicals [112]. Hyperhomocysteinemia was also found to be associated with decreased pregnancy and implantation rates in IVF patients as well as increased abortion rates [113]. Hence, defects in the metabolism pathways of folate lead to distorted levels of homocysteine explaining the development of unexplained infertility [108].

ROS levels were also investigated in the peritoneal fluid of women with unexplained infertility. In one study, Wang et al. detected higher levels of ROS in the peritoneal fluid of patients with unexplained infertility in comparison to those with endometriosis and tubal ligation. Moreover, this elevation did not affect fertility in the other two groups, but played a significant role in those with unexplained infertility further suggesting an association between OS and unexplained infertility [105]. Increased OS has also been documented in the follicular fluid of patients with unexplained infertility undergoing IVF [114]. It has been observed that increased OS damages the oocyte's cell membrane through lipid peroxidation. This damage leads to poor oocyte quality, which in turn impacts embryo quality [20, 115].

Pekel et al. detected lower levels of sFas (an apoptosis marker) in the serum of unexplained infertility patients (2.85 ± 0.44 pg/mL) in comparison to the control group (2.90 ± 1.01 pg/mL, $p < 0.01$) [116]. Decreased levels of this marker of apoptosis were associated with an increase in apoptosis as well as down regulation of the immune system of the affected patients [117]. Also, a remarkable decrease was noticed in the total antioxidant capacity levels in the follicular fluid of the study group (0.88 ± 0.16 mmol/L) when compared to the controls (1.31 ± 0.63 mmol/L, $p < 0.05$). These findings indicate an increase in apoptosis as well as a decrease in the antioxidant scavenging system in these patients, correlating OS to unexplained infertility [116].

To further validate the role of OS in unexplained infertility, the role of vitamin E supplementation, an antioxidant, has been studied on such patients undergoing controlled ovarian stimulation. 400 IU/day of vitamin E was administered to the study group during the period of ovarian stimulation until the day in which the human chorionic gonadotropin injection to trigger ovulation. It has been reported that endometrial thickness and receptivity as well as implantation and pregnancy rates were increased in the group of patients with unexplained infertility treated with vitamin E [118].

6.9 Conclusion

In conclusion, OS has been recognized as contributory to the pathophysiology of infertility in women with unexplained infertility; however the exact mechanistic pathways are still not known. Antioxidant supplementation has been reported to alleviate infertility symptoms, but the ideal regimens are still to be determined.

References

1. Sunderam S et al (2015) Assisted reproductive technology surveillance – United States, 2013. MMWR Surveill Summ 64(11):1–25
2. Sunderam S et al (2012) Assisted reproductive technology surveillance – United States, 2009. MMWR Surveill Summ 61(7):1–23
3. Benjamin D, Sharma RK, Moazzam A, Agarwal A (2012) Methods for the detection of ROS in human sperm samples. Studies on men's health and fertility. Humana, New York, pp 257–274
4. Min J et al (2006) Paraoxonase gene polymorphism and vitamin levels during pregnancy: relationship with maternal oxidative stress and neonatal birthweights. Reprod Toxicol 22(3):418–424
5. Min J et al (2009) Effect of oxidative stress on birth sizes: consideration of window from mid pregnancy to delivery. Placenta 30(5):418–423
6. Clerici G et al (2012) Oxidative stress in pathological pregnancies. J Obstet Gynaecol 32(2):124–127
7. Verit FF, Erel O (2008) Oxidative stress in nonobese women with polycystic ovary syndrome: correlations with endocrine and screening parameters. Gynecol Obstet Invest 65(4): 233–239
8. Karadeniz M et al (2008) Oxidative stress markers in young patients with polycystic ovary syndrome, the relationship between insulin resistances. Exp Clin Endocrinol Diab 116(4):231–235
9. Costello MF et al (2007) Metformin versus oral contraceptive pill in polycystic ovary syndrome: a cochrane review. Hum Reprod 22(5):1200–1209
10. Khan KA, Stas S, Kurukulasuriya LR (2006) Polycystic ovarian syndrome. J Cardiometab Syndr 1(2):125–132
11. Tandulwadkar SR, Lodha PA, Mangeshikar NT (2014) Obstetric complications in women with IVF conceived pregnancies and polycystic ovarian syndrome. J Hum Reprod Sci 7(1):13
12. Teissier M et al (2000) Comparison of follicle steroidogenesis from normal and polycystic ovaries in women undergoing IVF: relationship between steroid concentrations, follicle size, oocyte quality and fecundability. Hum Reprod 15(12):2471–2477
13. Cohen Y et al (2004) Spindle imaging: a new marker for optimal timing of ICSI? Hum Reprod 19(3):649–654
14. De Santis L et al (2005) Polar body morphology and spindle imaging as predictors of oocyte quality. Reprod Biomed Online 11(1):36–42
15. Chattopadhayay R et al (2010) Effect of follicular fluid oxidative stress on meiotic spindle formation in infertile women with polycystic ovarian syndrome. Gynecol Obstet Invest 69(3):197–202
16. Wang W et al (2001) Developmental ability of human oocytes with or without birefringent spindles imaged by Polscope before insemination. Hum Reprod 16(7):1464–1468
17. Rienzi L et al (2005) Meiotic spindle visualization in living human oocytes. Reprod Biomed Online 10(2):192–198
18. Moon JH et al (2003) Visualization of the metaphase II meiotic spindle in living human oocytes using the Polscope enables the prediction of embryonic developmental competence after ICSI. Hum Reprod 18(4):817–820
19. Rajani S et al (2012) Assessment of oocyte quality in polycystic ovarian syndrome and endometriosis by spindle imaging and reactive oxygen species levels in follicular fluid and its relationship with IVF-ET outcome. J Hum Reprod Sci 5(2):187
20. Das S et al (2006) Reactive oxygen species level in follicular fluid – embryo quality marker in IVF? Hum Reprod 21(9):2403–2407
21. Kaya C et al (2009) Advanced oxidation protein products are increased in women with polycystic ovary syndrome: relationship with traditional and nontraditional cardiovascular risk factors in patients with polycystic ovary syndrome. Fertil Steril 92(4):1372–1377

22. Boutzios G et al (2013) Polycystic ovary syndrome offspring display increased oxidative stress markers comparable to gestational diabetes offspring. Fertil Steril 99(3):943–950
23. Bierhaus A et al (1997) Advanced glycation end product-induced activation of NF-κB is suppressed by α-lipoic acid in cultured endothelial cells. Diabetes 46(9):1481–1490
24. Descamps-Latscha B et al (2005) Advanced oxidation protein products as risk factors for atherosclerotic cardiovascular events in nondiabetic predialysis patients. Am J Kidney Dis 45(1):39–47
25. Seleem AK et al (2014) Superoxide dismutase in polycystic ovary syndrome patients undergoing intracytoplasmic sperm injection. J Assist Reprod Genet 31(4):499–504
26. Lopes HF et al (2003) DASH diet lowers blood pressure and lipid-induced oxidative stress in obesity. Hypertension 41(3):422–430
27. Asemi Z et al (2013) A randomized controlled clinical trial investigating the effect of DASH diet on insulin resistance, inflammation, and oxidative stress in gestational diabetes. Nutrition 29(4):619–624
28. Asemi Z et al (2014) Effects of DASH diet on lipid profiles and biomarkers of oxidative stress in overweight and obese women with polycystic ovary syndrome: a randomized clinical trial. Nutrition 30(11-12):1287–1293
29. Ishii T et al (2014) Genetically induced oxidative stress in mice causes thrombocytosis, splenomegaly and placental angiodysplasia that leads to recurrent abortion. Redox Biol 2:679–685
30. Seet RC-S et al (2010) Markers of oxidative damage are not elevated in otherwise healthy individuals with the metabolic syndrome. Diabetes Care 33(5):1140–1142
31. Burton GJ, Hempstock J, Jauniaux E (2003) Oxygen, early embryonic metabolism and free radical-mediated embryopathies. Reprod Biomed Online 6(1):84–96
32. Şimşek M et al (1998) Blood plasma levels of lipoperoxides, glutathione peroxidase, beta carotene, vitamin A and E in women with habitual abortion. Cell Biochem Funct 16(4):227–231
33. Miller H et al (2000) Glutathione levels and miscarriage. Fertil Steril 74(6):1257–1258
34. Gao H-J et al (2012) Endoplasmic reticulum stress induced by oxidative stress in decidual cells: a possible mechanism of early pregnancy loss. Mol Biol Rep 39(9):9179–9186
35. Kwak JY et al (1999) Immunopathology of the implantation site utilizing monoclonal antibodies to natural killer cells in women with recurrent pregnancy losses. Am J Reprod Immunol 41(1):91–98
36. Plaisier M et al (2009) Decidual vascularization and the expression of angiogenic growth factors and proteases in first trimester spontaneous abortions. Hum Reprod 24(1):185–197
37. Quenby S et al (2009) Uterine natural killer cells and angiogenesis in recurrent reproductive failure. Hum Reprod 24(1):45–54
38. Hempstock J et al (2003) The contribution of placental oxidative stress to early pregnancy failure. Hum Pathol 34(12):1265–1275
39. Kwak JY et al (1995) Up-regulated expression of CD56+, CD56+/CD16+, and CD19+ cells in peripheral blood lymphocytes in women with recurrent pregnancy losses. Clin Immunol Newslett 15(2):18–22
40. Lee SK et al (2013) Determination of clinical cellular immune markers in women with recurrent pregnancy loss. Am J Reprod Immunol 70(5):398–411
41. Kwak-Kim J et al (2003) Increased T helper 1 cytokine responses by circulating T cells are present in women with recurrent pregnancy losses and in infertile women with multiple implantation failures after IVF. Hum Reprod 18(4):767–773
42. Matsubayashi H et al (2001) Increased natural killer-cell activity is associated with infertile women. Am J Reprod Immunol 46(5):318–322
43. Talukdar A et al (2015) Effect of coenzyme Q10 on Th1/Th2 paradigm in females with idiopathic recurrent pregnancy loss. Am J Reprod Immunol 74(2):169–180
44. Sánchez-Aranguren LC et al (2014) Endothelial dysfunction and preeclampsia: role of oxidative stress. Front Physiol 5:372
45. Matsubara K et al (2010) Role of nitric oxide and reactive oxygen species in the pathogenesis of preeclampsia. J Obstet Gynaecol Res 36(2):239–247

46. Burton GJ, Yung H-W (2011) Endoplasmic reticulum stress in the pathogenesis of early-onset pre-eclampsia. Pregnancy Hypertens 1(1):72–78
47. Webster R, Roberts V, Myatt L (2008) Protein nitration in placenta–functional significance. Placenta 29(12):985–994
48. Wallace K et al (2014) CD4+ T cells are important mediators of oxidative stress that cause hypertension in response to placental ischemia. Hypertension 64(5):1151–1158
49. Kalyanaraman B (2013) Teaching the basics of redox biology to medical and graduate students: oxidants, antioxidants and disease mechanisms. Redox Biol 1(1):244–257
50. Palmer RM, Ashton D, Moncada S (1988) Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature 333(6174):664–666
51. Qian J, Fulton D (2013) Post-translational regulation of endothelial nitric oxide synthase in vascular endothelium. Front Physiol 4:347
52. Many A, Hubel C, Roberts J (1996) Hyperuricemia and xanthine oxidase in preeclampsia, revisited. Am J Obstet Gynecol 174(1):288–291
53. Farrow KN et al (2008) Superoxide dismutase restores eNOS expression and function in resistance pulmonary arteries from neonatal lambs with persistent pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 295(6):L979–L987
54. Cindrova-Davies T (2014) The therapeutic potential of antioxidants, ER chaperones, NO and H2S donors, and statins for treatment of preeclampsia. Front Pharmacol 5:119
55. Mishra GD, Kuh D (2012) Health symptoms during midlife in relation to menopausal transition: British prospective cohort study. BMJ 344
56. Te Velde E et al (1998) Developmental and endocrine aspects of normal ovarian aging. Mol Cell Endocrinol 145(1):67–73
57. Su HI, Freeman EW (2009) Hormone changes associated with the menopausal transition. Minerva Ginecol 61(6):483–489
58. Nathan L, Chaudhuri G (1998) Antioxidant and prooxidant actions of estrogens: potential physiological and clinical implications. Semin Reprod Endocrinol 16(4):309–314
59. Doshi SB, Agarwal A (2013) The role of oxidative stress in menopause. J Midlife Health 4(3):140–146
60. Abu Hashim H (2014) Potential role of aromatase inhibitors in the treatment of endometriosis. Int J Womens Health 6:671–680
61. Giudice LC (2010) Clinical practice. Endometriosis. N Engl J Med 362(25):2389–2398
62. Macer ML, Taylor HS (2012) Endometriosis and infertility: a review of the pathogenesis and treatment of endometriosis-associated infertility. Obstet Gynecol Clin North Am 39(4):535–549
63. Wolfler MM et al (2011) Two-dimensional gel electrophoresis in peritoneal fluid samples identifies differential protein regulation in patients suffering from peritoneal or ovarian endometriosis. Fertil Steril 95(8):2764–2768
64. Soave I et al (2015) Environment and endometriosis: a toxic relationship. Eur Rev Med Pharmacol Sci 19(11):1964–1972
65. Guo SW, Wang Y (2006) The prevalence of endometriosis in women with chronic pelvic pain. Gynecol Obstet Invest 62(3):121–130
66. Andrade SS et al (2013) 17beta-Estradiol and steady-state concentrations of H2O2: antiapoptotic effect in endometrial cells from patients with endometriosis. Free Radic Biol Med 60:63–72
67. Jana SK et al (2013) 1H NMR based targeted metabolite profiling for understanding the complex relationship connecting oxidative stress with endometriosis. Biomed Res Int 2013:329058
68. Ferguson BR, Bennington JL, Haber SL (1969) Histochemistry of mucosubstances and histology of mixed mullerian pelvic lymph node glandular inclusions. Evidence for histogenesis by mullerian metaplasia of coelomic epithelium. Obstet Gynecol 33(5):617–625
69. Halme J et al (1984) Retrograde menstruation in healthy women and in patients with endometriosis. Obstet Gynecol 64(2):151–154
70. Jeung IC et al (2015) Effect of helixor A on natural killer cell activity in endometriosis. Int J Med Sci 12(1):42–47

71. Defrere S et al (2008) Potential involvement of iron in the pathogenesis of peritoneal endometriosis. Mol Hum Reprod 14(7):377–385
72. Gupta S et al (2008) Pathogenic mechanisms in endometriosis-associated infertility. Fertil Steril 90(2):247–257
73. Sharma I et al (2010) Role of 8-iso-prostaglandin F2alpha and 25-hydroxycholesterol in the pathophysiology of endometriosis. Fertil Steril 94(1):63–70
74. Gupta S et al (2014) Power of proteomics in linking oxidative stress and female infertility. Biomed Res Int 2014:916212
75. Prieto L et al (2012) Analysis of follicular fluid and serum markers of oxidative stress in women with infertility related to endometriosis. Fertil Steril 98(1):126–130
76. Seeber BE et al (2010) The vitamin E-binding protein afamin is altered significantly in the peritoneal fluid of women with endometriosis. Fertil Steril 94(7):2923–2926
77. Wolfler MM et al (2013) Reduced hemopexin levels in peritoneal fluid of patients with endometriosis. Fertil Steril 100(3):777–781
78. Seo SK et al (2010) The roles of thioredoxin and thioredoxin-binding protein-2 in endometriosis. Hum Reprod 25(5):1251–1258
79. Lambrinoudaki IV et al (2009) Measurable serum markers of oxidative stress response in women with endometriosis. Fertil Steril 91(1):46–50
80. Matsuzaki S et al (2004) DNA microarray analysis of gene expression profiles in deep endometriosis using laser capture microdissection. Mol Hum Reprod 10(10):719–728
81. Fowler PA et al (2007) An investigation of the effects of endometriosis on the proteome of human eutopic endometrium: a heterogeneous tissue with a complex disease. Proteomics 7(1):130–142
82. Witko-Sarsat V et al (2003) AOPP-induced activation of human neutrophil and monocyte oxidative metabolism: a potential target for N-acetylcysteine treatment in dialysis patients. Kidney Int 64(1):82–91
83. Tamura H et al (2014) A pilot study to search possible mechanisms of ultralong gonadotropin-releasing hormone agonist therapy in IVF-ET patients with endometriosis. J Ovarian Res 7:100
84. Nothnick WB (2011) The emerging use of aromatase inhibitors for endometriosis treatment. Reprod Biol Endocrinol 9:87
85. Mier-Cabrera J et al (2008) Effect of vitamins C and E supplementation on peripheral oxidative stress markers and pregnancy rate in women with endometriosis. Int J Gynaecol Obstet 100(3):252–256
86. International association of diabetes and pregnancy et al (2010) International association of diabetes and pregnancy study groups recommendations on the diagnosis and classification of hyperglycemia in pregnancy. Diabetes Care 33(3):676–682
87. Correa PJ et al (2014) Prediction of gestational diabetes early in pregnancy: targeting the long-term complications. Gynecol Obstet Invest 77(3):145–149
88. Zhu C et al (2015) Association of oxidative stress biomarkers with gestational diabetes mellitus in pregnant women: a case-control study. PLoS One 10(4), e0126490
89. Teede HJ et al (2011) Gestational diabetes: development of an early risk prediction tool to facilitate opportunities for prevention. Aust N Z J Obstet Gynaecol 51(6):499–504
90. Asemi Z et al (2013) Effects of vitamin D supplementation on glucose metabolism, lipid concentrations, inflammation, and oxidative stress in gestational diabetes: a double-blind randomized controlled clinical trial. Am J Clin Nutr 98(6):1425–1432
91. Abell SK et al (2015) Inflammatory and other biomarkers: role in pathophysiology and prediction of gestational diabetes mellitus. Int J Mol Sci 16(6):13442–13473
92. Shah BR, Retnakaran R, Booth GL (2008) Increased risk of cardiovascular disease in young women following gestational diabetes mellitus. Diabetes Care 31(8):1668–1669
93. Buchanan TA, Xiang AH (2005) Gestational diabetes mellitus. J Clin Invest 115(3): 485–491
94. Lappas M et al (2011) The role of oxidative stress in the pathophysiology of gestational diabetes mellitus. Antioxid Redox Signal 15(12):3061–3100

95. Daci A, Elshani B, Giangiacomo B (2013) Gestational diabetes mellitus (GDM) in the Republic of Kosovo: a retrospective pilot study. Med Arch 67(2):88–90
96. Biri A et al (2006) Oxidant status in maternal and cord plasma and placental tissue in gestational diabetes. Placenta 27(2-3):327–332
97. Domellof M, Thorsdottir I, Thorstensen K (2013) Health effects of different dietary iron intakes: a systematic literature review for the 5th Nordic Nutrition Recommendations. Food Nutr Res 57
98. Bowers K et al (2011) A prospective study of prepregnancy dietary iron intake and risk for gestational diabetes mellitus. Diabetes Care 34(7):1557–1563
99. Qiu C et al (2011) Gestational diabetes mellitus in relation to maternal dietary heme iron and nonheme iron intake. Diabetes Care 34(7):1564–1569
100. Zein S, Rachidi S, Hininger-Favier I (2014) Is oxidative stress induced by iron status associated with gestational diabetes mellitus? J Trace Elem Med Biol 28(1):65–69
101. Asemi Z et al (2013) Favourable effects of the dietary approaches to stop hypertension diet on glucose tolerance and lipid profiles in gestational diabetes: a randomised clinical trial. Br J Nutr 109(11):2024–2030
102. Smith S, Pfeifer SM, Collins JA (2003) Diagnosis and management of female infertility. JAMA 290(13):1767–1770
103. Aboulghar MA et al (2003) Diagnosis and management of unexplained infertility: an update. Arch Gynecol Obstet 267(4):177–188
104. Ruder EH et al (2008) Oxidative stress and antioxidants: exposure and impact on female fertility. Hum Reprod Update 14(4):345–357
105. Wang Y et al (1997) Importance of reactive oxygen species in the peritoneal fluid of women with endometriosis or idiopathic infertility. Fertil Steril 68(5):826–830
106. Badawy A, Baker El Nashar A, El Totongy M (2006) Clomiphene citrate plus N-acetyl cysteine versus clomiphene citrate for augmenting ovulation in the management of unexplained infertility: a randomized double-blind controlled trial. Fertil Steril 86(3):647–650
107. Tamura T, Picciano MF (2006) Folate and human reproduction. Am J Clin Nutr 83(5):993–1016
108. Altmae S et al (2010) Variations in folate pathway genes are associated with unexplained female infertility. Fertil Steril 94(1):130–137
109. Yamada K et al (2005) Regulation of human methylenetetrahydrofolate reductase by phosphorylation. Proc Natl Acad Sci U S A 102(30):10454–10459
110. Frosst P et al (1995) A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 10(1):111–113
111. Harmon DL et al (1996) The common 'thermolabile' variant of methylene tetrahydrofolate reductase is a major determinant of mild hyperhomocysteinaemia. QJM 89(8):571–577
112. Weiss N (2005) Mechanisms of increased vascular oxidant stress in hyperhomocys-teinemia and its impact on endothelial function. Curr Drug Metab 6(1):27–36
113. Pacchiarotti A et al (2007) The possible role of hyperhomocysteinemia on IVF outcome. J Assist Reprod Genet 24(10):459–462
114. Attaran M et al (2000) The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. Int J Fertil Womens Med 45(5):314–320
115. Pasqualotto EB et al (2004) Effect of oxidative stress in follicular fluid on the outcome of assisted reproductive procedures. Fertil Steril 81(4):973–976
116. Pekel A et al (2015) Changes of sFas and sFasL, oxidative stress markers in serum and follicular fluid of patients undergoing IVF. J Assist Reprod Genet 32(2):233–241
117. Sarandakou A et al (2003) Apoptosis and proliferation factors in serum and follicular fluid from women undergoing in vitro fertilization. Fertil Steril 79(3):634–636
118. Cicek N et al (2012) Vitamin E effect on controlled ovarian stimulation of unexplained infertile women. J Assist Reprod Genet 29(4):325–328

Chapter 7
Therapeutic Role of Antioxidants (AOX) in the Treatment of Infertility

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7.1 Introduction

Infertility affects approximately 15 % of couples worldwide [1]. During normal cellular metabolism, reactive oxygen species (ROS) are generated on a continuous basis, either by endogenous sources (see Chap. 1) or exogenous sources (see Chap. 5). ROS are essential for many processes in the human body, including essential intracellular signaling pathways [2]. Notwithstanding, an elevation in ROS eventually leads to oxidative stress (OS), defined as the imbalance between oxidants and antioxidants [3]. Oxidative stress can result in subfertility by negatively affecting multiple processes in the male and female reproductive systems.

The main role of antioxidants (AOX) is to neutralize ROS [4]. An antioxidant by definition is "any substance that delays, prevents or removes oxidative damage to a target molecule" [5]. There are two types of AOX, namely non-enzymatic and enzymatic AOX, respectively. Non-enzymatic AOXs are natural substances that can be found in diets rich in vegetables and fruits; they are also known as exogenous AOX. Examples of these include vitamin C (ascorbic acid), vitamin E (tocopherol),

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pyruvate, glutathione (GSH), L-carnitine, taurine and hypotaurine, zync, selenium, beta-carotene, and carotene. In contrast, enzymatic (also known as endogenous) AOX represent the main antioxidant system and include superoxide dismutase (SOD), catalase and glutathione peroxidase (Table 7.1) [6].

In general, AOX help to maintain a balance between ROS and oxidative damage by acting as a defense mechanism. Any deficiency in AOX may lead to imbalance in this process [7]. In addition to the known diseases leading to infertility, lifestyle choices, including diet, alcohol consumption and smoking may contribute to an increased risk of AOX deficiency. Overall, infertility patients have either an increase in ROS levels, which lead to OS, lipid peroxidation and cellular damage, or a decrease in the antioxidant capacity, or both [1, 8–10].

Based on the AOX properties of defending against ROS, their use has gained increasing interest among practitioners as adjuvant agents to counteract the detrimental effects of OS [9, 11–14]. In this chapter, we will discuss the role of AOX as potential agents to alleviate OS-related male and female infertility.

7.2 Therapeutic Role of Antioxidants (AOX) in the Treatment of Infertility in the Male

7.2.1 Evidence from In Vitro Studies

7.2.1.1 Superoxide Dismutase (SOD)

The testes contain enzymatic antioxidants that protect spermatozoa from excessive ROS. An example is SOD, which is an endogenous enzyme that converts superoxide radicals ($^{\bullet}O2^{-}$) to hydrogen peroxide (H_2O_2) [15]. Hence, SOD neutralizes intra and extracellular superoxide anions.

SOD was shown to preserve sperm motility and reduce lipid peroxidation, as evidenced by the decrease in malondialdehyde (MDA) concentrations. In one study, sperm incubation with high concentrations of SOD (2000 μ/ml) preserved motility in mouse spermatozoa and enhanced mice embryo development. On the contrary, lower concentrations of SOD (<2000 μ/ml) had no remarkable effect [16]. In another study evaluating the effects of SOD on sperm viability, motility and morphology, the authors found that SOD was associated with improved semen quality [17]. Interestingly, a study conducted by de Lamirande et al., which evaluated sperm capacitation, hyperactivation and acrosome reaction, demonstrated that a higher proportion of sperm exposed to superoxide anion exhibited capacitation and hyperactivation as opposed to those unexposed to SOD [18].

7.2.1.2 Vitamin C

Vitamin C (ascorbic acid) is a water-soluble vitamin present in dietary sources. It is considered a synthetic antioxidant because it cannot be produced by the human body enzymatically. Vitamin C is found at ten times higher concentrations in the

Table 7.1 Role of antioxidants in ameliorating the effects of oxidative stress in assisted reproduction [50]

Antioxidant(s)	Study	Treatment dosage (per treatment or day)	Treatment details	Parameters improved
SOD	Rossi et al. [19]	SOD (100 U/ml) and catalase (100 U/ml)	Added to fresh semen before freezing	• Semen parameter, especially progressive motility) (SOD and catalase)—due to their combined and simultaneous action on superoxide anion and hydrogen peroxide
Catalase	Li et al. [20]	Ascorbate (300 or 600 μM) and catalase (200 or 400 IU/ml)	Added to semen before freezing	• Reduced ROS levels and ROS-induced damages in post-thaw spermatozoa [ascorbate (300 μM) and catalase (200 and 400 IU/l)]
Catalase	Chi et al. [21]	Catalase (1, 10, 100 U/ml) or EDTA (1, 10, 100 μM/ml)	Added to medium used during sperm wash	• Sperm motility (10 μM/ml EDTA) • Acrosome reaction rate of the spermatozoa (catalase) • Decreased DNA fragmentation rate of the spermatozoa (EDTA and catalase)
Vitamin E	Kalthur et al. [22]	Vitamin E 5 mM	Added to cryomedia prior to freeze-thaw	• Post-thaw motility • DNA integrity
Vitamin E	Taylor et al. [23]	Vitamin E 100 or 200 μmol	Added to cryomedia	• Post-thaw motility
Vitamin E	Cicek et al. [24]	Vitamin E 400 IU orally to women with unexplained infertility undergoing ovarian stimulation and IUI	Between day 3 and 5 of menstrual cycle until hCG injection	• Endometrial thickness on hCG day
Vitamin E+selenium	Moslemi and Tavanbakhsh [25]	Vitamin E 400 IU+Selenium 200 μg orally in men with idiopathic asthenozoospermia	100 days	• Sperm motility • Sperm morphology • Spontaneous pregnancy rates
Vitamin C	Akmal et al. [26]	2000 mg vitamin C in oligozoospermic men	2 months	• Mean sperm count • Sperm motility • Sperm morphology

(continued)

Table 7.1 (continued)

Antioxidant(s)	Study	Treatment dosage (per treatment or day)	Treatment details	Parameters improved
Vitamin C + vitamin E	Greco et al. [27]	Oral 1 g vitamin C and 1 g vitamin E in men with elevated sperm DNA fragmentation (15 %) and prior failed ICSI attempt	2 months	• Reduced DNA damaged sperm • Implantation rates • Implantation rates • Clinical pregnancy rates
Vitamin C	Henmi et al. [28]	Oral ascorbic acid 750 mg in women with luteal phase defects	1st day of the third cycle until a positive urinary pregnancy test	• Clinical pregnancy rates
Vitamin C	Crha et al. [29]	Vitamin C 500 mg in women undergoing IVF-ET	Gradual release over 8–12 h	• Improved pregnancy rates
Vitamin C	Branco et al. [30]	Ascorbic acid 10 mM	Semen before freezing	• Reduced DNA damage
Melatonin	Eryilmaz et al. [31]	Melatonin 3 mg in IVF-ET patients	3rd to the 5th day of the menstrual cycle until hCG	• Mean number of retrieved oocytes • Mean number of MII oocyte counts • G1 embryo ratio
Melatonin	Tamura et al. [32]	Melatonin 3 mg in women with prior IVF-ET failure	Between the 3rd and the 5th day of the menstrual cycle until hCG injection	• Oocyte quality • Fertilization rates
Melatonin	Unfer et al. [33]	Myo-inositol 4 g and melatonin 3 mg and folic acid 400 mcg in women with failed IVF cycle	3 months	• Number of MII oocytes retrieved • Total and top-quality embryos transferred • Fertilization rate
Coenzyme Q10	Nadjarzadeh et al. [34]	CoQ10 200 mg in iOAT patients	12 weeks	• Increased TAC • Reduced LPO
Coenzyme Q10	Safarinejad [35]	CoQ10 600 mg in iOAT patients	12 months	• Sperm quality (concentration, progressive motility, morphology) • Pregnancy rates
L-Carnitine	Lenzi et al. [36]	L-Carnitine 2 g	2 months	• Semen quality

L-Carnitine and acetyl L-Carnitine	Cavallini et al. [37]	L-Carnitine 2 g and acetyl-L-Carnitine 1 g	6 months	• Semen quality • Pregnancy rates
L-Carnitine with Vitamin E	Wang et al. [38]	L-Carnitine 2 g and vitamin E	3 months	• Percentage of forward motile sperm after the treatment • Pregnancy rate
L-Carnitine and acetyl L-Carnitine	Vicari and Calogero [39]	L-Carnitine 2 g and acetyl-L-Carnitine 1 g	3 months	• Sperm forward motility • Sperm viability • Reduced ROS production
L-Carnitine	Khademi et al. [40]	L-Carnitine 3 g in men with idiopathic sperm abnormalities	3 months	• Percentile of motile and grade A sperm • Percentile of normal-shaped sperms decreased significantly
L-Carnitine	Abdelrazik et al. [41]	L-Carnitine 0.3 and 0.6 mg/ml	Added to cryomedia	• Blastocyst development rate • Reduced DNA damage
Folic acid and zinc sulphate	Wong et al. [42]	Folic acid 5 mg and zinc sulphate 66 mg in subfertile men	26 weeks	• Folate concentrations in seminal plasma • Total normal sperm count
Myo-inositol and folic acid	Papaleo et al. [43]	Myo-inositol 4 g and 400 μg folic acid twice a day	Continuous from the day of GnRH administration	• Reduced mean number of immature oocytes at pick up • Reduced mean number of degenerated oocytes at pick up • Number of retrieved oocytes maintained
Myo-inositol and folic acid	Ciotta et al. [44]	Myo-inositol 4 g and 400 μg folic acid twice a day	3 months	• Number of oocytes recovered at pick-up • Reduced number of immature oocytes
Combination of antioxidants	Wirleitner et al. [45]	FertilovitRMplus twice daily	2 months	• Sperm motility • Reduced percentage of immotile sperm cells • Total sperm count • Percentage of class I sperm

(continued)

Table 7.1 (continued)

Antioxidant(s)	Study	Treatment dosage (per treatment or day)	Treatment details	Parameters improved
Combination of antioxidants	Tunc et al. [46]	1 Menevit capsule	3 months	• Pregnancy outcome
Combination of antioxidants	Tremellen et al. [47]	Menevit	3 months	• Viable pregnancy rate
Combination of antioxidants	Omu et al. [48]	Zinc 5 mg, Vitamin E + zinc 10 mg and Zinc + Vitamin E + C 200 mg	3 months	• Sperm parameters
Combination of antioxidants	Rizzo et al. [49]	Myo-inositol 4 g and folic acid 200 mg and melatonin 3 mg	Continuous from the day of GnRH administration	• Oocyte quality • Number of morphologically mature oocytes at ovum pick up

Note: Reprinted from "Strategies to Ameliorate Oxidative Stress during Assisted Reproduction," by Agarwal et al., 2014, Springer, p. 25–28. Copyright (2014) by Springer [50]

seminal fluid of fertile men than in infertile men and is therefore assumed to play an important role as a seminal AOX [51].

Notwithstanding, the effects of vitamin C on semen parameters have yielded conflicting results. A study by Askari et al. demonstrated that 10 mmol/l of vitamin C added to TEST yolk buffer failed to preserve sperm motility during the cryopreservation process [52]. On the other hand, Verma et al., reported a dose-dependent effect of vitamin C on sperm motility. In their study, sperm motility reached its peak after six hours incubation with vitamin C at a concentration of *800 μmol/l* [53]. Not only did vitamin C enhance sperm motility, but it also played a role in protecting sperm DNA from oxidative damage. Donnelly et al. demonstrated that in vitro sperm incubation with vitamin C at concentrations ranging from 300 to 600 *μmol/l* provided full protection to sperm against damage inflicted by H_2O_2 [54].

Fraga et al. reported an inverse association between vitamin C and sperm DNA damage. By testing normal semen samples, it was noted that increased 8-OHdG levels in the seminal fluid was associated with decreased levels of vitamin C. When administration of vitamin C reduced from 250 mg to 5 mg/day, a marked increase in 8-OHdG accompanied by a decrease in vitamin C levels in seminal fluid was noted. Similarly, saturation of seminal fluid by vitamin C led to a decrease of 8-OHdG levels and increase in vitamin C levels [55]. This result suggested that vitamin C may protect sperm from OS-induced DNA damage.

7.2.1.3 Vitamin E

Vitamin E actually pertains to a family of vitamins in the tocopherol group. The major type within this group is α-tocopherol. Vitamin E is a fat-soluble vitamin essential for reproduction. Like other AOX, vitamin E plays a role in protection of the cell membrane, prevention of protein modification and protection against DNA damage. With regards to male infertility, the function of vitamin E depends on selenium levels. During the scavenging process vitamin E utilizes glutathione peroxidase, a selenium-dependent enzyme, to reduce hydrogen peroxide molecules [56].

In an in vitro study performed by de Lamirande and Gagnon, vitamin E was shown to protect spermatozoa from damage by OS [57]. Moreover, addition of vitamin E to semen during cryopreservation also protected spermatozoa from OS [23]. In a series of studies by Aitken and colleagues, the role of vitamin E as a sperm motility enhancer and AOX was investigated. Using a 10 mmol dose, vitamin E decreased lipid peroxidation and preserved sperm motility [58, 59]. Furthermore, Verma and Kanwar reported that that 1 and 2 mmol/l dosages were related to improvements in sperm motility in vitro in a dose-dependent manner [60].

Addition of vitamin E to the cryomedia was also shown to preserve sperm motility during the freezing-thawing process [61, 62]. In contrast, vitamin E at a dose of 1 mmol had no effect on lipid peroxidation [63]. When combined with vitamin C, vitamin E may have pro-oxidative effects as shown by Hughes and colleagues [64]. Others have found contrary results in which the combination of vitamin E and C resulted in lower sperm DNA damage in normal and asthenozoospermic men [54, 65, 66].

7.2.1.4 Carnitine

L-Carnitine (LC) is a non-enzymatic AOX. It is produced by the liver and transported to the epididymis through blood circulation [67], where it is found in high concentrations. It has been suggested that LC is a key factor in promoting sperm motility during epididymis transit [12, 68]. Both LC and acetyl-L-carnitine (ALC) play a major role in providing energy for sperm, thereby affecting sperm function.

Several animal and human studies were conducted to explore the role of carnitine on sperm motility in vitro. L-Carnitine, alone or combined with ALC, was shown to increase sperm motility when added to sperm medium [69]. It seems though that L-carnitine has to be converted to ALC to exert its effects [70–72].

7.2.1.5 Carotenoids

Carotenoids are mainly xanthophyll and carotene. Lycopene, found in vegetables, fruits, as well as in the testes and seminal plasma, is the most powerful AOX within this group [73]. Lycopene exerts its effects by donating electrons to free radicals, thus balancing and neutralizing the latter [74]. Another mechanism of action is regulation of the cell cycle [74, 75].

Zini et al. showed that sperm chromatin was protected by adding lycopene at a concentration of 5 mmol to sperm medium in vitro [76]. Addition of lycopene was shown to protect sperm previously exposed to hydrogen peroxide from DNA damage at a concentration of 5 μmol/l but not 2 μmol/l [76].

7.2.1.6 Glutathione (GSH)

Glutathione, supplied by N-acetyl cysteine (NAC) during removal of free radicals, is found in large quantities in the epididymis [7, 67, 77]. In an early study, sperm incubation with NAC for 20 min enhanced motility and decrease ROS levels [78]. Subsequently, the combination of GSH and hypotaurine was tested in both asthenozoospermic and normal sperm at different concentrations and incubation periods, but no effect on sperm motility was noted [79].

7.2.2 Evidence from In Vivo *Studies*

7.2.2.1 Vitamin C

In a randomized control trial conducted on 30 men suffering from infertility but otherwise healthy, patients were divided into three groups according to the following prescription given for one month: (1) Vitamin C 1000 mg/day; (2) Vitamin C 200 mg/day; and (3) placebo. At both high and low dosages vitamin C was shown to improve sperm motility, viability and morphology compared to placebo [80]. In

another study involving smokers, 75 healthy men were divided into three groups and treated as aforementioned. Semen analysis revealed that only morphology was improved on those individuals who received high dosages [81]. In two subsequent studies exploring the dosage of 200 mg/day, vitamin C was given for 6 months but no improvements on semen parameters were noted [82, 83].

7.2.2.2 Vitamin E

Vitamin E was shown to improve sperm parameters and protect testis from damage in rats [84]. In humans, Moreover, 100 mg vitamin E given three times/day for 24 weeks to asthenozoospermic men resulted in motility enhancement and decreased lipid peroxidation [85].

7.2.2.3 Carnitine

Carnitine was also shown to improve sperm parameters in the mice [86, 87]. In humans, one study involving 100 infertile men treated by either placebo or LC (2 g/day) for 6 months revealed that sperm concentration and motility were improved [36]. In another study involving 47 infertile men treated for 3 months with LC (3 g/day), similar effects were observed with regard to sperm count and motility [88]. On the contrary, no detectable differences were noted in semen parameters of 20 men with idiopathic infertility treated with ALC (4 g/day) for sixty days [89].

7.2.2.4 Carotenoids

In an experimental study involving rats, lycopene (7 mg/kg) was shown to protect against cisplatin toxicity [90]. In humans, lycopene at a dosage of 4 mg/day given to 30 infertile men for 3 months resulted in a positive effect on sperm motility, morphology, and concentration [91]. Furthermore, in a randomized clinical trial involving 30 men with unexplained infertility treated with either 16 mg/day of astaxanthin or placebo for three months, sperm motility was increased whereas ROS levels were decreased. However, there was no impact on sperm morphology or concentration [92].

7.2.2.5 Glutathione (GSH)

In a placebo-controlled, double blind, cross-over trial of glutathione therapy involving 20 infertile men, Lenzi et al. observed that patients receiving 600 μg of GSH daily for two months had a positive impact on sperm morphology and motility [93]. These authors' results were corroborated by another study involving 11 infertile men who have taken the same GSH scheme [94]. In general, GSH seems to have a positive impact on semen parameters, but further studies are needed to evaluate its effects on male fertility.

7.2.2.6 Combined Antioxidant Treatment

The synergistic effect of combined AOX therapy has been explored in a few studies. In one study involving 54 infertile men, selenium (225 μg/day) and vitamin E (400 mg/day) were given to 25 patients whereas vitamin B group (4.5 g/day) was administered to the remaining 26 men. The authors observed that the combination of selenium and vitamin E resulted in increased sperm motility and viability and decreased lipid peroxidation [95]. In another study, Piomboni et al. examined the effects of AOX combination (vitamin C, lactoferrin and vitamin E) given to a group of 51 asthenoteratozoospermic patients. After a 3-month treatment period, sperm motility and morphology was increased whereas sperm DNA damage was reduced [96]. Comhaire et al. also examined the effect of AOX combination in a group of 27 infertile men. Different formulation involving N-acetyl-cysteine, vitamins A with E and a combination of fatty acids were given for 6 months. The authors noted an increase in sperm concentration and decrease in ROS levels with no change on sperm morphology or motility [97].

Several studies investigated the combination of vitamin C and vitamin E and their role in DNA protection and enhancement of male fertility. In a study conducted by Greco and colleagues, 64 infertile men with high levels of sperm DNA damage were randomized to receive 1000 mg/day of both vitamin C and E for two months or placebo. Although no differences were observed in conventional semen parameters, the group receiving AOX combination was shown to have lower levels of sperm DNA damage [27].

7.3 Assisted Reproductive Technology (ART) and AOX Therapy

Assisted reproductive technology (ART) has been widely used to treat infertility due to severe male factor [98]. In these settings, the use of AOX has been suggested to reduce the levels of ROS generated from many sources such as immature spermatozoa and leucocytes [99, 100]. Reduction in DNA fragmentation [30], improvement in sperm motility [101] and viability [102] after adding vitamin C to sperm culture media during ART have been reported. Catalase added to sperm culture media was also shown to be associated with reduction in ROS levels, DNA fragmentation and increase in acrosome reaction rate [21].

In addition, antioxidants have been used as cryomedia supplements aimed at protecting sperm from the freeze-thawing process as AOX can antagonize the ROS by preventing its formation or removing the already formed ROS [13, 99, 103]. The addition of vitamin E to sperm cryomedia was shown to have positive effects on sperm motility [25, 95] and morphology (Fig. 7.1) [23].

AOX can also be given as oral supplements during or prior to ART to reduce the levels of endogenous and exogenous sources of ROS [104–108]. Improvement in sperm motility [85, 109] and decrease in lipid peroxidation after vitamin E administration have been reported in previous prospective studies and RCTs. Similarly,

positive effects were found with folic acid and zinc sulphate on sperm concentration [42]. Furthermore, oral intake of coenzyme Q_{10} resulted in increased sperm concentration and motility [110].

7.4 Therapeutic Role of Antioxidants (AOX) in the Treatment of Female Infertility

In this section, the focus will be on the therapeutic role that antioxidants play in the female reproductive system and how these interventions can affect the fertility status of infertile women suffering from different reproductive diseases, either by increasing the chances of regular conception or increasing the chances of successful ART (Table 7.1).

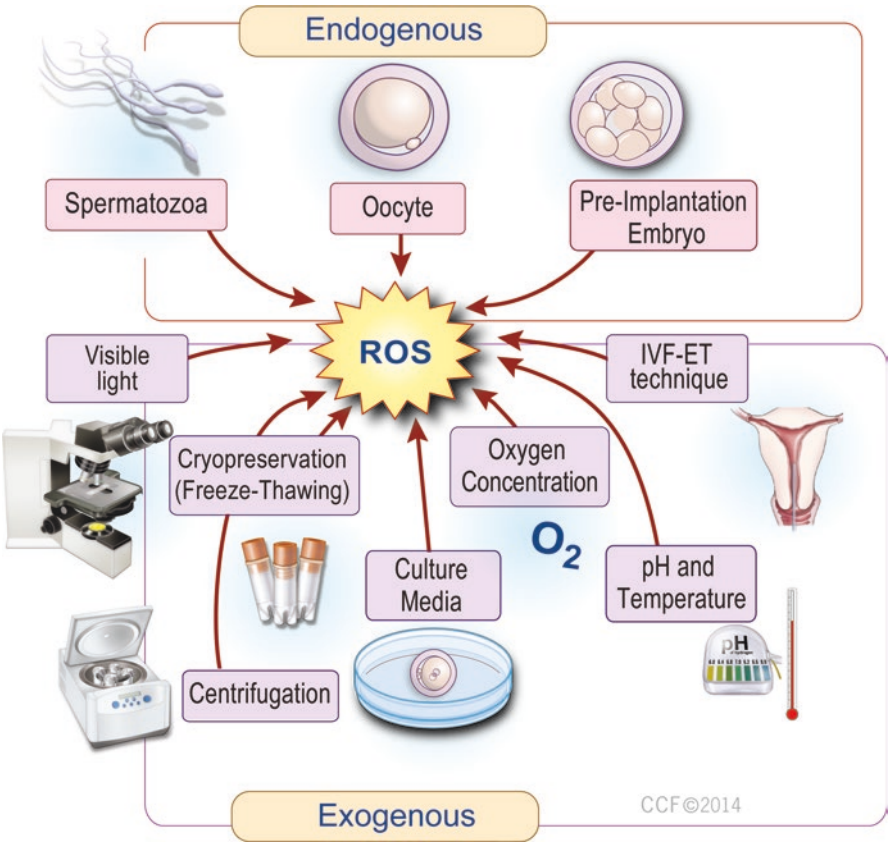

Fig. 7.1 Sources of ROS in ART

ROS act as stimulants for oocyte maturation and are required for meiosis I completion in the dominant oocyte. Although ROS are needed for the completion of meiosis I, antioxidants are essential for the initiation and completion of meiosis II [111]. Follicle stimulating hormone acts on pathways leading to catalase generation, which has a protective effect against the pro-apoptotic effects of ROS. Glutathione has also a role in neutralizing ROS.

7.4.1 Endometriosis

Endometriosis is a female reproductive disease in which the endometrium grows outside of the uterine cavity. The most common site of endometriosis is presence in the ovaries or peritoneum [112]. Endometriosis is a very common cause of infertility and is prevalent in 30 % of infertile women who are diagnosed with this condition [113]. It remains unclear how endometriosis has an effect on infertility; however, Sampson's well established theory suggests that the presence of endometrial tissue in the peritoneum can be explained by retrograde menstruation [114]. There are different treatment strategies to treat endometriosis related infertility; therapeutic laparoscopy and ART are the most widely used approaches [115].

Many studies suggest that women suffering from endometriosis have increased oxidative stress [116]. This can be explained by increased numbers of macrophages present in the peritoneum as a result of inflammatory response by endometrial tissue. Metabolic processes occurring in the macrophages are believed to be the cause of excessive ROS production leading to oxidative stress [116]. In addition to excessive ROS production, other inflammatory mediators such as cytokines, chemokines, metalloproteinases and prostaglandins are involved in the pathophysiology of endometriosis-related infertility [117]. Similar to a positive feedback mechanism, inflammatory processes allow for the development of endometrial tissue by means of increased ROS which leads to even further production of ROS thus making OS levels very high [118]. Nevertheless, the association between endometriosis patients and oxidative stress in the peritoneum is not unequivocal [119].

To further understand the relationship between endometriosis and OS, studies were conducted to test whether the levels of antioxidants in the peritoneal and follicular fluids of endometriosis patients were decreased. Overall, the levels of superoxide dismutase and glutathione peroxidase are usually decreased in endometriosis patients compared to controls [120–122]. Animal models have been used to test the effects of low molecular weight antioxidants such as vitamins C and E on induced endometriotic implants. In one study, vitamin C decreased the number of natural killer cells thus resulting in decreased levels of OS [123]. Another well-established method to measure the effect of antioxidants on OS is through the various OS markers such as malondialdehyde (MDA) (Table 7.1).

In a randomized controlled trial conducted by Mier-Cabrera et al. involving endometriosis patient, vitamins C and E were shown to decrease the levels of MDA and lipid hydroperoxides [124]. In another study conducted by Santanam

et al. the authors tested the effect of various antioxidant vitamins administered for 8 weeks to women with history of endometriosis and infertility in a randomized placebo-controlled trial. Several inflammatory markers were measured in the peritoneal fluid, including interleukin 6 (IL6), monocyte chemotactic protein (MCP), normal T-cell expressed and secreted (RANTES) [125]. In addition to that, pelvic pain was measured using a pain scale. The results of this study indicate that antioxidant treatment significantly decreases inflammatory markers in the peritoneal cavity of women suffering from endometriosis. Regarding the effects of antioxidants on pelvic pain, 43 % of those in the antioxidant group reported decreased pain in contrast to 0 % in the placebo group. Moreover, 52 % of patients in the antioxidant group reported no change in pain compared to 100 % of the placebo group. While the study lacked any comments on the relieving of infertility in endometriosis patient, the reduction in the levels of inflammatory markers suggests a corresponding decrease in OS, which could result in alleviation of infertility symptoms.

Many studies have demonstrated the effects of epigallocatechin-3-gallate (EGCG) on the size of endometriotic lesions. EGCG is a polyphenol that is present abundantly in green tea [126]. It can inhibit mitotic divisions as well as angiogenesis. In addition to that, it has been shown to induce apoptosis in endometriotic implants [127–129]. Its effects as an antioxidant have been studied in endometriosis patients. In one study, the *in vitro* effects of EGCG were compared to N-acetylcysteine (NAC)—a non-enzymatic antioxidant. Both NAC and EGCG reduced endometrial proliferation, but only EGCG showed any significant results on cell migration, invasion of endometrial tissue and reduction of alpha-smooth muscle actin (aSMA; a marker of myofibroblastic activity) [130]. These findings suggest a possible inhibition of the TGF-β1 signaling pathway leading to undifferentiation of myofibroblasts thus leading to reduction in endometriotic implants [131, 132]. Further studies on the effects of EGCG in treating endometriosis patients are needed to validate its reported beneficial effects, which might be hampered by its very low bioavailability [133, 134]

To conclude, oxidative stress has been implicated in the pathophysiology of endometriosis and antioxidants have shown to alleviate its effects. However, further studies are needed to investigate the effects of antioxidants on fertility parameters and assisted reproduction outcomes.

7.4.2 Polycystic Ovarian Syndrome

Polycystic ovarian syndrome (PCOS) is one of the most prevalent endocrine disorders in women at reproductive age. It is characterized by variable manifestations, including hyperandrogenism, hyperinsulinemia (insulin resistance) and hirsutism [135]. The underlying cause of PCOS seems to be multifactorial, including oxidative stress [136, 137]. It has been argued that many of the clinical symptoms of PCOS,

including central adiposity, hyperandrogenemia, obesity and infertility are interconnected with elevated levels of ROS [138, 139].

A study conducted on clomiphene-resistant PCOS women outlined the effect of L-carnitine on ovulation and pregnancy rates. L-Carnitine is a mitochondrial carrier that transports fatty acids into mitochondria for β-oxidation (Table 7.1). It has the ability to act as an antioxidant reducing intracellular metabolic stress. The group treated with both clomiphene citrate (CC) and L-carnitine showed significantly higher ovulation (64.4 % vs 17.4 %) and pregnancy rates (51.5 % vs 5.8 %) than the groups treated with clomiphene citrate alone or placebo. Secondary findings included faster follicle maturation rate as well as thicker endometrium at the time of hCG administration in the former [140]. This is an important finding given the reported detrimental effect of clomiphene citrate on estradiol levels and endometrial thickness [141]. Miscarriage rates were also lower in the experimental group when compared to the control; albeit of no statistical significance.

N-acetyl-cysteine (NAC) is an essential antioxidant that has the property of restoring glutathione to its reduced form (active form) [142–145]. Its effects on PCOS patients have been mixed. In one study, Salehpour et al. tested the effects of oral NAC as an adjuvant to clomiphene citrate on ovulation induction in PCOS patients [145]. The authors found that NAC had a positive effect on ovulation and pregnancy rates, which might be explained by NAC influence on insulin resistance and this effect has been reported by other investigators as well [146]. On the contrary, Elnashar et al. found that NAC had no effect of ovulation induction in CC-resistant PCOS patients [147].

Bahmani et al. investigated the effects of different doses of folate versus placebo on inflammatory factors and oxidative stress biomarkers in obese and overweight women suffering from PCOS [148]. The groups were given 1 and 5 mg/day of folate for 8 weeks, and a placebo group was included. OS markers including total antioxidant capacity (TAC), GSH, and MDA were measured prior to and after the completion of the trial. The results were as hypothesized; in the groups treated with folate there was an increase in TAC and GSH levels and reduction in MDA in a dose-dependent manner [149]. Folate supplementation has been shown to alleviate OS by decreasing ROS production [150]. Interestingly, folate administration had no noticeable effects on catalase or nitric oxide levels in the plasma.

7.4.3 Unexplained Infertility

Many studies have attempted to provide an explanation for the pathophysiology of unexplained infertility; increased OS in the peritoneum has been noticed in some of these patients [119]. A study conducted by Ruder et al. tested the relation between dietary antioxidant intake and time to pregnancy (TTP) in unexplained infertility

patients. Depending on age and body mass index (BMI) some antioxidants led to shorter TTP. In women with BMI < 25 kg/m^2 vitamin C was found to correlate with shorter TTP. In those women with BMI ≥ 25 kg/m^2 β-carotene was shown to shorten TTP. Women aged <35 years had shorter TTP when treated with either vitamin C or β-carotene although the difference was not statistically significant. Furthermore, women aged >35 years had shorter TTP with vitamin E supplementation although it was not statistically significant. β-Carotene was found to actually increase TTP in women >35 years [151]. Furthermore, it has been suggested that vitamin C alone might not be strong enough as an antioxidant to quench the elevated ROS levels seen in both women at advanced age or and those with high BMI [152, 153]. These patients are of particular interest since they represent the subgroups most likely to experience longer TTP and increased miscarriage rates [154, 155]. Overall the results of the study of Ruder et al. suggest that administration of antioxidants to women suffering from unexplained infertility may enhance their fertility status. In contrast, a study conducted by Youssef et al. on women suffering from unexplained infertility tested the effect of oral antioxidant supplementation (multivitamins and amino acid chelated minerals) on IVF/ICSI outcomes [156]. Specifically, the authors assessed oocyte maturity and pregnancy rates as the main outcome measures. There was no significant difference between the control group and experimental group in terms of oocyte quality or pregnancy rates suggesting that antioxidant supplementation administration had no effect on oocyte maturity.

7.4.4 Role of Antioxidants Added to Culture Media in ART

During the various steps in ART, ROS production could potentially increase to extreme levels causing oxidative stress, which is harmful to gametes and resulting embryos [9]. This effect can be potentially augmented by the fact that gametes and embryos have minimal antioxidant defense mechanisms (Table 7.1). As a result, leading to decrease in the chances of ARTs success [99]. Antioxidants can be integrated into the media used in ART to minimize oxidative stress.

Melatonin has been one of the most investigated antioxidants in this regard. Melatonin can act as a direct antioxidant and an indirect one. Melatonin has the ability not only to directly scavenge ROS but also to stimulate other enzymatic antioxidants (Table 7.1) [157, 158]. In one study, Unfer et al. tested the effect of melatonin combined with myo-inositol (a molecule essential for the phosphatidylinositol signal transduction pathway) [33]. The authors found that fertilization rates and the number of embryos available to transfer were higher in treated patients. Further studies are needed to better understand the type of AOX that would better benefit culture conditions during ART.

7.5 Conclusion

Subfertility can be a consequence of oxidative stress, which negatively impacts multiple cellular processes in the male and female reproductive functions. AOX supplementation plays an important role in decreasing the effect of OS. In the male, in vivo and in vitro studies suggest that AOX may improve semen parameters and decrease sperm DNA damage as well as at the cellular level decrease membrane lipid peroxidation. In women, it is suggested that AOX can enhance fecundity and reduce time-to-pregnancy. Although current data is not conclusive and results vary with type and duration of AOX administration, the literature overall concurs that AOXs are useful to reduce ROS levels, but there are some reported apprehensions regarding adverse effects of AOX. Most investigators agree that further clinical trials with adequate power are needed to conclusively demonstrate the beneficial effect of AOX in both male and female fertility, but so far results are promising and AOX may be used selectively in conjunction with other fertility treatments for individuals with male or female factor infertility.

References

1. Lombardo F et al (2011) The role of antioxidant therapy in the treatment of male infertility: an overview. Asian J Androl 13(5):690–697
2. Droge W (2002) Free radicals in the physiological control of cell function. Physiol Rev 82(1):47–95
3. Halliwell B (1999) Antioxidant defence mechanisms: from the beginning to the end (of the beginning). Free Radic Res 31(4):261–272
4. Perkins AV (2006) Endogenous anti-oxidants in pregnancy and preeclampsia. Austr N Z J Obstet Gynaecol 46(2):77–83
5. Shebis Y, Iluz D, Kinel-Tahan Y, Dubinsky Z, Yehoshua Y (2013) Natural antioxidants: function and sources food and nutrition sciences. Sci Res 4:643–649
6. Pasqualotto EB et al (2004) Effect of oxidative stress in follicular fluid on the outcome of assisted reproductive procedures. Fertil Steril 81(40):973–976
7. Agarwal A et al (2004) Role of antioxidants in treatment of male infertility: an overview of the literature. Reprod Biomed Online 8(6):616–627
8. Olooto WE (2012) Infertility in male; risk factors, causes and management – a review. J Microbiol Biotechnol Res 2(4):641–645
9. Agarwal A, Gupta S, Sikka S (2006) The role of free radicals and antioxidants in reproduction. Curr Opin Obstet Gynecol 18(3):325–332
10. Agarwal A et al (2014) Effect of oxidative stress on male reproduction. World J Mens Health 32(1):1–17
11. Ruder EH, Hartman TJ, Goldman MB (2009) Impact of oxidative stress on female fertility. Curr Opin Obstet Gynecol 21(3):219
12. Giancarlo Balercia AM, Gian Paolo Littarru (2012) Natural antioxidants in Male infertility. In: Sijo J Parekattil (ed). pp 369–380
13. Zini A, Al-Hathal N (2011) Antioxidant therapy in male infertility: fact or fiction? Asian J Androl 13(3):374–381
14. Walczak-Jedrzejowska R, Wolski JK, Slowikowska-Hilczer J (2013) The role of oxidative stress and antioxidants in male fertility. Cent Eur J Urol 66(1):60–67

15. Tsunoda S et al (2012) Impaired fertilizing ability of superoxide dismutase 1-deficient mouse sperm during in vitro fertilization. Biol Reprod 87(5):121
16. Nonogaki T et al (1992) Effects of superoxide dismutase on mouse in vitro fertilization and embryo culture system. J Assist Reprod Genet 9(3):274–280
17. Perumal P (2014) Effect of superoxide dismutase on semen parameters and antioxidant enzyme activities of liquid stored (5°C) Mithun (Bos frontalis) semen. J Animals 2014:9
18. de Lamirande E et al (1997) Reactive oxygen species and sperm physiology. Rev Reprod 2(1):48–54
19. Rossi T et al (2001) Improved human sperm recovery using superoxide dismutase and catalase supplementation in semen cryopreservation procedure. Cell Tissue Bank 2(1):9–13
20. Li Z et al (2010) Protective effects of ascorbate and catalase on human spermatozoa during cryopreservation. J Androl 31(5):437–444
21. Chi HJ et al (2008) Protective effect of antioxidant supplementation in sperm-preparation medium against oxidative stress in human spermatozoa. Hum Reprod 23(5):1023–1028
22. Kalthur G et al (2011) Vitamin E supplementation in semen-freezing medium improves the motility and protects sperm from freeze-thaw-induced DNA damage. Fertil Steril 95(3):1149–1151
23. Taylor K et al (2009) Effect of antioxidant supplementation of cryopreservation medium on post-thaw integrity of human spermatozoa. Reprod Biomed Online 18(2):184–189
24. Cicek N et al (2012) Vitamin E effect on controlled ovarian stimulation of unexplained infertile women. J Assist Reprod Genet 29(4):325–328
25. Moslemi MK, Tavanbakhsh S (2011) Selenium-vitamin E supplementation in infertile men: effects on semen parameters and pregnancy rate. Int J Gen Med 4:99–104
26. Akmal M et al (2006) Improvement in human semen quality after oral supplementation of vitamin C. J Med Food 9(3):440–442
27. Greco E et al (2005) Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. J Androl 26(3):349–353
28. Henmi H et al (2003) Effects of ascorbic acid supplementation on serum progesterone levels in patients with a luteal phase defect. Fertil Steril 80(2):459–461
29. Crha I et al (2003) Ascorbic acid and infertility treatment. Cent Eur J Public Health 11(2):63–67
30. Branco CS et al (2010) Resveratrol and ascorbic acid prevent DNA damage induced by cryopreservation in human semen. Cryobiology 60(2):235–237
31. Eryilmaz OG et al (2011) Melatonin improves the oocyte and the embryo in IVF patients with sleep disturbances, but does not improve the sleeping problems. J Assist Reprod Genet 28(9):815–820
32. Tamura H et al (2008) Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. J Pineal Res 44(3):280–287
33. Unfer V et al (2011) Effect of a supplementation with myo-inositol plus melatonin on oocyte quality in women who failed to conceive in previous in vitro fertilization cycles for poor oocyte quality: a prospective, longitudinal, cohort study. Gynecol Endocrinol 27(11):857–861
34. Nadjarzadeh A et al (2011) Coenzyme Q10 improves seminal oxidative defense but does not affect on semen parameters in idiopathic oligoasthenoteratozoospermia: a randomized double-blind, placebo controlled trial. J Endocrinol Invest 34(8):e224–e228
35. Safarinejad MR (2012) The effect of coenzyme Q(1)(0) supplementation on partner pregnancy rate in infertile men with idiopathic oligoasthenoteratozoospermia: an open-label prospective study. Int Urol Nephrol 44(3):689–700
36. Lenzi A et al (2003) Use of carnitine therapy in selected cases of male factor infertility: a double-blind crossover trial. Fertil Steril 79(2):292–300
37. Cavallini G et al (2012) Reduction in sperm aneuploidy levels in severe oligoasthenoteratospermic patients after medical therapy: a preliminary report. Asian J Androl 14(4):591–598
38. Wang YX et al (2010) L-carnitine: safe and effective for asthenozoospermia. Zhonghua Nan Ke Xue 16(5):420–422

39. Vicari E et al (2001) Antioxidant therapeutic efficiency after the use of carnitine in infertile patients with bacterial or non bacterial prostato-vesiculo-epididymitis. Arch Ital Urol Androl 73(1):15–25
40. Khademi A et al (2005) The effects of L-carnitine on sperm parameters in smoker and non-smoker patients with idiopathic sperm abnormalities. J Assist Reprod Genet 22(11-12):395–399
41. Abdelrazik H et al (2009) L-carnitine decreases DNA damage and improves the in vitro blastocyst development rate in mouse embryos. Fertil Steril 91(2):589–596
42. Wong WY et al (2002) Effects of folic acid and zinc sulfate on male factor subfertility: a double-blind, randomized, placebo-controlled trial. Fertil Steril 77(3):491–498
43. Papaleo E et al (2009) Myo-inositol may improve oocyte quality in intracytoplasmic sperm injection cycles. A prospective, controlled, randomized trial. Fertil Steril 91(5):1750–1754
44. Ciotta L et al (2011) Effects of myo-inositol supplementation on oocyte's quality in PCOS patients: a double blind trial. Eur Rev Med Pharmacol Sci 15(5):509–514
45. Wirleitner B et al (2012) Dietary supplementation of antioxidants improves semen quality of IVF patients in terms of motility, sperm count, and nuclear vacuolization. Int J Vitam Nutr Res 82(6):391–398
46. Tunc O, Thompson J, Tremellen K (2009) *Improvement in sperm DNA quality using an oral antioxidant therapy*. Reprod Biomed Online 18(6):761–768
47. Tremellen K et al (2007) A randomised control trial examining the effect of an antioxidant (Menevit) on pregnancy outcome during IVF-ICSI treatment. Aust N Z J Obstet Gynaecol 47(3):216–221
48. Omu AE et al (2008) Indications of the mechanisms involved in improved sperm parameters by zinc therapy. Med Princ Pract 17(2):108–116
49. RizzoP RE, Benedetto V (2010) Effect of the treatment with myo-inositol plus folic acid plus melatonin in comparison with a treatment with myo-inositol plus folic acid on oocyte quality and pregnancy outcome in IVF cycles. A prospective, clinical trial. Eur Rev Med Pharmacol Sci 14(6):555–561
50. Agarwal A et al (2014) Strategies to ameliorate oxidative stress during assisted reproduction. Springer, New York
51. Ko EY, Kefer JC, Agarwal A, Sabanegh E (2012) Synthetic antioxidants. In: Male infertility: contemporary clinical approaches, andrology, ART and antioxidants. Springer, New York, pp 381–388. doi:10.1007/978-1-4614-3335-4_36
52. Askari HA et al (1994) Effect of natural antioxidants tocopherol and ascorbic acids in maintenance of sperm activity during freeze-thaw process. Arch Androl 33(1):11–15
53. Verma A, Kanwar KC (1998) Human sperm motility and lipid peroxidation in different ascorbic acid concentrations: an in vitro analysis. Andrologia 30(6):325–329
54. Donnelly ET, McClure N, Lewis SE (1999) The effect of ascorbate and alpha-tocopherol supplementation in vitro on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. Mutagenesis 14(5):505–512
55. Fraga CG et al (1991) Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. Proc Natl Acad Sci U S A 88(24):11003–11006
56. Brigelius-Flohe R, Traber MG (1999) Vitamin E: function and metabolism. FASEB J 13(10):1145–1155
57. de Lamirande E, Gagnon C (1992) Reactive oxygen species and human spermatozoa. I. Effects on the motility of intact spermatozoa and on sperm axonemes. J Androl 13(5):368–378
58. Aitken RJ, Clarkson JS, Fishel S (1989) Generation of reactive oxygen species, lipid peroxidation, and human sperm function. Biol Reprod 41(1):183–197
59. Aitken RJ, Clarkson JS (1988) Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. J Androl 9(6):367–376
60. Verma A, Kanwar KC (1999) Effect of vitamin E on human sperm motility and lipid peroxidation in vitro. Asian J Androl 1(3):151–154
61. Critser JK et al (1988) Cryopreservation of human spermatozoa. III. The effect of cryoprotectants on motility. Fertil Steril 50(2):314–320

62. Mazzilli F et al (1995) Human sperm cryopreservation and reactive oxygen species (ROS) production. Acta Eur Fertil 26(4):145–148
63. Twigg J et al (1998) Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. Hum Reprod 13(6):1429–1436
64. Hughes CM et al (1998) The effects of antioxidant supplementation during Percoll preparation on human sperm DNA integrity. Hum Reprod 13(5):1240–1247
65. Donnelly ET, McClure N, Lewis SE (1999) Antioxidant supplementation in vitro does not improve human sperm motility. Fertil Steril 72(3):484–495
66. Sierens J et al (2002) In vitro isoflavone supplementation reduces hydrogen peroxide-induced DNA damage in sperm. Teratog Carcinog Mutagen 22(3):227–234
67. Tremellen K (2012) Antioxidant therapy for the enhancement of male reproductive health: a critical review of the literature in male infertility. Springer, New York, pp 389–399, A.A. Sijo J. Parekattil (ed)
68. Jeulin C, Lewin LM (1996) Role of free L-carnitine and acetyl-L-carnitine in post-gonadal maturation of mammalian spermatozoa. Hum Reprod Update 2(2):87–102
69. Tanphaichitr N (1977) In vitro stimulation of human sperm motility by acetylcarnitine. Int J Fertil 22(2):85–91
70. Jeulin C, Soufir JC (1992) Reversible intracellular ATP changes in intact rat spermatozoa and effects on flagellar sperm movement. Cell Motil Cytoskeleton 21(3):210–222
71. Jeulin C, Dacheux JL, Soufir JC (1994) Uptake and release of free L-carnitine by boar epididymal spermatozoa in vitro and subsequent acetylation rate. J Reprod Fertil 100(1):263–271
72. Agarwal A, Said TM (2004) Carnitines and male infertility. Reprod Biomed Online 8(4):376–384
73. Klebanov GI et al (1998) The antioxidant properties of lycopene. Membr Cell Biol 12(2):287–300
74. Palozza P et al (2012) Lycopene as a guardian of redox signalling. Acta Biochim Pol 59(1):21–25
75. Rao AV, Ray MR, Rao LG (2006) Lycopene. Adv Food Nutr Res 51:99–164
76. Zini A, San Gabriel M, Libman J (2010) Lycopene supplementation in vitro can protect human sperm deoxyribonucleic acid from oxidative damage. Fertil Steril 94(3):1033–1036
77. Gressier B et al (1994) Decrease of hypochlorous acid and hydroxyl radical generated by stimulated human neutrophils: comparison in vitro of some thiol-containing drugs. Methods Find Exp Clin Pharmacol 16(1):9–13
78. Oeda T et al (1997) Scavenging effect of N-acetyl-L-cysteine against reactive oxygen species in human semen: a possible therapeutic modality for male factor infertility? Andrologia 29(3):125–131
79. Donnelly ET, McClure N, Lewis SE (2000) Glutathione and hypotaurine in vitro: effects on human sperm motility, DNA integrity and production of reactive oxygen species. Mutagenesis 15(1):61–68
80. Dawson EB et al (1987) Effect of ascorbic acid on male fertility. Ann N Y Acad Sci 498:312–323
81. Dawson EB et al (1992) Effect of ascorbic acid supplementation on the sperm quality of smokers. Fertil Steril 58(5):1034–1039
82. Abel BJ et al (1982) Randomised trial of clomiphene citrate treatment and vitamin C for male infertility. Br J Urol 54(6):780–784
83. Hargreave TB et al (1984) Randomised trial of mesterolone versus vitamin C for male infertility. Scottish Infertility Group. Br J Urol 56(6):740–744
84. Zhou DX et al (2006) The protective effect of vitamin E against oxidative damage caused by formaldehyde in the testes of adult rats. Asian J Androl 8(5):584–588
85. Suleiman SA et al (1996) Lipid peroxidation and human sperm motility: protective role of vitamin E. J Androl 17(5):530–537

86. Amendola R et al (1989) Effects of L-acetylcarnitine (LAC) on the post-injury recovery of mouse spermatogenesis monitored by flow cytometry. 1. Recovery after X-irradiation. Andrologia 21(6):568–575
87. Ramadan LA et al (2002) Testicular toxicity effects of magnetic field exposure and prophylactic role of coenzyme Q10 and L-carnitine in mice. Pharmacol Res 46(4):363–370
88. Vitali G, Parente R, Melotti C (1995) Carnitine supplementation in human idiopathic asthenospermia: clinical results. Drugs Exp Clin Res 21(4):157–159
89. Moncada ML et al (1992) Effect of acetylcarnitine treatment in oligoasthenospermic patients. Acta Eur Fertil 23(5):221–224
90. Atessahin A et al (2006) Protective role of lycopene on cisplatin-induced changes in sperm characteristics, testicular damage and oxidative stress in rats. Reprod Toxicol 21(1):42–47
91. Gupta NP, Kumar R (2002) Lycopene therapy in idiopathic male infertility – a preliminary report. Int Urol Nephrol 34(3):369–372
92. Comhaire FH et al (2005) Combined conventional/antioxidant "Astaxanthin" treatment for male infertility: a double blind, randomized trial. Asian J Androl 7(3):257–262
93. Lenzi A et al (1993) Placebo-controlled, double-blind, cross-over trial of glutathione therapy in male infertility. Hum Reprod 8(10):1657–1662
94. Lenzi A et al (1992) Glutathione therapy for male infertility. Arch Androl 29(1):65–68
95. Keskes-Ammar L et al (2003) Sperm oxidative stress and the effect of an oral vitamin E and selenium supplement on semen quality in infertile men. Arch Androl 49(2):83–94
96. Piomboni P et al (2008) Sperm quality improvement after natural anti-oxidant treatment of asthenoteratospermic men with leukocytospermia. Asian J Androl 10(2):201–206
97. Comhaire FH et al (2000) The effects of combined conventional treatment, oral antioxidants and essential fatty acids on sperm biology in subfertile men. Prostaglandins Leukot Essent Fatty Acids 63(3):159–165
98. Agarwal A, Durairajanayagam D, du Plessis SS (2014) Utility of antioxidants during assisted reproductive techniques: an evidence based review. Reprod Biol Endocrinol 12:112
99. Lampiao F (2012) Free radicals generation in an in vitro fertilization setting and how to minimize them. World J Obstet Gynecol 1(3):29–34
100. Pegg DE (2007) Principles of cryopreservation. Methods Mol Biol 368:39–57
101. Jenkins TG, Aston KI, Carrell DT (2011) Supplementation of cryomedium with ascorbic acid-2-glucoside (AA2G) improves human sperm post-thaw motility. Fertil Steril 95(6):2001–2004
102. Fanaei H et al (2014) Effects of ascorbic acid on sperm motility, viability, acrosome reaction and DNA integrity in teratozoospermic samples. Iran J Reprod Med 12(2):103–110
103. Bansal AK, Bilaspuri GS (2010) Impacts of oxidative stress and antioxidants on semen functions. Vet Med Int 2010:1–10
104. Hammadeh ME et al (2008) Reactive oxygen species, total antioxidant concentration of seminal plasma and their effect on sperm parameters and outcome of IVF/ICSI patients. Arch Gynecol Obstet 277(6):515–526
105. Tavilani H et al (2008) Activity of antioxidant enzymes in seminal plasma and their relationship with lipid peroxidation of spermatozoa. Int Braz J Urol 34(4):485–491
106. Fatma Atig MR, Ali HB, Ben H, Abdelhamid K, Saad A, Ajina M (2012) Altered antioxidant status and increased lipid per-oxidation in seminal plasma of Tunisian infertile men. Int J Biol Sci 1:139–149
107. Marzec-Wroblewska U et al (2011) Zinc and iron concentration and SOD activity in human semen and seminal plasma. Biol Trace Elem Res 143(1):167–177
108. Yan L et al (2014) Seminal superoxide dismutase activity and its relationship with semen quality and SOD gene polymorphism. J Assist Reprod Genet 31(5):549–554
109. Ghanem H, Shaeer O, El-Segini A (2010) Combination clomiphene citrate and antioxidant therapy for idiopathic male infertility: a randomized controlled trial. Fertil Steril 93(7):2232–2235
110. Lafuente R et al (2013) Coenzyme Q10 and male infertility: a meta-analysis. J Assist Reprod Genet 30(9):1147–1156

111. Behrman HR et al (2001) Oxidative stress and the ovary. J Soc Gynecol Investig 8(1 suppl):S40–S42
112. Ozkan S, Murk W, Arici A (2008) Endometriosis and infertility. Ann N Y Acad Sci 1127(1):92–100
113. Allaire C (2006) Endometriosis and infertility: a review. J Reprod Med 51(3):164–168
114. Halme J et al (1984) Retrograde menstruation in healthy women and in patients with endometriosis. Obstet Gynecol 64(2):151–154
115. Damewood M (1989) The role of the new reproductive technologies including IVF and GIFT in endometriosis. Obstet Gynecol Clin North Am 16(1):179–191
116. Augoulea A et al (2009) The role of the oxidative-stress in the endometriosis-related infertility. Gynecol Endocrinol 25(2):75–81
117. Balunas MJ et al (2009) Isolation and characterization of aromatase inhibitors from Brassaiopsis glomerulata (Araliaceae). Phytochem Lett 2(1):29–33
118. Santulli P et al (2014) Protein oxidative stress markers in peritoneal fluids of women with deep infiltrating endometriosis are increased. Hum Reprod. pp deu290
119. Polak G et al (2001) Total antioxidant status of peritoneal fluid in infertile women. Eur J Obstet Gynecol Reprod Biol 94(2):261–263
120. Yi L, Lilan L, Haibo Z (2001) Levels of lipid perioxides and superoxide dismutase in peritoneal fluid of patients with endometriosis. J Tongji Med Univ 21(2):166–167
121. Shu J et al (2013) The effect of peritoneal fluid from patients with endometriosis on mitochondrial function and development of early mouse embryos. PLoS One 8(12), e82334
122. Szczepanska M et al (2003) Oxidative stress may be a piece in the endometriosis puzzle. Fertil Steril 79(6):1288–1293
123. Durak Y et al (2013) Effect of vitamin C on the growth of experimentally induced endometriotic cysts. J Obstet Gynaecol Res 39(7):1253–1258
124. Mier-Cabrera J et al (2011) Quantitative and qualitative peritoneal immune profiles, T-cell apoptosis and oxidative stress-associated characteristics in women with minimal and mild endometriosis. BJOG 118(1):6–16
125. Santanam N et al (2013) Antioxidant supplementation reduces endometriosis-related pelvic pain in humans. Transl Res 161(3):189–195
126. Kada T et al (1985) Detection and chemical identification of natural bio-antimutagens: a case of the green tea factor. Mutat Res 150(1):127–132
127. Laschke MW et al (2008) Epigallocatechin-3-gallate inhibits estrogen-induced activation of endometrial cells in vitro and causes regression of endometriotic lesions in vivo. Hum Reprod 23(10):2308–2318
128. Xu H et al (2009) Anti-angiogenic effects of green tea catechin on an experimental endometriosis mouse model. Hum Reprod 24(3):608–618
129. Ricci A et al (2013) Natural therapies assessment for the treatment of endometriosis. Hum Reprod 28(1):178–188
130. Matsuzaki S, Darcha C (2014) Antifibrotic properties of epigallocatechin-3-gallate in endometriosis. Hum Reprod 29(8):1677–1687
131. Tomasek JJ et al (2002) Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol 3(5):349–363
132. Hinz B et al (2007) The myofibroblast: one function, multiple origins. Am J Pathol 170(6):1807–1816
133. Chow HS et al (2005) Effects of dosing condition on the oral bioavailability of green tea catechins after single-dose administration of Polyphenon E in healthy individuals. Clin Cancer Res 11(12):4627–4633
134. Yang CS et al (2008) Bioavailability issues in studying the health effects of plant polyphenolic compounds. Mol Nutr Food Res 52(S1):S139–S151
135. Khan KA, Stas S, Kurukulasuriya LR (2006) Polycystic ovarian syndrome. J Cardiometab Syndr 1(2):125–132
136. Norman RJ et al (2007) Polycystic ovary syndrome. Lancet 370(9588):685–697

137. Nardo L, Patchava S, Laing I (2008) Polycystic ovary syndrome: pathophysiology, molecular aspects and clinical implications. Panminerva Med 50(4):267–278
138. Olusi S (2002) Obesity is an independent risk factor for plasma lipid peroxidation and depletion of erythrocyte cytoprotectic enzymes in humans. Int J Obes Relat Metab Disord 26(9):1159–1164
139. Verit FF, Erel O (2008) Oxidative stress in nonobese women with polycystic ovary syndrome: correlations with endocrine and screening parameters. Gynecol Obstet Invest 65(4):233–239
140. Ismail AM et al (2014) Adding L-carnitine to clomiphene resistant PCOS women improves the quality of ovulation and the pregnancy rate. A randomized clinical trial. Eur J Obstet Gynecol Reprod Biol 180:148–152
141. Unfer V et al (2004) High dose of phytoestrogens can reverse the antiestrogenic effects of clomiphene citrate on the endometrium in patients undergoing intrauterine insemination: a randomized trial. J Soc Gynecol Investig 11(5):323–328
142. Borgström L, Kågedal B, Paulsen O (1986) Pharmacokinetics of N-acetylcysteine in man. Eur J Clin Pharmacol 31(2):217–222
143. Wentzel P, Thunberg L, Eriksson UJ (1997) Teratogenic effect of diabetic serum is prevented by supplementation of superoxide dismutase and N-acetylcysteine in rat embryo culture. Diabetologia 40(1):7–14
144. Fulghesu AM et al (2002) N-acetyl-cysteine treatment improves insulin sensitivity in women with polycystic ovary syndrome. Fertil Steril 77(6):1128–1135
145. Salehpour S et al (2009) N acetyl cysteine, a novel remedy for poly cystic ovarian syndrome. Int J Fertil Steril 3(2):66–73
146. Holte J et al (1994) Enhanced early insulin response to glucose in relation to insulin resistance in women with polycystic ovary syndrome and normal glucose tolerance. J Clin Endocrinol Metabol 78(5):1052–1058
147. Elnashar A et al (2007) N-acetyl cysteine vs. metformin in treatment of clomiphene citrate–resistant polycystic ovary syndrome: a prospective randomized controlled study. Fertil Steril 88(2):406–409
148. Bahmani F et al (2014) The effects of folate supplementation on inflammatory factors and biomarkers of oxidative stress in overweight and obese women with polycystic ovary syndrome: a randomized, double-blind, placebo-controlled clinical trial. Clin Endocrinol (Oxf) 81(4):582–587
149. Aghamohammadi V, Gargari BP, Aliasgharzadeh A (2011) Effect of folic acid supplementation on homocysteine, serum total antioxidant capacity, and malondialdehyde in patients with type 2 diabetes mellitus. J Am Coll Nutr 30(3):210–215
150. Ullegaddi R, Powers HJ, Gariballa SE (2004) B-group vitamin supplementation mitigates oxidative damage after acute ischaemic stroke. Clin Sci 107(5):477–484
151. Ruder EH et al (2014) Female dietary antioxidant intake and time to pregnancy among couples treated for unexplained infertility. Fertil Steril 101(3):759–766
152. Brown LA et al (2009) Oxidant stress in healthy normal-weight, overweight, and obese individuals. Obesity 17(3):460–466
153. Tatone C et al (2008) Cellular and molecular aspects of ovarian follicle ageing. Hum Reprod Update 14(2):131–142
154. Lashen H, Fear K, Sturdee D (2004) Obesity is associated with increased risk of first trimester and recurrent miscarriage: matched case–control study. Hum Reprod 19(7):1644–1646
155. Kemmann E, Pasquale SA, Skaf R (1983) Amenorrhea associated with carotenemia. JAMA 249(7):926–929
156. Youssef MA et al (2015) Oral antioxidants supplementation for women with unexplained infertility undergoing ICSI/IVF: randomized controlled trial. Hum Fertil 18(1):38–42
157. Reiter RJ, Tan DX, Maldonado MD (2005) Melatonin as an antioxidant: physiology versus pharmacology. J Pineal Res 39(2):215–216
158. Das S et al (2006) Reactive oxygen species level in follicular fluid – embryo quality marker in IVF? Hum Reprod 21(9):2403–2407

Chapter 8
Compendium of Oxidative Stress-Related Research from Cleveland Clinic (1993–2016)

Damayanthi Durairajanayagam, Amolak S. Dhillon, Rian Salasin, Anthony Kashou, and Narasimhan Kothandaraman

8.1 Introduction

Over the last two decades and more, the American Center for Reproductive Medicine (ACRM) at Cleveland Clinic has made great contributions to medicine that have helped in deepening our understanding of oxidative stress (OS) and its effects on human reproduction, particularly in the male. In this chapter, we will explore the findings of past research conducted by the Cleveland Clinic researchers to date, which spans from reactive oxygen species (ROS), OS and its measurement, effects of OS on sperm preparation and cryopreservation during assisted reproduction procedures as well as on semen parameters, the physiological and pathophysiological effects (both direct and indirect) of endogenous and exogenous ROS, the role of antioxidants in reversing the effects of oxidants and finally, the use of proteomics and bioinformatics tools in discovering the biological processes and pathways underlying oxidative stress-induced infertility.

A state of OS is said to be present when there is an imbalance between the production of ROS and the body's ability to neutralize them via antioxidant defense

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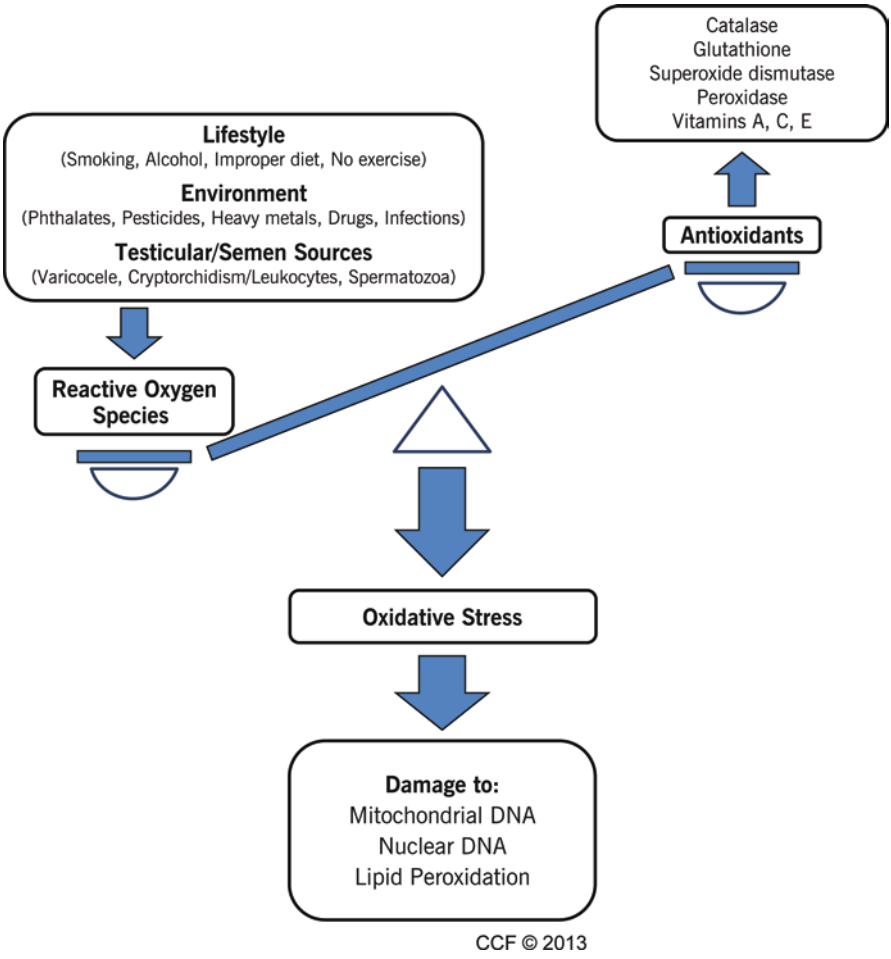

Fig. 8.1 The imbalance caused by the accumulation of reactive oxygen species due to various factors and depletion of antioxidants leads to a state of oxidative stress. In the spermatozoa, high oxidative stress levels cause membrane and DNA damage

mechanisms (Fig. 8.1) [1, 2]. Derived from oxygen, ROS are generated as a byproduct of cellular metabolism, and its levels are maintained within a physiological range by the actions of endogenous antioxidants. Excessive intracellular levels of ROS can damage mitochondrial and sperm nuclear DNA, and thus it is associated with the etiology of numerous diseases and reproductive complications [3]. For example, high levels of ROS have been correlated with poor fertility outcomes seen in assisted reproduction using assisted reproductive technology (ART) [4, 5]. In fact, up to 50–60 % of recurrent pregnancy loss may be attributed to OS [6]. It is important to note that seminal OS evaluation is thought to have prognostic capabilities beyond tests for sperm quality and function [7]. An animal-based study looking at ROS generation and antioxidative mechanisms in the rat kidney demonstrated

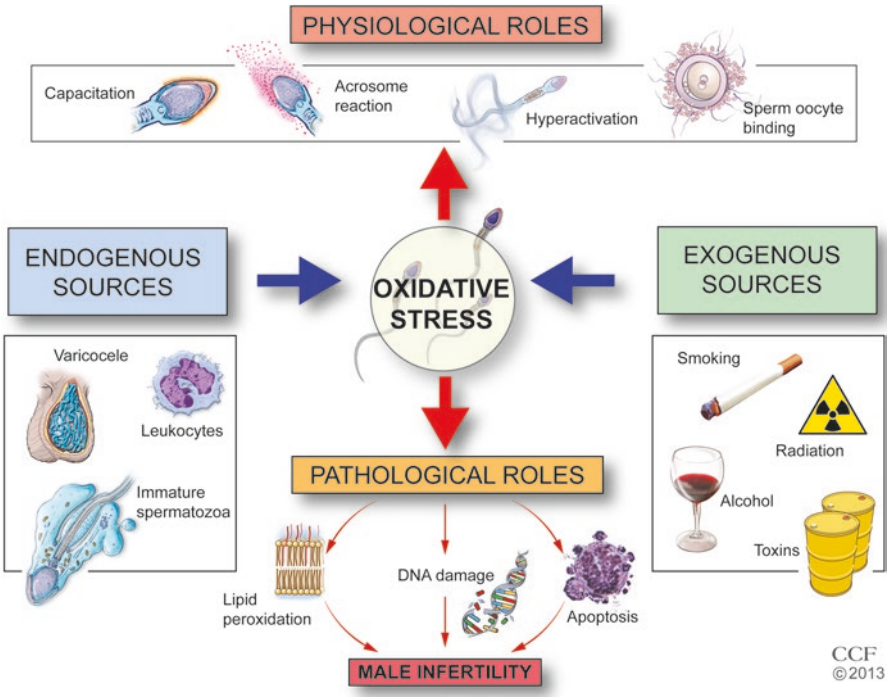

Fig. 8.2 Sources and roles of oxidative stress in male infertility

that antioxidants play a predominant role in the protection against the deleterious effects of ROS [8].

There are two types of ROS sources that could account for its presence within a cell–exogenous and endogenous. Some exogenous sources include smoking and toxins, while endogenous sources include varicocele and NADPH oxidase activation in immature spermatozoa (Fig. 8.2) [7, 9]. In immature spermatozoa, the mitochondria serve as a primary source of ROS. This is because superoxide is constantly being produced as a byproduct of cellular respiration due to electron leakage in the electron transport chain (specifically when electrons are passing through complexes I–IV) [3]. Under physiological conditions, this superoxide is converted to hydrogen peroxide (Fig. 8.3).

8.2 Physiological Roles of ROS

ROS are known to be involved in normal physiological processes, such as sperm maturation, capacitation, hyperactivation, acrosome reaction, and sperm-oocyte fusion [1, 10–15]. In a prospective controlled study, researchers at the Cleveland Clinic have looked into, intracellular basal and induced levels of ROS and their

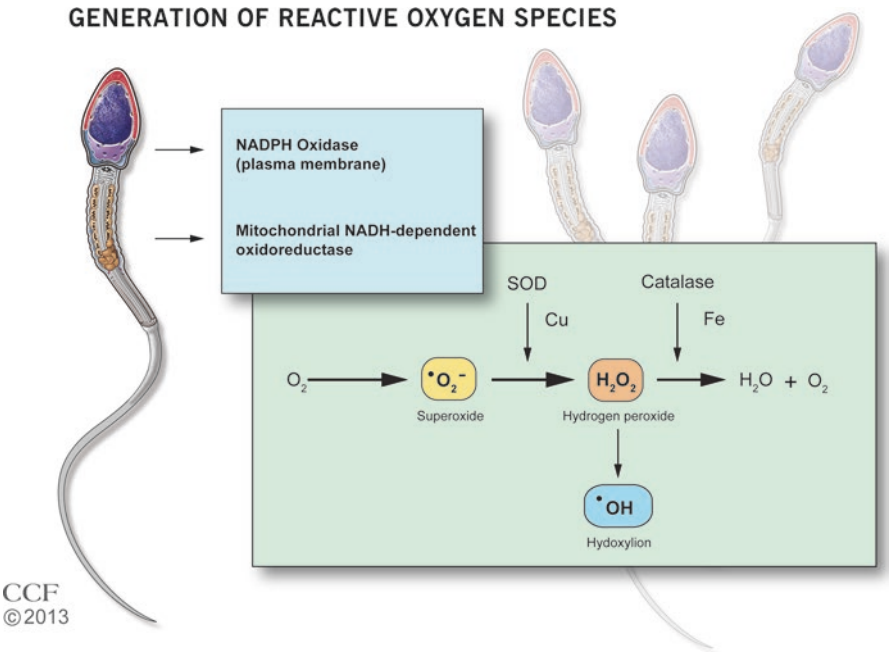

Fig. 8.3 Generation of reactive oxygen species. *NADPH* nicotinamide adenine dinucleotide phosphate, *NADH* nicotinamide adenine dinucleotide, *SOD* superoxide dismutase, *Cu* copper, *Fe* iron

relationship with sperm quality (viability and apoptosis) in neat, mature and immature sperm fractions from healthy volunteers of unproven fertility [16]. They found that sperm processing, particularly centrifugation may cause higher intracellular levels of hydrogen peroxide and thus oxidative stress, as seen in mature and immature sperm fractions compared to neat spermatozoa. In fact, sperm preparation may cause a differential shift in intracellular levels of hydrogen peroxide and superoxide, which could negatively impact sperm quality.

Several studies have been conducted to determine the physiologic levels of ROS in infertile men and to determine the cut off values for OS that distinguish between fertile and infertile men [17, 18]. The most recent results from a 2015 study showed that the appropriate cut off value for OS was 102.2 RLU/s/10^6 sperm, which is defined as the basal ROS level in infertile men [19]. This value is significant because it defines a normal level of ROS generation that allows for essential reproductive functions to occur physiologically without the harmful effect of OS. This value, obtained using a luminol-based chemiluminescence assay, also represents the optimal cutoff value to discriminate between normal and infertile men and may be used in a clinical setting during routine diagnostic screening to test for male infertility [19].

The determination of the ROS cutoff values is also related to a study that had explored the relationship between early embryonic development and levels of ROS in culture media on the first day after insemination [20]. Findings of this study suggested that ROS may be an important chemical marker for early embryonic

growth and that high embryonic fragmentation and a slow cleavage rate may be due in part to high levels of ROS in intracytoplasmic sperm injection (ICSI) cycles. In a follow up study, embryonic development was also compared with day one total antioxidant levels in culture media [21]. Cleveland Clinic researchers found that day one total antioxidant capacity (TAC) in culture media was an important biochemical marker for early embryonic growth considering that the TAC parameter seemed to be partially related to decrease embryonic fragmentation, and enhanced cleavage and blastocyst development rates.

8.3 Generation of OS

With an established background in understanding the production of ROS, the effects of high levels of ROS and OS on sperm preparation, cryopreservation, and assisted reproductive procedures were assessed.

8.3.1 Sperm Preparation

Research studies by the Cleveland Clinic have looked into the relationship between ROS levels and the time interval (1, 3, 5 and 24 h) between semen collection and analysis, as well as how levels of ROS generation fluctuate at different sperm concentrations (7.5, 15, 30 and 60×10^6 ml) [22]. This study was conducted on semen samples from patients suspected of infertility and showed that ROS levels decreased significantly over time. This suggests that the time between sperm collection and analysis negatively affects sperm motility and viability, which could contribute to the decreasing ROS levels. In addition, ROS levels from the same specimen were found to vary when measured at different sperm concentrations. Thus, to obtain an accurate measurement of the actual amount of ROS generated, ROS levels in whole semen samples must be evaluated within an hour of sample collection. Following this study, sperm preparation techniques including centrifugation (time and g-force), resuspension, L4 filtration, swim-up, and washing were investigated to see if these techniques influenced ROS levels and/or damaged sperm [23–25]. Results showed that continuous washing, centrifugation, and vortexing of samples generated high levels of ROS; with washing causing the most harm [2, 23, 24]. These findings suggest that the overuse of these sperm preparation techniques should be avoided during ART procedures. However, sperm samples processed using techniques such as L4 filtration and swim-up yielded significantly lower ROS levels and with little to no sperm damage compared to that of the unprocessed sample. Both these methods were able to separate the highly motile sperm that produced less ROS from the original sample. In fact, ROS levels from samples processed using the L4 filtration and swim-up methods showed no statistically significant differences in ROS levels compared to each other [25].

Another study by Cleveland Clinic researchers compared post-wash and post-thaw (24 h) parameters of sperm to test the efficacy of the double density gradient media, PureSperm and swim-up (sperm migration), which are density gradient and motility checking techniques that help isolate sperm that are morphologically normal and motile [26]. The use of PureSperm provided more mature and motile sperm in fresh and cryopreserved semen compared to swim up. In another similar study, the differences between processing media (PureCeption, ISolate and SpermGrad-125) used during sperm preparation by density gradient were studied using samples from normal, healthy donors. All three gradients were found to produce sperm samples of good quality as they improved the percent motility, total motile sperm, percent recovery and DNA damage. However, the semen quality and percent recovery of morphologically normal sperm differed between these different commercially-available density gradient media. The DNA fragmentation that resulted from sperm preparation was negligible and did not differ significantly when different media was used in this controlled trial [27].

8.3.2 Cryopreservation

Cryopreservation of sperm cells is a common step involved in assisted reproduction procedures. However as a result of the freeze-thaw process, cryopreserved sperm are found to have damaged membranes due to membrane stress or lipid peroxidation. In one study, Cleveland Clinic researchers investigated the extent of lipid peroxidation (LPO) during cryopreservation, and the relationship between sperm concentration, motility and morphology with LPO in semen from healthy donors [28]. Results indicated that (1) cryopreservation-induced membrane damage was not related to LPO; (2) malonaldehyde (an indicator of lipid peroxidation) levels increased as sperm concentration increased; and (3) there was no correlation between motility, morphology, and malonaldehyde levels. As such, sperm handling, sperm freezing and the freezing medium seemed to not increase lipid peroxidation in the semen of normal men.

8.3.3 Assisted Reproductive Procedures (ART)

Assisted reproductive procedures (ART) is a common treatment for couples with infertility issues, however, the induction of OS can compromise the efficacy of these procedures [10]. Increasing levels of ROS without a simultaneous rise in antioxidant levels result in OS and consequently lipid, protein, and DNA damage. This oxidative state is thought to result in poor ART success rates [29]. In fact, ROS can have a significant impact on assisted reproduction outcomes and cause IVF failure if not kept in check (Fig. 8.4) [30]. In a prospective study, follicular fluid from patients undergoing IVF was studied to examine the association between LPO, TAC, oocyte

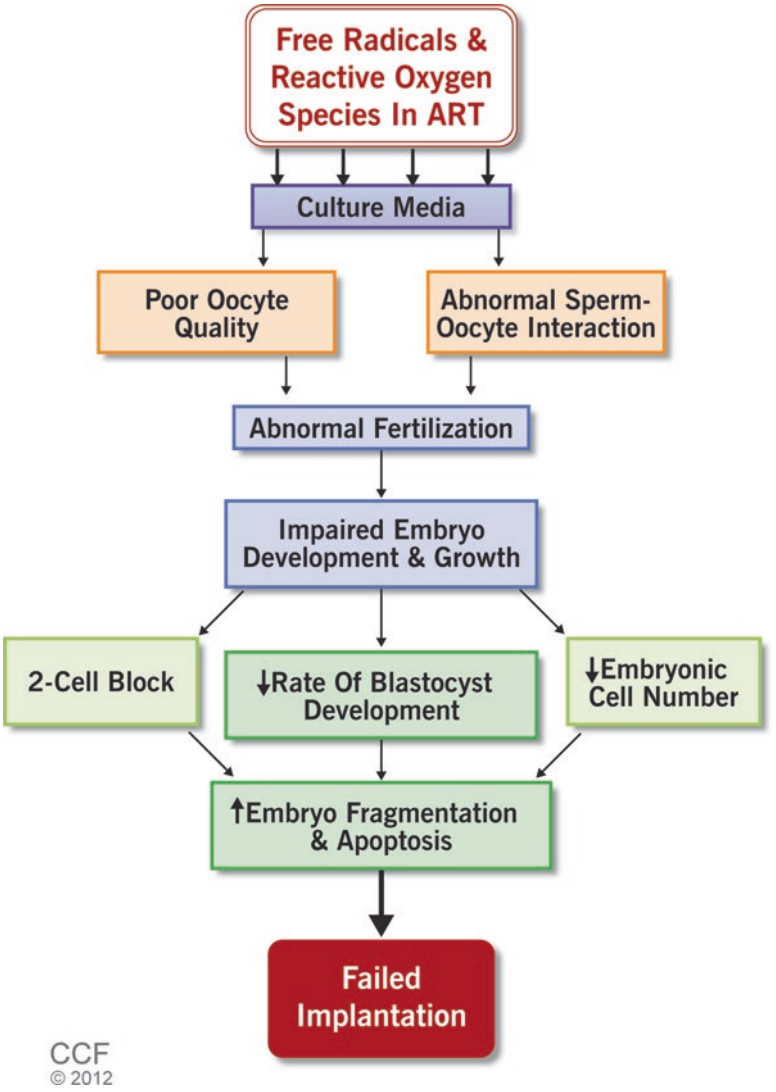

Fig. 8.4 The effects of free radicals and ROS present during ART procedures on embryo and blastocyst development leading to failed implantation

maturity, embryo quality, fertilization, cleavage, and pregnancy rates [31]. LPO and TAC were positively correlated with pregnancy rates, and it was surmised that some amounts of LPO might be needed for pregnancy to be established. Furthermore, levels of LPO and TAC in patients who achieved a pregnancy through IVF could perhaps act as an indicator of the minimal metabolic activity needed within the preovulatory follicle in order to be able to establish a pregnancy. In other studies by Cleveland Clinic researchers, the presence of ROS in follicular fluid and its role in

pregnancy outcome, the relationship between levels of ROS in seminal fluid and fertilization rates, and role of cytokines in peri-ovulatory follicular fluid were all determined during and after IVF cycles [18, 32, 33]. Results showed that (1) ROS levels in spermatozoa had a significant correlation with fertilization rates after IVF [18]; (2) follicular fluid ROS at low concentrations might be predictive of a successful pregnancy in these IVF patients [32]; (3) high levels of IL-12 and low levels of IL-6 were associated with negative IVF outcomes; (4) IL-13 and tumor necrosis factor alpha (TNF-alpha) were absent in all patients; and (5) IL-1β was not significantly different for pregnant and non-pregnant cycles [33].

In cases of male infertility when IVF does not work, the ICSI method may be employed in which a sperm cell is injected into the oocyte's cytoplasm [34]. Back in 2009, in-vitro maturation (IVM) emerged as a popular replacement for IVF because it reduces the need for ovarian priming and stimulation, which is a routine protocol in the conventional IVF process, thus reducing the risk of hyperstimulation syndrome [35].

In two studies by Cleveland Clinic researchers, the relationship between early human embryonic development parameters to day 1 ROS levels and day 3 ROS levels in culture media were examined in patients undergoing IVF and ICSI. ROS levels were measured by chemiluminescence using luminol as the probe. High day 1 ROS levels in culture media resulted in low blastocyst rate, fertilization rate, and cleavage rate respectively, as well as high embryonic fragmentation with ICSI but not with conventional IVF. Thus, lower pregnancy rates were observed in both IVF and ICSI cycles that was associated to high day 1 ROS levels. Similarly, high day 3 ROS levels in culture media correlated negatively with blastocyst development rate and pregnancy rate respectively. Researchers found day 3 ROS levels to be low in the pregnant cycles of IVF and ICSI patients. In addition, each 10 unit increase in day 3 ROS levels could decrease pregnancy rates by 41 % in these patients [20, 36]. Oxidative stress levels in the follicular fluid of ICSI patients was further assessed in another study which evaluated the association between the follicular fluid ROS (FFROS), TAC, ROS-TAC score and pregnancy after ICSI [37]. Higher follicular fluid TAC (FF TAC), higher FF ROS-TAC scores and lower FF ROS levels were associated with pregnancy after ICSI. These studies demonstrate that oxidative stress parameters may act as markers of metabolic activity within the follicle. However, supplementation with antioxidants can aid in reducing OS during sperm preparation techniques and thus increase the success of ART [38] (see Sect. 8.13).

8.4 Measurement of OS

Overproduction of ROS in semen has been associated with reduced sperm function and fertility potential [39, 40]. Moreover, infertile men have shown reduced semen parameters (concentration, motility, and morphology) and elevated ROS levels when compared to fertile men [41]. Routine semen analysis along with measurement of ROS and TAC in seminal ejaculate are essential in the assessment

Table 8.1 Normal range of ROS generation to distinguish between fertile and infertile men

Year	Normal range of ROS generation	Specificity, %	Sensitivity, %	Reference
1995	0–5.5×10^4 counted photons per minute at a sperm concentration of 20×10^6 ml^{-1}	N/A	N/A	[45]
2007	0.55×10^4 counted photons per minute (neat samples) and 10.0×10^4 counted photons per minute (washed samples)	N/A	N/A	[40]
2009	0.0185×10^6 counted photons per minute/20×10^6 sperm	82	78	[17]
2014	91.9 RLU/s/10^6 sperm	68.8	93.8	[41]
2015	<24.1 RLU/s/10^6 sperm	87.2	80.5	[46]
2015	102.2 RLU/s/10^6 sperm	53.3	76.4	[19]

All measurements were performed using the luminol-based chemiluminescence method

of sperm and semen quality [42], as they can provide insight into the etiology of male infertility.

The main purpose of translational medicine is to transform the knowledge gathered from research into clinical practice [43]. However, it is important to consider that with new guidelines and parameters being defined by the WHO in 2010, more men would be categorized as fertile while in reality, there may be a rise in the number of infertile men that goes unnoticed [44]. Therefore the Cleveland Clinic advises caution when using WHO reference values to discriminate infertile and fertile men.

Different techniques have been employed to assess OS status and run quality control studies [43]. As mentioned previously, positive correlations between ROS production and sperm concentration have indicated the importance of concentration adjustment before comparing ROS levels between different specimens [45]. However, accurate measurement of ROS formation has been hindered by the lack of standardization and confounding variables, causing the "normal" range to vary over the years (Table 8.1) [17, 40, 41, 45, 46].

Determination of a normal range of ROS generation using a simple, cost-effective assay can be incorporated into routine diagnostic testing which may assist in predicting male fertility status. Specialized sperm function tests (including the Endtz tests, TAC, chemiluminescence, sperm deformity index (SDI) and other sperm oxidative stress assessments) offer an important opportunity to delve deeper into sperm dysfunction during routine semen analysis [47].

8.4.1 Endtz Test

A study conducted by Cleveland Clinic using the Endtz test demonstrated a strong positive correlation between ROS generation and polymorphonuclear granulocyte concentration, thus adding to the discourse about the controversial role of leukocytospermia in semen [48]. The results signified that the Endtz test is a simple and cost-effective test that indicates the excessive generation of ROS in semen.

8.4.2 Total Antioxidant Capacity (TAC)

In studies conducted by Cleveland Clinic, the efficacy of measuring total antioxidant capacity (TAC) to discriminate between fertile and infertile patients was tested. In these studies, proven fertile donors have higher TAC scores than infertile patients [49, 50], thus suggesting that measuring TAC in semen is an effective and simple test for diagnosing and managing male infertility. The colorimetric assay was also established as a cheaper, quicker and reliable method of measuring TAC when compared to the conventional chemiluminescence assay [51]. Further studies by Cleveland Clinic researchers found that a ROS-TAC score was superior to simply measuring ROS and TAC alone when distinguishing fertile and infertile men [52]. In the female reproductive system, increased blastocyst development rate, decreased embryonic fragmentation and enhanced cleavage rate were to some extent related to day 1 TAC in the culture media. As such, TAC in day 1 culture media can be a significant biochemical marker for early embryonic growth [21].

8.4.3 Chemiluminescence

Accurate assessment of ROS levels in semen are essential to determine because they can aid in the diagnosis of OS-induced infertility [53]. The chemiluminescence assay is a commonly used method for the direct measurement of intracellular and extracelluluar ROS generation [42]. Studies conducted by the Cleveland Clinic researchers have validated the chemiluminescence assay as a reliable and accurate diagnostic test that minimizes intra- or inter-assay variation and prevents interference from extraneous factors [54]. Moreover, the Clinic researchers have specified that the assay is both accurate and reliable only when the sperm concentration is greater than 1×10^6 ml^{-1} and the samples are analyzed within the first hour after specimen collection [55].

Further studies conducted by Cleveland Clinic researchers have found that in certain instances, flow cytometry has a higher specificity for intracellular ROS production by spermatozoa and thus some samples which tested negative by chemiluminescence might still have high levels of intracellular hydrogen peroxide [56]. In cases such as these, flow cytometry would be a more accurate method to measure ROS generation.

Further insight into ROS generation in semen can be provided by the nitroblue tetrazolium reduction test, which is an effective method of assessing the contributions of defective spermatozoa and seminal leucocytes to the overall ROS generation in semen. Moreover, the NBT reduction test is easily available and performed, has high sensitivity and is cost-effective [57].

8.4.4 Sperm Deformity Index (SDI)

The sperm deformity index (SDI) score indicates the quality of sperm morphology, which can serve as a more powerful predictor of male factor fertility and of IVF outcome compared to the assessment of the percentage of sperm with normal morphology [58]. High SDI scores are associated with OS [59, 60] and apoptosis [61] in spermatozoa.

Thus, the SDI is also a useful method to identify infertile men with abnormal levels of OS-induced DNA damage in spermatozoa [59, 62]. Researchers at Cleveland Clinic have found that SDI scores were positively correlated with the percentage increase in sperm DNA damage when sperm samples treated with NADPH (which is known to cause an increase in ROS production) were incubated for 24 h; thus validating the SDI [59]. In an *in vitro* study, exposure of sperm to peritoneal fluid from patients with endometriosis was associated with significantly increased DNA damage after 24 h of incubation. The SDI scores assessed showed significant correlation with sperm DNA damage at 24 h post-incubation [63].

8.5 Sperm Parameters

Cleveland Clinic researchers have extensively examined the relationship between OS and semen parameters. Research from the Clinic has indicated that ROS levels and reduced mitochondrial membrane potential are positively correlated with abnormal semen parameters [64–66]. Semen samples with a large percentage of immotile sperm have higher seminal ROS levels than semen samples with motile spermatozoa [9, 22, 40, 64]. In fact, for every tenfold increase in ROS, there is a 9 % decrease in sperm motility [67]. Infertile men with high ROS levels have poorer motility and a higher incidence of DNA fragmentation than infertile patients with low ROS levels [9, 68].

On the other hand, in healthy fertile males, ROS levels are independent of sperm concentration, motility, and abstinence duration [68]. It was hypothesized that the little fluctuation in ROS levels in healthy sperm donors might be related to physiologic changes in spermatogenesis [68]. Another experiment was conducted to study the pathology of ROS in a healthy fertile man. A fluctuation of ROS levels in his semen was observed, however; this fluctuation in seminal ROS did not affect sperm concentration and motility. It was concluded that healthy men have adequate anti-oxidative defense mechanisms to deal with the physiological changes in ROS levels [69]. The link between ROS levels and impaired semen parameters suggests that routine semen analysis, including the determination of levels and sources of ROS generation, should be included in the routine evaluation of subfertile men [70].

8.6 Immature Sperm

Studies by Cleveland Clinic researchers have shown that there is a significant difference between the amounts of ROS produced by spermatozoa at different stages of maturation [65]. Sperm samples with abnormal morphology (and a greater number of immature sperm) generated more ROS than samples with morphologically normal spermatozoa [22, 64, 71]. A positive correlation exists between excessive generation of seminal ROS and the number of defective spermatozoa with tail defects, mid-piece defects, amorphous heads, and cytoplasmic droplets. Specifically, ROS production increases most when spermatozoa exhibit the retention of excess cytoplasm within their mid-piece (Fig. 8.5) [57, 62, 65]. This condition is termed excess residual cytoplasm (ERC). ERC occurs during an arrest in spermiogenesis where all the cytoplasm in the spermatozoa is not extruded from the mid-piece. ERC is characterized by high levels of glucose-6-phosphate dehydrogenase, which in turn increases the production of NADPH via the hexose monophosphate shunt [72]. NADPH is the substrate for NADPH oxidase, which is responsible for the monovalent reduction of oxygen to the superoxide anion [73]. In essence, ERC (which is exhibited in many immature sperm) can result in a burst of superoxide production.

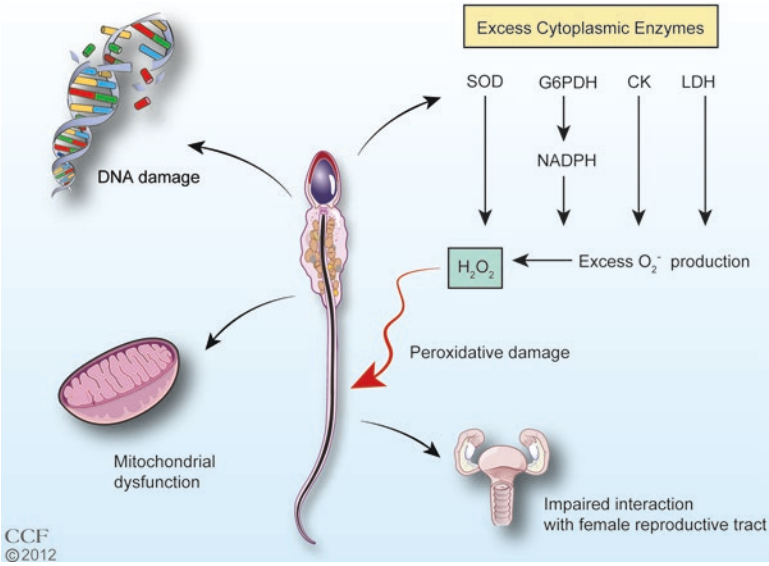

Fig. 8.5 Pathological effects of excess residual cytoplasm include peroxidative damage to the sperm membrane, DNA damage, mitochondrial dysfunction, and impaired sperm function within the female reproductive tract. *SOD* superoxide dismutase, *G6PDH* Glucose-6-phosphate dehydrogenase, *CK* creatine kinase, *LDH* lactate dehydrogenase, *NADPH* nicotinamide adenine dinucleotide phosphate, H_2O_2 hydrogen peroxide

8.7 Semen Quality

Cleveland Clinic researchers have investigated the prospect of using a semen quality (SQ) score rather than the conventional method of measuring semen parameters to identify infertile males. A SQ score based on clinical trials was found to be a better method to identify individuals with male factor infertility than measuring sperm parameters based on WHO guidelines. A cutoff value of ≤93.1 for the SQ score had provided an optimum sensitivity and specificity and was therefore established as the SQ cutoff value that was able to correctly identify ~80 % of patients with male factor infertility. Semen quality scores were negatively correlated with ROS levels [74].

8.8 Oligoasthenoteratozoospermia

Cleveland Clinic researchers have extensively looked at the link between oligoasthenoteratozoospermia (OAT) and OS. When compared to both infertile patients and healthy donors, oligo-(O), astheno-(A), and teratozoospermic (T) patients had higher ROS values and a lower SQ score [39, 74, 75]. Infertile patients with oligoasthenozoospermia (OA) have elevated levels of malondialdehyde (MDA) indicating LPO [75]. One study found that thiobarbituric sperm from teratozoospermic patients produced a significantly higher amount of ROS than mature sperm from normozoospermic patients [76, 77]. Another study identified asthenozoospermia as a primary culprit for the excessive production of nitric oxide (NO) [78]. This study found that the mRNA from endothelial nitric oxide synthase (NOS) was expressed more in leukocytes that were isolated from asthenozoospermic semen samples when compared to that from normozoospermic semen samples. The increase in NO expression was also associated with an increase in immotile sperm [78]. In OAT patients, sperm motility and morphology decreased as seminal ROS levels increased [39]. Furthermore, patients with OAT have reduced acrosin activity as well as higher levels of thiobarbituric acid reactive substances (TBARS), an indicator of LPO, in their semen than that of normospermic individuals [79].

8.9 Idiopathic Infertility

Through research by the Cleveland Clinic, the overproduction of ROS has been associated with numerous male fertility complications including idiopathic infertility [5]. Patients with idiopathic infertility have higher ROS values and lower TAC when compared to infertile patients with varicocele and vasectomy reversal [71]. Similarly, when compared to healthy fertile controls, patients with idiopathic infertility have reduced sperm parameters and TAC as well as elevated ROS levels [50, 80, 81]. Female patients with idiopathic infertility also demonstrated higher ROS levels than female patients with endometriosis and healthy female controls [80].

8.10 Negative Effects of ROS

8.10.1 Lipid Peroxidation

The oxidative insult to spermatozoa due to the over production of ROS can result in LPO [5]. During LPO, over 60 % of the fatty acid chains in the plasma membrane can deteriorate thus reducing sperm membrane integrity [13, 82]. One of the byproducts of LPO is MDA, which has been used in numerous studies by the Cleveland Clinic to monitor the degree of peroxidative damage to sperm membranes. One such study observed that as sperm concentration increased, so did MDA levels, thus indicating that sperm concentration is positively correlated with LPO [28]. Antioxidants that break free radical chain reactions, such as Vitamin E, inhibit LPO [73].

8.10.2 Mitochondrial Membrane Potential

A controlled prospective study conducted by Cleveland Clinic researchers compared the mitochondrial membrane potential (MMP) with ROS levels in nineteen infertile men and seven healthy donors. MMP is an important indicator of the functional integrity of the spermatozoa, and is positively correlated with sperm motility and viability. Results of the aforementioned study showed that abnormal semen parameters and ROS levels were negatively correlated with MMP, thus indicating that the measurement of MMP in spermatozoa provides useful insight into a man's fertility potential [66].

8.10.3 Sperm Chromatin Integrity

Sperm DNA damage is observed more frequently in infertile men than in healthy fertile men thus suggesting that it could be a major cause of male infertility [83]. In a study at the Cleveland Clinic, the sperm chromatin structure assay was used to assess DNA damage in human spermatozoa at different stages of maturation from males undergoing infertility evaluation [84]. The study showed that chromatin alterations were interconnected with leukocyte concentration in immature and mature sperm as well as with immature germ cell concentration and abnormal forms of semen. In other studies, Cleveland Clinic researchers reviewed the relationship between sperm chromatin integrity, hormone levels, seminal plasma TAC, and sperm parameters in men with male factor and non-male factor infertility. These studies suggested that male factor infertility associated with sperm chromatin damage may be related to sperm protamination and to serum DHEA [85, 86].

8.10.4 Sperm DNA Damage

Sperm DNA damage has been implicated as a leading cause of male infertility. In two studies by Cleveland Clinic researchers, the effect of ROS generation on DNA damage and apoptosis (by stimulating DNA damage) in men classified as idiopathic infertile was examined [87, 88]. As apoptosis showed little correlation with sperm DNA damage, it seemed unlikely that apoptosis was the cause of ROS-induced DNA damage in these studies. Instead, it was ROS generation that showed a significant contribution towards DNA damage in spermatozoa.

In a further study conducted by the Clinic, the correlation between abnormal sperm morphology ROS levels and sperm DNA damage was evaluated using the SDI [59]. NADPH (a primary source of ROS) was found to play a key role in ROS-mediated DNA damage in infertile patients' semen samples containing high levels of abnormal spermatozoa. Other studies by Cleveland Clinic researchers have also found that immature spermatozoa have higher incidences of NADPH-induced DNA damage [77]. In fact, the relative proportion of immature sperm producing ROS was directly correlated with increased DNA damage in mature sperm. However, the researchers found no correlation between DNA damage and sperm morphology in mature sperm. They concluded that the increased DNA damage in mature sperm could be due to oxidative damage resulting from ROS producing (immature) sperm that occurs as sperm migrates from the seminiferous tubules to the epididymis [89].

Finally, levels of sperm DNA damage and OS in infertile men with varicocele were studied. Results showed significantly high levels of DNA damage, which seemed to be related to the high levels of OS found in the patients' semen [90].

8.10.5 Cytokines

Research conducted at the Cleveland Clinic has linked oxidative stress and the production of ROS with the expression of cytokines. In such studies, infertile patients with varicocele and patients having undergone vasectomy reversal had elevated levels of IL-6, which was positively correlated with ROS production [91, 92]. Another study found that alteration to microtubule spindle structure and chromosomal alignment in mouse metaphase II oocytes due to tumor necrosis factor is augmented by the induction of oxidative stress [93].

8.10.6 Apoptosis

Numerous studies have been conducted by Cleveland Clinic researchers to examine the relationship between apoptosis and ROS (Fig. 8.6). In one study, a positive correlation between ROS levels and the percentage of cells undergoing apoptosis was observed [87]. Another study examined the mechanism by which ROS might

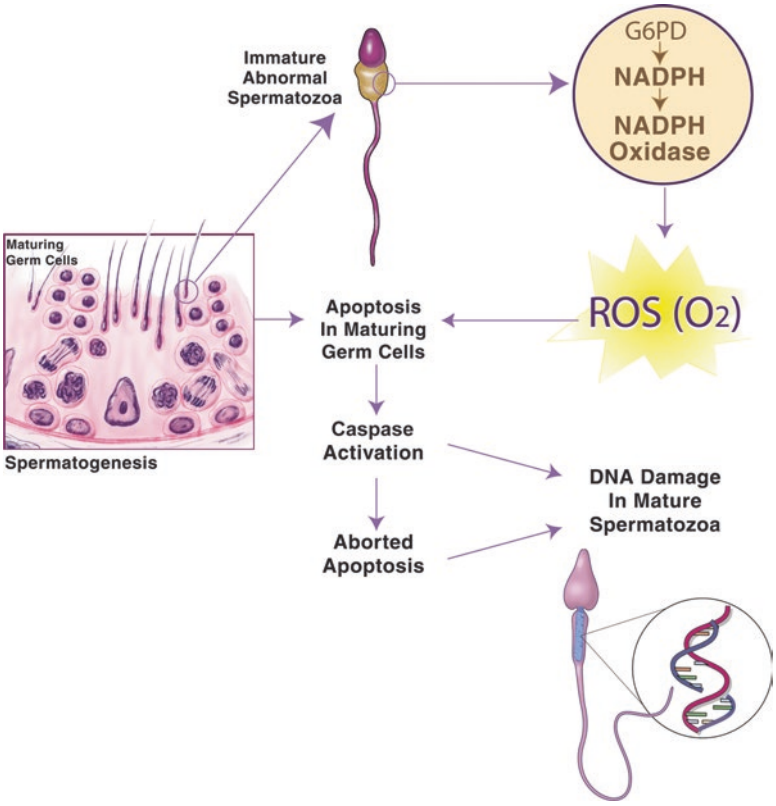

Fig. 8.6 The mechanisms involved in oxidative stress-induced cell apoptosis and DNA damage. *G6PD* Glucose-6-phosphate dehydrogenase, *NADPH* nicotinamide adenine dinucleotide phosphate, *ROS* reactive oxygen species

increase apoptotic cell death. Researchers reported that an increase in ROS levels was associated with an increase in caspase 3, caspase 9, and cytochrome c, which are proteins that mediate apoptosis [94]. Moreover, the significant positive correlation between ROS and these apoptosis-mediating proteins also suggested the possibility of DNA damage through increased ROS production. Anti-heat shock protein antibodies (anti-HSP) also have an adverse effect on embryonic cell development by increasing the incidence of apoptosis in these cells [95]. However, ROS levels were unaffected by variation in anti-HSP antibodies. Thus, the increased incidence of apoptosis occurred independent of ROS level fluctuation [95].

The presence of L-carnitine (an amino acid derivative) in the peritoneal fluid of endometriosis patients lowered the incidence of mouse embryo apoptosis, therefore suggesting that carnitines could play a protective role against apoptotic cell death. The improvement seen in the level of embryo apoptosis after the addition of L-carnitine may be due to the potent antioxidant properties of L-carnitine and its role in downregulating cytokines present in the peritoneal fluid of endometriosis patients [96]. Besides protection from apoptotic cell death, L-carnitine has free radical-scavenging

activity and inhibits LPO, thereby protecting the cell membrane and DNA against damage induced by free oxygen radicals. Another study using mouse pre-implantation embryos showed that the incidence of apoptosis was reduced by supplementation of culture media with protein (serum substitute). In addition, protein supplementation reduced ROS levels and helped improve the hatching rate in these embryos [97].

8.10.7 Poly ADP-Ribose Polymerase

Research by the Cleveland Clinic indicates an active role for poly (ADP-Ribose) polymerase (PARP) in maintaining normal sperm cell physiology by ensuring normal sperm maturation and preventing OS as well as apoptosis [98, 99]. PARP is an abundant nuclear enzyme that plays a role in DNA repair and transcriptional regulation. It also helps detect DNA strand breaks caused by ROS. Cleaved poly (ADP-ribose) polymerase (cPARP) plays a significant role in interrupting the DNA repair process and activating caspase-3, thus inducing apoptotic cell death. Cleaved PARP is present in ejaculated human spermatozoa and plays a role in OS-induced damage of spermatozoa [99].

8.10.8 Embryo Development

The Cleveland Clinic researchers have looked at the effect of OS on the developing embryo. In one study, ROS was positively correlated with blastocyst development rate [100], thus suggesting that ROS has a physiological role in embryonic development. However, another study found that when embryonic cells were cultured, high day 1 ROS levels were associated with low blastocyst development rate, embryonic fragmentation, and low fertilization rate in intra-cytoplasmic injection (ICSI) cycles [20]. In essence, while ROS play a physiological role in oocyte development and maturation, excessive ROS production can be harmful to embryonic cells [2, 35, 101]. This is because OS can alter steroidogenesis in the ovaries, which increases androgen production and disturbs follicular development [82]. Moreover, excess ROS levels in the oocyte, follicular fluid and embryo result in poor oocyte quality, alter the activity of cumulus mass cells and result in embryonic development block and retardation [10].

8.10.9 Embryo Fragmentation

Patients undergoing assisted reproduction using conventional IVF or ICSI cycles were included in a study by Cleveland Clinic researchers. The results showed that high day 1 ROS levels were associated with high embryonic fragmentation only during ICSI cycles and not IVF, due to the early exposure of embryos to ROS in ICSI cycles [60].

8.10.10 Hydrosalpingeal Fluid

For the first time, Cleveland Clinic researchers characterized the presence of ROS, TAC and LPO in hydrosalpingeal fluid (HSF) aspirated from infertile women undergoing laparoscopic salpingectomy. The blastocyst development rate of mouse embryos incubated in human HSF correlated positively to concentrations of ROS but did not correlate significantly with LPO. It was found that increasing concentrations of HSF decreased the blastocyst development rate, thus suggesting a possible role for OS in the embryotoxicity of HSF [100]. In a follow up study, the researchers demonstrated for the first time the presence of cytokines IL-1beta, IL-8, IL-6 and TNF-alpha, and the absence of IL-13 in human HSF. Of these cytokines, IL-6 correlated positively to the blastocyst development rate of mouse embryos [102].

8.11 Indirect Effects of ROS

8.11.1 Temperature

Spermatogenesis is a temperature-dependent process, which occurs ideally at temperatures slightly lower than that of the body. As such, thermoregulation is essential to maintain an adequate testicular temperature. A higher testicular temperature negatively effects spermatogenesis and the resulting spermatozoa. Therefore, thermoregulatory failure leading to testicular heat stress can result in poor sperm quality and thus increase the risk of infertility [103]. Moreover, elevated temperature in the testis can result in germinal atrophy, spermatogenic arrest as well as germ cell apoptosis [103]. One study by Cleveland Clinic researchers that confirms these heat-induced changes observed that sperm motility decreased dramatically after sperm samples were incubated at low temperatures (4 °C) [67] However, this decrease in percentage motile sperm was less dramatic when the sperm sample was incubated at 27 and 37 °C. The maintenance of sperm motility at higher temperatures was partially explained by the observation that seminal ROS levels were lowest in the sperm sample incubated at 37 °C. Thus, it can be surmised that when the temperature of a sperm sample deviates from the physiological temperature of the body, spermatozoa are adversely affected largely due to an increase in ROS production [67].

8.11.2 Exposure to Hydrogen Peroxide

Cleveland Clinic researchers have investigated the effect of hydrogen peroxide (H_2O_2) exposure on spermatozoa and found that exogenous H_2O_2 increases intracellular ROS and NO production, and subsequently, decreases sperm motility [104].

A study was conducted to test the effect of H_2O_2 on mouse metaphase II oocyte microtubular spindle structure [93]. It was recorded that even at low concentration, H_2O_2 exposure increased the odds of abnormal microtubule and chromosomal alignment by 93 %. Furthermore, the intensity and probability of the damage to the microtubules and chromosomal alignment increased as the incubation period increased. It was also noted that chromosomal alignment in metaphase II oocytes was more resistant to the effects of H_2O_2-induced OS compared with microtubules [93]. In another study, Mahfouz et al. further examined the effects of H_2O_2 exposure on immature and mature sperm fractions from samples by healthy volunteers of unproven fertility [16]. Exposure to H_2O_2 decreased the viability in both sperm fractions and increased the number of apoptotic sperm. Thus, this study indicated that intracellular H_2O_2 might induce apoptosis [16]. The pathology of H_2O_2 is related to the ability of it to diffuse through the membrane of sperm cells and reduce the activity of certain enzymes [73]. However, under high ROS conditions, intracellular H_2O_2 was positively correlated with the number of viable sperm. This is because high ROS conditions may lead to an increase in defense mechanisms in spermatozoa, which help to counter and adapt to the increased presence of H_2O_2 [16].

8.11.3 Varicocele

Varicocele is the dilation of veins around the spermatic cord [13]. This condition has been closely linked with OS, because the testis responds to varicocele-associated stressors at the expense of ROS production [105, 106]. Contemporary evidence points towards a dominant role of ROS and OS in the pathogenesis of varicocele-associated male infertility despite the actual mechanisms not being elucidated yet. Excessive ROS present in varicocele patients is associated with sperm DNA fragmentation, both of which could result in poor sperm function and fertilization outcome [107]. The latest systematic review and meta-analysis by Cleveland Clinic researchers showed that varicocele is a significant risk factor that exerts a negative impact on semen quality [108].

Studies by the Cleveland Clinic researchers have found that semen samples from fertile donors have higher sperm concentration and motility than those from varicocele patients [88, 92, 109]. One clinical trial found that infertile patients with varicocele had a greater degree of impaired sperm parameters when compared to infertile patients without varicocele [90]. This study clarifies that although infertile men produce excessive amounts of ROS regardless of the presence of varicocele, varicocele exacerbates the condition of OS in infertile patients [110]. When compared to infertile patients with vasectomy reversal and idiopathic infertility, infertile patients with varicocele had the highest levels of ROS [71]. Another study compared infertile patients with varicocele to fertile patients with varicocele using healthy fertile donors as controls [111]. It was found that the infertile and fertile patients with varicocele had significantly higher ROS levels, lower TAC levels, lower ROS-TAC scores and lower semen quality scores compared to the controls; however, the ROS-TAC score between

the fertile and infertile varicocele patients respectively did not differ [111]. These findings indicate that the presence of varicocele exclusively impairs sperm function regardless of the whether the patient was originally fertile or not.

Furthermore, a specific study found that the grading of varicocele affects the levels of seminal OS present in patients with varicocele [109, 112]. Patients with grade 3 varicocele had higher seminal ROS levels than those with grade 1 varicocele [109, 112]. Literature on Cleveland Clinic studies also suggests that the contribution of varicocele to increase OS levels may be limited to only infertile patients. One study analyzed the semen from fertile patients with and without varicocele and found that there was no significant difference in seminal ROS levels between the two groups. Thus, this study indicates that the adverse effect of increased ROS levels on sperm parameters in varicocele patients may be predictable depending on whether the patient is initially fertile. Moreover, this study demonstrates that the presence of clinical varicocele does not associate with higher seminal ROS levels or abnormal semen parameters in fertile men. In fact, in these fertile men, ROS levels were not correlated with varicocele grade or testicular volume [113].

The mechanism by which varicocele leads to the extensive generation of ROS is partly explained by studies which found that infertile varicocele patients (compared to fertile donors) have elevated ROS levels and inflammatory cytokines, such as IL-6, as well as decreased TAC [71, 90–92, 109, 114, 115]. An increase in pro-inflammatory cytokines leads to higher seminal ROS levels due to a significant decrease in TAC [13]. On the other hand, functional proteomic analysis conducted by the Cleveland Clinic has provided insight into the mechanisms by which varicocele can impair sperm function. Infertile men with unilateral and bilateral varicocele have various overexpressed and underexpressed proteins which impair proteins involved in fundamental reproductive processes such as capacitation, hyperactivation, sperm-zona binding and acrosome reaction [116, 117]. These proteins can serve as potential biomarkers for unilateral and bilateral varicocele.

Varicocele treatment reduces ROS levels as well as improves semen parameters and fertilization rates [118]. The most effective treatment option for infertile men with varicocele is microsurgical spermatic vein ligation [47].

8.11.4 Spinal Cord Injury

Over 90% of men with spinal cord injury are infertile, indicating that spinal cord injury is related to infertility and possibly OS [13]. In one study, the Cleveland Clinic investigated the relationship between the generation of ROS and semen characteristics in men with spinal cord injury. To stimulate a burst of ROS production from leukocytes, N-formyl-methionyl-leucylphenylalanine (FMLP) was used, while ROS production from spermatozoa was stimulated by 12-myristate 13-acetate phorbol ester (PMA) in the semen of both male patients with spinal cord injury and normal men [119]. When the semen was compared, it was found that the levels of ROS and the

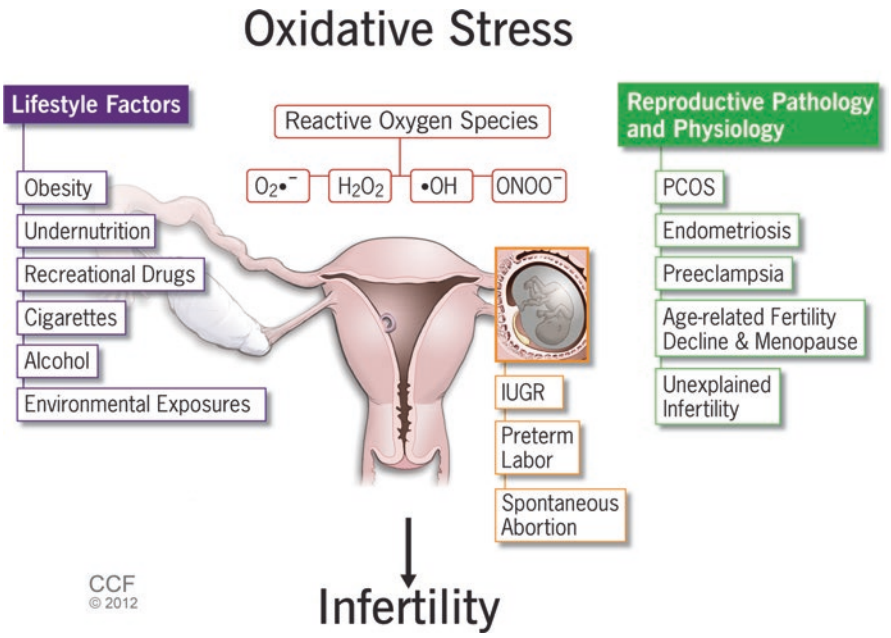

Fig. 8.7 Factors contributing to the development of oxidative stress and their impact on female reproduction (*PCOS* polycystic ovary syndrome, *IUGR* intrauterine growth restriction)

white blood cell count were higher in spinal cord injury patients than in normal men. Sperm morphology and motility were also lower in spinal cord injury patients [119]. These results indicate that a link exists between spinal cord injury and OS.

8.11.5 Endometriosis

ROS is present in both fertile and infertile females with endometriosis (Fig. 8.7) [80]. Studies by Cleveland Clinic researchers have revealed that a vicious cycle exists in the case of endometriosis whereby OS is induced which in turn causes the implantation of the ectopic endometrium (a primary characteristic of endometriosis). This action triggers the generation of more ROS which further triggers OS [120]. However, it is important to note that some studies found that the presence of ROS in the peritoneal fluid of females with endometriosis is not statistically different from the ROS production from healthy female donors [80]. Thus, the presence of ROS in the peritoneal fluid may not have a significant effect on females with endometriosis in the context of infertility. In contrast, other studies observed increased levels of OS markers (MDA and pro-inflammatory cytokines) in the peritoneal fluid of patients with endometriosis [121] thus suggesting that endometriosis can induce OS.

Antioxidants, such as vitamins C and E neutralize ROS and thus could be a form of treatment for patients with endometriosis [120].

8.11.6 Vasectomy Reversal

Vasectomy reversal patients have elevated ROS and IL-6 levels when compared to normal controls [91, 122, 123], thus indicating that vasectomy reversal may induce seminal OS. ROS levels are also higher in infertile vasectomy reversal patients than fertile vasectomy reversal patients [123]. However, vasectomy reversal does not seem to affect TAC levels [122]. As expected due to the excessive generation of ROS, the patients who have undergone vasectomy reversal have decreased sperm concentration and motility [91]. Moreover, levels of creatine kinase (CK), a biochemical marker for cellular maturity were elevated in infertile patients with vasectomy reversal when compared to patients with idiopathic infertility, testicular cancer, and normal fertile donors. This indicates that spermatozoa from post-vasectomy reversal patients may be biochemically immature, and therefore have a lesser chance of fertilizing the ovum [124]. This study conducted by Cleveland Clinic researchers suggests that vasectomy reversal may be associated with OS.

8.11.7 Age

Studies reported by the Cleveland Clinic researchers have linked poor semen quality, DNA mutations [125] and an increase in disorders such as autism and schizophrenia, with increased paternal age [126]. Elevated ROS levels related to aging and infertility can result from decreased antioxidant levels and impaired Leydig cell function [3]. A study on 98 fertile men who were candidates for vasectomy was conducted to evaluate the effect of age on ROS generation [127]. Participants in the study were divided into two groups: individuals under the age of 40 (n = 78) and individuals over the age of 40 (n = 20). Positive controls consisted of 46 infertile patients. The mean age of the men was 35.1 ± 5.6 years. The group of individuals above the age of 40 had higher ROS levels than the younger group of men and the controls. Thus, ROS levels in the whole ejaculate have a positive age-dependent correlation among fertile men [127].

8.11.8 Smoking

Cigarettes have over 4000 chemicals, some of which can cause excessive generation of ROS, thus inducing a state of OS [39]. Levels of OS were evaluated in infertile men with a history of smoking, which demonstrated that infertile men who smoked had higher OS levels compared to non-smoking infertile men [128]. In this study,

smoking was associated with increased ROS levels, seminal leukocyte concentrations, and decreased ROS-TAC score. Another study by the Cleveland Clinic analyzed the semen from infertile smokers, infertile non-smokers, and healthy fertile donors [128]. It was determined that smoking increased seminal leukocyte concentrations by 48 %, increased ROS levels by 107 % and lowered ROS-TAC scores by 10 points [39, 128]. In the female reproductive system, smoking can also adversely alter follicular and oocyte maturation [129]. A recent evidence based review by Cleveland Clinic researchers studied the data on smoking and male fertility and reiterated that couples who are trying to conceive in particular should adhere to the preventive approach of discouraging smoking and eliminating exposure to tobacco smoke [130].

Aside from smoking tobacco, smoking marijuana may also be associated with ROS generation because it disturbs the endocannabinoid system (ECS), which is involved in the regulation of the male reproductive system. Therefore, marijuana may disrupt the process of spermatogenesis and impair sperm function including motility, capacitation and acrosome function [131].

8.11.9 Cell Phones

The non-thermal effects of mobile phones on male reproduction includes the excessive generation of seminal ROS and reduction of antioxidant enzymes [132]. In a study conducted at the Cleveland Clinic, a semen sample was exposed to 1 h of radio frequency electromagnetic waves with one unaffected semen sample acting as a control [133]. Higher seminal ROS values and a decrease in sperm motility and viability were observed in the semen sample exposed to the electromagnetic waves. However, TAC remained unaffected and DNA damage was not induced [133]. These results confirm that exposure to radiofrequency electromagnetic waves (RF-EMW) radiation may negatively impact sperm quality by generating ROS [132]. Cell phone radiation also targets the plasma membrane of spermatozoa and increases caspase activation [132], thus resulting in an increase in apoptotic cell death and activation of NADPH oxidases, causing a burst of superoxide production [134].

8.11.10 Testicular Cancer

Cancer therapies such as chemotherapy, radiotherapy and/or surgery are known to have detrimental effects on the male reproductive system. Moreover, patients with testicular cancer, often have poor semen quality even prior to treatment [135]. Thus in cancer patients, male fertility preservation has great importance and the only reliable method to do so is by sperm banking, where the patient's spermatozoa are cryopreserved for use at a later time [136].

Research from the Cleveland Clinic has demonstrated that CK levels in cancer patients (both testicular and non-testicular) were not significantly different from CK

levels in normal healthy donors, suggesting that sperm produced by cancer patients are biochemically mature [124, 137]. Furthermore, another study analyzed whether lipid peroxidation affects semen quality in cancer patients. Results showed that the MDA levels in frozen-thawed (cryopreserved) semen samples among testicular or non-testicular cancer patients did not differ from that of control subjects. These findings support the suggestion that poor post-thaw semen quality in patients with testicular or non-testicular cancer is not associated with lipid peroxidation, but could instead be due to other stress factors induced during the freeze-thaw process [137, 138].

8.11.11 Preeclampsia

Preeclampsia is a multisystem disorder that is associated with placental OS [139, 140]. This condition is characterized by an increase in OS markers, such as LPO, elevated ROS levels, and a decrease in antioxidants [139, 141]. The induction of OS in pre-eclampsia is associated with neutrophil activation [142]. Placental OS triggers the release of prostaglandins and cytokines, which in turn impair endothelial cell function and contribute to preeclampsia [143].

8.11.12 Erectile Dysfunction

Penile erectile function is associated with the integrity of the endothelium, which is dependent upon nitric oxide (NO) concentrations. Excessive generation of superoxide reduces NO levels, thus, implicating a role for OS in the pathophysiology of erectile dysfunction [144].

8.11.13 Menopause

During menopause, a decline in estradiol levels creates a favorable environment for oxidation and, hence, OS can be observed. An increase in pro-inflammatory cytokines indicates that OS occurs in a post-menopausal state [145].

8.12 Infections

8.12.1 Leukocytes

Leukocytospermia is believed to directly affect ROS levels [146]. A study by Cleveland Clinic researchers found that both neat and washed semen samples exhibiting leukocytospermia have higher seminal ROS levels than fertile donors whose

semen does not contain leukocytes [40, 57, 147]. However, even in a fertile healthy population, ROS production is positively correlated to leukocyte concentration in semen [40]. Other studies have observed a negative correlation between sperm motility and leukocyte concentration and, further, found that leukocytes were positively correlated with abnormal sperm morphology [60, 79, 147]. Specifically, one study found that thiobarbituric acid reactive substances (TBARS) production was positively correlated with leukocyte concentration, indicating that leukocytospermia could induce lipid peroxidation in spermatozoa [79]. In addition, semen samples contaminated by leukocytes also exhibit a higher extent of spermatozoa with damaged DNA [84, 147].

The mechanism by which leukocytes can generate ROS is partly attributed to the fact that leukocyte activation increases the production of NADPH via the hexose monophosphate shunt, which in turn increases the concentration of the superoxide anion [73].

Numerous publications from the Cleveland Clinic have evaluated the WHO guidelines for leukocytospermia and the minimal leukocyte concentration in semen that is indicative of OS. One study found that semen samples with no leukocytes had significantly lower seminal ROS levels than semen samples with even a low concentration of leukocytes [148]. This study concluded that it was difficult to determine an exact leukocyte concentration cutoff value, because any semen sample exhibiting leukocytospermia (whether low or high concentration) produced excessive amounts of ROS [148]. A more specific and defined study evaluated the WHO value of 1×10^6 leukocytes/ml as a marker for OS [146]. Participants in the study were divided into three groups: no leukocytospermia (control), low-level leukocytospermia ($<1 \times 10^6$ leukocytes/ml), and high-level leukocytospermia ($>1 \times 10^6$ leukocytes/ml). Both groups exhibiting leukocytopsermia had higher seminal ROS levels than controls; however, the amount of ROS between the two groups was not significantly different. These findings corroborate the claim that low-level leukocytospermia induces OS and that the WHO guidelines need to be re-evaluated [146].

High seminal leucocyte concentration can also cause semen hyperviscosity (SHV), which prevents the travel of normal spermatozoa in the female reproductive tract [149].

8.12.2 Prostatitis

Research from the Cleveland Clinic has linked prostatitis with OS. In one study, Pasqualotto et al. divided patients into three groups: (1) prostatitis with leukocytes in semen, (2) prostatitis without leukocytes in semen, and (3) healthy fertile donors as controls [32]. Both patient groups with prostatitis had higher seminal ROS levels and lower TAC than controls. However, sperm parameters did not differ between the three groups, indicating that prostatitis may not have an adverse effect on sperm function. The patient group with prostatitis and leukocytes in their semen had the highest ROS levels. This study concluded that men with chronic prostatitis demonstrate seminal OS and that the presence of OS cannot be predicted by the patients' leukocytospermic status [32].

8.12.3 Ureaplasma urealyticum

Ureaplasma urealyticum is a commensal bacterium found in the flora of the lower genitourinary tract in sexually active adults. Ureaplasma infection is sexually transmitted and is involved in the pathology of various reproductive diseases such as pelvic inflammatory disease, chronic prostatitis and urethritis. Infected patients have higher seminal ROS levels than infertile patients and healthy controls who do not have the bacteria [150]. Leukocytes were found in only one of the 17 semen samples with positive U. urealyticum cultures, indicating that the increase in ROS generation is induced by the bacteria itself and is not associated with an increase in leukocyte production [150].

8.13 Antioxidants

8.13.1 Antioxidants Mechanisms

Antioxidants are defense mechanisms that neutralize and prevent the overproduction of ROS. Maintaining physiological levels of ROS is important because spermatozoa are very sensitive to free radicals due to their high levels of polyunsaturated fatty acids in their membranes, which are easily oxidized [151].

A variety of antioxidants have been tested by the Cleveland Clinic researchers in relation to male infertility. For example, Cleveland Clinic researchers have looked at the potential protective effects of free-radical scavengers on testicular damage induced by cadmium chloride. Cadmium chloride not only induced testicular impairment, but also reduced testicular lactate dehydrogenase-X (LDH-X) activity. Oxypurinol or superoxide dismutase (SOD) prevented testicular damage except when cadmium chloride was administered at high doses [152]. Red palm oil was also studied as an agent to reduce the effects of OS on rat spermatozoa [153]. The study showed that red palm oil administration is able to reduce the effect of OS-induced sperm damage caused by organic hydroperoxide exposure. In another study, the evaluation of antioxidants intake in male patients whose spouses had a history of recurrent embryo loss was conducted. Of these men, those who had high DNA damage or lipid peroxidation were put on an antioxidant supplementation regime. Spouses of six of the nine men, who were on antioxidant therapy for at least 3 months, were able to conceive. Couples whose male partners received antioxidant supplementation achieved a successful pregnancy [154]. With its potent singlet oxygen quencher properties, the carotenoid lycopene, is another possible treatment option for male infertility [155]. The usage of oral antioxidant supplementation, has been accepted clinically, and is currently being used to treat some infertile men [156].

8.13.2 Endogenous Sources

Two main sources of endogenous ROS in semen are leukocytes and immature spermatozoa, with leukocytes as the primary source [11, 157]. One possible strategy to counteract the endogenous production of ROS is to increase the oxidant scavenging capacity of the seminal plasma [158]. The seminal plasma protects against ROS damage because it contains enzymes (such as SOD and catalase) that scavenge ROS [11]. Seminal plasma also plays an important protective role against the adverse effects of endogenous ROS on sperm capacitation. In fact, seminal plasma is effective in reversing premature sperm capacitation induced by excessive ROS as well [159].

8.13.3 Exogenous Sources

Carnitines and vitamins C and E have been shown to be effective in improving fertility rates and may be considered as a first-line treatment [10, 160–162]. However, vitamin C therapy is controversial because vitamin C can also act as a pro-oxidant, and could thus contribute to intracellular ROS generation [160].

In two studies conducted by Cleveland Clinic researchers, the antioxidant capabilities of Vitamins C, and E and pentoxifyline were studied. The first study evaluated the unfavorable effects of exogenously induced ROS on mouse embryo development using a 12-phorbol 13-myristate acetate (PMA)-activated leukocyte model. The study demonstrated that exposure of the embryos to ROS over extended periods of time resulted in embryo toxicity [163]. The administration of antioxidants (Vitamin C and E) reversed the ROS-induced embryonic toxicity; however, Vitamin C was shown to have more effective antioxidant effect than vitamin E. In a similar study, mouse embryo was exposed to hydrogen peroxide and pentoxifyline was administered to assess its antioxidant potential on the blastocyst development rate [164]. Pentoxifylline administration was beneficial in reducing the hydrogen peroxide-induced embryo damage and in improving IVF outcome.

The efficacy of L-carnitine (LC) in improving sperm motility, vitality, and reducing DNA oxidation was made evident by a Cleveland Clinic study which found that LC significantly improved sperm motility and vitality, while not affecting DNA oxidation levels [165]. The combined L-carnitine and L-acetyl-carnitine therapy (LC + ALC; 2 months wash out, 6 months therapy/placebo, 2 months follow up) in 56 infertile males with OAT was also tested in a placebo-controlled double-blind randomized trial by Cleveland Clinic researchers [166]. Results showed an increase in all sperm parameters after combined LC + ALC treatment; with sperm motility increasing the most in patients who had very low motility to begin with.

Moreover, the protective effect of L-carnitine (LC) against deleterious substances present in peritoneal fluid (PF) of patients with endometriosis was studied by Cleveland Clinic researchers [96]. The findings showed PF from endometriosis

patients caused damage to oocyte microtubules and chromosomes as well as increased embryo apoptosis when compared to controls (PF from tubal ligation patients). However, supplementation with LC in the peritoneal fluid of endometriosis patients improved the microtubule and chromosome structures, and decreased the level of embryo apoptosis; while supplementation with LC in embryo culture medium successfully blocked the apoptotic effects of actinomycin-D (AD), and decreased DNA damage; thus suggesting LC supplementation as a possible treatment for patients with endometriosis and as a treatment to improve embryogenesis [96, 167]. Within the male reproductive tract, sperm metabolism and maturation are significantly impacted by the endogenous presence of high concentrations of LC and acetyl-L-carnitine (ALC) in the epididymis and spermatozoa [168].

8.14 Proteomics

Conventional semen analysis has a limited ability to evaluate the adverse effects of oxidative stress-related diagnoses on fertility. Specialized laboratory tests such as measurement of OS markers and sperm DNA fragmentation analysis provide diagnostic and prognostic information that complement the routine semen analysis. In addition, the developing areas of genomics, metabolomics, and in particular proteomics, have the potential to deepen our understanding of reproductive physiology and the pathophysiology of diseases that could compromise the male fertility potential, such as varicocele [169].

Over the recent decades, advances in molecular biology have gleaned insight into links between protein activity, function, and its encoded gene. This novel approach, known as proteomics, has allowed for the identification and characterization of proteins via qualitative and quantitative analysis. Cleveland Clinic researchers have embarked on proteomics studies in the last 5 years, with a focus on investigating the effects of elevated ROS levels/OS on spermatozoa [170–172] and seminal plasma [173–175] in both fertile and infertile males as well as in infertile men with varicocele [116, 176, 177].

Proteomic techniques, such as two-dimensional electrophoresis (2-DE), liquid chromatography mass spectrometry (LC-MS), and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Fig. 8.8), have provided insight into sperm function and dysfunction [178]. Alterations in seminal plasma and sperm proteins in men with OS are now thought to be associated with stress or metabolic responses and regulatory pathways [179]. Differentially expressed proteins (DEPs) in semen of fertile and infertile men have also been identified [170, 172, 174, 180]. Four predominant proteins have been found in the semen of healthy males, including semenogelin II precursor (SEMG2), prolactin-induced protein (PIP), clusterin isoform 1 (CLU), and prostate-specific antigen isoform 1 preproprotein (KLK3); however, semen of infertile men lacked SEMG2 and CLU [180].

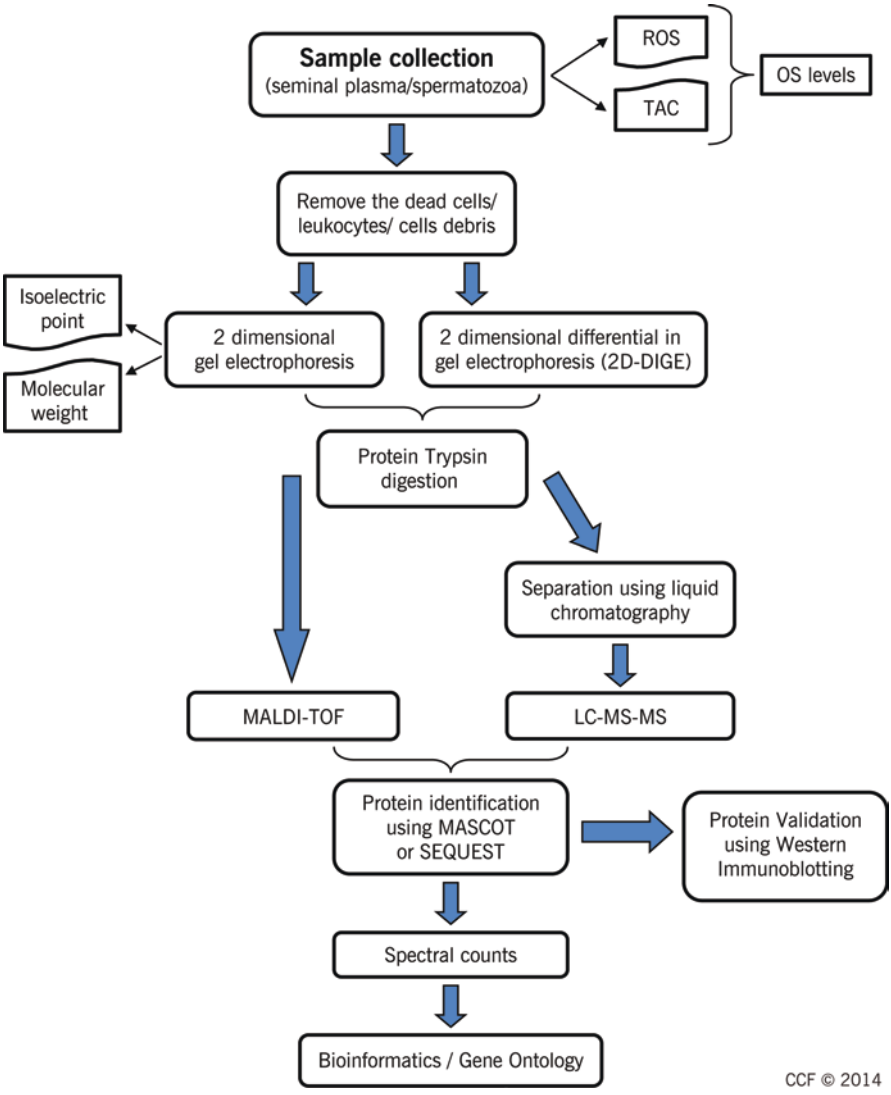

Fig. 8.8 An overview of the general methodology of protein isolation and identification from seminal plasma and spermatozoa samples. Oxidative stress levels are determined by measuring the ROS and/or the TAC levels. This is followed by protein separation by electrophoresis and protein identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS) or matrix-assisted laser desorption ionization-time of flight (MALDI-TOF). Data analysis programs (MASCOT, SEQUEST) are used to analyze the isolated proteins. Protein expressions are quantified based on spectral counts. The protein lists that are generated are then analyzed using bioinformatics tools. Gene ontology provides the information on specific gene function in cellular pathways. Proteins of interest can be validated using Western blotting

To learn more about the underlying pathology of unilateral varicocele, Cleveland Clinic researchers investigated the proteomic changes in infertile men with unilateral varicocele to identify and analyze proteins of interest in these men compared to fertile men [116]. In this study, proteins were extracted and separated by 1-D SDS-PAGE, then identified on a LTQ-Orbitrap Elite hybrid mass spectrometer system. Researchers found that 369 proteins were differentially expressed between both groups, and the major functional pathways of the DEPs related to the unilateral varicocele group were associated with gene expression, signal transduction and apoptosis among others. In addition, 38 proteins were unique to the unilateral varicocele group [e.g. cysteine-rich secretory protein 2 precursor (CRISP2) and arginase-2 (ARG2)] and 120 proteins were unique to the fertile group. When compared with fertile men, 114 proteins were overexpressed while 97 proteins were underexpressed in the unilateral varicocele patients.

Functional proteomic profiling has been used by the Cleveland Clinic researchers to compare the protein profiles between infertile men with unilateral and bilateral varicocele. Proteomic analysis in this study was done using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the Linear Trap Quadrupole-Orbitrap Elite hybrid mass spectrophotometer system. They found that 64 proteins in the bilateral group and 31 proteins in the unilateral group were uniquely expressed. Among the core functions associated with the key protein interaction networks were post-translational modification (PTM; mainly acetylation), protein folding, free radical scavenging, and cell death. The main molecular and cellular functions were protein degradation, free radical scavenging, and PTMs; while the major biological pathways for the 253 DEPs were metabolism, apoptosis, and signal transduction. These findings provide some insight into the mechanistic implications of varicocele-related male infertility [176].

The Cleveland Clinic researchers performed a comparative proteomics study to elucidate the key differences in the distribution and expression of spermatozoa proteins in infertile men with varicocele. They reported 99 proteins were differentially expressed in the varicocele group, including the Protein kinase A-R1A (PKAR1A), Adenylate kinase 7 (AK7), T-complex protein 1 subunit zeta-2 (CCT6B), Heat shock-related 70 kDa protein 2 (HSPA2), and Outer dense fiber of sperm tails 2 (ODF2) proteins that are involved in stress response and sperm function, including molecular chaperones. The major protein functions that were compromised in the varicocele group were spermatogenesis, sperm motility, and mitochondrial dysfunction [177].

Examination of the proteomic profile of spermatozoa from infertile or fertile men with increased ROS levels (ROS positive) in seminal plasma identified 74 proteins, of which ten proteins were overexpressed and five were underexpressed in the ROS positive group. Metabolic processes that were negatively impacted in this group of infertile men were protein modifications and OS regulation, energy metabolism and regulation, as well as gluconeogenesis and glycolysis [172]. When the ROS positive seminal plasma of men (infertile or fertile) was analyzed, 14 proteins were identified with seven of these common and unique proteins identified in both the ROS positive and ROS negative (low ROS levels) groups. ROS positive men presented with a

more abundant expression of prolactin-induced protein (PIP). Moreover, men with increased ROS levels in their seminal ejaculate express unique proteins that are either downregulated or oxidatively modified [173].

Another novel proteomics study by the Cleveland Clinic researchers looked into the differential expression of proteins in the seminal plasma of four groups of men based on sperm count and morphology: (1) normal sperm count and normal morphology (NN); (2) normal sperm count and abnormal morphology (NA); (3) oligozoospermia and normal morphology (ON); and (4) oligozoospermia and abnormal morphology (OA). Proteomics analysis was done using LC-MS/MS followed by functional bioinformatics analysis to compare the protein distribution in the NA, ON and OA groups with that of the NN group. Among the 4 groups, there were 20 DEPs that were identified. Of the unique proteins expressed, some proteins were downregulated—three in the NA group, one each in the ON and OA groups respectively; while some proteins were upregulated—two each in the ON and OA groups respectively. The functional analysis showed that the biological regulation was found to be the major process affected [175].

A comparative proteomic analysis conducted at the Clinic revealed that a pathway involving 35 DEPs controlling protein folding and protein degradation were overexpressed in the infertile high ROS group when compared to fertile controls [174]. These overexpressed proteins, such as metallo-endopeptidase (MME), DJ-1 (PARK7), PIP, lactotransferrin (LTF), and peroxiredoxin (PRDX), may serve as potential biomarkers of OS-induced male infertility [179]. Further, six differentially expressed proteins [Calmegin (CLGN), Tripeptidyl peptidase II (TPPII), Dynein intermediate chain 2, axonemal (DNAI2), Early endosome antigen 1 (EEA1), Heat shock 70 kDa protein 4 L (HSPA4L) and Plasma serine protease inhibitor (SERPINA5)] with distinct reproductive functions have been identified in men with low, medium, or high ROS levels [170]. Protective proteins against OS have also been recognized [171, 172].

In another novel proteomics study among fertile men, Cleveland Clinic researchers employed a 3-layer density gradient method to obtain four fractions of spermatozoa at different maturation stages. These fractions were then subjected to SDS-PAGE and proteomics analysis using the Finnigan LTQ-Orbitrap Elite hybrid mass spectrometer system, followed by bioinformatics analysis. This study provided essential information about the proteins and biological processes that are involved in spermatozoa maturation. Researchers identified 1469 proteins from all fractions and found that the number of detected proteins decreased in proportion to the spermatozoa maturation stage. And as the spermatozoa mature, the expression of proteins involved in response to OS processes (such as HSP 70 1A) as well as protein biosynthesis, transport, and ubiquitination, was found to decrease. Conversely, the expression levels of proteins involved in gamete generation, cell motility (such as Tektin 2 and Tektin 3), energy metabolism and oxidative phosphorylation processes showed an increasing trend. This study illustrated the proteins that are crucial for spermatozoa maturation, motility, and fertilization capacity as well as biological processes that are enhanced or suppressed during the spermatozoa maturation. This knowledge will be useful in recognizing any proteins and pathways that may be altered or modified in infertile men [181].

With the tremendous advancements in the expanding field of sperm proteomics, the ability to identify causative proteins and treat unexplained male infertility is promising and could certainly revolutionize an andrologist's diagnostic armamentarium. Validating sperm function biomarkers can have tremendous diagnostic and prognostic value, specifically for males with unexplained infertility in which routine semen analysis fails to detect subcellular sperm dysfunctions.

Not only in males, but the deleterious effects of OS on proteins and nucleic acids is hypothesized to be one of the major mechanistic pathways in the differential expression of proteins in endometriosis, PCOS, and unexplained infertility in females [182]. Thus, molecular pathways deciphered through proteomics analysis may play a key role in the early detection and management of both female and male infertility-related conditions [179, 182].

Certainly, the ability to assess sperm DNA damage and OS via protein identification and expression provides for a relatively independent measure of fertility potential [183]. Future studies should evaluate the variation in post-translational modifications of proteins in healthy and idiopathic infertile men [184]. Furthermore, advances in gene and protein interactions using global proteomics and transcriptomics technologies in male infertility studies will contribute greatly to the knowledge pool in treating the complex and multi factorial nature of male infertility [185].

That being said, while there is a real need to develop andrologic diagnostic techniques, it is essential to translate and weigh the cost-effectiveness and applicability of proteomics in clinical practice. In the meantime, unraveling the causes of idiopathic male infertility and unveiling of novel molecular targets will help advance the development of new pharmacologic agents in the treatment of male infertility [186].

8.15 Conclusion and Future Outlook

Arising from high concentrations of free radicals and/or suppressed antioxidant potential, OS is now well established as an important underlying cause of human infertility. It has also been implicated in various disorders and diseases associated with male and female infertility. Over the past 23 years, Cleveland Clinic researchers have been dedicated towards better understanding the causes and consequences of OS on the human fertility potential, particularly in the male. Findings of these research studies, along with that of other research laboratories have contributed greatly to the founding and expansion of the body of knowledge on OS and its related conditions. Assessment of oxidative stress levels in infertile patients, along with other specialized tests can be used to predict the severity of its impact on the fertility potential.

With over more than two decades of experience in the physiological and pathological roles of high ROS levels on the male gametes, as well as measures to assess and alleviate the adverse effects of high levels of ROS on male fertility, the research focus of the Cleveland Clinic researchers in the recent years have shifted towards unraveling the changes in the molecular processes and pathways that lead up to

infertility. Of late, proteomics studies are beginning to reveal the severe consequences of oxidative stress at a subcellular level, which can bring about alterations in the protein expression in seminal plasma and/or the spermatozoa. By characterizing these protein profiles of seminal ejaculate from men with different clinical conditions, such as high oxidative stress, it is hoped that selected biomarkers may emerge that will better equip the clinician with treatment options that will best benefit the patient.

References

1. Makker K, Agarwal A, Sharma R (2009) Oxidative stress & male infertility. Indian J Med Res 129(4):357–367
2. Agarwal A, Saleh RA (2002) Role of oxidants in male infertility: rationale, significance, and treatment. Urol Clin North Am 29(4):817–827
3. Desai N et al (2010) Free radical theory of aging: implications in male infertility. Urology 75(1):14–19
4. Agarwal A, Gupta S, Sikka S (2006) The role of free radicals and antioxidants in reproduction. Curr Opin Obstet Gynecol 18(3):325–332
5. Du Plessis SS et al (2015) Contemporary evidence on the physiological role of reactive oxygen species in human sperm function. J Assist Reprod Genet 32(4):509–520
6. Sekhon L et al (2010) Female infertility and antioxidants. Curr Womens Health Rev 6:84–95
7. Saleh RA, Agarwal A (2002) Oxidative stress and male infertility: from research bench to clinical practice. J Androl 23(6):737–752
8. Gupta A et al (1999) Profile of reactive oxygen species generation and antioxidative mechanisms in the maturing rat kidney. J Appl Toxicol 19(1):55–59
9. Gunes S, Al-Sadaan M, Agarwal A (2015) Spermatogenesis, DNA damage and DNA repair mechanisms in male infertility. Reprod Biomed Online 31(3):309–319
10. Agarwal A, Durairajanayagam D, du Plessis SS (2014) Utility of antioxidants during assisted reproductive techniques: an evidence based review. Reprod Biol Endocrinol 12:112
11. Sharma RK, Agarwal A (1996) Role of reactive oxygen species in male infertility. Urology 48(6):835–850
12. Ko EY, Sabanegh ES Jr, Agarwal A (2014) Male infertility testing: reactive oxygen species and antioxidant capacity. Fertil Steril 102(6):1518–1527
13. Kothari S et al (2010) Free radicals: their beneficial and detrimental effects on sperm function. Indian J Exp Biol 48(5):425–435
14. Agarwal A et al (2008) Redox considerations in female reproductive function and assisted reproduction: from molecular mechanisms to health implications. Antioxid Redox Signal 10(8):1375–1403
15. Agarwal A, Prabakaran S, Allamaneni S (2006) What an andrologist/urologist should know about free radicals and why. Urology 67(1):2–8
16. Mahfouz RZ et al (2010) Sperm viability, apoptosis, and intracellular reactive oxygen species levels in human spermatozoa before and after induction of oxidative stress. Fertil Steril 93(3):814–821
17. Desai N et al (2009) Physiologic and pathologic levels of reactive oxygen species in neat semen of infertile men. Fertil Steril 92(5):1626–1631
18. Agarwal A et al (2005) Correlation of reactive oxygen species levels with the fertilization rate after in vitro fertilization: a qualified meta-analysis. Fertil Steril 84(1):228–231
19. Agarwal A, Ahmad G, Sharma R (2015) Reference values of reactive oxygen species in seminal ejaculates using chemiluminescence assay. J Assist Reprod Genet 32(12):1721–1729

20. Bedaiwy MA et al (2004) Differential growth of human embryos in vitro: role of reactive oxygen species. Fertil Steril 82(3):593–600
21. Bedaiwy M et al (2006) Role of total antioxidant capacity in the differential growth of human embryos in vitro. Fertil Steril 86(2):304–309
22. Shekarriz M, Thomas AJ Jr, Agarwal A (1995) Effects of time and sperm concentration on reactive oxygen species formation in human semen. Arch Androl 34(2):69–75
23. Agarwal A, Ikemoto I, Loughlin KR (1994) Effect of sperm washing on levels of reactive oxygen species in semen. Arch Androl 33(3):157–162
24. Shekarriz M et al (1995) A method of human semen centrifugation to minimize the iatrogenic sperm injuries caused by reactive oxygen species. Eur Urol 28(1):31–35
25. Agarwal A, Ikemoto I, Loughlin KR (1994) Levels of reactive oxygen species before and after sperm preparation: comparison of swim-up and L4 filtration. Arch Androl 32(3):169–174
26. Allamaneni SS et al (2005) Comparative study on density gradients and swim-up preparation techniques utilizing neat and cryopreserved spermatozoa. Asian J Androl 7(1):86–92
27. Malvezzi H et al (2014) Sperm quality after density gradient centrifugation with three commercially available media: a controlled trial. Reprod Biol Endocrinol 12:121
28. Wang Y, Sharma RK, Agarwal A (1997) Effect of cryopreservation and sperm concentration on lipid peroxidation in human semen. Urology 50(3):409–413
29. Agarwal A et al (2006) Oxidative stress in an assisted reproductive techniques setting. Fertil Steril 86(3):503–512
30. du Plessis S et al (2008) Impact of oxidative stress on IVF. Exp Rev Obstet Gynecol 3(4):539–554
31. Pasqualotto EB et al (2004) Effect of oxidative stress in follicular fluid on the outcome of assisted reproductive procedures. Fertil Steril 81(4):973–976
32. Attaran M et al (2000) The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. Int J Fertil Womens Med 45(5):314–320
33. Bedaiwy M et al (2007) Differential expression of follicular fluid cytokines: relationship to subsequent pregnancy in IVF cycles. Reprod Biomed Online 15(3):321–325
34. Premkumar BJ, Agarwal A (2012) Female infertility and assisted reproduction: impact of oxidative stress - an update. Curr Womens Health Rev 8:183–207
35. Combelles CM, Gupta S, Agarwal A (2009) Could oxidative stress influence the in-vitro maturation of oocytes? Reprod Biomed Online 18(6):864–880
36. Bedaiwy MA et al (2010) Relationship of reactive oxygen species levels in day 3 culture media to the outcome of in vitro fertilization/intracytoplasmic sperm injection cycles. Fertil Steril 94(6):2037–2042
37. Bedaiwy MA et al (2012) Effect of follicular fluid oxidative stress parameters on intracytoplasmic sperm injection outcome. Gynecol Endocrinol 28(1):51–55
38. Agarwal A, Allamaneni SS (2011) Free radicals and male reproduction. J Indian Med Assoc 109(3):184–187
39. Agarwal A et al (2014) Mechanisms of oligozoospermia: an oxidative stress perspective. Syst Biol Reprod Med 60(4):206–216
40. Athayde KS et al (2007) Development of normal reference values for seminal reactive oxygen species and their correlation with leukocytes and semen parameters in a fertile population. J Androl 28(4):613–620
41. Agarwal A et al (2014) Characterizing semen parameters and their association with reactive oxygen species in infertile men. Reprod Biol Endocrinol 12:33
42. Kashou AH, Sharma R, Agarwal A (2013) Assessment of oxidative stress in sperm and semen. Methods Mol Biol 927:351–361
43. Agarwal A, Saleh RA (2002) Utility of oxidative stress test in the male infertility clinic. Zhonghua Nan Ke Xue 8(1):1–9
44. Agarwal A et al (2015) A unique view on male infertility around the globe. Reprod Biol Endocrinol 13:37
45. Shekarriz M, Thomas AJ Jr, Agarwal A (1995) Incidence and level of seminal reactive oxygen species in normal men. Urology 45(1):103–107

46. Homa ST et al (2015) Reactive Oxygen Species (ROS) in human semen: determination of a reference range. J Assist Reprod Genet 32(5):757–764
47. Agarwal A, Hamada A, Esteves SC (2015) Engaging practicing gynecologists in the management of infertile men. J Obstet Gynaecol India 65(2):75–87
48. Shekarriz M et al (1995) Positive myeloperoxidase staining (Endtz test) as an indicator of excessive reactive oxygen species formation in semen. J Assist Reprod Genet 12(2):70–74
49. Mahfouz R et al (2009) Diagnostic value of the total antioxidant capacity (TAC) in human seminal plasma. Fertil Steril 91(3):805–811
50. Pasqualotto FF et al (2008) Poor semen quality and ROS-TAC scores in patients with idiopathic infertility. Urol Int 81(3):263–270
51. Said TM et al (2003) Enhanced chemiluminescence assay vs colorimetric assay for measurement of the total antioxidant capacity of human seminal plasma. J Androl 24(5):676–680
52. Sharma RK et al (1999) The reactive oxygen species-total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. Hum Reprod 14(11):2801–2807
53. Agarwal A, Allamaneni SS, Said TM (2004) Chemiluminescence technique for measuring reactive oxygen species. Reprod Biomed Online 9(4):466–468
54. Vessey W et al (2014) Reactive oxygen species in human semen: validation and qualification of a chemiluminescence assay. Fertil Steril 102(6):1576–1583, e4
55. Kobayashi H et al (2001) Quality control of reactive oxygen species measurement by luminol-dependent chemiluminescence assay. J Androl 22(4):568–574
56. Mahfouz R et al (2009) Evaluation of chemiluminescence and flow cytometry as tools in assessing production of hydrogen peroxide and superoxide anion in human spermatozoa. Fertil Steril 92(2):819–827
57. Esfandiari N et al (2003) Utility of the nitroblue tetrazolium reduction test for assessment of reactive oxygen species production by seminal leukocytes and spermatozoa. J Androl 24(6):862–870
58. Aziz N et al (1996) The sperm deformity index: a reliable predictor of the outcome of oocyte fertilization in vitro. Fertil Steril 66(6):1000–1008
59. Said TM et al (2005) Novel association between sperm deformity index and oxidative stress-induced DNA damage in infertile male patients. Asian J Androl 7(2):121–126
60. Aziz N et al (2004) Novel associations between specific sperm morphological defects and leukocytospermia. Fertil Steril 82(3):621–627
61. Aziz N et al (2007) The relationship between human sperm apoptosis, morphology and the sperm deformity index. Hum Reprod 22(5):1413–1419
62. Aziz N et al (2004) Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index. Fertil Steril 81(2):349–354
63. Mansour G et al (2009) The impact of peritoneal fluid from healthy women and from women with endometriosis on sperm DNA and its relationship to the sperm deformity index. Fertil Steril 92(1):61–67
64. Agarwal A, Ikemoto I, Loughlin KR (1994) Relationship of sperm parameters with levels of reactive oxygen species in semen specimens. J Urol 152(1):107–110
65. Gil-Guzman E et al (2001) Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation. Hum Reprod 16(9):1922–1930
66. Wang X et al (2003) Alterations in mitochondria membrane potential and oxidative stress in infertile men: a prospective observational study. Fertil Steril 80(Suppl 2):844–850
67. Esfandiari N et al (2002) Effects of temperature on sperm motion characteristics and reactive oxygen species. Int J Fertil Womens Med 47(5):227–233
68. Mahfouz R et al (2010) Semen characteristics and sperm DNA fragmentation in infertile men with low and high levels of seminal reactive oxygen species. Fertil Steril 94(6):2141–2146
69. Desai NR et al (2010) Reactive oxygen species levels are independent of sperm concentration, motility, and abstinence in a normal, healthy, proven fertile man: a longitudinal study. Fertil Steril 94(4):1541–1543

70. Deepinder F, Cocuzza M, Agarwal A (2008) Should seminal oxidative stress measurement be offered routinely to men presenting for infertility evaluation? Endocr Pract 14(4):484–491
71. Pasqualotto FF et al (2000) Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation. Fertil Steril 73(3):459–464
72. Rengan AK et al (2012) An investigation of excess residual cytoplasm in human spermatozoa and its distinction from the cytoplasmic droplet. Reprod Biol Endocrinol 10:92
73. Agarwal A, Saleh RA, Bedaiwy MA (2003) Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril 79(4):829–843
74. Nallella KP et al (2005) Identification of male factor infertility using a novel semen quality score and reactive oxygen species levels. Clinics (Sao Paulo) 60(4):317–324
75. Kumar R et al (2009) Oxidative stress and sperm mitochondrial DNA mutation in idiopathic oligoasthenozoospermic men. Indian J Biochem Biophys 46(2):172–177
76. Agarwal A, Tvrda E, Sharma R (2014) Relationship amongst teratozoospermia, seminal oxidative stress and male infertility. Reprod Biol Endocrinol 12:45
77. Said TM et al (2005) Impact of sperm morphology on DNA damage caused by oxidative stress induced by beta-nicotinamide adenine dinucleotide phosphate. Fertil Steril 83(1):95–103
78. Buldreghini E et al (2014) Human leucocytes in asthenozoospermic patients: endothelial nitric oxide synthase expression. Andrologia 46(10):1176–1182
79. Zalata AA et al (2004) Relationship between acrosin activity of human spermatozoa and oxidative stress. Asian J Androl 6(4):313–318
80. Wang Y et al (1997) Importance of reactive oxygen species in the peritoneal fluid of women with endometriosis or idiopathic infertility. Fertil Steril 68(5):826–830
81. Venkatesh S et al (2009) Clinical significance of reactive oxygen species in semen of infertile Indian men. Andrologia 41(4):251–256
82. Agarwal A, Makker K, Sharma R (2008) Clinical relevance of oxidative stress in male factor infertility: an update. Am J Reprod Immunol 59(1):2–11
83. Cocuzza M et al (2007) Clinical relevance of oxidative stress and sperm chromatin damage in male infertility: an evidence based analysis. Int Braz J Urol 33(5):603–621
84. Alvarez JG et al (2002) Increased DNA damage in sperm from leukocytospermic semen samples as determined by the sperm chromatin structure assay. Fertil Steril 78(2):319–329
85. Agarwal A, Varghese AC, Sharma RK (2009) Markers of oxidative stress and sperm chromatin integrity. Methods Mol Biol 590:377–402
86. Lakpour N et al (2012) Relationship of seminal plasma antioxidants and serum male hormones with sperm chromatin status in male factor infertility. Syst Biol Reprod Med 58(5):236–244
87. Moustafa MH et al (2004) Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. Hum Reprod 19(1):129–138
88. Saleh RA et al (2003) Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. Fertil Steril 79(Suppl 3):1597–1605
89. Ollero M et al (2001) Characterization of subsets of human spermatozoa at different stages of maturation: implications in the diagnosis and treatment of male infertility. Hum Reprod 16(9):1912–1921
90. Saleh RA et al (2003) Evaluation of nuclear DNA damage in spermatozoa from infertile men with varicocele. Fertil Steril 80(6):1431–1436
91. Nandipati KC et al (2005) Relationship of interleukin-6 with semen characteristics and oxidative stress in vasectomy reversal patients. Andrologia 37(4):131–134
92. Nallella KP et al (2004) Relationship of interleukin-6 with semen characteristics and oxidative stress in patients with varicocele. Urology 64(5):1010–1013
93. Choi WJ et al (2007) Oxidative stress and tumor necrosis factor-alpha-induced alterations in metaphase II mouse oocyte spindle structure. Fertil Steril 88(4 Suppl):1220–1231
94. Wang X et al (2003) Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. Fertil Steril 80(3):531–535

95. Esfandiari N et al (2007) Heat-shock proteins modulate the incidence of apoptosis and oxidative stress in preimplantation mouse embryos. Fertil Steril 87(5):1214–1217
96. Mansour G et al (2009) L-carnitine supplementation reduces oocyte cytoskeleton damage and embryo apoptosis induced by incubation in peritoneal fluid from patients with endometriosis. Fertil Steril 91(5 Suppl):2079–2086
97. Esfandiari N et al (2005) Protein supplementation and the incidence of apoptosis and oxidative stress in mouse embryos. Obstet Gynecol 105(3):653–660
98. Jha R et al (2009) Determination of Poly (ADP-ribose) polymerase (PARP) homologues in human ejaculated sperm and its correlation with sperm maturation. Fertil Steril 91(3):782–790
99. Mahfouz RZ et al (2009) Evaluation of poly(ADP-ribose) polymerase cleavage (cPARP) in ejaculated human sperm fractions after induction of apoptosis. Fertil Steril 91(5 Suppl):2210–2220
100. Bedaiwy MA et al (2002) Relationship between oxidative stress and embryotoxicity of hydrosalpingeal fluid. Hum Reprod 17(3):601–604
101. Agarwal A, Gupta S, Sharma R (2005) Oxidative stress and its implications in female infertility – a clinician's perspective. Reprod Biomed Online 11(5):641–650
102. Bedaiwy MA et al (2005) Relationship between cytokines and the embryotoxicity of hydrosalpingeal fluid. J Assist Reprod Genet 22(4):161–165
103. Durairajanayagam D, Agarwal A, Ong C (2015) Causes, effects and molecular mechanisms of testicular heat stress. Reprod Biomed Online 30(1):14–27
104. du Plessis SS et al (2010) Effects of H(2)O(2) exposure on human sperm motility parameters, reactive oxygen species levels and nitric oxide levels. Andrologia 42(3):206–210
105. Agarwal A, Hamada A, Esteves SC (2012) Insight into oxidative stress in varicocele-associated male infertility: part 1. Nat Rev Urol 9(12):678–690
106. Doshi SB et al (2012) Role of reactive nitrogen species in male infertility. Reprod Biol Endocrinol 10:109
107. Cho CL, Agarwal A, Esteves SC (2015) Novel insights into the pathophysiology of varicocele and its association with reactive oxygen species and sperm DNA fragmentation. Asian J Androl 18(2):186–193
108. Agarwal A et al (2016) Effect of varicocele on semen characteristics according to the new 2010 World Health Organization criteria: a systematic review and meta-analysis. Asian J Androl 18(2):163–170
109. Abd-Elmoaty MA et al (2010) Increased levels of oxidants and reduced antioxidants in semen of infertile men with varicocele. Fertil Steril 94(4):1531–1534
110. Hamada A, Esteves SC, Agarwal A (2013) Insight into oxidative stress in varicocele-associated male infertility: part 2. Nat Rev Urol 10(1):26–37
111. Pasqualotto FF et al (2008) Semen quality and oxidative stress scores in fertile and infertile patients with varicocele. Fertil Steril 89(3):602–607
112. Allamaneni SS et al (2004) Increased seminal reactive oxygen species levels in patients with varicoceles correlate with varicocele grade but not with testis size. Fertil Steril 82(6):1684–1686
113. Cocuzza M et al (2008) Impact of clinical varicocele and testis size on seminal reactive oxygen species levels in a fertile population: a prospective controlled study. Fertil Steril 90(4):1103–1108
114. Pasqualotto FF et al (2001) Oxidative stress in normospermic men undergoing infertility evaluation. J Androl 22(2):316–322
115. Agarwal A, Prabakaran S, Allamaneni SS (2006) Relationship between oxidative stress, varicocele and infertility: a meta-analysis. Reprod Biomed Online 12(5):630–633
116. Agarwal A et al (2015) Major protein alterations in spermatozoa from infertile men with unilateral varicocele. Reprod Biol Endocrinol 13:8
117. Agarwal A et al (2016) Spermatozoa protein alterations in infertile men with bilateral varicocele. Asian J Androl 18(1):43–53

118. Agarwal A et al (2009) Role of oxidative stress in pathogenesis of varicocele and infertility. Urology 73(3):461–469
119. Padron OF et al (1997) Seminal reactive oxygen species and sperm motility and morphology in men with spinal cord injury. Fertil Steril 67(6):1115–1120
120. Harlev A, Gupta S, Agarwal A (2015) Targeting oxidative stress to treat endometriosis. Expert Opin Ther Targets 19(11):1447–1464
121. Benjamin D, Sharma RK, Moazzam A, Agarwal A (2012) Methods for the detection of ROS in human sperm samples. Studies on men's health and fertility. Humana, New York, pp 257–274
122. Kolettis PN et al (1999) Effect of seminal oxidative stress on fertility after vasectomy reversal. Fertil Steril 71(2):249–255
123. Sharma R et al (2000) Oxidative stress and IL-6 levels in patients undergoing vasectomy reversal. J Androl 21(2 Supp/1):19
124. Sidhu RS et al (1998) Relationship between creatine kinase levels and clinical diagnosis of infertility. J Assist Reprod Genet 15(4):188–192
125. Alshahrani S et al (2014) Infertile men older than 40 years are at higher risk of sperm DNA damage. Reprod Biol Endocrinol 12:103
126. Sharma R et al (2015) Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. Reprod Biol Endocrinol 13:35
127. Cocuzza M et al (2008) Age-related increase of reactive oxygen species in neat semen in healthy fertile men. Urology 71(3):490–494
128. Saleh RA et al (2002) Effect of cigarette smoking on levels of seminal oxidative stress in infertile men: a prospective study. Fertil Steril 78(3):491–499
129. Varghese AC et al (2011) Oocyte developmental competence and embryo development: impact of lifestyle and environmental risk factors. Reprod Biomed Online 22(5):410–420
130. Harlev A et al (2015) Smoking and male infertility: an evidence-based review. World J Mens Health 33(3):143–160
131. du Plessis SS, Agarwal A, Syriac A (2015) Marijuana, phytocannabinoids, the endocannabinoid system, and male fertility. J Assist Reprod Genet 32(11):1575–1588
132. Agarwal A, Durairajanayagam D (2015) Are men talking their reproductive health away? Asian J Androl 17(3):433–434
133. Agarwal A et al (2009) Effects of radiofrequency electromagnetic waves (RF-EMW) from cellular phones on human ejaculated semen: an in vitro pilot study. Fertil Steril 92(4):1318–1325
134. Desai NR, Kesari KK, Agarwal A (2009) Pathophysiology of cell phone radiation: oxidative stress and carcinogenesis with focus on male reproductive system. Reprod Biol Endocrinol 7:114
135. Tvrda E, Agarwal A, Alkuhaimi N (2015) Male reproductive cancers and infertility: a mutual relationship. Int J Mol Sci 16(4):7230–7260
136. Agarwal A, Ong C, Durairajanayagam D (2014) Contemporary and future insights into fertility preservation in male cancer patients. Transl Androl Urol 3(1):27–40
137. Sidhu RS, Wang Y, Agarwal A (1997) Creatine kinase level and lipid peroxidation rate in human spermatozoa from patients with cancer. J Assist Reprod Genet 14(9):538–542
138. Wang Y, Sharma RK, Agarwal A (1997) Effect of lipid peroxidation on cryopreserved semen quality in patients with testicular or nontesticular cancer. Urology 50(3):414–417
139. Gupta S, Agarwal A, Sharma RK (2005) The role of placental oxidative stress and lipid peroxidation in preeclampsia. Obstet Gynecol Surv 60(12):807–816
140. Agarwal A, Gupta S (2005) Role of reactive oxygen species in female reproduction. Part I. Oxidative stress: a general overview. AgroFOOD Industry Hi-Tech 16(1):21–25
141. Gupta S et al (2009) Lipid peroxidation and antioxidant status in preeclampsia: a systematic review. Obstet Gynecol Surv 64(11):750–759
142. Sharma R, Agarwal A (2004) Role of reactive oxygen species in gynecologic diseases. Reprod Med Biol 3:177–199
143. Agarwal A, Gupta S, Sharma RK (2005) Role of oxidative stress in female reproduction. Reprod Biol Endocrinol 3:28

144. Agarwal A et al (2006) Role of oxidative stress in the pathophysiological mechanism of erectile dysfunction. J Androl 27(3):335–347
145. Doshi SB, Agarwal A (2013) The role of oxidative stress in menopause. J Midlife Health 4(3):140–146
146. Agarwal A et al (2014) Reactive oxygen species and sperm DNA damage in infertile men presenting with low level leukocytospermia. Reprod Biol Endocrinol 12:126
147. Saleh RA et al (2002) Leukocytospermia is associated with increased reactive oxygen species production by human spermatozoa. Fertil Steril 78(6):1215–1224
148. Sharma RK et al (2001) Relationship between seminal white blood cell counts and oxidative stress in men treated at an infertility clinic. J Androl 22(4):575–583
149. Du Plessis SS, Gokul S, Agarwal A (2013) Semen hyperviscosity: causes, consequences, and cures. Front Biosci 5:224–231
150. Potts JM et al (2000) Association of ureaplasma urealyticum with abnormal reactive oxygen species levels and absence of leukocytospermia. J Urol 163(6):1775–1778
151. Agarwal A, Prabakaran SA (2005) Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. Indian J Exp Biol 43(11):963–974
152. Agarwal A, Ikemoto I, Loughlin KR (1997) Prevention of testicular damage by free-radical scavengers. Urology 50(5):759–763
153. Aboua YG et al (2012) A red palm oil diet can reduce the effects of oxidative stress on rat spermatozoa. Andrologia 44(Suppl 1):32–40
154. Gil-Villa AM et al (2009) Role of male factor in early recurrent embryo loss: do antioxidants have any effect? Fertil Steril 92(2):565–571
155. Durairajanayagam D et al (2014) Lycopene and male infertility. Asian J Androl 16(3):420–425
156. Gupta S et al (2010) The role of oxidative stress and antioxidants in assisted reproduction. Curr Womens Health Rev 6:227–238
157. Agarwal A, Prabakaran SA, Said TM (2005) Prevention of oxidative stress injury to sperm. J Androl 26(6):654–660
158. Agarwal A et al (2004) Role of antioxidants in treatment of male infertility: an overview of the literature. Reprod Biomed Online 8(6):616–627
159. Villegas J et al (2003) Reactive oxygen species induce reversible capacitation in human spermatozoa. Andrologia 35(4):227–232
160. Agarwal A, Sekhon LH (2011) Oxidative stress and antioxidants for idiopathic oligoasthenoteratospermia: is it justified? Indian J Urol 27(1):74–85
161. Agarwal A, Sekhon LH (2010) The role of antioxidant therapy in the treatment of male infertility. Hum Fertil (Camb) 13(4):217–225
162. Agarwal A, Gupta S (2005) Role of reactive oxygen species in female reproduction and the effects of antioxidant supplementation – Part 2. AgroFOOD industry hi-tech. pp 38–41
163. Wang X et al (2002) Vitamin C and vitamin E supplementation reduce oxidative stress-induced embryo toxicity and improve the blastocyst development rate. Fertil Steril 78(6):1272–1277
164. Zhang X et al (2005) Effect of pentoxifylline in reducing oxidative stress-induced embryotoxicity. J Assist Reprod Genet 22(11-12):415–417
165. Banihani S et al (2014) Cryoprotective effect of L-carnitine on motility, vitality and DNA oxidation of human spermatozoa. Andrologia 46(6):637–641
166. Lenzi A et al (2004) A placebo-controlled double-blind randomized trial of the use of combined l-carnitine and l-acetyl-carnitine treatment in men with asthenozoospermia. Fertil Steril 81(6):1578–1584
167. Abdelrazik H et al (2009) L-carnitine decreases DNA damage and improves the in vitro blastocyst development rate in mouse embryos. Fertil Steril 91(2):589–596
168. Agarwal A, Said TM (2004) Carnitines and male infertility. Reprod Biomed Online 8(4):376–384
169. Majzoub A et al (2016) Specialized sperm function tests in varicocele and the future of andrology laboratory. Asian J Androl 18(2):205–212

170. Ayaz A et al (2015) Impact of precise modulation of reactive oxygen species levels on spermatozoa proteins in infertile men. Clin Proteomics 12(1):4
171. Hamada A et al (2013) Two-dimensional differential in-gel electrophoresis-based proteomics of male gametes in relation to oxidative stress. Fertil Steril 99(5):1216–1226, e2
172. Sharma R et al (2013) Proteomic analysis of human spermatozoa proteins with oxidative stress. Reprod Biol Endocrinol 11:48
173. Sharma R et al (2013) Proteomic analysis of seminal fluid from men exhibiting oxidative stress. Reprod Biol Endocrinol 11:85
174. Agarwal A et al (2015) Comparative proteomic network signatures in seminal plasma of infertile men as a function of reactive oxygen species. Clin Proteomics 12(1):23
175. Sharma R et al (2013) Functional proteomic analysis of seminal plasma proteins in men with various semen parameters. Reprod Biol Endocrinol 11:38
176. Esteves SC et al (2015) Diagnostic accuracy of sperm DNA degradation index (DDSi) as a potential noninvasive biomarker to identify men with varicocele-associated infertility. Int Urol Nephrol 47(9):1471–1477
177. Agarwal A et al (2015) Proteomic signatures of infertile men with clinical varicocele and their validation studies reveal mitochondrial dysfunction leading to infertility. Asian J Androl 18(2):282–291
178. du Plessis SS et al (2011) Proteomics: a subcellular look at spermatozoa. Reprod Biol Endocrinol 9:36
179. Agarwal A et al (2014) Proteomics, oxidative stress and male infertility. Reprod Biomed Online 29(1):32–58
180. Thacker S et al (2011) Evaluation of sperm proteins in infertile men: a proteomic approach. Fertil Steril 95(8):2745–2748
181. Cui Z, Sharma R, Agarwal A (2015) Proteomic analysis of mature and immature ejaculated spermatozoa from fertile men. Asian J Androl 18(5):735–746
182. Gupta S et al (2014) Power of proteomics in linking oxidative stress and female infertility. Biomed Res Int 2014:916212
183. Esteves SC et al (2014) A translational medicine appraisal of specialized andrology testing in unexplained male infertility. Int Urol Nephrol 46(6):1037–1052
184. Barazani Y, Agarwal A, Sabanegh ES Jr (2014) Functional sperm testing and the role of proteomics in the evaluation of male infertility. Urology 84(2):255–261
185. Tahmasbpour E, Balasubramanian D, Agarwal A (2014) A multi-faceted approach to understanding male infertility: gene mutations, molecular defects and assisted reproductive techniques (ART). J Assist Reprod Genet 31(9):1115–1137
186. Hamada AJ, Montgomery B, Agarwal A (2012) Male infertility: a critical review of pharmacologic management. Expert Opin Pharmacother 13(17):2511–2531