Development of In Vitro Maturation for Human Oocytes

Natural and Mild Approaches to Clinical Infertility Treatment

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Preface

Assisted reproductive technology (ART) has helped several million women overcome childlessness due to infertility. Initial attempts in human in vitro fertilization (IVF) in the 1930s used in vitro matured (IVM) oocytes [1–4], because it was impossible to obtain human in vivo matured oocytes at that time. The landmark work on IVM of human immature oocytes was carried out in 1960s [5, 6]; the human IVF techniques were also established with IVM oocytes [7–10]. Therefore, we can say all current advanced ART for infertility treatment is based on the early development work of IVM.

In the 1970s, laparoscopy was introduced to collect human mature oocytes from preovulatory follicles [11], resulting in the first reported case of in vivo matured oocytes in IVF [12]. Although the first human live birth resulting from IVF was produced by natural cycle IVF [13], this procedure was gradually replaced by ovarian hyperstimulation combined with IVF treatment, because the number of oocytes retrieved determined the embryos available for transfer, which, in turn, directly affected the chance of successful pregnancy [14–16]. Initially, clomiphene citrate (CC) was used as a single ovarian stimulation agent [17–19]. Subsequently, it was utilized in combination with human menopausal gonadotropin (HMG) to generate multiple follicle developments and to increase the yield of more than one oocyte [20–22]. To prevent the problem of premature ovulation, gonadotropins (recombinant or HMG) were combined with pituitary downregulation with LHRH agonists (referred at the time as controlled ovarian hyperstimulation or COH, but now called conventional IVF) with the aim of obtaining an average of 10–15 mature oocytes per retrieval from each woman.

In recent years, however, the protocols for ovarian stimulation with IVF treatment have undergone considerable changes, especially following the introduction of LHRH antagonists, which block LH for a few days within the woman's natural cycle and permit milder forms of stimulation (mild IVF) with the aim of reducing complications and focusing on the quality rather than quantity of the oocytes.

Although high-dose gonadotropin COH cycles are associated with more oocytes collected, this approach is associated with a number of adverse short- and long-term side effects, including greater risks of ovarian hyperstimulation syndrome (OHSS) [23]; pulmonary embolus; maternal, fetal, and neonatal complications, such as preterm labor, preterm delivery, and low birthweight babies (both premature and SGA); and lastly, greater inconvenience and

increased cost. Thus, mild and natural cycle IVF, as well as IVM treatment, has become appealing options to more and more infertile couples.

Today, given the efficiency of IVF and improvements in the culture system, natural cycle IVF or mild stimulation may be more suitable for women undergoing IVF treatment. Natural cycle, without any gonadotropin stimulation, is encumbered by a number of problems, including an increased risk of failure to retrieve oocytes and an absence of embryo available for transfer. Nevertheless, there has been a resurgence of interest in natural cycle IVF treatment in recent years because the efficiency of IVF technology has improved markedly [24–26], including modifications, such as the addition of GnRH antagonist and FSH add-back (modified natural IVF). With these adjustments, premature ovulation is no longer a problem. Several studies have shown that natural cycle IVF treatment has advantages over conventional COH IVF treatment with downregulation, particularly in the management of women with low ovarian reserve [27, 28].

In contrast to conventional IVF treatment, the aim of mild stimulation is to develop safer and more patient-friendly protocols where the risks of the treatment are minimized. Mild stimulation is defined as administration of low-dose exogenous gonadotropins, and/or for a shorter duration in GnRH antagonist co-treated cycles, or when oral compounds (CC, aromatase inhibitors) are used for ovarian stimulation, with the aim of retrieving fewer than eight oocytes [29, 30]. Mild stimulation using CC in combination with low doses of gonadotropins can also be considered a realistic option for good prognosis patients undergoing IVF [31].

Interestingly, despite theoretical advantages, mild IVF treatment has not become a mainstream treatment approach in the USA at the present time. Although mild ovarian stimulation is an appropriate option to consider for certain patient groups or based on patient preference [32], current evidence pointing to fewer cryopreserved embryos and lower success rates per cycle could be regarded as potential disadvantages and limit its acceptability for patients [33]. A recent large retrospective study found a significant decrease in live birth rate associated with increasing FSH dose regardless of the number of oocytes retrieved [34], cautioning against high doses of FSH in IVF treatment cycles albeit falling short of recommending mild IVF treatment. There is also evidence that mild stimulation or modified natural cycle protocols may have equal or even improved success rates compared with conventional IVF in women with a history of poor ovarian response [35].

Recovery of immature oocytes followed by IVM of these oocytes is a potentially useful treatment for women with infertility. This method is particularly effective for women with polycystic ovaries (PCO) or polycystic ovarian syndrome (PCOS)-related infertility, because there are numerous antral follicles within the ovaries of this group of patients [36–38]. Apart from women with PCOS, IVM treatment may be also offered to women who are delayed responders or who are over responding during stimulation in IVF cycles as an alternative to cancelation with acceptable pregnancy and live birth rates [39, 40]. To date, IVM treatment has been mainly applied to women with PCOS and is not regarded as applicable to all types of infertility. However, there is a growing number of women requiring IVF treatment

where ovarian stimulation is either rejected by the women due to concerns about side effects or contraindicated, such as in women with a previously treated estrogen-dependent cancer.

As the development of IVM treatment continues, one very attractive possibility for enhancing the successful outcome is combining natural cycle IVF treatment with immature egg retrieval followed by IVM of those immature oocytes [41]. It has been proven that the use of IVM technology can thus be broadened to treat women suffering from all causes of infertility with acceptable pregnancy and live birth rates [42–45].

More recently, Paulson et al. [46] postulated that one of the barriers to access to fertility care is the relative complexity of fertility treatments. If these treatment processes can be simplified, more infertile women may be able to take advantage of the treatments. A more simplified, milder IVF treatment approach represents a viable alternative to standard treatment. As we accumulate more experience and outcome data, mild stimulation IVF and IVM may prove to be not just alternatives to standard treatments, but potentially first-line treatment choices. All these exciting new treatment options are explored in depth in this book. The aim of this book is to share our experience and protocols with the ART fraternity.

Part I covers the scientific rationale for follicular development by outlining ovarian endocrinology and how somatic cells interact with oocytes during follicular development: the mechanism of oocyte maturation, and how these have led to understand the current concept and protocols for oocyte maturation in vivo and in vitro. Also discussed is the importance of mitochondrial changes during oocyte growth and maturation. Here, we emphasize that follicular maturation (or growth) and oocyte maturation are two totally different concepts. Follicular maturation (or growth) refers to the relatively lengthy process developing over several weeks from primordial follicle to preovulatory follicle; oocyte maturation is triggered by LH surge in vivo and refers to the maturation from the fully grown oocyte from germinal vesicle (GV) stage to metaphase-II (M-II) stage, in order to receive sperm for fertilization. Oocyte maturation can occur spontaneously in vitro after releasing from follicles with suitable culture conditions.

Part II covers the differences between natural cycle IVF treatment and stimulated IVF cycles and the different hormone profiles from follicular fluid in natural cycle IVF treatment. It also covers the standard ovarian stimulation protocols and their outcome in general, including cumulative success rates with natural cycle IVF treatment. Also discussed are how to prevent and manage ovarian hyperstimulation syndrome (OHSS) and which patients are suitable for natural cycle IVF treatment.

Part III covers mild stimulated IVF treatments both with exogenous gonadotropins and aromatase inhibitors. It also covers mild stimulation protocols for fertility preservation in women at risk of infertility following cancer treatment. An alternative treatment, INVO procedure, is described, and accessible infertility care and genetic aspect of recurrent implantation failure are also discussed.

Finally, Part IV covers IVM as clinical treatment for women with PCOS and how to avoid the severe OHSS with IVM treatment. It also covers the

methodology of immature oocyte retrieval and all laboratory and clinical aspects of IVM treatment. Also discussed are obstetrical and congenital outcomes of IVM babies and how the development of IVM treatment may be applied to all types of infertile women with natural cycle IVF combined with IVM treatment.

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Part I
Ovarian Function

Ovarian Endocrinology

1

Seido Takae, MD, PhD and Nao Suzuki, MD, PhD

Factors Regulate Ovarian Cycles

The following factors are involved in the ovarian cycle (i.e., follicular development, ovulation, and corpus luteum formation) and the regulation of menstruation as a result of luteal regression. All these factors are so-called "classical hormones" that are secreted by specific cells or organs. These factors affect target organs via the bloodstream and constitute the core of the feedback mechanism of the diencephalon (hypothalamus)–pituitary–ovary–uterus system.

A. Gonadotropin-releasing hormone (GnRH);
B. Gonadotropins: follicle-stimulating hormone (FSH) and luteinizing hormone (LH);
C. Steroid hormones: estrogen, androgens, progesterone, glucocorticoids, and mineralocorticoids; and
D. Glycoproteins: inhibin, activin, follistatin, and anti-Müllerian hormone (AMH).

In this chapter, particularly important aspects of hormones A–D are selected and described.

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GnRH

The menstrual cycle is controlled via regulation of GnRH, which is synthesized by the hypothalamus. GnRH, a polypeptide composed of 10 amino acids (Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), is a hormone that is secreted by the hypothalamus and that has an extremely short half-life (2–3 min). The GnRH-1 gene (*GNRH1*) is located on human chromosome 8 (8p11.2-p21) and produces a 92-amino acid precursor peptide called prepro-GnRH, which includes a signal sequence (23 amino acids), GnRH (10 amino acids), a proteolytic processing site (3 amino acids), and GnRH-associated peptide (56 amino acids) (Fig. 1.1) [1].

Most GnRH neurons are located in the preoptic area of the hypothalamus and project their axons to the median eminence. GnRH molecules that are stored in secretory granules in the nerve endings of the neuron are released via neuroendocrine mechanisms, travel through the hypophyseal portal system, bind to GnRH receptors that are primarily expressed on anterior pituitary cells, and stimulate the secretion of gonadotropins such as FSH and LH from the anterior pituitary gland (Fig. 1.2) [1].

a. Rhythmic secretion of GnRH

GnRH is secreted in a rhythmic fashion, and in response to this, gonadotropins are also secreted rhythmically from the pituitary. GnRH secretion is extremely crucial in the maintenance of the menstrual cycle. In the follicular phase of the

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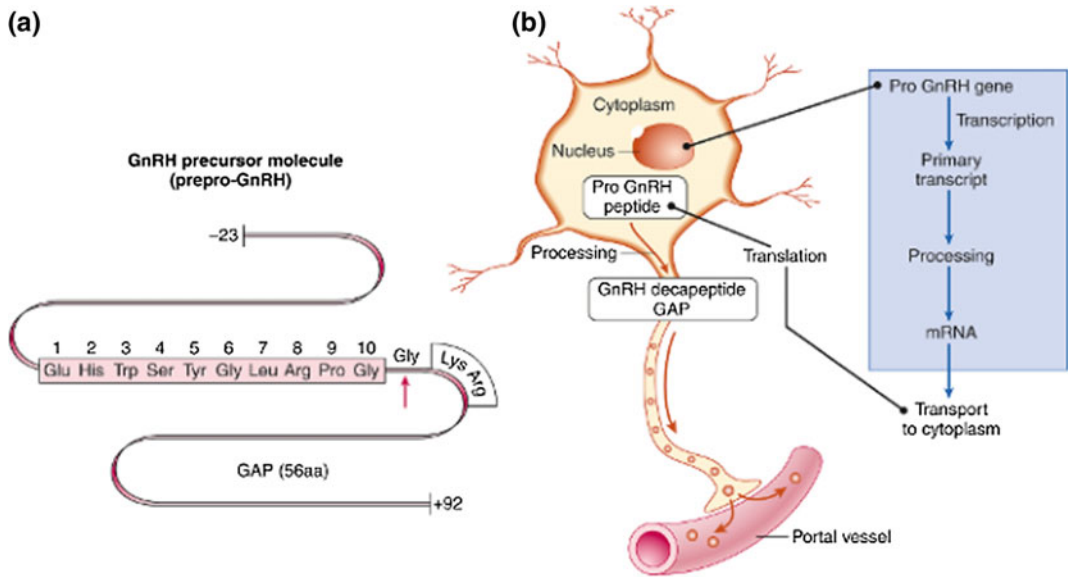

Fig. 1.1 Schematic of GnRH synthesis. A, Representation of prepro-GnRH, including a 23-amino acid signal sequence, GnRH, a proteolytic processing site (Gly-Lys-Arg), and GnRH-associated peptide (GAP). The *arrow* indicates the site of proteolytic cleavage and C-amidation. B, Schematic of neuronal GnRH synthesis and secretion. Reproduction with the permission [1]

menstrual cycle, GnRH pulses occur every 60–90 min. Under such physiological conditions, GnRH induces a priming effect of gonadotropin-secreting cells in the pituitary, consequently upregulating GnRH receptors and enhancing their responsiveness to GnRH molecules. However, when GnRH is activated with a continual pulse or with pulses that occur at a greater frequency than physiological levels, GnRH receptors decline and responsiveness also decreases (downregulation) (Fig. 1.3) [1]. In the latter half of the follicular phase, GnRH secretion cycles become shorter, and the amount of secretion increases as ovulation approaches; however, in the luteal phase, these cycles rapidly become longer and the amount of secretion further increases.

b. Mechanism of regulation of GnRH secretion

GnRH secretion is regulated by neurotransmitters, such as noradrenaline, dopamine, and opioid peptides. External stimuli influence the cell body of GnRH neurons in the preoptic area and arcuate nucleus via noradrenaline synapses in the optic nerve or brain stem. Furthermore, other substances including opioids and dopamine in the arcuate nucleus of the hypothalamus act as neurotransmitters and affect the GnRH neuron cell body either directly or via a synapse. Some of these substances include molecules that are associated with appetite, sleep, and emotion, and it is postulated that these substances are involved in the onset of menstrual disorders that are observed under extreme stress. In addition, prolactin secreted from the anterior pituitary gland increases the dopamine neuron activity via a short feedback loop and subsequently suppresses GnRH secretion. This signifies that under hyperprolactinemic conditions, GnRH secretion decreases, triggering the onset of hypothalamic–pituitary dysfunction.

c. GnRH secretion regulated by kisspeptin

There is growing interest in the newly discovered molecule kisspeptin, which is a neuropeptide involved in the ovarian cycle. The rhythmic secretion or surge of GnRH is thought to occur due to the positive and negative feedback of

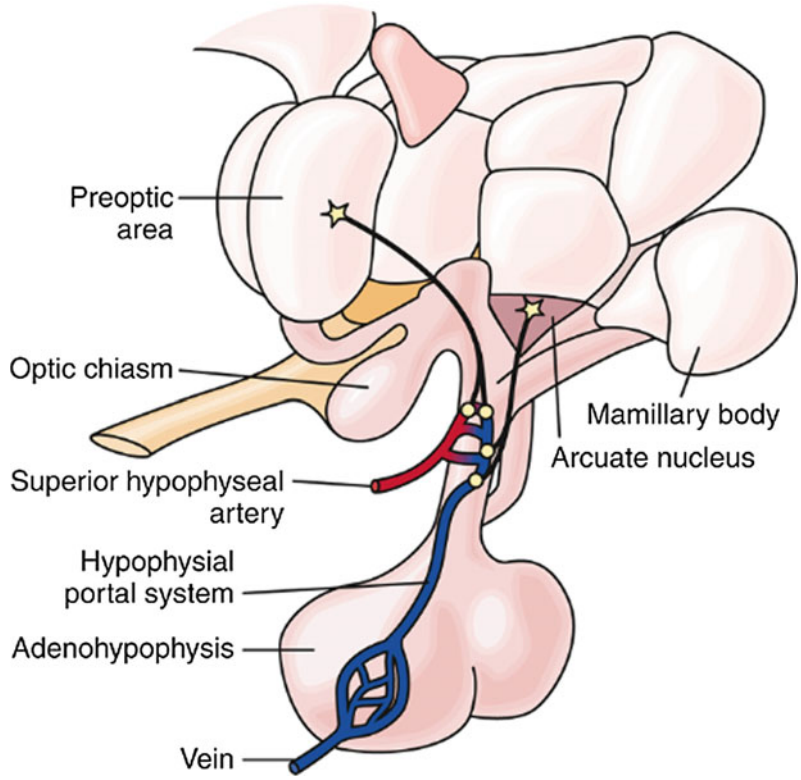

Fig. 1.2 Anatomical relationship between hypothalamic GnRH neurons and their target cell populations in the adenohypophysis (anterior pituitary). GnRH neuron cell bodies are located in the preoptic area and the mediobasal hypothalamus. GnRH axonal projections terminate at the median eminence, where GnRH is secreted into the hypophyseal portal system. Reproduction with the permission [1]

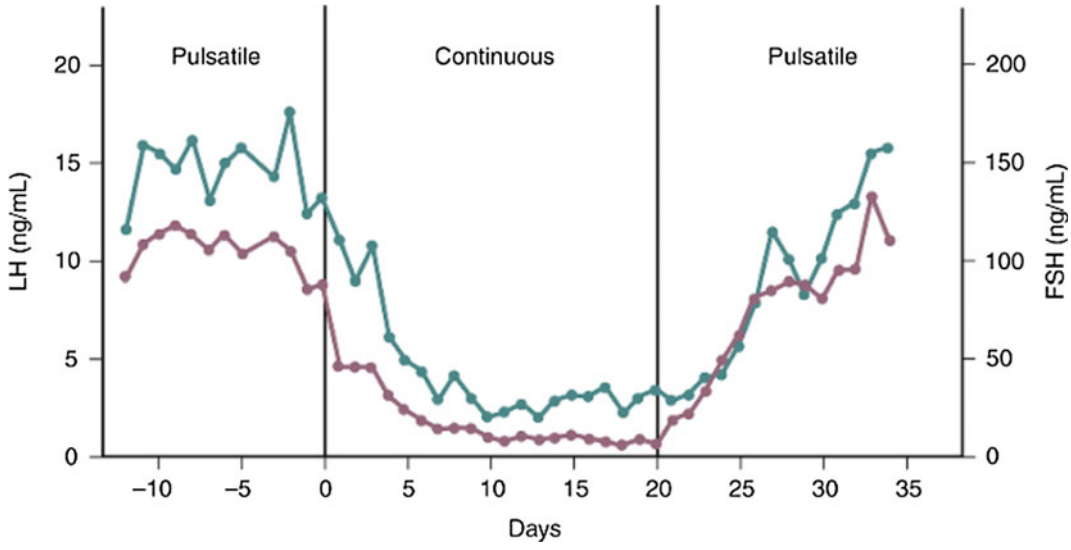

Fig. 1.3 The influence of pulsatile versus continuous GnRH administration to GnRH-deficient monkeys. Intermittent exogenous GnRH administration reconstitutes normal gonadotropin secretion. However, continuous GnRH infusion leads to a marked reduction (downregulation) in luteinizing hormone (*green*) and follicle-stimulating hormone (*purple*) concentrations. Resumption of pulsatile GnRH administration restores LH and FSH secretions. Reproduction with the permission [1]

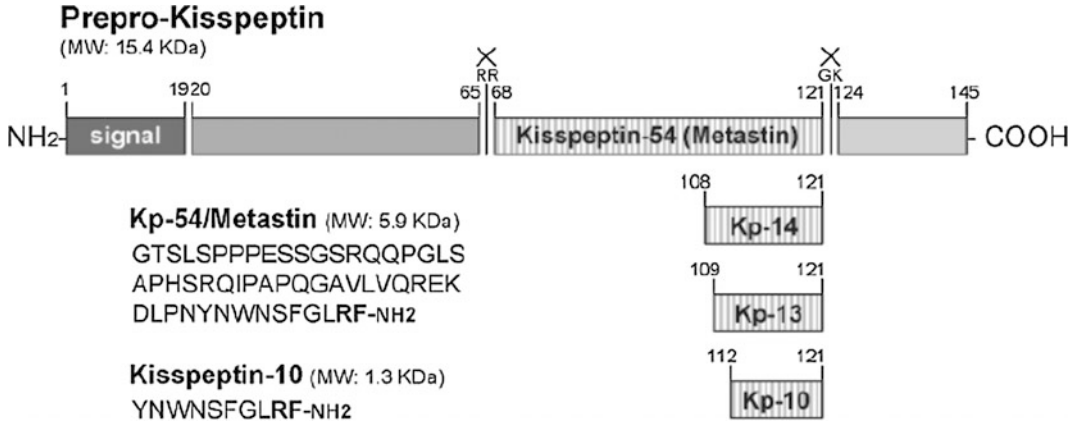

Fig. 1.4 Structural features of human kisspeptins generated by cleavage form a common precursor, the prepro-kisspeptin. Prepro-kisspeptin, encoded by the *KiSS-1* gene, is a 145-amino acid protein that contains a 19-amino acid signal peptide and central 54-amino acid region, flanked by two consensus cleavage sites (denoted by *X*), which gives rise to metastin or kisspeptin-54. Further cleavage of metastin generates kisspeptins of lower molecular weight: kisspeptin-14 (Kp-14), Kp-13, and Kp-10. All kisspeptins are able to bind and activate GPR54. Besides general structural organization, the complete amino acid sequences of human metastin and kisspeptin-10 are shown. The consensus C-terminal RF-amide motif, hallmark of this peptide superfamily, is indicated in bold (with the permission reproduced from [3])

estrogen; however, estrogen receptors are virtually nonexistent on GnRH neurons, and the specific mechanism of GnRH secretion control has been a mystery for a long time. As a molecule that may underlie the details of such a mechanism, kisspeptin has recently become a molecule of interest. Moreover, because the biological neural network that produces kisspeptin also produces neurokinin B as well as opioid dynorphin, this network is currently called the kisspeptin–neurokinin B–dynorphin (KNDy) network [2].

Kisspeptin is a peptide encoded by *Kiss1*, and its human version is composed of 54 amino acids (Fig. 1.4) [3]. The receptor for this peptide is GPR54, an orphan G-protein-coupled receptor. Based on structural similarities, these were globally termed kisspeptins, as they are derived from differential proteolytic processing of a common precursor. In humans, the KiSS-1 precursor contains 145 amino acids, with a putative 19-amino acid signal sequence, two potential dibasic cleavage sites (at amino acids 57 and 67), and one putative site for terminal cleavage and amidation [3]. Kisspeptin was originally discovered in 1996 as a suppressor of metastasis of human malignant melanoma [4]. The peptide is named after the famous Kisses chocolate as it was discovered in Hershey, Pennsylvania, and the "SS" portion also means "suppressor sequence" [5]. In addition, it is sometimes called "metastin" due to its characteristic of suppressing cancer metastasis [6].

GnRH neurons extend from the preoptic area to the infundibular nucleus (homologous to the arcuate nucleus in other species) of the hypothalamus in humans, whereas in rodents, GnRH neurons reside predominantly in the preoptic area [5]. Kisspeptin neurons are localized in the anteroventral periventricular nucleus and arcuate nucleus in the preoptic area of the hypothalamus in rodents such as rats (Fig. 1.5) [5]. Similarly, kisspeptin neurons are located in the rostral preoptic area and the infundibular nucleus in the human hypothalamus [7]. In humans, the majority of kisspeptin cell bodies are found in the infundibular nucleus, but a second dense population of kisspeptin cells has been identified in the rostral preoptic area [7]. Although kisspeptin neurons are located in the

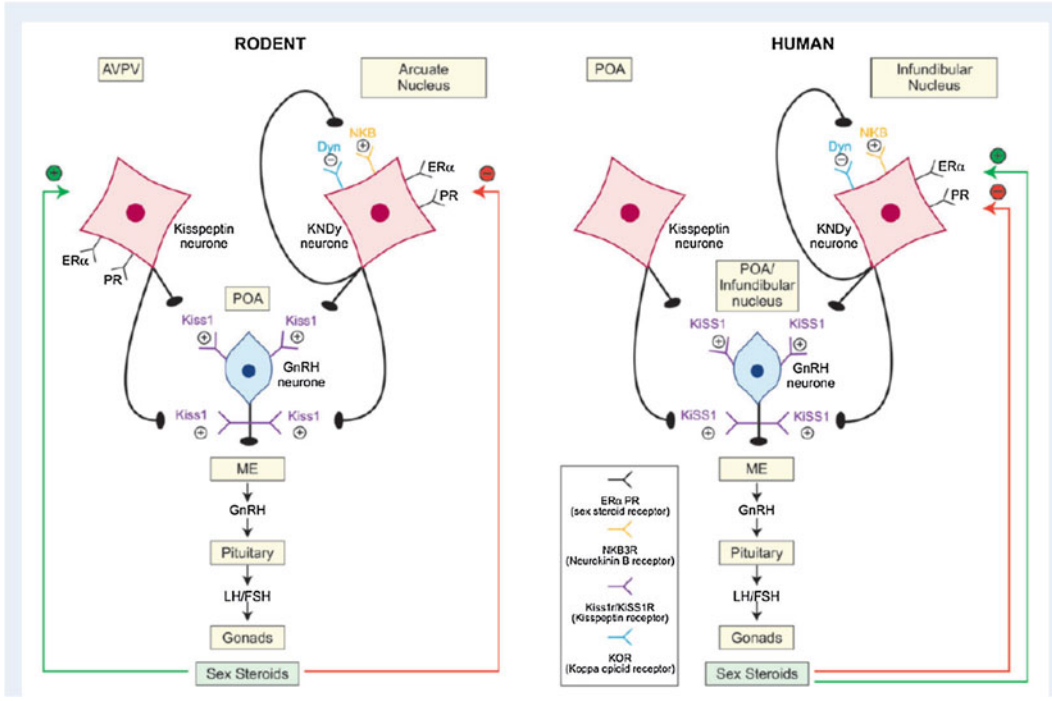

Fig. 1.5 Schematic diagram showing the neuroanatomy of the kisspeptin–GnRH pathway and the relationship between KNDy neurons and GnRH neurons in humans and rodents. Kisspeptin signals directly to the GnRH neurons, which express kisspeptin receptor. The location of kisspeptin neurone populations within the hypothalamus is species specific, residing within the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus in rodents, and within the preoptic area (POA) and the infundibular nucleus in humans. Kisspeptin neurons in the infundibular (humans)/arcuate (rodents) nucleus coexpress neurokinin B and dynorphin (KNDy neurons), which via neurokinin B receptor and kappa opioid peptide receptor autosynaptically regulate pulsatile kisspeptin secretion, with neurokinin B being stimulatory and dynorphin inhibitory. Negative (*red*) and positive (*green*) sex steroid feedback is mediated via distinct kisspeptin populations in rodents, via the AVPV and the arcuate nucleus, respectively. In humans, KNDy neurons in the infundibular nucleus relay both negative (*red*) and positive (*green*) feedback. The role of the POA kisspeptin population in mediating sex steroid feedback in humans is incompletely explored. ME, median eminence; +, stimulatory; −, inhibitory; ERα, estrogen receptor alpha; PR, progesterone receptor; Kiss I/kiSS I, kisspeptin; NKB, neurokinin B; and Dyn, dynorphin (with the permission reproduced from [5])

infundibular/arcuate nucleus across all species including humans, the rostral population is species specific [5, 7]. In rodents, the rostral population is located in the anteroventral periventricular nucleus, the periventricular nucleus, and the continuum of this region, which is known as the rostral periventricular region of the third ventricle (Fig. 1.5) [5].

Kisspeptin stimulates the secretion of both LH and FSH in humans [8]. Kisspeptin 54 has an immediate and dose-dependent effect with a half-life of 26.6 min; in contrast, kisspeptin 10 has an extremely short half-life of 4 min [9]. The effects of kisspeptin differ depending on the type of exposure, route of administration, gender, and isoform [5, 8, 9]. Some studies suggest that kisspeptin directly stimulates pituitary gonadotrophs to release LH and FSH, based on the expression of *Kiss1* and *Kiss1r* in gonadotrophs and the secretion of gonadotropins from pituitary explants treated with kisspeptin [5, 10]. Because GnRH secretion is pulsatile, the effect of kisspeptin on the characteristics of that pulsatility (as reflected in LH pulses) has been investigated. Intravenous infusion of kisspeptin 54 [subcutaneous bolus 0.3 nmol/kg (1.76 mg/kg) and

0.6 nmol/kg (3.5 mg/kg)] in healthy women increases the LH pulse frequency and amplitude [11].

Kisspeptin in the infundibular nucleus mediates negative feedback of estrogen in humans (Fig. 1.5). In postmenopausal women, kisspeptin neurons in the infundibular nucleus become hypertrophied and express more KISS1 mRNA than in premenopausal women [12]. These hypertrophied neurons express both ESR1 (encoding estrogen receptor alpha) and neurokinin B mRNA, show increased expression of neurokinin B, and show a similar distribution as that of kisspeptin neurons [13]. Kisspeptin and neurokinin B in the infundibular nucleus may act synergistically to mediate estrogen-negative feedback [5]. Estrogen may mediate negative feedback by suppressing kisspeptin and neurokinin B release from KNDy neurons, which reduces their stimulatory input to GnRH neurons (Fig. 1.5) [5].

In addition, kisspeptin may mediate estrogenic positive feedback. Estrogen feedback switches from negative to positive in the late follicular phase to induce the GnRH/LH surge at the time of ovulation. However, the neuroendocrine mechanisms involved in this critical physiological event are unclear. Emerging data suggest that although the negative feedback of sex steroids is mediated by KNDy neurons in the infundibular/arcuate nucleus, the positive feedback of sex steroids is more site specific and species specific (Fig. 1.5) [5]. The expression of *Kiss1* mRNA in the anteroventral periventricular nucleus is dramatically increased after estrogen replacement and at the time of the GnRH/LH surge [14]. KNDy neurons may play a role in positive estrogen feedback [5]. Furthermore, kisspeptin neurons in the anteroventral periventricular nucleus are activated during ovulation. It has also been shown that the expression of kisspeptin in the arcuate nucleus increases with the removal of the ovaries and decreases with the presence of estrogen. Based on these findings, the high concentration of estrogen secreted by mature follicles acts on kisspeptin neurons in the anteroventral periventricular nucleus, and the activated kisspeptin neurons influence the preoptic area and stimulate GnRH neuron cell bodies. Through these mechanisms, the positive feedback loop of estrogen is formed, evoking the LH surge after the GnRH surge [5].

Gonadotropins

Gonadotropin is a collective term for FSH and LH that are secreted from the anterior pituitary gland. These hormones promote follicular development in the ovary, elicit ovulation of a mature egg through the LH surge, and induce follicle luteinization after ovulation, indicating that they play many different roles in the body. Gonadotropins bind to FSH receptors and LH receptors that are present in the theca cells and granulosa cells of the ovary, and they regulate steroid production in the ovary through this mechanism. In addition, steroid hormones and inhibin secreted from the ovary through these stimuli affect the central nervous system, thereby influencing gonadotropin secretion. As described here, the interaction of the hypothalamus–pituitary–ovary axis is essential in the regulation of ovarian function, and in particular, gonadotropins and their receptors play a pivotal role in this pathway.

a. Construction of Gonadotropins

Gonadotropin is a glycoprotein hormone that is synthesized and secreted by the anterior pituitary gland and is used as a general term for FSH and LH. These hormones are heterodimers formed by a covalently bound α-chain and β-chain. The α-chain is common between the hormones, and the same α-chain is also found in non-gonadotropin molecules such as thyroid-stimulating hormone and human chorionic gonadotropin. This indicates that the β-chain determines the function of each hormone (Fig. 1.6) [15].

b. Gonadotropin receptors

The cDNA sequence of the gonadotropin receptor, specifically the LH receptor, was first

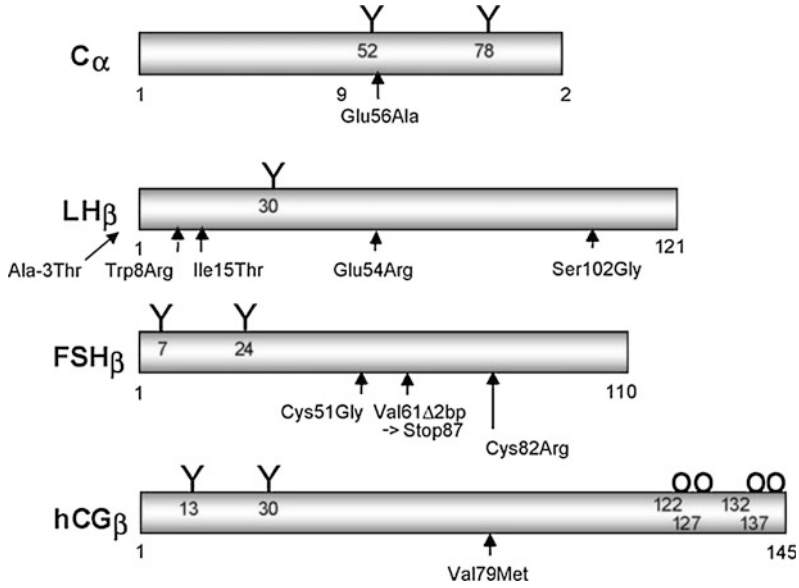

Fig. 1.6 Schematic presentation of sizes, locations of the carbohydrate side chains, and currently known mutations and polymorphisms in the gonadotropin subunits (i.e., common α-subunit [Ca], LHβ, FSHβ, and hCGβ). The numbers below the right ends of the *bars* indicate the number of amino acids in the mature subunit proteins. Symbols "Y" and "O" indicate the locations of N-linked and O-linked carbohydrate side chains, respectively. The *arrows* below the *bars* indicate the locations of point mutations and polymorphisms (with the permission reproduced from [15])

identified in rats and pigs in 1989 [16]. Subsequently, the cDNA sequences of gonadotropin receptors from various species including humans were determined [17]. Both FSH and LH receptors are G-protein-coupled receptors and form a subgroup together with thyroid-stimulating hormone receptors [15].

The FSH receptor consists of 17 single peptides and 678 amino acids, and its molecular weight is predicted to be about 75,500. The human FSH receptor gene is located on the short arm of chromosome 2 (2p21). The LH receptor is composed of 26 single peptides and 673 amino acids, and its molecular weight is predicted to be approximately 75,000. However, with posttranslational modifications with sugar residues, the actual molecular weight is considered to be about 85,000–92,000. The human LH receptor gene is located on the short arm of chromosome 2 (2p21), similar to the FSH receptor [15].

It is generally considered that gonadotropin receptors are activated in the following manner: The long extracellular N-terminal domain recognizes the β-chain of gonadotropins, and the seven-pass transmembrane domain subsequently forms a ringlike pocket where the α-chain of the gonadotropins binds, which in turn activates the receptor. When the receptor becomes activated in this manner, the intracellular domain binds to a G-protein, which subsequently becomes activated. As a result, adenylate cyclase, a target enzyme of G-proteins, becomes activated, and cAMP is synthesized intracellularly. These cAMP molecules are thought to act as intracellular second messengers in intracellular signal transduction and subsequently activate protein kinase A and affect gene transcription regulation [18].

In addition, gonadotropin receptors are involved in cell proliferation via Ras-mediated activation of MAP (mitogen-activated protein) kinase [19]. Furthermore, gonadotropic receptor-mediated activation of the inositol triphosphate pathway leads to an elevation in the intracellular Ca^{2+} concentration, suggesting that they may also play a role in the activation of protein kinase

C [20]. It is generally considered that cAMP and Ca^{2+} do not act independently, but rather in concert with each other in the G-protein-coupled receptor signaling cascade, and this is also considered to be true of gonadotropin receptors.

c. **Localization of gonadotropin receptors**

In the ovary, FSH receptors are present only on granulosa cells and are absent on theca cells. In human ovaries, the expression of FSH receptors increases in the early and middle stages of the follicular phase but rapidly decreases after ovulation. In other words, FSH receptor expression declines with the progression of luteinization of granulosa cells that occurs after the LH surge and consequent ovulation. In contrast, LH receptors are expressed in theca cells, and their expression is upregulated with follicular development. Furthermore, due to FSH stimulation, the expression of LH receptors is also upregulated in granulosa cells of mature follicles immediately before ovulation [15].

These gonadotropin receptors are thought to function by influencing each other, and this phenomenon is classically known as the "two-cell theory" (Fig. 1.7). In the early stages of steroid hormone production in the ovary, androgen synthesis increases within the theca cells due to LH stimulation of these cells. These androgen molecules are then transported to the granulosa cells and are synthesized into estrogen by aromatase actions. The activity of this aromatase is augmented by FSH stimulation. The synthesized estrogen molecules act together with FSH and further enhance the efficiency of FSH and LH stimulation to augment the expression of FSH and LH receptors.

d. **Regulation of gonadotropin secretion (effects of estrogen on the central nervous system)**

LH is secreted in two different ways, basic secretion (pulse secretion) and ovulatory secretion (surge secretion), and GnRH released by the hypothalamus controls such secretion through the central nervous system. GnRH is also thought to be secreted via two different methods, pulse and surge, and the aforementioned kisspeptin has also been indicated to control GnRH through the central nervous system.

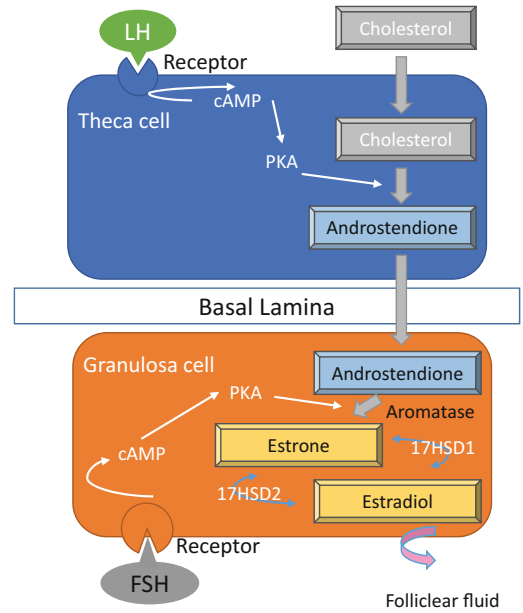

Fig. 1.7 The schema of "two-cell theory." Androgen synthesis increases within the theca cells due to LH stimulation. These androgen molecules are then transported to the granulosa cells and are synthesized into estrogen by aromatase actions. The activity of this aromatase is augmented by FSH stimulation. The synthesized estrogen molecules act together with FSH and further enhance the efficiency of FSH and LH stimulation to augment the expression of FSH and LH receptors

Steroid Hormones

Steroid hormones are a type of steroids with a steroid nucleus structure (Fig. 1.8) and are typically synthesized from cholesterol in the adrenal glands and gonads [21]. These hormones are broadly classified into the following five types depending on the specific receptors to which they bind. From the perspective of the synthetic mechanisms of steroid hormone metabolism, they are precursors, intermediate products, or metabolites of each other.

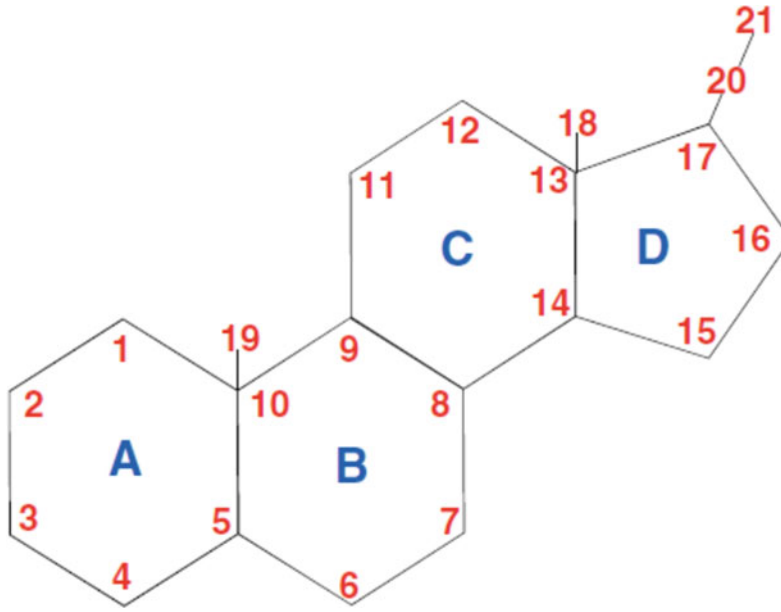

Fig. 1.8 Basic steroid structure showing a fully saturated 21 carbon steroid with the alphabetical naming of the individual rings and the numbering sequence of the carbon atoms. All steroids share the same basic 17 carbon structure with the presence of four linked rings (three six sided and one five sided) known as the cyclopentanophenanthrene (or cyclopentanoperhydrophenanthrene) ring. The rings are alphabetically labeled with the carbon atoms which are numbered sequentially. Cholesterol is recognized as the parent steroid and contains 27 carbon atoms, whereas the three main groups of steroids of interest in clinical endocrinology consist of 18, 19, or 21 carbon atoms, representing the estrange, androstane, and pregnane skeleton (with the permission reproduced from [21])

a. Progestogen (gestagens);
b. Androgens (androgenic hormones);
c. Estrogens;
d. Glucocorticoids; and
e. Mineralocorticoids.

Of these, a–c are sex steroid hormones, and d and e are called corticoids. Mineralocorticoids are a collective term for molecules with aldosterone-like actions that regulate osmotic pressure, predominantly by affecting the salt concentration balance. Glucocorticoids play a role in glucose metabolism. Unlike proteins, steroids are fat soluble and can diffuse both intra- and extracellularly. For this reason, unlike peptide hormones that transmit signals via cell-surface receptors, steroid hormones can bind directly to receptors that are expressed intracellularly.

In addition, each of the steroid hormones undergoes various modifications, such as hydroxylation, sulfation, methoxylation, and glucuronidation, thereby becoming metabolized into a low-activity state and eliminated into the bile or urine. Furthermore, the actions and activities of steroid hormones differ greatly from molecule to molecule, even if the differences in side-chain modifications are minute. Figure 1.9 shows the structures and metabolic pathways of steroids [21]. In addition, each sex steroid hormone is described.

Sex Steroid Hormones

a. **Progestogens**

This class of hormones is composed of a basic structure of 21 carbons called the "pregnane backbone" (C21 pregnane) and is produced by a variety of organs including the ovary (primarily the corpus luteum), placenta, adrenal cortex, and testis. Progestogens, as the name suggests, play an important role in the maintenance of pregnancy. Their levels

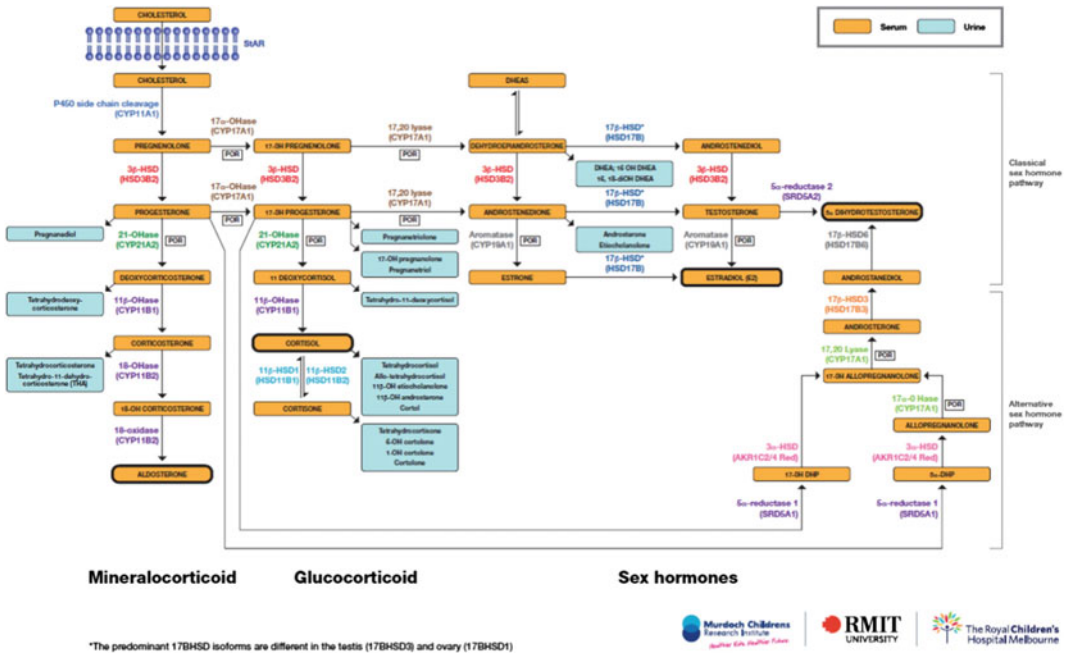

Fig. 1.9 The structures and metabolic pathways of steroids. Mineralocorticoids are typified by aldosterone that regulates osmotic pressure. Glucocorticoids play a role in glucose metabolism. In addition, each of the steroid hormones undergoes various modifications, such as hydroxylation, sulfation, methoxylation, and glucuronidation. The actions and activities of steroid hormones differ greatly. And also, contrary to adrenal cortex, androgens are converted to estrogens by aromatase on the ovary. (with the permission reproduced from [21])

significantly fluctuate within the menstrual cycle during the non-pregnant state. Progestogens are present in men at a level similar to those in women during the follicular phase. Progestogens are the most upstream molecules of the steroid metabolic pathway, and thus, they are considered to be precursors of all steroid hormones. In the first step of steroid metabolism, pregnenolone is synthesized from cholesterol. Progestogens are primarily metabolized by the liver in the form of pregnanediol and eliminated in urine. Therefore, the urine pregnanediol concentration reflects the function of progestogen-producing organs. Representative progestogens are shown below.

i. ***Progesterone* (*Prog: P4*)**

Molecular formula (MF): $C_{21}H_{30}O_2$, molecular weight (MW): 314.46, biological half-life (t1/2): 34.8–55.13 h

This hormone is produced primarily by the corpus luteum of the ovary, the adrenal gland, and the placenta and is also secreted by adipose tissue. The blood concentration of progesterone changes during the menstrual cycle. Although progesterone levels are low from the follicular phase to the ovulation phase, they rapidly increase during the luteal phase due to secretion from luteinized granulosa cells. Subsequently, with luteal regression, progesterone levels decrease. When pregnancy is established, the placenta begins to produce progesterone, contributing to the maintenance of pregnancy.

ii. ***Pregnenolone* (*Preg: P5*)**

MF: $C_{21}H_{32}O_2$, MW: 316.483

Pregnenolone is the furthest upstream in the steroid hormone metabolic pathway and is a precursor of all steroid hormones. Pregnenolone is synthesized in the mitochondria of adrenal glands, testes, ovarian theca cells, and the placenta via side-chain cleavage of cholesterol.

iii. ***17α-hydroxyprogesterone* (*17-OH progesterone: 17P4 or 17-OHP*)**

MF: $C_{21}H_{30}O_3$, MW: 330.46

Although this hormone is synthesized primarily in the adrenal glands, it is also produced by the corpus luteum. Its blood concentration during pregnancy is 10–1000 times greater than the P4 concentration during the normal menstrual cycle. Measurement of 17-OHP is important for evaluating the state of luteal function during pregnancy.

iv. ***17α-hydroxypregnenolone* (*17-OH pregnenolone: 17P5 or 17-OHP*)**

MF: $C_{21}H_{32}O_3$, MW: 332.48

This hormone is produced in the adrenal glands and gonads. Measurement of 17-OHP is useful for diagnosing congenital adrenocortical hyperplasia, which is caused by mutations in steroid hormone conversion enzymes such as HSD3β2 and CYP17A1.

b. **Androgens**

The structure of androgens is an androstane backbone consisting of 19 carbons (C19 androstane). Androgens are primarily produced in the testis, ovary, and adrenal cortex, and their synthesizing enzymes are found in the smooth endoplasmic reticulum. Androgens are metabolized predominantly by the liver. Their physiological roles include proliferation of cells in the prostate gland, seminal vesicle, and epididymis, promotion of spermatogenesis in the seminiferous tubules, promotion of renal tubule function in the kidneys, increase in the glomerular filtration rate, promotion of sebum sebaceous matter secretion from the sebaceous glands, proliferation of muscle and bone cells, suppression of LH secretion from the anterior lobe of the hypophysis, and suppression of GnRH secretion from the hypothalamus.

i. ***Testosterone***

MF: $C_{19}H_{28}O_2$, MW: 288.42, t1/2: 2–4 h

Testosterone is predominantly produced in the testicles of men during puberty and later. Its blood concentration ranges from 3–13 ng/ml, although this level decreases slightly with age. It is also produced by the ovaries in women, although its blood concentration is 0.2–1 ng/ml, which is lower than in men. In addition, the adrenal glands produce a small amount of testosterone. The majority of testosterone molecules are bound to globulin and albumin in the blood. Unbound active testosterone accounts for only 1–2% of the overall amount.

ii. ***Dehydroepiandrosterone* (*DHEA*)**

MF: $C_{19}H_{28}O_2$, MW: 288.424, t1/2: 12 h

Mainly produced in the adrenal glands, DHEA is the most abundant steroid hormone and has the highest blood concentration of all the steroid hormones in humans. Synthetic levels of DHEA peak in the early 20 s and decrease with age. DHEA possesses weak androgenic properties, comprising 3–34% of the activity of testosterone.

iii. ***Androstenedione* (*andro: A4: AE*)**

MF: $C_{19}H_{26}O_2$, MW: 286.4

Androstenedione is produced in the testis, ovary, and adrenal cortex. It possesses weak androgenic properties that account for 20–40% of the activity of testosterone. In premenopausal women, a total of approximately 3 mg/day of androstenedione is synthesized in the adrenal glands and ovaries in nearly equivalent amounts. For this reason, androstenedione levels are reduced by half after menopause. This hormone is also used as a supplement in steroid replacement therapy.

iv. ***5α-dihydrotestosterone* (*DHT: 5α-DHT*)**

MF: $C_{19}H_{30}O_2$, MW: 290.42

Approximately 7% of testosterone is converted to this hormone in the testis, adrenal cortex, and hair root. Because estrogen cannot be directly synthesized from 5α-DHT, it is frequently used in experiments involving the androgen receptor. This hormone is catabolized in the body to 3α- and 3β-androstanediol. It

exhibits the strongest androgenic properties of all the androgens, approximately 2.5 times greater than that of testosterone.

c. Estrogens

Estrogens are steroid hormones that possess an estrane backbone consisting of 18 carbons (C18 estrane). Estrogens play a key role in female reproduction in all vertebrate animals. Estrogen synthesis occurs irreversibly by aromatase using androgens as substrates. Estrogen is predominantly produced in developing follicles, the corpus luteum, and the placenta. Synthesis and secretion are promoted by gonadotropins, which are released from the anterior lobe of the hypophysis. In women, blood estrogen levels fluctuate throughout the menstrual cycle, and similar to progesterone, estrogen levels increase with the number of gestational weeks. Furthermore, aromatase is present in adipocytes, the liver, adrenal glands, testes, mammary glands, and brain, and these cells and tissues also produce small amounts of estrogen. Therefore, although no periodicity in estrogen production occurs, estradiol levels of about 100 pM (30 ng/L) are maintained in both postmenopausal women and men. In addition, when estrogen is supplemented orally, most of it is rapidly degraded by the liver through the portal vein.

Specific actions of estrogens include the promotion of secondary sex characteristics, maintenance of germ cells, and especially, the development and maturation of the female reproductive organs. Estrogens also affect the bones, liver, and brain and promote feminization. Primary examples of estrogens are indicated below.

i. *Estrone* (*oestrone: E1*)

MF: $C_{18}H_{22}O_2$, MW: 270.366, t1/2: 19 h

Estrone is synthesized irreversibly from androstenedione and reversibly from estradiol and also exists in a sulfation state, making it less vulnerable to metabolism. Estrone sulfate, estrone, and estradiol can easily be converted to each other in the body, and these characteristics are thought to be essential for the regulation of estrogenic activities. In addition, estrone sulfate is the primary component of estrogens used in hormone replacement therapy; its administration is conducted with the assumption that it will be converted to estradiol. Similar to estradiol, blood estrone levels fluctuate depending on the menstrual cycle. Moreover, blood estrone levels during pregnancy also gradually increase with increasing gestational weeks. The estrogenic activities of estrones are weak and exhibit 12.5% of the estrogenic activities of estradiol in rats.

ii. *Estradiol* (*17s-estradiol: oestradiol: E2*)

MF: $C_{18}H_{24}O_2$, MW: 272.38, t1/2: $\sim$13 h

Estradiol is irreversibly synthesized in the granulosa cells of the ovary, adrenal cortex, and testis by the aromatization of testosterone and from estrone. Blood estradiol levels change throughout the menstrual cycle, peaking before the ovulation phase, and also increase during pregnancy. Of the molecules synthesized in the body, estradiol has the greatest estrogenic activity. However, because it is degraded relatively quickly, estradiol is also administered in the form of estrone.

iii. *Estriol* (*oestriol: E3*)

MF: $C_{18}H_{24}O_3$, MW: 288.38

Pregnenolone, which transfers from the mother to the fetus via the placenta, is sulfated in the fetal adrenal glands, hydroxylated in the fetal liver, and finally aromatized upon return to the placenta, thereby completing estriol synthesis. This series of reactions is essential for the development of the fetal liver and placenta. The pregnenolone level rapidly begins to increase, later than other estrogens, at 12 weeks of gestation. Additionally, because E3 and its metabolites are abundantly present in the urine of pregnant women, it is utilized as an index of fetal development. Normally, estriol levels are very low in both men and women. The activity of

estriol is extremely weak and comprises about 1% of the activity of estradiol.

Key Molecules in the Biosynthesis and Catabolism of Steroid Hormones

The important molecules involved in the synthesis and metabolic regulation of steroid hormones are listed below [1] and summarized in Table 1.1 [21].

Steroidogenic Acute Regulatory Protein (StAR/StARD1; encoded by *STARD1*) [1]

Human STARD1 is a 285-amino acid protein and is the primary regulator of steroidogenesis in gonads and the adrenal gland. The critical step in steroidogenesis is the transfer of cholesterol from outer to inner mitochondrial membranes [22]. This transfer is enhanced by STARD1, which has

Table 1.1 The important molecules involved in the synthesis and metabolic regulation of steroid hormones, reproduction with the permission [21]

Enzyme	Gene	Chromosome locus	Tissue/organs of expression	Major function	Role in human steroidogenesis
P450 scc	CYP11A1	15q23-q24	All layers of adrenal cortex, Leydig cells, theca cells, brain	22-hydroxylation 20-hydroxylation 20,22-desmolase	Converts cholesterol to pregnenolone
3β-HSD1	HSD3B1	1p13.1	Placenta, breast, liver, brain	3β-dehydrogenase Δ5-Δ4 isomerase	Perioheral conversion of Δ5 compounds to Δ4
3β-HSD2	HSD3B2	1p13.1	All layers of adrenal cortex, Leydig cells, theca cells	3β-dehydrogenase Δ5-Δ4 isomerase	Conversion of Δ5 compounds to Δ4 in adrenal and gonads
17-hydroxylase/ 17,20-lyase	CYP17A1	10q24.3	ZF, ZR, Leydig cells, theca cells, brain	17α-hydroxylase 17,20 lyase	Conversion of pregnenolone and progesterone to 17-hydroxylated products, conversion of 17-OH-Preg to DHEA and 17-OHP to androstenedione
P450-oxidoreductase	POR	7q11.2	Widely expressed in human tissues	Electron transfer	Electron donor for 17-hydroxylase, 21-hydroxylase and aromatase
21-hydroxylase (21α-hydroxylase)	CYP21A2	6p21.1	ZG, ZF	21-hydroxylation	Conversion of progesterone to DOC and 17-OHP to 11-deoxycortisol
11β-hydroxylase	CYP11B1	8q21-q22	ZF, to a lesser extent in ZR, brain	11β-hydroxylation	11-Deoxycortisol to cortisol, 11-DOC to corticosterone
Aldosterone synthase	CYP11B2	8q21-q22	ZG, brain	11β-hydroxylation 18-hydroxylase 18-oxidation	DOC to aldosterone in 3 reactions
17β-HSD1	HSD17B1	17q11-q21	Placenta, granulosa cells	17β-ketosteroid reductase	Oestrone to oestradiol
17β-HSD2	HSD17B2	16q24.1-q24.2	Endometrium, placenta, ovary	17β-hydroxysteroid dehydrogenase	Oestradiol to oestrone, testosterone to androstenedione, DHT to 5α-androstanediol

(continued)

Table 1.1 (continued)

Enzyme	Gene	Chromosome locus	Tissue/organs of expression	Major function	Role in human steroidogenesis
17β-HSD3	HSD17B3	9q22	Leydig cells	17β-ketosteroid reductase	Androstenedione to testosterone
17β-HSD5	HSD17B5 (AKR1C3)		ZR, fetal adrenal, liver, prostate	17β-ketosteroid reductase	Androstenedione to testosterone
17β-HSD6	HSD17B6	12q13.3	Prostate, probable role in alternative pathway	Dehydrogenase	Androstanediol to DHT
P450 aromatase	CYP19A1	15q21.1	Granulosa cells, placenta, fat, growing bones	Oxidative demethylation	Androstenedione and testosterone to oestradiol
5α-reductase1	SRD5A1	5p15	Scalp, peripheral tissues	5α-reduction	Metabolism of multiple steroids, peripheral conversion of testosterone to DHT
5α-reductase2	SRD5A2	2p23	Fetal genital skin, prostate	5α-reduction	Testosterone to DHT
Reductive 3α-HSDs	AKR1C 1,2,3,4	10p14-p15	Multiple tissues	3α-ketosteroid reductase 17β-ketosteroid reductase 20α-reduction of pregnanes	Inactivation of multiple steroids in liver, reduction of 5α-DHP to allopregnanolone in brain
11β-HSD1	HSD11B1	1q32-q41	Liver, testis, lung, fat, PCT	Reduction (in vivo)	Cortisone to cortisol
11β-HSD2	HSD11B2	16q22	Distal nephron, placenta	Dehydrogenase	Cortisol to cortisone

a short biological half-life. STARD1 is also a key player in substrate flux to the side-chain cleavage system. Because neither *STARD1* mRNA nor protein is expressed in the human placenta, trophoblast cholesterol side-chain cleavage is independent of STARD1. Mutation in the gene that encodes STARD1 causes a rare congenital autosomal recessive disease called lipoid adrenal hyperplasia, and this mutation accounts for at least 5% of congenital adrenal hyperplasia cases [23].

Other START domain proteins [1]

A family of proteins was identified that shares a domain that is similar to the C-terminus of STARD1. These proteins are called StAR-related lipid transfer (START) domain proteins [24]. The human placenta produces high levels of pregnenolone, and the absence of STARD1 expression in this organ demonstrates STARD1-independent steroidogenesis and suggests that another protein such as STARD3 may

perform the function of STARD1 in the placenta. Other START domain proteins (STARD4, STARD5) present in the cytoplasm may function as sterol carrier proteins to transport cholesterol to the mitochondria, although the details of their roles in sterol trafficking remain unknown.

The Cholesterol Side-Chain Cleavage Enzyme (P450scc Encoded by *CYP11A1*) [1]

The cleavage of cholesterol side chains is catalyzed by cytochrome P450scc and the associated electron transport system. This cleavage reaction occurs in three catalytic cycles: The first two cycles add hydroxyl groups to positions C22 and C20, and the third cycle cleaves the side chain between these carbons. Cholesterol binding to cytochrome P450scc increases its affinity for reduced ferredoxin, and this complex increases the shuttling of electrons to the substrate-bound enzyme.

CYP11A1 contains nine exons, similar to other mitochondrial steroidogenic P450 enzymes such as 11β-hydroxylase and aldosterone synthase, and is located on chromosome 15q23-q24. Mutations in *CYP11A1* significantly diminish cleavage of cholesterol side chains and are associated with adrenal insufficiency and XY sex reversal. The phenotypes of these diseases are similar to phenotypes seen in the presence of *STARD1* inactivating mutations [23, 25–27].

17α-Hydroxylase/17,20-Lyase (P450c17; *CYP17A1*) [1]

The endoplasmic reticulum enzyme P450c17 catalyzes two reactions: hydroxylation of pregnenolone and progesterone at C17 and conversion of pregnenolone into C19 steroids. The human enzyme also converts progesterone, but to a much lesser extent [23]. In addition, P450c17 catalyzes 16α-hydroxylation of progesterone and dehydroepiandrosterone. 17α-hydroxylation requires one pair of electrons and molecular oxygen, and the lyase reaction requires another pair of electrons and molecular oxygen. The reducing equivalents are transferred from NADPH to the heme iron of P450c17 by NADPH cytochrome P450 reductase (POR). POR-deficient individuals have a type of autosomal recessive congenital adrenal hyperplasia, demonstrating the importance of POR in steroid metabolism catalyzed by cytochrome P450 enzymes located in the endoplasmic reticulum [28]. The steroid profile in such individuals suggests a deficiency in both 21-hydroxylase and 17-hydroxylase/17–20 lyase. A range of phenotypes is observed, including adrenal insufficiency, ambiguous genitalia, and Antley–Bixler skeletal malformation syndrome.

CYP17A1 is located on band 10q24.3 and includes eight exons. Mutations in *CYP17A1* produce combined or individual states of deficiency for P450c17 activities [23, 28]. Individuals with combined deficiency show reduced production of C19 and C18 steroids, low levels of cortisol resulting in elevated ACTH (Adrenocorticotropic hormone) secretion, and abnormally high levels of steroids upstream of the P40c17 reaction. Hypertension due to sodium retention and hypokalemia results from elevated levels of 11-deoxycorticosterone. The absence of sex steroid hormones prevents adrenarche and puberty in females and leads to incomplete or no development of male genitalia (46, XY DSD).

Aromatase (P450aro; *CYP19A1*) [1]

Aromatase is also an endoplasmic reticulum enzyme that uses three molecules each of NADPH and molecular oxygen to catalyze three sequential hydroxylation reactions at C19 to produce one molecule of C18 steroid with a phenolic A ring [23, 29]. This reaction occurs at a single active site on the enzyme, and the reducing equivalents are transferred by POR to P450aro.

Aromatase is encoded by *CYP19A1*, which is a large gene located on band 15q21.1. Different promoters produce cell-specific transcripts [23, 30]. The promoter that directs transcription of aromatase in the ovary is adjacent to the exon that encodes the translation start site (promoter IIa). In granulosa cells, FSH stimulates transcription of

genes that encode aromatase and POR, which provides its reducing equivalents.

Several cases of aromatase deficiency have been reported [23, 31–33]. Pregnancies with aromatase deficiency of the fetus show low estrogen excretion in the maternal urine, maternal virilization, and ambiguous genitalia or female pseudohermaphroditism (46, XX DSD) in affected female fetuses. This maternal and fetal virilization in the absence of aromatase activity in the placenta shows the importance of this structure for converting maternal and fetal androgens into estrogens.

An 87-bp insertion in the splice junction between exon 6 and intron 6 of *CYP19A1* causes addition of 29 in-frame amino acid residues. This mutant protein has less than 3% of the normal aromatase activity. The expression of mutant cDNA confirmed that this protein has extremely low aromatase activity. Most other mutations are missense or nonsense mutations in exons 4, 9, and 10. In patients with aromatase deficiency, compound heterozygous mutations in coding sequences produce proteins with very low activity. Aromatase activity in placentas from fetuses with *CYP19A1* mutations is reduced to 21% of normal values.

Families with autosomal dominant over-expression of aromatase show excess estrogen [34–36], leading to severe prepubertal gynecomastia in males and macromastia and premature puberty in females. In some families, aromatase over-expression is due to heterozygous genomic rearrangements.

11β-Hydroxylases (P450c11β and P450c11AS; *CYP11B1* and *CYP11B2*) [1]

Two genes located on band 8q24.3 encode related mitochondrial enzymes that are involved in 11β-hydroxylation and aldosterone synthesis. These enzymes are P45011β, which is encoded by *CYP11B1*, and P450c11AS (also called "P450aldo," "P450c18," or "P450cmo"), which is encoded by *CYP11B2*, respectively [23]. Both enzymes have 11β-hydroxylase activity, but P450c11AS can also catalyze two oxygenation steps at C18 that are required for aldosterone synthesis. Transcription of *CYP11B1* is induced by cAMP signaling pathways that are induced by ACTH. P45011β is expressed in the zonae fasciculata and reticularis of the adrenal cortex. On the other hand, *CYP11B2* is only expressed in the zona glomerulosa. Angiotensin II induces protein kinase C signaling pathways, leading to the transcription of *CYP11B2*.

Mutations in *CYP11B1* produce deficiency in 11β-hydroxylase, whereas mutations in *CYP11B2* produce deficiencies in 18-hydroxylase or corticosterone methyl oxidase I and 18-oxidase or corticosterone methyl oxidase II [23, 37]. Insufficient 11β-hydroxylase activity is characterized by high levels of 11-deoxycortisol and deoxycorticosterone, which lead to salt retention and hypertension. Affected females are virilized due to abnormally high production of adrenal androgens derived from elevated ACTH levels. *CYP11B1* mutations that cause deficiency in 11β-hydroxylase include non-synonymous amino acid substitutions and a premature stop codon. Deficiency in corticosterone methyl oxidase I is due to the complete absence of P450c11AS activity. In such cases, no aldosterone synthesis occurs, but normal production of corticosterone and cortisol remains.

21-Hydroxylase (P450c21; *CYP21A2*) [1]

The endoplasmic reticulum enzyme P450c21 is expressed in the adrenal gland and catalyzes 21-hydroxylation of progesterone and 17α-hydroxyprogesterone in the mineralocorticoid and glucocorticoid biosynthesis pathway [23, 37–39]. One mole of molecular oxygen and reducing equivalents (from NADPH through POR) are required for hydroxylation of C21. Mutations in which POR is inactivated produce a partial deficiency in 21-hydroxylase activity and in 17α-hydroxylase/17–20 lyase activity [23, 40]. ACTH is the main regulator of CYP21A2 expression in the zona fasciculata via cAMP-mediated signal transduction.

CYP21A2 is located within the human leukocyte antigen region on band 6p21.1. 21-hydroxylase deficiency is one of the most common (1:10,000 to 1:15,000 births) autosomal recessive metabolic diseases because of frequent unequal crossover events and gene conversions [38]. When deletions and/or gene conversions are extensive, the adjacent gene encoding tenascin-X may be affected. When this happens and both alleles are mutated, a form of Ehlers–Danlos syndrome results [39]. The symptoms of congenital adrenal hyperplasia due to 21-hydroxylase deficiency are caused by deficits in cortisol (absence of conversion of 17α-hydroxyprogesterone into 11-deoxycortisol) and aldosterone (absence of conversion of progesterone into deoxycorticosterone). The accumulation of adrenal androgens due to elevated ACTH levels also contributes to the phenotype because of the loss of cortisol-negative feedback on the hypothalamic–corticotrophin axis. However, the clinical phenotypes are variable and depend on the extent of the deficiency in 21-hydroxylase [23, 38].

Various forms including the non-salt-wasting, salt-wasting, and non-classic subtypes are associated with mutations that determine the amount of residual 21-hydroxylase activity. The salt-wasting form is the characteristic of severe enzyme deficiency due to deletions and large gene conversions. The simple virilizing, non-salt-wasting subtype involves mutations that reduce activity (e.g., a missense mutation results in the non-synonymous amino acid substitution Ile172Asp), and the non-classic or late-onset subtype is due to mutations that only minimally affect the level of P450c21 expression or activity (e.g., Val28Leu and Pro30Leu).

Hydroxysteroid Dehydrogenases and Reductases [1]

Hydroxysteroid dehydrogenases (HSDs), also known as oxidoreductases, catalyze the interconversion of alcohol and carbonyl functions on the steroid nucleus and side chains according to the position and in a stereospecific manner. These enzymes use oxidized (+) or reduced (H) nicotinamide adenine dinucleotide (NAD (H)) or nicotinamide adenine dinucleotide phosphate (NADP(H)) as cofactors [23, 41, 42]. HSDs catalyze both oxidation and reduction in different in vitro conditions (e.g., substrate, pH, and cofactor). However, in vivo, catalysis is unidirectional, and they are classified as dehydrogenases or reductases.

The importance of cofactors in HSD activity is demonstrated by inactivating mutations in the gene encoding hexose-6-phosphate dehydrogenase, which regenerates NADPH in the endoplasmic reticulum, which is required for the HSD11B1 reaction. Patients with mutations in this gene are deficient in cortisone reductase activity due to impaired HSD11B1 activity [23, 43].

Multiple isoforms of HSDs and their tissue-specific expression determine the ability of specific enzymes to mainly act as reductases (ketone reduction) or dehydrogenases (alcohol oxidation). In tissues in which steroids are synthesized, HSDs catalyze the final steps in the biosynthesis of progestin, androgen, and estrogen. In tissues regulated by steroids, HSDs regulate steroid hormone receptor occupancy by converting active steroid hormones into inactive metabolites or relatively inactive steroids into molecules with increased binding activity.

3β-Hydroxysteroid Dehydrogenase/Δ5-4 Isomerases [1]

The 3β-HSD/Δ5-4 isomerases are localized in the endoplasmic reticulum and mitochondria, are membrane-bound, and utilize the cofactor NAD^+. These isomerases dehydrogenate the 3β-hydroxyl group and then isomerize the Δ5 olefinic bond to produce a Δ4 ketone structure. These enzymes convert pregnenolone into progesterone, 3-, 17α-hydroxypregnenolone into 17α-hydroxyprogesterone, and dehydroepiandrosterone into androstenedione [23]. Both reactions, dehydrogenase and isomerase,

occur at a single bifunctional catalytic site with different conformations for each activity. The rate-limiting step of the overall reaction is the 3β-hydroxysteroid dehydrogenase step, and the NADH formed as a result of this reaction likely changes the conformation of the enzyme to promote isomerization.

Five unprocessed pseudogenes that are closely related to *HSD3B1* and *HSD3B2* are present on band 1p13.1, and two are located between the expressed genes. The sequences of the exons of the two active genes are quite similar, and the resulting proteins are different by only 23-amino acid residues. HSD3Bs are expressed in the inner mitochondrial membrane in some cells and may act on pregnenolone that is produced by the cholesterol side-chain cleavage system.

The 3β-HSD/Δ5-4 isomerases appear to not be rate-limiting enzymes. However, mutations that produce a deficiency in HSD3B2 cause a type of congenital adrenal hyperplasia in which steroidogenesis in the gonads and adrenal glands is impaired, leading to the accumulation of Δ5 steroids in the blood.

The most severe type of HSD3B2 deficiency produces salt wasting due to insufficient mineralocorticoid production [23, 37]. Kinetic analysis of mutant proteins associated with salt-wasting and non-salt-wasting types of disease has demonstrated a fourfold to 40-fold reduction in the conversion of pregnenolone into progesterone. The salt-wasting phenotype is associated with frameshift mutations that produce a truncated protein as well as several missense mutations that affect the affinity for the cofactor and the stability of the enzyme. The different clinical phenotypes appear to be due to greater instability of the mutant proteins in patients with salt-wasting disease compared with proteins in patients with the non-salt-wasting form.

A late-onset or attenuated subtype of 3β-HSD deficiency, which is diagnosed by measuring steroid levels, has been reported, but no mutations in the genes encoding HSD3B1 and HSD3B2 have been found in such patients. Other possible genetic explanations include mutations in the distal promoter or epigenetic factors that affect enzyme expression. The apparent reduction in 3β-HSD activity may also be due to changes in the membrane that affect catalytic activity or posttranslational modifications of the enzyme that reduce its activity.

As mentioned above, mutations in *HSD3B1* have not been detected, but sequence variants with no known functional significance have been described. Because HSD3B1 is the main 3β-HSD/Δ5-4 isomerase expressed in the placenta, mutations that inactivate HSD3B1 may be responsible for miscarriage or preterm birth because of insufficient production of progesterone in the placenta.

11β-Hydroxysteroid Dehydrogenases [1]

The biological activity of cortisol in target tissues is mediated by the activity of two 11β-hydroxysteroid dehydrogenases that are short-chain alcohol dehydrogenases. These enzymes interconvert active glucocorticoids and inactive 11-keto metabolites [23, 44]. HSD11B2 is a type 2 enzyme found in the endoplasmic reticulum. The enzyme has reversible oxidoreductase activity in vitro, but in vivo, the enzyme preferentially catalyzes the reduction in the 11-keto group using NADPH as a cofactor. HSD11B2 regenerates cortisol from 11-ketosteroids in the liver, lung, adipose tissue, brain, vascular tissue, and gonads. HSD11B1 also affects glucocorticoid pharmacology. HSD11B1 in the liver converts the inactive prohormones cortisone and prednisone into active cortisol and prednisolone, respectively. As described above, mutations in hexose-6-phosphate dehydrogenase produce a syndrome in which high ratios of cortisone to cortisol are observed as well as impaired cortisol-negative feedback that leads to elevated ACTH secretion. In addition, individuals with these mutations have increased production of adrenal androgens leading to hyperandrogenism, sexual precocity, and polycystic ovary syndrome.

HSD11B2, which is also expressed in the endoplasmic reticulum, has a higher affinity for its substrate than HSD11B1. HSD11B2 catalyzes oxidation of cortisol, using the cofactor NAD^+. Renal mineralocorticoid receptors cannot distinguish cortisol or corticosterone from aldosterone, leading to inappropriate activation by glucocorticoids. HSD11B2 protects

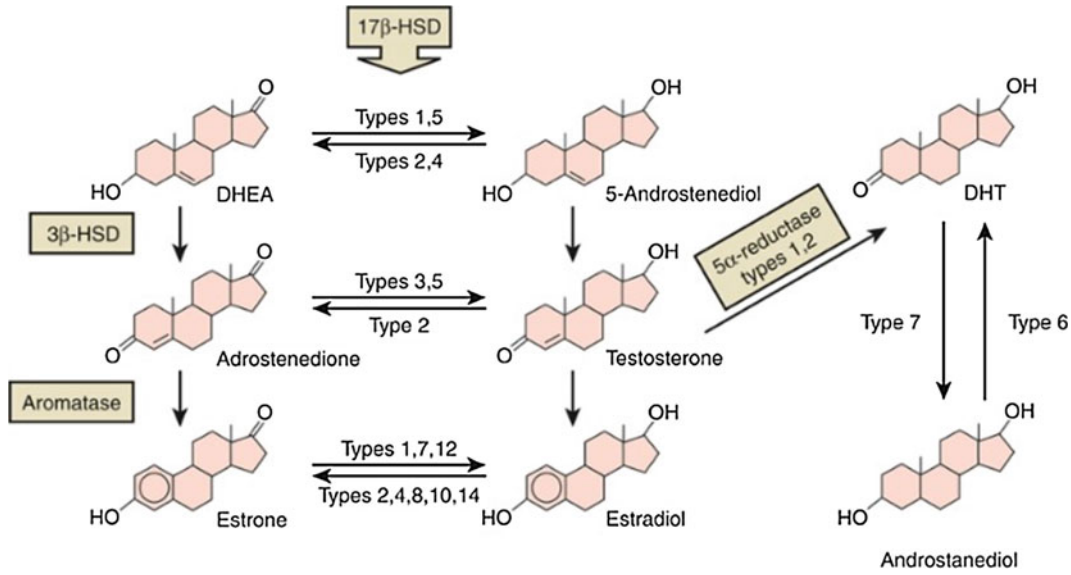

Fig. 1.10 The activity of 17β-hydroxysteroid dehydrogenases (17β-HSDs). On the adrenal cortex and ovary, 17β-HSDs act as dehydrogenase–reductases. They can convert DHEA to androstenediol, androstenedione to testosterone, and estrone to estradiol each other. (with the permission reproduced from [1])

mineralocorticoid receptors in the kidney because it converts cortisol and corticosterone to 11-keto compounds.

Inactivating mutations in HSD11B2 produce a disorder in humans that is characterized by apparent excessive mineralocorticoids. Similarly, mice that lack this enzyme show hypertension, hypokalemia, and renal structural abnormalities. Competitive inhibitors of HSD11B2 include glycyrrhizic acid, a component of licorice, and its metabolite carbenoxolone. In vivo administration of these compounds induces reduced expression of *HSD11B2* mRNA, producing apparent excessive mineralocorticoids similar to inactivation of the gene [45, 46].

17β-Hydroxysteroid Dehydrogenases [1]

17-ketosteroids are reduced, resulting in higher-potency 17β-hydroxysteroids in the adrenals, gonads, and placenta. Target tissues generally inactivate 17β-hydroxysteroids by oxidizing them [23, 42, 47–49]. Approximately seven of the 14 known mammalian 17β-HSDs (types 1 through 14) mediate this metabolism in humans (Fig. 1.10) [1]. Except for type 5, which is an aldo-keto reductase, these 14 17β-HSDs are members of the family of short-chain dehydrogenase–reductases. The enzymes use different cofactors and show various substrate specificities, including different specificities for nonsteroids, different subcellular locations, and various tissue-specific expression patterns.

HSD17B1, the type 1 enzyme, is called an "estrogenic" 17β-HSD due to its activity in catalyzing the final step in estrogen biosynthesis. HSD17B1 reduces estrone, a weak estrogen, to produce 17β-estradiol, which is much more potent This enzyme is expressed in the cytoplasm, uses either NADH or NADPH as a cofactor, and has 100-fold higher affinity for C18 compared to C19 steroids.

The gene that encodes HSD17B1 is located on bands 17q11-12 in tandem with a highly homologous pseudogene. HSD17B1 is expressed in ovarian granulosa cells and the placental syncytiotrophoblast and is also expressed at

higher levels in breast cancer cells than HSD17B2, which converts estradiol to estrone. Amplification of HSD17B1 in estrogen receptor-positive breast cancers is associated with lower survival than in patients without amplification.

3α- and 20α-Hydroxysteroid Dehydrogenase Activities [1]

Several HSDs such as AKR1C1 catalyze reactions at the 3 and 20 positions of steroid hormones [23, 41]. Tandemly duplicated genes on chromosome 10p14-p15 encode 3α-reductases, which are members of the aldo-keto reductase family that are expressed in liver, prostate, breast, uterus, testis, and adrenals.

Enzymes of the aldo-keto reductase family with 20α-HSD activity reduce progesterone to yield 20α-hydroxyprogesterone, which is inactive. These enzymes have a molecular weight of approximately 34 kDa, are found in the cytoplasm, and are expressed in human keratinocytes and cells in the liver, prostate, testis, adrenal gland, brain, uterus, and mammary gland.

Another group of enzymes of the short-chain dehydrogenase/reductase family has 3α-hydroxysteroid oxidative activity. Enzymes of the short-chain dehydrogenase–reductase family possess 20α-HSD activity, including HSD17B1 and HSD17B2, the latter of which preferentially oxidizes 20α-hydroxyprogesterone to produce progesterone.

$\Delta^{4\text{-}5}$ Reductases [1]

$\Delta^{4\text{-}5}$ reductases are membrane-associated enzymes that catalyze hydride transfer from NADPH to the carbon 5 position of steroid hormones, thereby reducing the Δ5-4 double bond [23, 50, 51]. The resulting products are either 5α or 5β-dihydrosteroids.

5α-Reductases

Humans express two 29-kDa 5α-reductases that share 50% similarity in amino acid sequence [52, 53]. Each gene, type 1 (*SRD5A1*) and type 2 (*SRD5A2*), for these 5α-reductases has five exons. *SRD5A2* is located on chromosome 2p23, and *SRA5A1* is located on chromosome 5p15; a pseudogene is present on Xq24-qter. SRD5A2 is predominantly expressed in male genital tissue, such as genital skin and the prostate, and reduces testosterone to produce the androgen, 5α-dihydrotestosterone, which is more potent. SRD5A1 catalyzes similar reactions on C21 and C19 of steroid hormones and is found in the liver, kidneys, skin, and brain. SRD5A1 can also synthesize 5α-dihydrotestosterone, but its tissue distribution indicates that inactivation of steroid hormones may be its main function. Mutations that inactivate *SRD5A2* lead to male pseudohermaphroditism (46, XY DSD) and abnormal ratios of testosterone to 5α-dihydrotestosterone.

Various severities of abnormal development of the external genitalia are seen, ranging from mild hypospadias to severely affected external genitalia that appear female. Wolffian ducts develop normally in the presence of sufficient quantities of testosterone. Females with mutations in *SRD5A2* are normal and have normal menstrual cycles. Such females have a low incidence of hirsutism and acne. Females also show low ratios of 5α- to 5β-dihydrosteroid metabolites in the urine, similar to males with these mutations. The infrequency of acne in both affected sexes, the rarity of hirsutism in affected females, the absence of male pattern baldness, and an atrophic prostate in affected males suggests that SRD5A2 is important for androgen metabolism in the skin and for androgen-dependent growth of the prostate.

Mutations in human *SRD5A1* have not been reported. However, targeted deletion of this gene in female mice produces reduced fecundity and a defect in parturition due to failed cervical ripening. This defect is rescued by 5α-androstanediol [54, 55]. The only human 5β-reductase (SRD5B1 or AKR1D1) that has been identified is a member of the aldo-keto reductase superfamily [56]. Its mechanism of catalysis is similar

to that of 5α-reductase. This enzyme plays a role in inactivating steroid hormones in the liver. Mutations in *SRD5B1* (AKR1D1), which is located on chromosome 7q32-q33, lead to abnormal synthesis of bile acids, a reduction in primary bile acids, and 5β-reduced steroid metabolites.

Sulfotransferases [1]

A group of enzymes has been identified that includes estrogen sulfotransferase (SULT1E1: encoded by a gene on chromosome 4q13.1), which sulfonates the 3-hydroxyl position of phenolic steroids, and hydroxysteroid sulfotransferases (encoded by *SULT2A1* and *SULT2B1*, which are closely linked and found on chromosome 19q13.4). These enzymes transfer a sulfonate (SO_3^-) anion from the activated donor, 3′-phosphoadenosine-5′-phosphosulfate, to a steroid hydroxyl acceptor, inactivating the hormone [23, 57, 58].

Steroid Sulfatase [1]

The sulfonate group on steroids is cleaved by steroid sulfatase, which is encoded by *STS* on chromosome Xp22.3 [23, 59–62]. This enzyme has an important function in controlling the synthesis of biologically active steroids from inactive sulfated molecules such as estrone sulfate and dehydroepiandrosterone sulfate. The syncytiotrophoblast expresses high levels of steroid sulfatase, which plays an important role in the synthesis of placental estrogen by producing sulfonated androgen precursors in the fetal compartment before aromatization. Steroid sulfatase also metabolizes cholesterol sulfate and sulfated estrogens in the skin.

A deficiency in sulfatase results in impaired estrogen synthesis in the placenta and ichthyosis after birth [61, 62]. This deficiency occurs most frequently in males (1:2000 to 1:6000 live male births) because the sulfatase gene is located on the X chromosome. Large deletions in *STS* are associated with mutations in the adjacent Kallmann syndrome gene (*KAL1*). In pregnancies with an affected fetus, maternal plasma estriol and urinary estriol excretion are characteristically present at approximately 5% of the levels found in normal pregnancies. Estrone and estradiol excretion is approximately 15% of normal levels. Higher levels of 16α-hydroxydehydroepiandrosterone in maternal serum are found. However, intravenous administration of dehydroepiandrosterone sulfate to the mother does not increase the estrogen excretion, but dehydroepiandrosterone does.

UDP-Glucuronosyl Transferases [1]

Glucuronidation is catalyzed by a family of uridine 5′-diphospho UDP-glucuronosyl transferases and participates in metabolic clearance of steroid hormones by hepatic and non-hepatic tissues [63–66]. Three subfamilies, UGT1A, UGT2A, and UGT2B, comprise 18 UDP-glucuronosyl transferases. Polymorphisms in genes encoding UDP-glucuronosyl transferases are associated with variations in estrogen levels, suggesting that these phase II biotransformation enzymes play a role in regulating the levels of bioactive steroids.

Glycoproteins

Inhibin, Activin, and Follistatin

Inhibin, activin, and bone morphogenic proteins are polypeptide hormones that belong to the transforming growth factor (TGF)-β superfamily and play important roles in the function and the development of many tissues [67]. A deficiency in these TGF-β superfamily molecules therefore triggers developmental and physiological impairment, occasionally leading to the appearance of endocrine or non-endocrine tumors [68].

Activins are dimeric proteins composed of two inhibin/activin β subunits [activin-A (βA, βA), activin-AB (βA, βB), activin-B (βB, βB)], whereas inhibins are hetero-dimers of an inhibin/activin β subunit and the structurally related inhibin/activin α subunit [inhibin A (αβA) and inhibin B (αβB)] [67, 69].

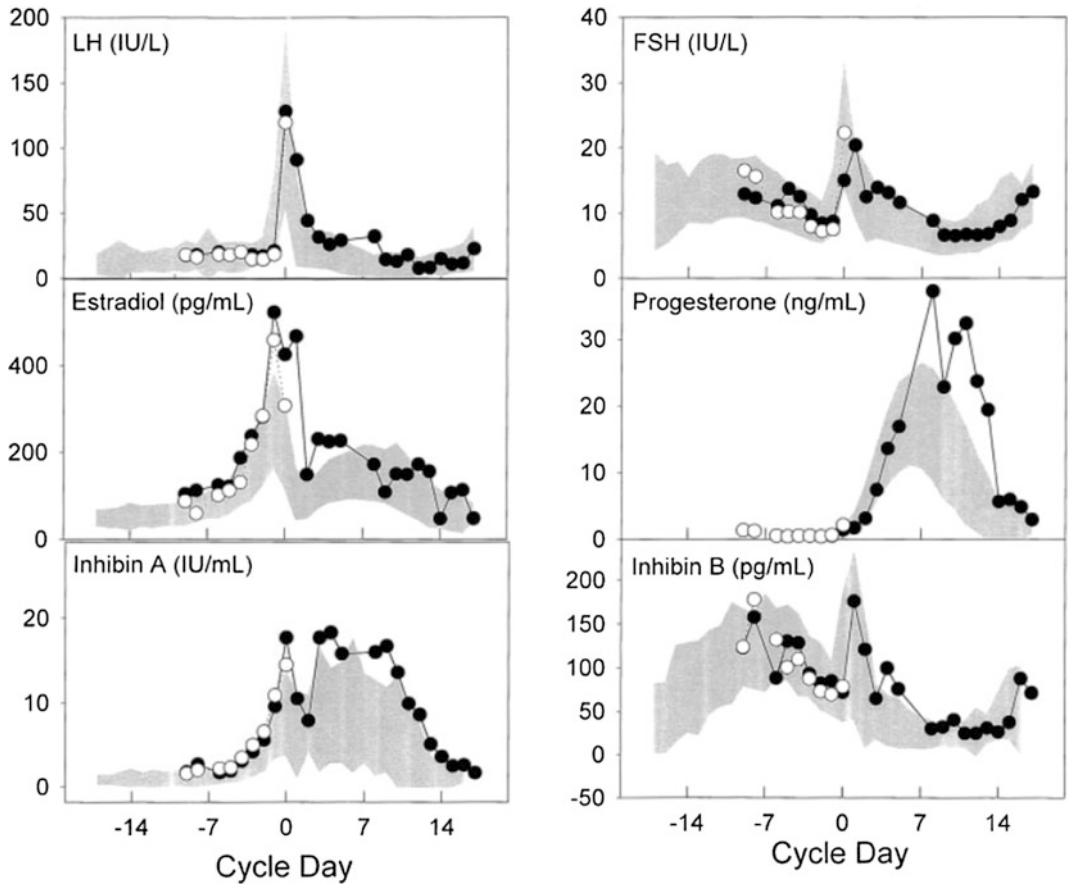

Fig. 1.11 Serum hormone levels in the first cycle compared to those in a subsequent aspiration cycle: determination of follicle maturity. Serum hormone levels from daily blood sampling for one patient are shown, including the results from one complete cycle (*closed circles*) and the subsequent aspiration cycle up to the day of aspiration (*open circles*). *Shaded areas* show the mean ±1 SD for normal women (n = 122 cycles for LH, FSH, estradiol, and progesterone and n = 23 cycles for inhibins A and B). Results for the aspiration cycle are nearly identical to the previous cycle, which allows estimation of the day of aspiration (follicle maturity). In this example, the follicle was aspirated on the day of the LH surge, designed day 0 in both serum and hFF analyses (with the permission reproduced from [70])

Follistatin is a single-chain glycoprotein hormone of 31–49 kDa, depending on alternative mRNA splicing and variable glycosylation of the protein [70].

Both inhibin A and inhibin B are secreted primarily by follicular granulosa cells and work together with estradiol to suppress FSH secretion from the pituitary gland. Since early studies using radioimmunoassays were first performed, an accurate method using ELISA has become available to distinguish inhibin A and inhibin B. The ELISA results of inhibin A are similar to previous measurements using radioimmunoassays. Inhibin B levels are highest in the early to mid-follicular phase and decline in the late follicular phase [70] (Fig. 1.11 [71]). Because preantral follicles only produce the βB subunit, in perimenopausal women who have a reduced

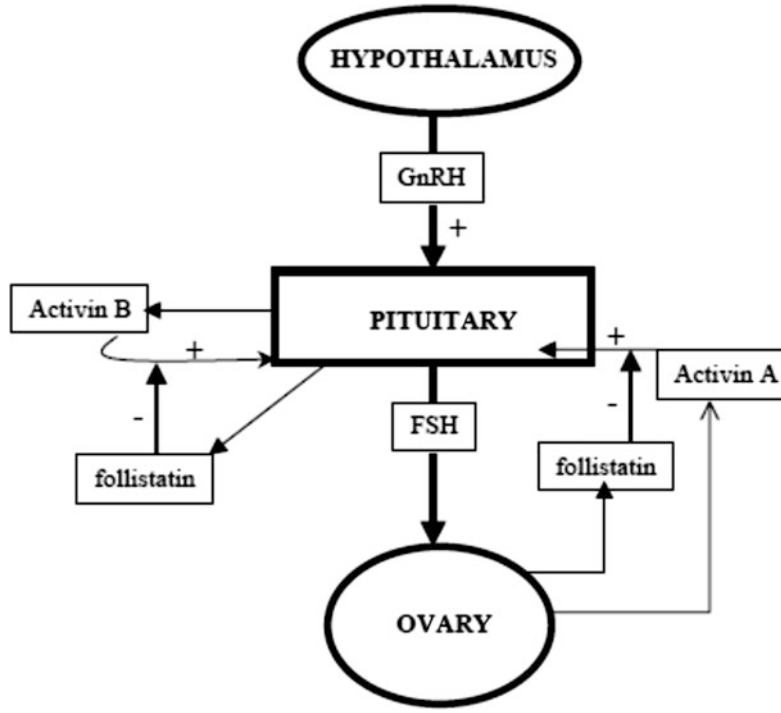

Fig. 1.12 Summary of autocrine/paracrine and endocrine actions of activins and follistatin in the pituitary–ovarian axis (with the permission reproduced from [73])

number of follicles, inhibin B decreases and is considered to consequently elevate FSH [72].

As the follicle population develops in preparation for ovulation, the dominant follicle within this population increases the production of the βA subunit. This results in an increase in inhibin A levels from the late follicular phase, with a peak at mid-cycle [73]. The LH surge decreases all inhibin subunits and subsequently induces the expression of βA subunits in the corpus luteum once again.

Activin is also primarily produced by follicular granulosa cells. It reaches a peak in concentration during the luteo-follicular transition period and promotes FSH secretion in the early follicular phase [74]. Activin is known to promote the expression of FSH receptors in undifferentiated granulosa cells [75]. This observation is important in understanding how follicles mature from being FSH independent to FSH dependent. These findings have also been verified in porcine and sheep [76, 77], and follistatin has been indicated to similarly neutralize activin actions in humans [78].

Of the activins, activin-B is produced by anterior pituitary cells in rats and has been suggested to affect gonadotropic production of the pituitary gland via autocrine or paracrine mechanisms [79]. Based on these findings, activin and follistatin are thought to function as depicted in Fig. 1.12 [74]. Activin is also known to gradually decrease, while inhibin A and follistatin increase with follicular development, and follicular development appears to be regulated through this series of mechanisms [71].

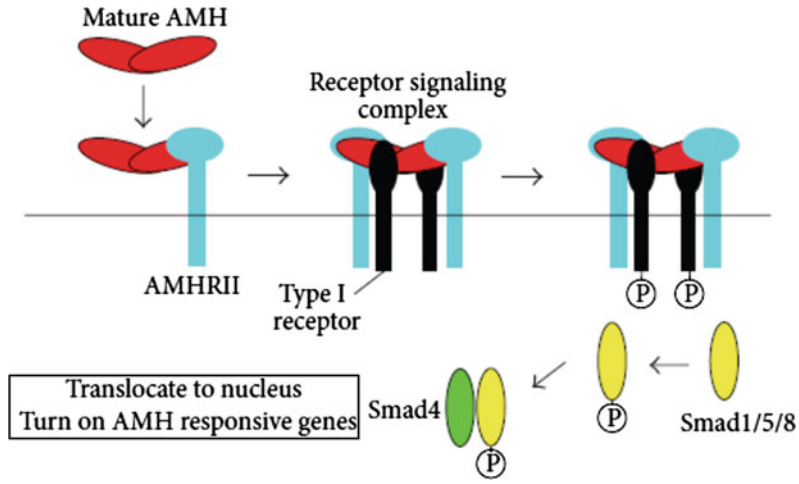

Fig. 1.13 The mechanism of AMH activity for AMH receptors. AMH binds to the extracellular domain of AMH type II receptors, phosphorylates AMH type I receptors, and activates intracellular Smad protein signaling. Ligand binding induces the formation of heteromeric complexes, in which type II receptors phosphorylate type I receptors. Type I receptors then activate Smad1/5/8, which associate with Co-Smad (Smad4). These Smad complexes move into the nucleus and regulate the expression of target genes in cooperation with transcription factors (with the permission reproduced from [83])

Anti-Müllerian Hormone (AMH)

AMH is a 140-kDa disulfide-linked homodimeric glycoprotein and, similar to inhibin and activin, belongs to the TGF-β superfamily. AMH is secreted by male Sertoli cells and is known to induce Müllerian duct regression for the differentiation of male gonads and to play a role in promoting the development of the Wolffian duct. It was first reported in the 1940s as a Müllerian-inhibiting substance [80].

It was subsequently reported that chicken AMH is produced not only during fetal stages, but also during adult stages from the follicular granulosa cells [81] and that AMH secretion begins postnatally in humans [82]. The mechanism of action of AMH is similar to that of other molecules belonging to the TGF-β superfamily. AMH binds to the extracellular domain of AMH type II receptors, phosphorylates AMH type I receptors, and activates intracellular Smad protein signaling [83] (Fig. 1.13) [84].

Animal experiments in AMH knockout mice revealed extremely crucial evidence that AMH suppresses the development of primordial follicles. This study showed that primordial follicle recruitment is promoted to a greater extent in AMH null mice than in control mice, and 4-month-old AMH null mice exhibit a greater number of preantral follicles and small antral follicles. However, it was also shown that due to a marked decrease in primordial follicles, the lack of AMH will ultimately lead to early depletion of follicles [85, 86]. Additionally, AMH is postulated to diminish FSH sensitivity in follicles and is consequently involved in the selection of dominant follicles [85, 87]. Furthermore, the increase in follicle size reduces AMH production, and this is considered to further increase the size of dominant follicles [88].

Although AMH expression is not found in the primordial follicles, it is found in follicles from primary follicles to 4-mm-sized antral follicles (Figs. 1.14 and 1.15) [89]. Its expression

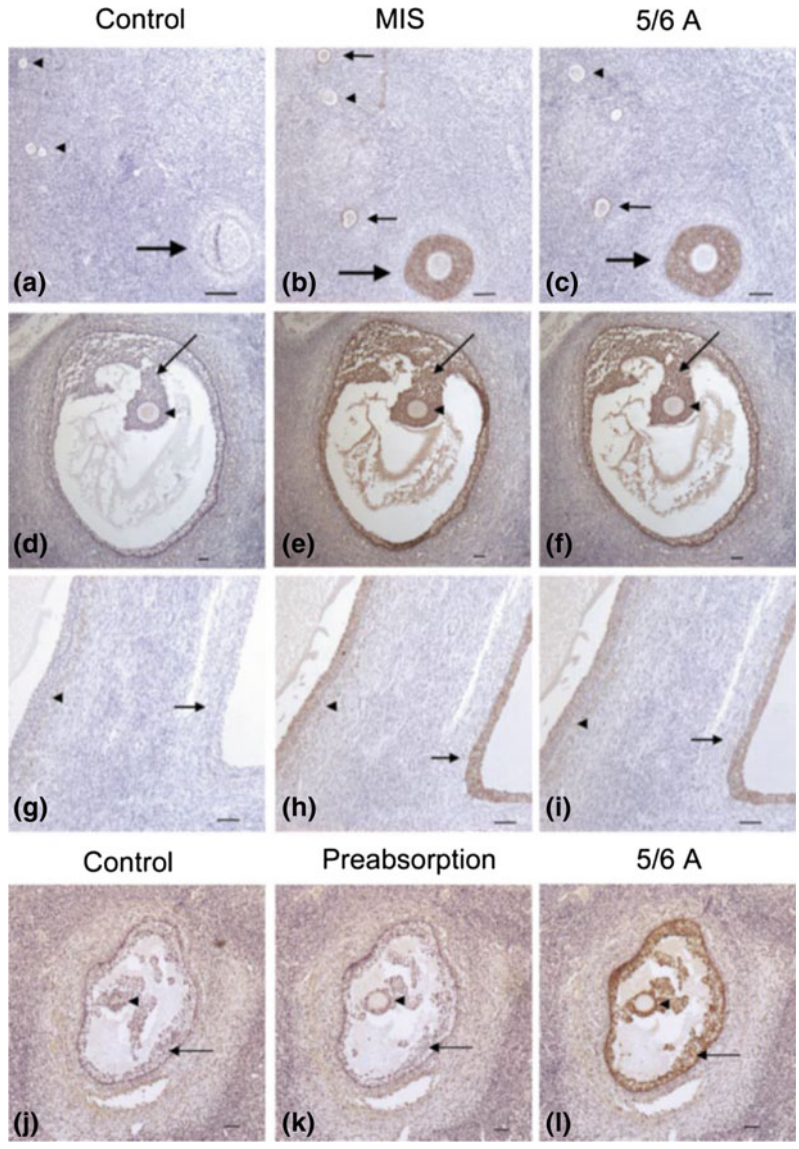

Fig. 1.14 Micrographs of anti-Mullerian hormone (AMH) immunohistochemical-stained human ovarian tissue sections. Specific (*brown*) AHM stain deposition is present in the cytoplasm of granulosa cells. Scale bar +100 μm. Sections **a**, **d,** and **g** are controls; sections **b**, **e,** and **h** were stained using the MIS C-20 antibody; sections **c**, **f,** and **i** use the 5/6A antibody. (**a**–**c**) Adjacent sections at X100 magnification with a primordial follicle (*arrowhead*), a primary follicle (*small arrow*), and a secondary follicle (*large arrow*). Primordial follicles show no immunostaining of the cytoplasm of the granulosa cells, whereas the primary follicles show normal staining (+) with both the MIS C-20 and 5/6A antibodies. Secondary follicle shows strong staining (++) with both antibodies. (**d**–**f**) Adjacent sections at X40 magnification with a small antral follicle <1 mm. The oocyte shows weak, non-specific brown staining (*arrowhead*), whereas the granulosa cells, especially of the cumulus (*arrow*), show strong staining (++) with both antibodies. (**g**–**i**) Adjacent sections at X100 magnification show two large antral follicles. The follicle on the *left side* with a diameter of 6.1 mm shows weak staining (±) of the granulosa cells (*arrowhead*), whereas the smaller follicle on the right side (*arrow*) with a diameter of 2.5 mm shows normal staining (+) with both antibodies. (**j**–**l**) Adjacent sections at X40 magnification with a small antral follicle <1 mm. The oocyte shows weak, non-specific brown staining (*arrowhead*). The granulosa cells show strong staining (++) with the 5/5A antibodies. When the peptide is added to the antibody, no immunohistochemical staining occurs (K) (with the permission reproduced from [88])

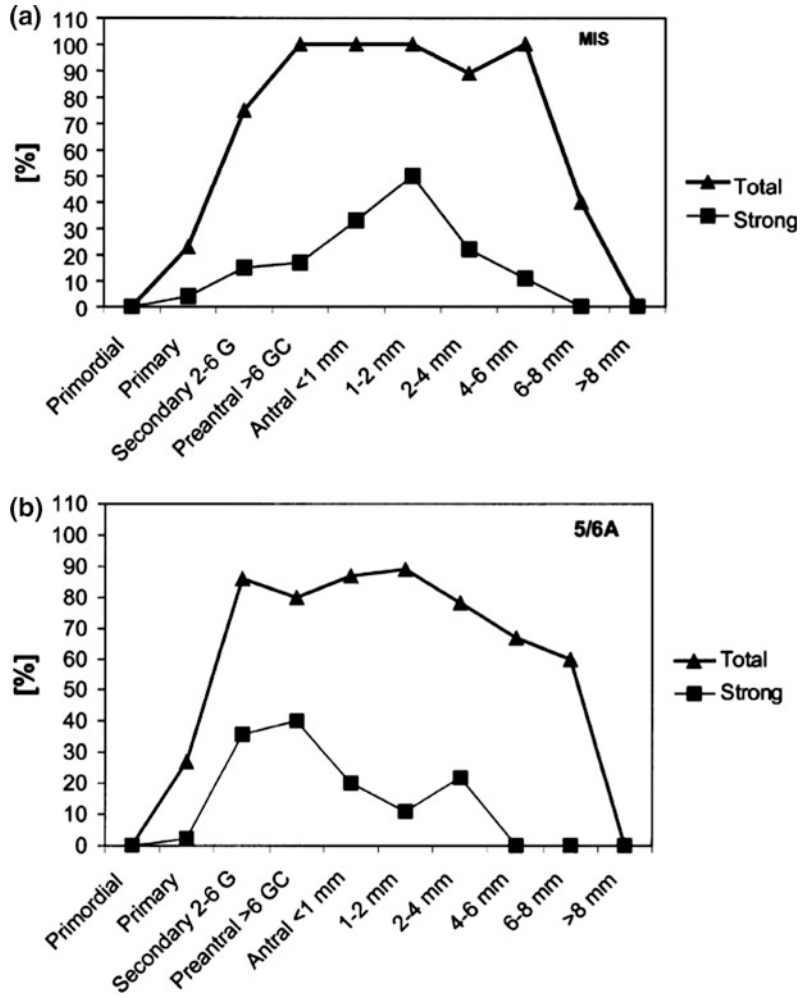

Fig. 1.15 Graphical summary of the immunohistochemical data in Table II and III. Only the percentages of the follicles with strong (++) and total staining (+ and ++) are shown. The total staining in graphical depictions represents an addition of the percentage of follicles within a certain class with normal (+) and strong (++) staining (total staining; *triangles*). For comparison, the percentage of follicles of a certain class that shows strong staining is depicted separately (strong staining; *squares*). Staining increases rapidly with the stage of the follicles and decreases when follicles are >4–6 mm. In the upper graph (**a**), staining with the MIS antibody is shown. In the lower graph (**b**), staining with the novel 5/6A antibody is shown (with the permission reproduced from [88])

decreases when the antral follicles are between 4 and 8 mm in size and eventually disappears [89]. Both gene expression and the follicular fluid concentration of AMH increase until the follicle size is 8 mm and subsequently decrease rapidly; these observations are consistent with the above findings. In addition, AMH secreted by 5- to 8-mm-sized follicles is known to account for 60% of all blood AMH [90]. Although the majority of AMHs are secreted by the antral follicles, AMH levels are correlated with the number of primordial follicles and indirectly

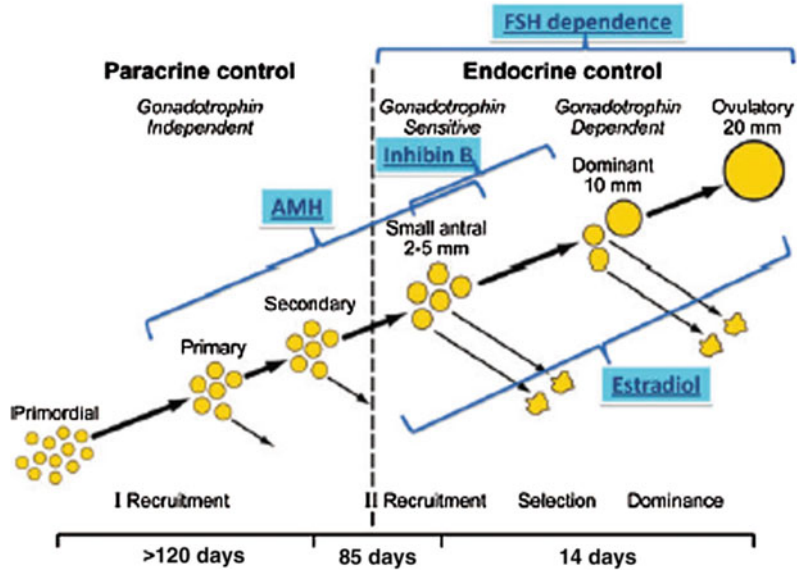

Fig. 1.16 Schematic representation of follicle development emphasizing that AMH is produced in early stages of follicle development (characterized by gonadotropin-independent growth), as opposed to inhibin A and estradiol produced by follicles at later stages of development where growth is FSH dependent (with the permission reproduced from [86])

represent the ovarian reserve. Because the development of secondary follicles and follicles from prior stages is FSH independent [91], serum AMH levels are not affected by the development of dominant follicles.

In addition, from the perspective that AMH is clinically used as a simple marker for ovarian aging and is not affected by the menstrual cycle, it is distinct from inhibin B, estradiol (E2), and FSH that are dependent on the menstrual cycle [92]. Figure 1.16 illustrates the development of follicles and the production of each ovarian reserve marker [87].

The AMH level and antral follicle count (AFC), which is assessed with transvaginal ultrasound, are strongly correlated with each other [93]. Both AMH and AFC have shown a correlation with the number of primordial follicles in ovarian tissue [94]. Figure 1.17 shows the correlation between AMH levels and the number of primordial follicles [94]. In addition, AMH levels begin to increase from early puberty, reach a plateau at age 20–25 years, and subsequently decline gradually with age toward menopause (Fig. 1.18) [95]. However, because the AMH level is affected by the number of antral follicles present in both ovaries [96], it is important to note that the level fluctuates widely [97].

The AMH level is also influenced by the administration of steroids such as oral contraceptives (OC), indicating that an individual's clinical background should be noted when measuring AMH [92, 98]. In particular, a large-scale study that included approximately 2300 participants found that AMH levels are lower in current OC users than in non-OC users [99]. Moreover, long-term GnRH agonist administration is known to reduce AMH levels [100]. For these reasons,

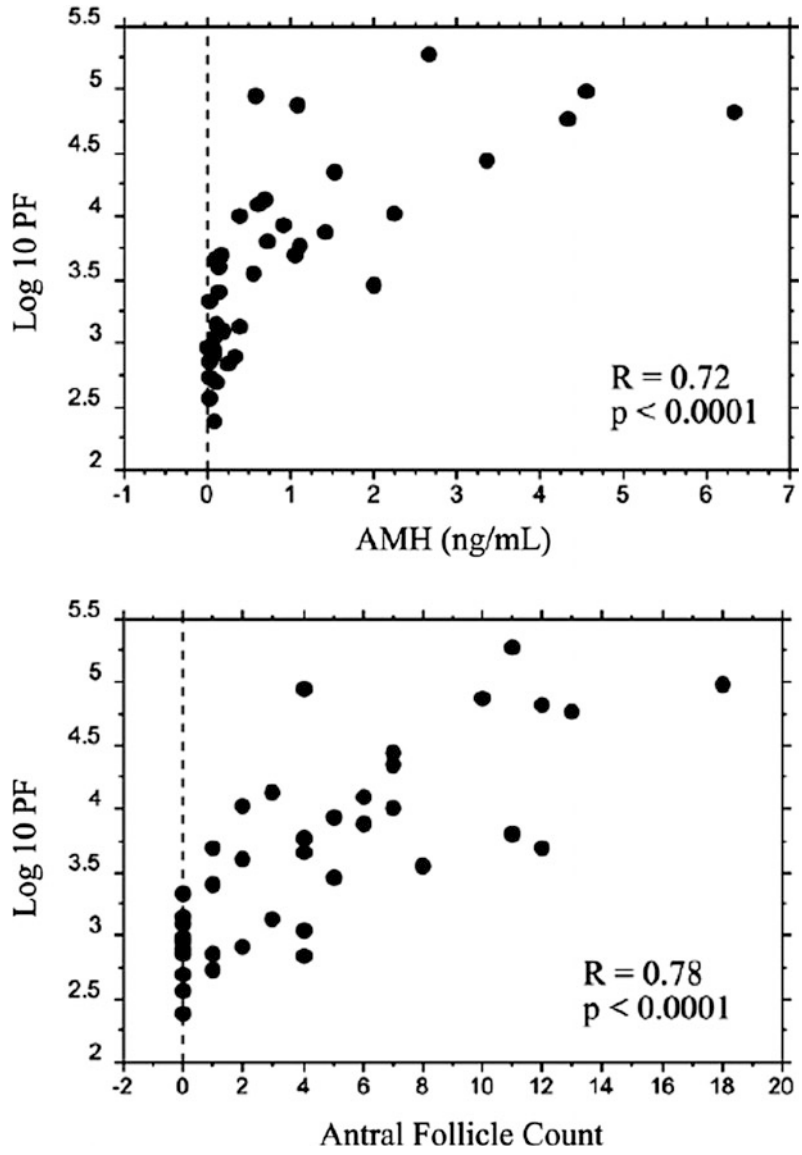

Fig. 1.17 The correlation between ovarian reserve test (serum AMH level and antral follicle count) and the number of primordial follicles. According to the result of follicle count using human ovary from patients who received oophorectomy due to benign gynecologic indications, the serum AMH level ($R = 0.72$, $P < 0.0001$) and ovarian AFC ($R = 0.78$, $P < 0.0001$) have shown strong correlation with the numbers of remaining primordial follicles. The numbers of remaining follicles were counted using fractionator/optical disector method (with the permission reproduced from [93])

when GnRHa is used, for example, for cancer treatment, the reliability of AMH as an ovarian reserve marker is low [92]. Furthermore, because AMH levels are also influenced by being overweight [101], ethnicity [102], vitamin D status [103], AMH polymorphism [104], and smoking [99], careful attention is necessary when estimating the ovarian reserve from AMH levels.

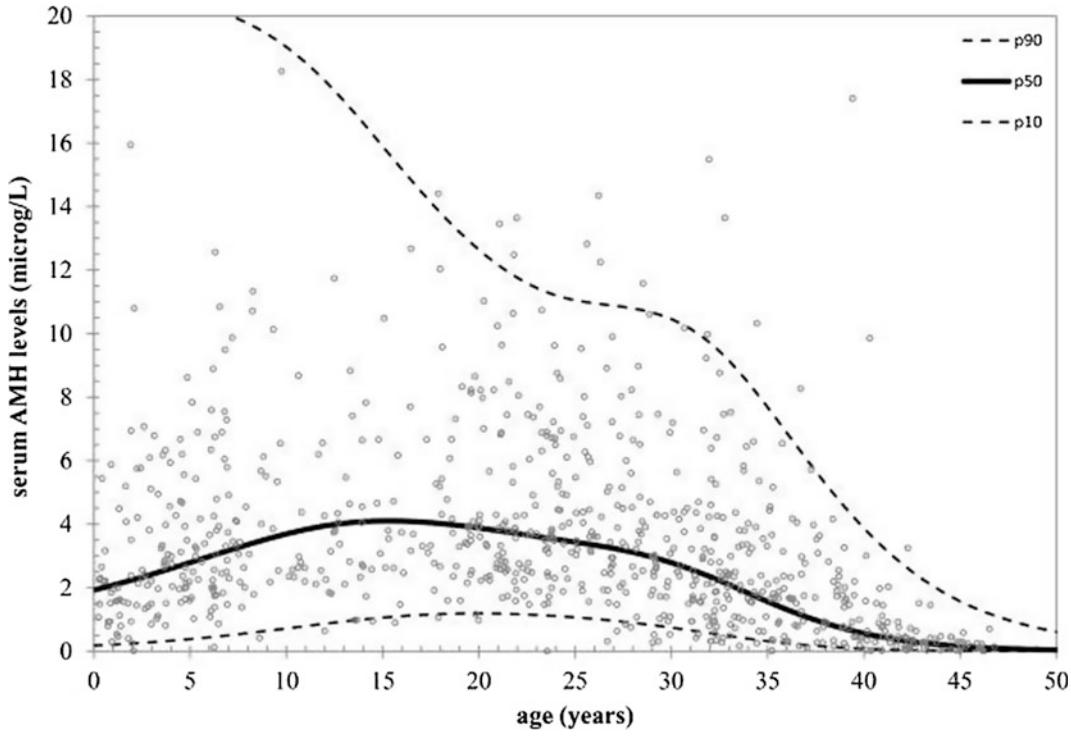

Fig. 1.18 AMH nomogram based on natural linear spline interpolation. According to the measurement of serum AMH level of 804 healthy women ranging infancy until the end of the reproductive period, AMH was inversely correlated with age ($r = 0.24$; $P < 0.001$). The age at which the maximum AMH value was attained was at 15.8 yr. Reference lines of serum AMH for the 10th, 50th, and 90th percentiles of predicted AMH values versus age (with permission reproduced from [94])

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Follicular Development and Oocyte Growth

2

Han Li and Ri-Cheng Chian, Msc, PhD

Introduction

Follicular development begins as early as the fourth month of fetal life [1]. At that time, the primordial germ cells (PGCs) have migrated from the yolk sac endoderm to the gonadal ridge, undergoing mitotic divisions. PGCs are called oogonia once they reach the gonads, then the oogonia enter the first meiotic division and become primary oocytes. Somatic cells originating from the primitive gonad surround the oogonia, forming primordial follicles [2]. These primordial follicles constitute the ovarian follicular reserve, which provides a woman with reproductive potential during her entire lifetime. It is a central dogma in reproductive biology that during the life of the individual there cannot be any increase in the number of primary oocytes beyond those originally laid down when the ovary was formed. However, a series of recent studies have challenged this dogma by showing regeneration of oocytes from putative germ cells in bone marrow and peripheral blood [3–5]. Future studies may address whether spontaneous neo-oogenesis takes place in the adult ovary.

The follicles develop through primordial, primary, and secondary stages before acquiring an antral cavity. At the antral stage, most follicles undergo atresia. After pubertal onset, maturation of the hypothalamus–pituitary–ovarian (HPO) axis results in pulsatile release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary, so a few of the antral follicles can be rescued by gonadotropins to continue growth and normally one antral follicle can reach the pre-ovulatory stage each month [6, 7]. In a natural menstrual cycle, there is only one follicle will be chosen to ovulate eventually while others going atresia under the accurate regulation of both HPO axis and intra-ovarian regulators, such as growth factors, cytokines, and gonadal steroids. But more recent results document that multiple follicle waves may exist during the human menstrual cycle, which has challenged the traditional notion [8].

During ovarian follicle development, oocytes also grow and differentiate, and a complex cytoplasmic organization is required [9]. The growth phase of the oocyte allows development of the zona pellucida and production of mRNA and proteins required for subsequent fertilization and early embryonic development. These factors must be stored within the oocyte, as resumption of meiosis results in transcriptional silencing [10]. Oocyte developmental competence, defined as the ability of the oocyte to resume and complete meiosis, and support pre-implantation embryonic development after fertilization, is acquired gradually during folliculogenesis.

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Follicular Development

From Primordial Follicles to Pre-antral Follicle

Formation of Primordial Follicles

In humans, primordial germ cells (PGCs) arrive in the gonadal ridge from the yolk sac endoderm by the seventh week of gestation to become oogonia, which proliferate by mitosis before differentiating into primary oocytes. PGCs are called oogonia once they reach the gonads, some oogonia become primary oocytes and enter the first stages of meiosis at around 11–12 weeks of gestation. Primordial follicle formation begins as early as 15th week of gestation when a single layer of flattened pre-granulosa cells surround each diplotene oocytes [1, 11]. After oocytes are within the primordial follicles, they remain arrested in the dictyate stage of meiosis I. The reproductive life span of women is determined by the number of primordial follicles in the ovary. Ovaries contain a maximum of six millions of germ cells during fetal development in woman, to 300,000 at puberty before the first ovulation. The age-related depletion of the resting follicles occurs as a result of two processes: atresia and entry in growth phase [6].

A dogma in biology of reproduction states that the pool of non-renewable primordial follicles serves as a source of developing follicles and oocytes that decline with age. This doctrine has been challenged by Jonathan Tilly's team in 2004 and 2005, whose research claimed that the adult mammalian ovary is not endowed with a finite number of oocytes, but instead possesses stem cells that contribute to their renewal [3–5]. The ability to isolate and promote the growth and development of such ovarian germ-line stem cells (GSCs) would provide a way to treat infertility in women. While such ovarian GSCs are characterized in non-mammalian model organisms, the findings that support the existence of adult ovarian GSCs in mammals have been controversial [12]. Although some studies claimed that mammalian ovary may contain some GSCs in vivo and would be reactivated under certain conditions in vitro and generate oocyte-like cells, perhaps these cells may not be main contributors to ovarian function [13]. The hypothesis of ovarian neo-oogenesis remains to be more convincingly demonstrated [14].

Initial Recruitment of Follicles

Initial recruitment is believed to be a continuous process that starts just after follicle formation, until the ovarian reserve is depleted [15]. During initial recruitment, some primordial follicles start to grow, whereas the rest of the follicles remain quiescent for months or years. Morphometric studies suggested that follicles initiate the growth based upon the order in which they were formed (Fig. 2.1). During this process, flattened granulosa cells of primordial follicles become cuboidal during transition into the primary stage along with an increase in oocyte diameter [16].

FSH is not required for this transition as primordial follicles do not express FSH receptors [17]. Resting follicles are likely to be under constant inhibitory influences of systemic or local origins to remain dormant [6]. A decrease of inhibitory influences or an increase of stimulatory factors allows the initiation of follicle growth. Anti-mullerian hormone (AMH) is involved in the control of primordial follicle activation by inhibiting the recruitment of primordial follicles into the growing pools. In adult females, AMH is produced by the granulosa cells of growing follicles, its expression decreasing in large antral follicles, and serum AMH is a useful biomarker of the ovarian reserve of growing follicles in human [18]. Recent studies on genetically modified mice have revealed that there are indeed some inhibitory signals that maintain primordial follicles in the dormant state. Loss of function of any of the inhibitory molecules for follicular activation, including Tsc-1, Pten, Foxo3a, and Foxl2, leads to premature activation of the primordial follicle pool [19–21]. With aging, as follicles continuously leave the resting pool, the number of growing follicles decreases, but the proportion of primary and early growing follicles increases in primates [22] as in mice [23]. This increase may be triggered by the progressive disappearance of an inhibitory influence on primary follicle [6].

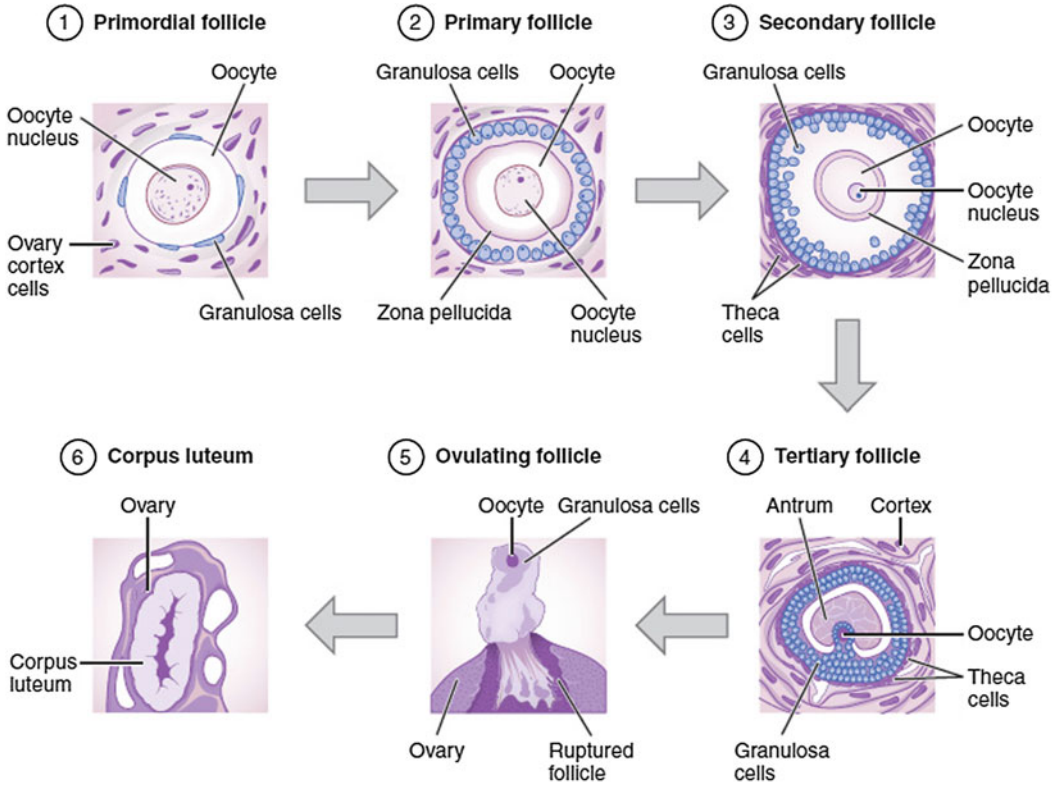

Fig. 2.1 The structures of different follicles and its development are shown in a clockwise direction proceeding from the primordial follicle to pre-ovulatory follicle as well from ovulating to corpus luteum formation. (*1*) The primordial follicle contains the oocyte surrounded by flat, squamous granulosa cells that are segregated from the oocyte's environment by the basal lamina. (*2*) The primary follicle begins with the change of granulosa cells from a flat to a cuboidal structure, and the oocyte genome is activated and genes become transcribed. In this stage, a glycoprotein polymer capsule, the zona pellucida, is formed around the oocyte, separating it from the surrounding granulosa cells. (*3*) The secondary follicle is surrounded by the outermost layer, the basal lamina, and undergoes cytodifferentiation to become the theca externa and theca interna. An intricate network of capillary vessels forms between these two thecal layers and begins to circulate blood to and from the follicle. (*4*) The tertiary follicle is the basic structure of antral follicle. Granulosa and theca cells continue to undergo mitosis concomitant with an increase in antrum volume. (*5*) The ovulating follicle is excreting the oocyte with a complement of cumulus cells by the surge of LH during menstrual cycle. (*6*) The corpus luteum is formed from the ruptured follicle, and a steroidogenic cluster of cells that maintains the endometrium of the uterus by the secretion of large amounts of progesterone. The figure was downloaded from the Web site. http://cnx.org/content/col11496/1.6/

In addition to inhibitory signals that inhibit premature activation of primordial follicles, there are some other signals in the ovary that promote the transition of primordial follicles to primary follicles. With synergistic actions of these signals, growth is initiated in primordial follicles [24]. According to studies on transgenic animal models and on the human ovary, several members of the TGF-β super family, such as BMP-4, BMP-7 [25, 26], and GDF-9, play critical roles in this process. Other growth factors and cytokines also act at the formation of primary follicles, such as kit-ligand, leukemia inhibitory factor (LIF) [27], basic fibroblast growth factor (bFGF) [28], and BMP-15 [29].

Several transcription factors that might regulate this early step of folliculogenesis have been identified, illuminating key signaling pathways

responsible for the maintenance at the resting stage or the recruitment of primordial follicles. Recently, four main transcription factors have been identified by using mutant mice: Nobox (newborn ovary homeobox), Sohlh-1 and Sohlh-2 (spermatogenesis and oogenesis helix–loop–helix 1), and Lhx8 [30–32]. The phenotype of the four gene mutants is very similar [31], as a failure in the primordial to primary follicle transition. Further studies are needed to reveal potential inhibitory factors or intra-ovarian stimulating factors that are involved in the initial stage of follicle recruitment.

Pre-antral Follicle Growth and Differentiation

Pre-antral follicular development includes the primary to secondary follicle transition and the development of secondary follicles to the per-antral stage. When follicles leave the resting pool, the granulosa cells become cuboidal and begin to express markers of cell proliferation. The transition from the primordial to primary stage can be very prolonged. When primary follicles enter the growth phase their size increases, both by enlargement of the oocyte and proliferation of granulosa cells, single-layered primary follicles are transformed into multilayered secondary follicles. As they enlarge, the surrounding layer of stroma cells stratifies and differentiates in two parts: the outer part is the theca externa and the inner part is the theca interna [33]. The oocyte continues to grow, the zona pellucida is formed, theca condenses around the pre-antral follicle, and the vascular supply develops [29]. The zona pellucida consisted by a glycoprotein polymer capsule around the oocyte, separating it from the surrounding granulosa cells. The zona pellucida, which remains with the oocyte after ovulation, contains enzymes that catalyze with sperm to allow penetration. From a follicular diameter of about 0.15 mm in humans [34], the theca interna commences its epithelioid differentiation and the follicle is defined as a secondary follicle and constitutes the first category of growing follicles in a classification based on morphological aspect and total number of granulosa in each individual follicle [34]. Stroma-like theca cells are recruited by oocyte-secreted signals. They surround the follicle's outermost layer, the basal lamina, and undergo cytodifferentiation to become the theca externa and theca interna. An intricate network of capillary vessels forms between these two thecal layers and begins to circulate blood to and from the follicle. The secondary follicle is marked histologically by a fully grown oocyte surrounded by a zona pellucida, approximately nine layers of granulosa cells, a basal lamina, a theca interna, a capillary net, and a theca externa.

The response of follicles to gonadotropins depends on the number of receptors for gonadotropins present on follicle cells and the transducing mechanisms to which these receptors couple. Available data in humans indicate that the number of FSH receptors on granulosa cells seem to be unchanged [10]. During this phase, follicles less than 2 mm exhibit a slight steroidogenic activity, the progressively increased mitotic activity of granulosa cells is not mediated by the actions of gonadotropins [35]. FSH may have a permissive role rather than being essential in pre-antral follicle growth [36]. In contrast to the debatable role of FSH in pre-antral follicle growth, there is firm evidence that certain members of the TGF-β super family locally produced from follicles [33, 37, 38], theca cells (BMP-4 and BMP-7) or both (TGF-β), or oocytes (GDF-9 and BMP-15), play crucial roles in the growth of primary follicles into pre-antral and antral stages [25, 39, 40]. Because EGF/TGF-α and their receptors, as well as IGF-R, have been detected in pre-antral and small antral human follicles, it is likely that these factors may also play a positive role in sustaining growth of small follicles [6].

BMP-4 and BMP-7 modulate FSH signaling in a way that promotes estradiol production while inhibiting progesterone synthesis, acting as a luteinization inhibitor [41]. In vitro exposure of ovarian cortical samples to oocyte-derived recombinant GDF-9 has been shown to increase the number of primary and secondary follicles in human and rodents suggesting an important role, at least under in vitro conditions, for this growth factor in the initiation and progression of follicle growth [27, 42]. Another research has found that

BMP-15 can stimulate granulosa cell mitosis in pre-antral follicles during the FSH-independent stages. BMP-15 can also inhibit FSH receptor expression [38].

From Antral to Pre-ovulatory Follicles

In humans, follicles pass from pre-antral to early antral stage at a follicular diameter comprised between 0.18 and 0.25 mm. It is also during this stage that the follicle begins to exhibit some fluid-filled spaces within the granulosa cell layers, which will coalesce to form the antral cavity, along with increased vascularization of the theca layer, continued growth of oocytes and proliferation of granulosa and theca cells [24]. Stroma-like theca cells are recruited by oocyte-secreted signals. They surround the follicle's outermost layer, the basal lamina, and undergo cytodifferentiation to become the theca externa and theca interna. An intricate network of capillary vessels forms between these two thecal layers and begins to circulate blood to and from the follicle.

The time required by a follicle to grow from the pre-antral stage to a size of 2-mm antral follicle is of about 70 days [2], and this part of folliculogenesis is named basal follicular growth. The formation of a fluid-filled cavity adjacent to the oocyte called the antrum designates the follicle as an antral follicle, in contrast to a so-called pre-antral follicle that still lacks an antrum. An antral follicle is also called a Graafian follicle. Once entering the growing pool, most growing follicles progress to the antral stage, at which point they inevitably undergo atresia. After pubertal onset, a small number of the antral follicles can be rescued by gonadotropins to continue growth, and normally only one antral follicle is further developed each month in preparation for ovulation. Antral follicles (2–5 mm diameter) develop into pre-ovulatory follicles (16–29 mm diameter) in 14 days during the follicular phase of the menstrual cycle (Fig. 2.2).

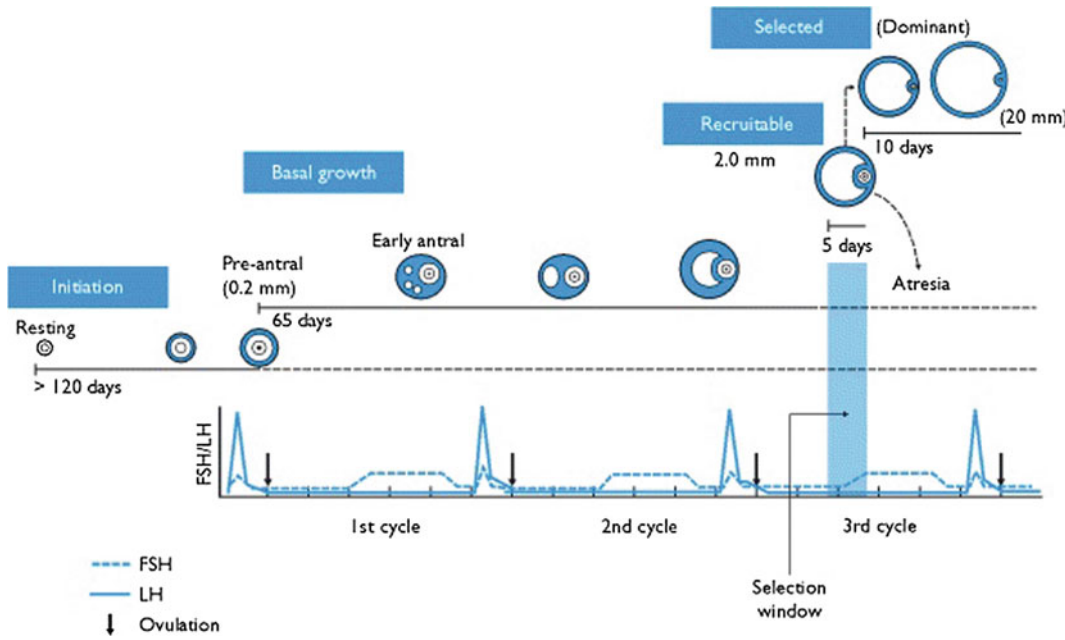

Fig. 2.2 Diagram for folliculogenesis. The development of a primordial follicle to a pre-ovulatory follicle takes in excess of 120 days. After it has become a pre-antral follicle of about 0.2 mm diameter, it takes about 65 days to develop into a pre-ovulatory follicle. Cohorts of follicles continually develop but only one is 'selected' and becomes the dominant follicle. All others undergo atresia. The figure was downloaded from the Web site. http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=endocrin.box.1226/

Antral Follicle Recruitment

Follicles in diameter of about 2–5 mm are present throughout the menstrual cycle. During the late luteal phase, the 2–5 mm follicles, which have entered the pre-antral stage 70 days earlier, become selectable follicles for further development [34]. Recruitment of the follicular cohort occurs in response to a transient elevation in circulating FSH. After increases in circulating FSH during the peri-menstrual period, a cohort of antral follicles escapes apoptosis due to the survival action of FSH (Fig. 2.2). In this period, the granulosa and theca cells continue to undergo mitosis concomitant with an increase in antrum volume, and those follicles are defined as tertiary follicles. In the tertiary follicle, the basic structure of the antral follicle has formed and no novel cells are detectable. Tertiary follicles can attain a tremendous size that is hampered only by the availability of FSH, which it is now dependent on.

Under action of an oocyte-secreted morphogenic gradient, the granulosa cells of the tertiary follicle undergo differentiation into four distinct subtypes: corona radiata, surrounding the zona pellucida; membrana, interior to the basal lamina; peri-antral, adjacent to the antrum; and cumulus oophorus, which connects the membrana and corona radiate granulosa cells together (Fig. 2.3). Each type of the cells behaves differently in response to FSH. Theca cells express receptors for luteinizing hormone (LH). LH induces the production of androgens by the theca cells, most notably androstenedione, which are aromatized by granulosa cells to produce estrogens, primarily estradiol. Consequently, estrogen levels begin to rise.

Although traditional thinking proposes a single wave of cyclic follicular recruitment and growth, recently it has been suggested that multiple waves of follicle development may occur in the human ovary [43]. The selectable follicles become more responsive to gonadotropins, but their FSH-induced aromatase remains poorly expressed [33]. Available data in humans indicate that the number of FSH receptors does not change during antral development, at least until 12 mm [44]. This does not signify that responsiveness of granulosa cells to FSH is unchanged, since mechanisms of autocrine/paracrine changes may occur [45]. Once the follicles reach antral and larger sizes, multiple intra-follicular factors are produced locally to ensure successful maturation and ovulation, such as factors produced from granulosa cells (activins, BMP-6), theca cells (BMP-2, BMP-4 and BMP-7) or both (TGF-β), or oocytes (GDF-9 and BMP-15). Those factors act alone or synergistically in human selectable follicles, having the potential to increase the FSH, and induce the proliferation of granulosa cells when FSH levels increase. The increasing follicular vascularization may also increasing the blood

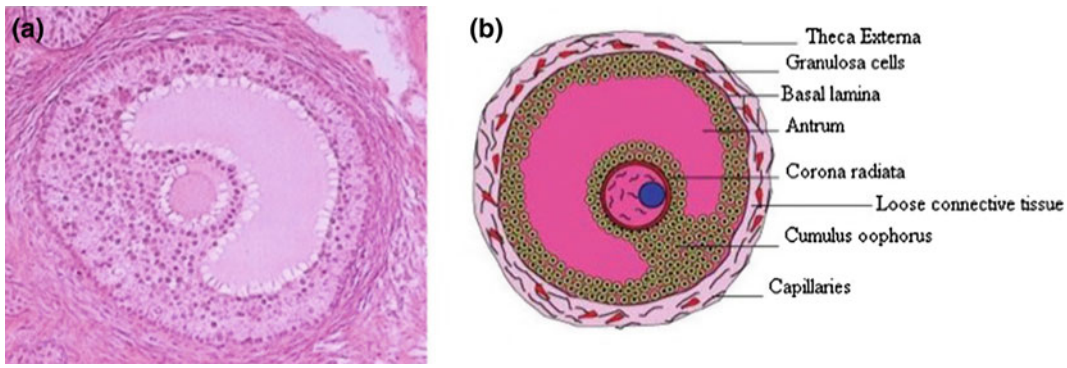

Fig. 2.3 The structure of human antral follicle. The figures were downloaded from Web site: **a** http://resources.ama.uk.com/glowm_www/uploads/1211558633_graafian_follicle.jpg; **b** http://biology4isc.weebly.com/1-human-reproduction.html

flow and then increase the amount of FSH reaching the follicle, all above mechanisms may enhance granulosa cell proliferation [46].

Dominant Follicle Selection

Follicle selection is the process by which a single dominant follicle is chosen from the recruited cohort for preferential growth [34, 47, 48]. At the time of selection, the dominant follicle continues to grow while the subordinate follicles undergo atresia [47, 49]. Divergence occurs when the dominant follicle reaches a diameter of about 10 mm on day 6–9 of the follicular phase in women [44, 47, 50, 51].

There is evidence in women that the dominant follicle has an early size advantage and the lowest FSH 'threshold' over subordinate follicles [52, 53]. Using the bovine model, it is now clear that each follicular wave is necessarily preceded by a surge in circulating FSH [54]. It has been suggested that the future dominant follicle may contain more granulosa cells and FSH receptors, making it more sensitive to FSH, compared with the subordinate follicles [55]. The increased responsiveness of dominant follicles to FSH stimulates the expression of both FSH and LH receptors in the granulosa cells of this follicle [56, 57]. The follicle destined to become dominant then has more LH receptors and the ability to respond to LH imbues the follicle with the ability to survive without FSH [54]. Additionally, this rapidly growing follicle also produces higher levels of autocrine/paracrine growth factors. Multiple studies have demonstrated the importance of insulin-like growth factors (IGFs), TGF-β super family, and other local factors in the amplification of FSH action [40, 47], which constituting a local positive selection mechanism [58, 59]. It is proposed that differential exposure to these signaling molecules may be one of the ways in which the dominant follicle is sensitized to FSH and thereby selecting for preferential growth. Another mechanism recently has been proposed for dominant follicle selection is the possible differential regulation of blood vessel formation and permeability in the theca layers of cohort follicles [60, 61].

The dominant follicle may also suppress the growth of the subordinates in the existing wave and suppresses the emergence of the next follicular wave [54]. The follicle destined for ovulation grows faster than the rest of the cohort and changes from an androgen- to an estrogen-producing structure by expressing its FSH-induced aromatase activity. Estrogens and inhibins produced by this follicle suppress pituitary FSH released during the mid-follicular phase [6]. Negative selection against subordinate follicles is a result of estrogen and inhibins, the remaining growing antral follicles are deprived of adequate FSH stimulation required for survival [62]. Subordinate follicles are not able to thrive in an environment of declining FSH and undergo atresia [11, 51, 63]. So the process of selection has been described as a phenomenon of avoiding atresia [64, 65]. Those follicles are defined as atretic follicles. Nevertheless, it does not mean that the subordinate follicles are started atresia immediately even though they were not selected to become the dominant follicle in the same wave of cohort follicular pool. The surge of LH during the menstrual cycle may trigger the atresia of those atretic follicles in the same wave of follicular pool, but it seems that the oocytes contained in those atretic follicles do not lose their developmental competence at once. However, the mechanism of atresia for those atretic follicles needs to be further confirmed.

Pre-ovulatory Follicle Development

The dominant follicle continues to develop after it is selected and reaches pre-ovulatory status at a diameter of 16–29 mm in the late-follicular phase [34, 66, 67]. In parallel with its increasing size, high proliferative activity of granulosa cells in the pre-ovulatory follicle also undergoes marked changes in steroidogenic activity [6].

The preferential growth of the dominant follicle is associated with increased aromatase activity and a rapid elevation of circulating and follicular fluid estradiol-17β [8, 43]. The process of folliculogenesis indicates that follicular responsiveness to gonadotropins increases progressively as the follicle develops from the pre-antral to pre-ovulatory stage [6]. Greater gonadotropin responsiveness in the dominant

follicle, compared with subordinate follicles, is responsible for mediating dominant follicle granulosa cell estradiol production, LH receptor expression, and continued pre-ovulatory growth [68, 69]. The dominant follicle is responsible for over 90% of the estrogen production in the pre-ovulatory period [64]. When folliculogenesis is completed, just before ovulation, the granulosa cells are highly differentiated in the pre-ovulatory follicle, having stopped to proliferate but producing high levels of steroids [6, 70].

Estradiol production from the dominant follicle peaks the day before the LH surge [2, 71] providing positive feedback at the hypothalamus and pituitary to stimulate the surge of LH necessary for inducing ovulation. The highly vascularized pre-ovulatory follicle, which has acquired LH receptors, is able to respond to the mid-cycle rise in LH [2]. Ovulation occurs, on average, within 24 h of the LH peak [62, 72]. Serum progesterone concentrations begin to rise after the pre-ovulatory estradiol peak but before the LH surge, and indicate the onset of follicular luteinization [73, 74].

Ovarian Follicular Wave Dynamics

Antral follicles 2–5 mm in diameter have been detected histologically and ultrasonographically throughout the human menstrual cycle [43]. The pattern of emergence of 2–5 mm follicles is a matter of long-standing debate. Some investigators have suggested that antral follicles 2–5 mm develop continuously, while others have proposed that 'cohorts' or 'waves' of antral follicles develop in a cyclic manner during the menstrual cycle [75]. A wave of follicular development has been defined as the synchronous growth of a group of antral follicles at regular intervals during the ovarian cycle. The traditional theory of human folliculogenesis holds that a single cohort of 4–14 antral follicles is recruited to grow in each ovary during the late luteal phase of the human menstrual cycle [1] and selection of one dominant follicle from this cohort for preferential growth in the early- to mid-follicular phase.

However, more recent results document that antral follicular growth may start in different phases of the menstrual cycle due to the balance of endocrine and intra-ovarian regulators, and selection of a dominant follicle can occur in anovulatory waves before the ovulatory follicle in women [44]. More research involving daily transvaginal ultrasonography and concurrent endocrine profiling has documented a wave pattern of antral follicle development during the menstrual cycle in women. A research has found that a cohort of 4–14 follicles 2–5 mm was recruited either two or three times during the inter-ovulatory interval in a study of 50 healthy women [8, 43]. The causes and consequences of two- or three-wave patterns are not understood clearly, but some researches found the correlate to the number of waves in an inter-ovulatory interval was the duration of follicular dominance of the first follicular wave after ovulation. Therefore, factors that influence the development of the dominant follicle of the first wave may be responsible for regulating the wave pattern. So far, it has not been established whether follicular wave dynamics are consistent within individual woman, are related with fertility, or change with age. The understanding of human ovarian folliculogenesis may have profound implications in ART and fertility preservation [76].

Follicle Atresia

In the humans, atresia causes the elimination of >90% of follicles entering the growth phase [6, 34, 77]. The phenomenon of atresia affects follicles at all stages of their development, may be considered as a normal process, which is a significant factor in determining the precise number of follicles that will ovulate in each cycle [34, 78]. Once a cohort of follicles is recruited to grow, they are destined to undergo apoptosis at the early antral stage unless rescued by survival factors. The selected follicles mature and ovulate in response to the pre-ovulatory gonadotropin surge. Following repeated cycles of recruitment, atresia, or ovulation, the follicle reserve is exhausted [79].

Changes of Follicular Morphology and Metabolism During Atresia

The healthy follicles and atretic follicles have difference between morphological criteria and follicular metabolism. Early changes of atretic follicles are irregular shape of the follicle and of the oocyte and nuclear pyknosis in the granulosa cell layers [80, 81]. The pyknosis of granulosa cells is an apoptotic process [33, 82, 83]. The first morphological evidence of apoptosis is condensation of nuclear chromatin into crescentic caps at the periphery of the nucleus, at the same time, cytoplasmic condensation results in a reduction of total cell volume and a related increase in cell density [6].

It is generally assumed that atretic follicles possess an intra-follicular androgenic milieu that distinguishes them from healthy follicles [2], and aromatase activity is poorly expressed [72]. On the contrary, the healthy follicles larger than 8 mm differ strongly from atretic follicles of similar size, and they possess aromatase. Other alterations in follicular metabolism including the appearance of lipid droplets, 3β-HSD, glucose-6-phosphate dehydrogenase, acid phosphatase, and aminopeptidase, as well as a profound decrease in the levels of lactate denydrogenase in follicular fluid of human atretic follicle [6].

Hormonal Regulation of Follicle Atresia

Ovarian follicular atresia is a hormonally controlled apoptotic process. Atresia occurs at all stages of follicle development, although follicle growth continues in the absence of circulating FSH during the pre-antral stage, and FSH is necessary for the development of follicle antrum [84, 85], because sufficient exposure of antral follicles to FSH is the most critical stimulus for the follicles to escape atresia and reach the pre-ovulatory follicle stage. During the menstrual cycle, circulating levels of FSH exhibit important variations: FSH is highest during the first half of the follicular phase and lowest during the mid-luteal phase, and during this last phase, selectable follicles exhibit their highest rate of atresia. In the absence of FSH, steroids, and growth factors [77, 84], follicles undergo atresia [72, 83, 86]. As mentioned above, the surge of LH during the menstrual cycle may trigger the rest of tertiary follicles for atresia. However, it does not mean that the oocytes obtained from the follicles during the mid-luteal phase have lower developmental competence. Another word, atresia is a long process, and the atresia of selectable follicles in the ovary may need several menstrual cycles to be completed.

Importantly, follicles can be rescued at early phases of atresia by exogenous gonadotropins [87, 88]. Gonadotropins are the major survival factors that suppress granulosa cell apoptosis through the activation of the cAMP-dependent pathway, which verifies that the signaling is via the protein kinase A pathway [89]. The apoptosis-suppressing action of gonadotropins is augmented by local factors including interleukin-1/nitric oxide, estrogens, and insulin-like growth factor-1, which in turn prevent apoptosis by activating the cGMP-dependent pathway, nuclear estrogen receptor, and tyrosine phosphorylation, respectively [79]. Another pituitary hormone, growth hormone (GH), also affects follicular growth and differentiation and often augments the action of gonadotropins [90, 91]. When tested in the follicle culture system, GH also suppresses the spontaneous onset of apoptosis [91].

Molecular Mechanisms of Follicle Cell Apoptosis

The initiation phase of apoptosis within the granulosa cells can be promoted by extrinsic factors such as cytokines [e.g., tumor necrosis factor, Fas ligand and tumor necrosis factor-related apoptosis inducing ligand (TRAIL)] or the withdrawal of growth factors and is often mediated by membrane death receptors (e.g., tumor necrosis receptor family) [92–94]. Alternatively, cell death can also be induced by intrinsic factors including oxidative stress or activation of tumor suppressor gene (e.g., p53) [11, 95].

Oxidative stress may induce the granulosa cell apoptosis via alteration of the cellular ionic environment, which activates a Ca^{2+}/Mg^{2+} -sensitive endonuclease resulting in activation of the apoptotic cascade [96, 97]. In addition, DNA damage,

such as that initiated by oxidative free radicals, may be a primary stimulus for increased p53 expression in the granulosa cells [98]. It has been suggested that p53 has the potential to amplify the negative effect of oxidative free radicals on granulosa viability. On the contrary, the apoptosis-suppressing action of gonadotropins is augmented by local factors including interleukin-1/nitric oxide, estrogens, and IGF-1, which in turn prevent apoptosis by activating the cGMP-dependent pathway, nuclear estrogen receptor, and tyrosine phosphorylation, respectively.

Although the exact signals, receptors, and intracellular signaling pathways leading to apoptosis within the granulosa cells are not understood completely, it is likely that multiple molecules include both survival (such as gonadotropins, insulin-like growth factor-1, interleukin-1, epidermal growth factor, basic fibroblast growth factor, TGF-α, bcl-2, and bcl-xlong) and atretogenic factors (TGF-β, interleukin-6, androgens, reactive oxygen species, bax, Fas antigens, p53, TNF, and caspases) are involved [82, 98–100], and these diverse hormonal signals probably converge on selective intracellular pathways (including genes of the bcl-2 and ICE families) to regulate apoptosis, the outcome depends upon a delicate balance between these molecules [79].

Oocyte Growth

Size of Oocytes and Follicles

During folliculogenesis, human oocyte grows from 35 to 120 μm in diameter [6]. Oocyte growth is interdependent with the development and differentiation of the follicles [101]. When follicles enter the growth phase, they enlarge, both by proliferation of granulosa cells and by an increase in size of the oocyte. At the end of oocyte growth, it has acquired the capacity to resume meiosis. Normally, it has been accepted the notion that the oocyte growth is already finalized at the antral stage, significantly before follicle development is completed. In fact, while for several days the antral follicle experiences a further expansion in preparation for ovulation, no increase in oocyte size is observed [102]. Early studies indicated that the size-dependent ability for meiotic competence depends not only on the sizes of the follicle and oocyte but also on the stage of the menstrual cycle.

Most mRNA and protein are synthesized during the period of oocyte growth. Meanwhile, macromolecules and organelles are produced and stored in very large amounts [103]. Normally, it is believed that the ability to complete maturation to metaphase II and developmental competence is acquired progressively with increasing follicular size. In mice, it has been reported that developmental competence is dependent on both the size of the follicle and the size of oocytes [104]. It has been reported that the human oocyte has a size-dependent ability to resume meiosis from 90 to 120 μm in diameter [105], non-full-size oocytes should not be considered when assessing developmental competence, because the non-full-size oocytes have less products (mRNA and protein) stored in the cytoplasm during oocyte growth. The relationship between oocyte size and methylation imprints could indicate that imprint establishment requires the accumulation of proteins involved in the enzymatic process, in support of this hypothesis, the expression of the DNA methyltransferase genes, Dnmt3a, Dnmt3b, and Dnmt3L, peaked in oocytes from postnatal day 15 ovaries [106].

In clinical treatment, the use of gonadotropins has resulted in asynchrony of follicular development [2]. The size of the leading follicle seems do not affect the fertilization and cleavage rates of cohort oocytes from gonadotropin stimulated cycles [107]. However, it has been reported that fertilization rates are lower in oocytes obtained from the size of follicles <10 mm in diameter than in those retrieved from larger follicles [108]. It must be noted that immature oocytes are retrieved frequently after human chronic hormone (HCG) administration even from the size of follicles >10 mm in diameter, and these immature oocytes can be matured and developed in vitro following in vitro fertilization [109]. Importantly, some observations suggest that

germinal vesicle (GV) stages of oocytes obtained from antral follicles are apparently morphologically similar, but in fact, they can be developed differently. This has important implications for human IVM because it underpins the concept that meiotic failure can reflect an intrinsic oocyte characteristic, irrespective of the ability of current IVM systems to fully support maturation.

Mechanism of Oocyte Maturation

Follicle development is accompanied by a parallel phase of oocyte growth, by which the oocyte reaches full size in preparation for fertilization and pre-implantation development. Fully growing oocytes prepare for fertilization and embryonic development by accumulating essential maternal materials and by undergoing genomic modifications during oocyte growth. The fully growing oocytes can be entered to maturation process in vivo by LH surge. Normally, the oocyte maturation can be divided into nuclear and cytoplasmic maturation, as well as membrane maturation and genomic maturation. Nuclear maturation refers to the resumption of meiosis and progression to metaphase II. Cytoplasmic maturation is a term that refers to preparation of oocyte cytoplasm for fertilization and embryonic development [109, 110]. Oocyte membrane maturation can be referred the process of the oocytes need to be primed with steroids to develop Ca^{2+} oscillations during maturation [111, 112]. Genomic maturation refers to the establishment of the correct epigenetic status. Modifications of the oocyte chromosome complement and rearrangements of cytoplasmic components are also crucial for the achievement of developmental competence. Several factors determine the ultimate competence of the oocyte, and these have been investigated and attempts made to mimic these conditions in vitro.

The details of mechanism for oocyte maturation will be discussed in Chap. 3; therefore, here we will briefly mention about the mechanism of oocyte maturation.

Nuclear Maturation

Nuclear maturation is defined as to the resumption of meiosis and completion of the first meiotic division from the GV stage to metaphase II (M-II). The oocyte acquires developmental competence during its growth within the follicle, and then, the pre-ovulatory surge of LH in vivo initiates germinal vesicle breakdown (GVBD). The mechanisms involved in GVBD are not fully understood. For many years, different lines of evidence generated mainly in the mouse model have indicated cAMP as the fundamental factor by which meiotic arrest is ensured before ovulation. It has been hypothesized that breakdown in gap junctional communication between the oocyte and granulosa cells at the time of the pre-ovulatory LH surge results in a decrease in cAMP levels within the oocyte, leading to the inactivation of the PKA pathway [110, 111, 113].

Many potential factors mediate the cumulus cell control of GVBD. The gap junctions permit regulatory molecules, such as steroids, Ca^{2+}, inositol 1,4,5-trisphosphate, cAMP, and purines, to pass freely between the cytoplasm of the oocyte and cumulus cells [114]. Some elements of this regulatory network (e.g., NPPC and EGF family members) have been identified in the mouse model and are under close scrutiny as candidates for the development of more advanced IVM systems [115]. Protein synthesis is needed for the progression of oocytes from the GV stage to M-II [114, 116, 117], as well as for the maintenance of M-II arrest [118]. Cytoplasmic proteins, maturation promoting factor (MPF), and cytostatic factor (CSF) regulate oocyte nuclear maturation [119]. Inhibition of protein synthesis in oocytes results in failure to activate MPF activity [120].

Throughout oocyte growth, prophase arrest was thought to be correlated with low levels of cell cycle regulatory proteins, such as MPF. Molecular characterization of MPF has shown that active MPF is a protein dimer composed of catalytic p34*cdc2* serine/threonine kinase, and regulatory cyclin B subunits [121]. The p34*cdc2* serine/threonine kinase is the product of the *cdc2* gene, and the p34*cdc*-cyclin heterodimer, a

protein kinase, has four phosphorylation sites that are regulated by kinase and phosphatase activities. The concentration of cyclin increases steadily through interphase, peaks at the G2/M phase transition, and falls precipitously at each mitosis. Cyclins have been divided into three classes, G1, A, and B, based on their amino acid similarity and timing of their appearance during the cell cycle [122]. Two isoforms of cyclin B have been described in the mouse [123]. The expression patterns of cyclin B1 and B2 differ, with the cyclin B1 isoform predominantly expressed in the oocytes. Cyclin B is phosphorylated and dephosphorylated during oocyte maturation [124]. It is known that the product of the *c-mos* proto-oncogene is a protein–serine/threonine kinase and has the same effect as CSF. The product of *c-mos* is expressed early in oocyte maturation and disappears immediately after fertilization [125]. Therefore, M-II arrest may be due to the transcription of *c-mos* as the oocyte mature.

Mitogen-activated protein kinase (MAPK) has been revealed as central to the regulation of meiotic arrest in oocytes. MAPK is also a serine/threonine kinase but is activated, not inhibited, by tyrosine phosphorylation. Activation of MAPK precedes activation of p34*cdc2*. Blocking MAPK activity prevents GVBD. However, MAPK is not necessarily required for GVBD in mouse oocytes [126]. A product of *c-mos* stimulates MAPK activity, but does not activate p34*cdc2* [127, 128]. The phosphorylation cascade of *c-mos* product and MAPK may play an important role in meiotic and mitotic cell cycles. In humans, MAPK is inactive in immature oocytes, active in mature oocytes, and the activity decreases after pronuclear formation after fertilization [129]. However, the mechanisms involved in GVBD, as well as the cell signaling pathways driving the oocyte into M-II in response to pre-ovulatory LH surge, are not fully understood [110].

Cytoplasmic and Membrane Maturation

Cytoplasmic maturation is a term that refers to preparation of oocyte cytoplasm for fertilization and embryonic development [87, 130]. RNA molecules, proteins, and imprinted genes are accumulated in the oocyte cytoplasm during its growth phase and are used to sustain the early phase of embryonic development before embryo DNA transcription begins [9, 33]. Rapid initiation of expression and high rates of transcription and translation during oocyte growth and folliculogenesis are followed by differential translation silencing and degradation of many mRNA species at the time of ovulation [131], so the oocyte and early pre-embryo are dependent upon the pool of mRNA and protein accumulated during the pre-ovulatory period [132]. Some maternal transcripts are even stored after the maternal to embryonic transition of gene expression has been completed [133]. Cytoplasmic factors of the oocyte may be responsible for maternal effects on de novo methylation and gene expression [99]. It is also known that insufficient cytoplasmic maturation of the oocyte will fail to promote male pronuclear formation and will thus increase chromosomal abnormalities after fertilization [134].

During oocyte growth, acute activation of a variety of signal transduction pathways and opening of ion channels has been observed in target cells within a few minutes of steroid exposure [135]. Many of these rapid steroid actions are non-genomic and initiated at the surface of the target cell by binding to membrane receptors [136]. It has been suggested that estrogen may act at the oocyte surface by producing changes in reactivity of its Ca^{2+} release system during cytoplasmic maturation [137, 138]. Oocytes need to be primed with estradiol to develop Ca^{2+} oscillations during maturation. Therefore, the process can be referred as oocyte membrane maturation [111, 112].

The preferential growth of the dominant follicle is associated with increased aromatase activity and a rapid elevation of circulating and follicular fluid estradiol-17β. The actions of estrogen are mediated through binding specifically to nuclear estrogen receptors, ligand-activated regulatory proteins that act as dimers on specific target genes containing defined DNA sequences called estrogen response elements [139]. Estrogen receptor binding to estrogen

response elements can result in induction or suppression of responsive genes. Therefore, estrogen may be involved in the events of cytoplasmic maturation of the oocyte.

It seems that oocytes also require a specific intra-follicular progesterone environment for the inductive signals of cytoplasmic and membrane maturation, because pre-ovulatory follicular fluid contains certain concentrations of progesterone [140, 141]. Besides non-genomic effects of progesterone, the actions of progesterone are mediated through binding specifically to nuclear progesterone receptors [142]. The maturation of granulosa cells is associated with stimulation of the phosphatidylinositol pathway, involving the mobilization of intracellular Ca^{2+} and an increase in protein kinase C, which together stimulate a reduction in progesterone. Therefore, oocyte maturation is associated with a shift from estradiol to progesterone production by the granulosa cells; it is possible that progesterone may be involved in the development of an oocyte membrane Ca^{2+} release system. However, the role of progesterone in the oocyte cytoplasmic and membrane maturation is not fully understood.

Endocrine control of oocyte growth by gonadotropins rests on a network of intra-follicular paracrine interactions. Normally, it has been thought that FSH is essential for ovarian follicular development, whereas LH is primarily responsible for ovulation and transformation of follicles into the corpus luteum. Although the importance of gonadotropins in gonadal development and reproductive function has been established, the mechanism of gonadotropins on follicle growth and oocyte maturation is not fully understood. In the ovary, FSH binds to FSH receptors located on mural granulosa cells and acts via the cAMP-dependent protein kinase pathway. In the follicle, the enhanced FSH responsiveness of pre-ovulatory follicles also appears to result from an increase in the content of the stimulatory G protein of the adenyl cyclase system [120, 143]. The induction of LH receptors by FSH is one of the hallmarks of the differentiating mural granulosa cells [119]. Theca cells constitutionally contain LH receptors. LH is capable of stimulating androgen substrate production from theca cells into FSH-stimulated granulosa cells to transform estrogen [144] and that the thecal layer is the major cellular source of follicular androgen. In addition, LH is thought to stimulate progesterone production of mural granulosa and cumulus cells in pre-ovulatory follicles [145, 146]. LH may synergize with FSH to sustain follicle development as well as to prepare it for the mid-cycle LH surge that triggers ovulation [147].

Oocyte–cumulus cell interactions are recognized as a founding element of oocyte maturation. Cumulus cells respond to gonadotropins and are known to secrete various substances. The protein synthesis pattern is different between oocytes with and without cumulus cells, and FSH modulates the protein synthesis pattern of cumulus cell-intact oocytes [148]. In fact, cumulus cells support a wide variety of functions of the maturing oocyte, including metabolism, meiotic arrest and resumption, and cytoskeletal rearrangements [58, 111]. Five types of interactions are recognized in the germinal-somatic regulatory loop: (1) direct contact-mediated signals in the absence of intercellular junctions, (2) typical ligand–receptor interactions, (3) paracrine signaling pathways, (4) gap junctions and other junctional contacts via transzonal projections (TZPs), and (5) receptor tyrosine kinases (RTKs) [149]. We have known that some intra-follicular paracrine factors secreted by oocytes and somatic cells regulate many important aspects of oocyte and follicular development, for example, the TGF-β family members GDF-9 and BMP-15; these agents are profoundly involved in oocyte–granulosa cell regulatory loops at early stages of oogenesis, and their action extends to the maturation phase [150]. This is also the evidence which supports a model for bidirectional communication, and oocyte growth is not just a reaction to stimulation, but it is driven by the oocyte itself. Oocytes and cumulus cells are dependent on each other for growth and survival throughout the different stages of follicular development [151].

Epigenetic Modification

An important aspect of oocyte maturation is the establishment of the correct epigenetic status. A number of imprinted genes are essential for fetal growth and development, including the functioning of the placenta. These genes are expressed in a parent-of-origin specific manner, as a result of the different epigenetic profiles acquired by imprinted genes during male and female gametogenesis. DNA methylation imprints are acquired progressively during the oocyte growth phase, as follicles progress from the primary to the antral stage, the methylation of specific genes is established at different stages in oocyte growth [152, 153]. Some researches suggest that primary maternal imprints are not yet established in immature oocytes [154]. Cytoplasmic factors of the oocyte are also dependent on maternal genetic background and may be responsible for maternal effects on de novo methylation and gene expression [99]. It is possible that certain methylation imprints are more susceptible to perturbation as a result of assisted reproductive technologies (ARTs) [155, 156]. A growing amount of evidence suggesting an association of imprinting disorders with ARTs highlights the need for further study of epigenetic defects associated with infertility treatments [106, 157].

Conclusion

Follicular development and oocyte growth are two different events that occurred at the same time during folliculogenesis. Folliculogenesis is a long process from primordial follicle, pre-antral (primary and secondary) follicle, to antral follicle (early tertiary follicle) and pre-ovulatory (late tertiary follicle) follicle stages. The duration of development from the primary follicles to the secondary follicles is required for about 120 days, and the development from the secondary follicles to the antral follicles is needed for approximately 71 days, whereas only 14 days are inquired to development from the antral follicles to a pre-ovulatory follicle (Fig. 2.4). The antral follicles that about 2–5 mm in diameter are presented throughout the menstrual cycle in both follicular and luteal phases. In a natural menstrual cycle, only one antral follicle will be selected as dominant follicle ($\geq$12 mm in diameter) for ovulation finally while all others will be undergone atresia.

The traditional theory of human follicular development indicates that a single wave of several antral follicles is recruited to grow in each ovary during the late luteal phase of the menstrual cycle and selection of one dominant follicle from this cohort wave for preferential

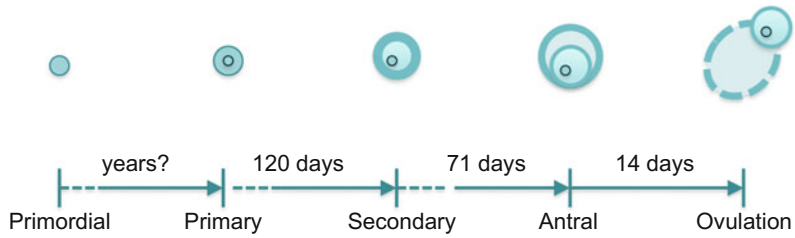

Fig. 2.4 Duration of follicular development in human ovary. The primordial follicles undergo initial recruitment to enter the growing pool of primary follicles; however, it is not clear that how long is the duration required for this step, in which maybe months or years are needed. More than 120 days are required for the development from the primary follicles to reach the secondary follicle stage, and 71 days are needed to growth from the secondary to the early antral follicle stage. Although from an antral follicle (2–5 mm in diameter) to pre-ovulatory follicle only takes 14 days for development and ovulation, there were several subordinating follicles developed throughout the menstrual cycle. Based on [158] with modification

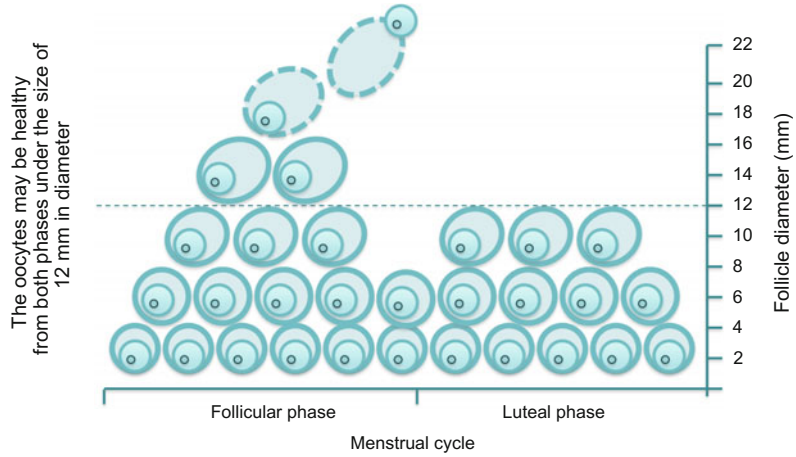

Fig. 2.5 Follicular development and atresia during menstrual cycle. The antral follicles that about 2–5 mm in diameter are presented throughout the menstrual cycle in both follicular and luteal phases. In a natural menstrual cycle, only one antral follicle will be selected to dominant follicle (≥12 mm in diameter) for ovulation finally while all others will be undergone atresia. Since the development of follicles from pre-antral to antral stage (2–5 mm in diameter) is required for approximately 70 days; therefore, the existing antral follicles may be appeared in several menstrual cycles. Although un-ovulating follicles will be undergone atresia eventually in the ovaries, the oocytes may be healthy when they were retrieved from the follicles under 12 mm in diameter in both follicular phase and luteal phase, unless the retrieved cumulus–oocyte complexes (COCs) showed degenerated morphology and the oocytes appeared with smaller size

growth in the early- to mid-follicular phase. Although it has been documented that a cohort of antral follicles in 2–5 mm diameter was recruited either two or three times during the inter-ovulatory interval in a study, the factors that influence the development of the dominant follicle of the first wave may be responsible for regulating the wave pattern. It is important to note that the oocytes retrieved from both follicular and luteal phases under 12 mm in diameter are healthy unless the size of oocytes was smaller than 120 μm, in which those oocytes were undergone for atresia already (Fig. 2.5).

During folliculogenesis, human oocyte grows from 35 μm to 120 μm in diameter. It is a common belief that the oocyte growth is already finalized at the antral stage, significantly before follicle development is completed. The concepts of follicular maturation and oocyte maturation are two different processes. Mature follicle is defined as the follicle developed to antral follicle stage, which contains immature oocyte inside. Oocyte maturation refers the oocyte completion of the second meiosis from GV stage to M-II stage following LH surge in pre-ovulatory in vivo. In natural cycles, although the existence of dominant follicle does not affect the oocyte development competence that retrieved from the subordinate follicles, it seems that there is lower developmental capacity for oocytes obtained from the smaller size of follicles than those retrieved from larger follicles.

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A New Understanding on the Regulation of Oocyte Meiotic Prophase Arrest and Resumption

3

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Meiotic Arrest

In mammalian germ cells of females, oocytes, meiosis is initiated during fetal life, but the process is arrested at the diplotene stage of the first meiotic prophase for a prolonged period [1], morphologically identified by a characteristic nucleus commonly known as the germinal vesicle (GV) with a prominent nucleolus and is associated with partial condensation of the chromosomes [1]. The prophase arrest of oocytes within preantral follicles is caused by inherent factors in the oocyte and correlates with low levels of activity by cell cycle regulatory proteins [2]. Once the growing oocyte reaches its full size and an antral space begins to form to divide the granulosa cells into two separate compartments, mural granulosa cells (MGCs) form the outer layers and the cumulus cells surround the oocyte, and the oocyte acquires the ability to complete meiosis [3–5]. However, these meiotically competent oocytes maintain meiotic prophase arrest from the early antral to preovulatory stages until the preovulatory surge of luteinizing hormone (LH) from the pituitary gland triggers the resumption of meiosis and ovulation during the estrous or menstrual cycle [6–8]. The mature oocytes (eggs) are then available for fertilization within the oviduct. Germinal vesicle breakdown (GVB) is the first change occurring and is widely used as an endpoint for assessing meiotic resumption or oocyte maturation started [9] (Fig. 3.1).

Oocyte Maturation Inhibitor

In 1935, it was discovered that oocytes or cumulus–oocyte complexes (COCs) could resume meiosis spontaneously without hormonal stimulation when they were liberated from rabbit antral follicles and cultured under simple nutritionally supportive conditions [10]. This original observation is confirmed by numerous studies with most mammalian, including human [11]. Interestingly, the time course of spontaneous maturation is similar to that of LH stimulation in vivo [11]. These observations lead to general acceptance of the hypothesis that the follicular granulosa cells prevent precocious resumption of meiosis until LH initiates oocyte maturation before ovulation. This hypothesis is further corroborated by the studies that co-culture of oocytes with follicular granulosa cells, granulosa cell extract and follicular fluid inhibits oocyte spontaneous maturation [12]. For a long time, many studies focus on identifying the factors participating in the maintenance of meiotic arrest and lead to partial characterization and purification of a factor, oocyte maturation inhibitor (OMI), from follicular fluids [13]. This OMI is a peptide of low molecular weight (~2000 Da), action on cumulus cells without species

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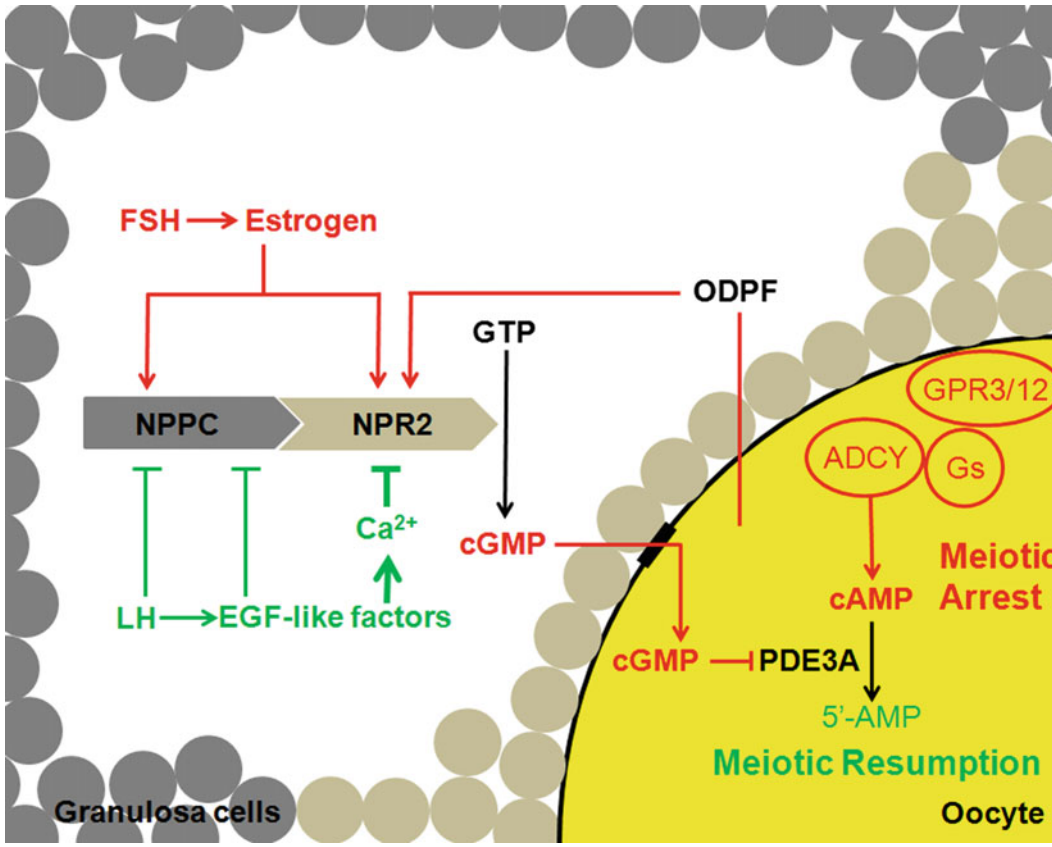

Fig. 3.1 The regulation of oocyte meiotic prophase arrest and resumption in mammals. NPPC produced by mural granulosa cells stimulates the generation of cGMP by NPR2 of cumulus cells. The cGMP then diffuses into oocytes and maintains meiotic prophase arrest by inhibiting oocyte-specific PDE3A activity and cAMP hydrolysis. Intraoocyte cAMP is produced by GPR3/12 activation of ADCY endogenous to the oocyte. Oocyte itself also promotes cumulus cell expression of NPR2 to elevate cGMP levels for meiotic arrest. FSH, through estrogen, enhances NPPC/NPR2 expression to ensure meiotic arrest during antral follicular development. LH-induced EGF-like growth factors decrease NPPC content and NPR2 activity, resulting in cGMP decrease and meiotic resumption. *NPPC* natriuretic peptide type C; *NPR2* natriuretic peptide receptor 2; *cGMP* cyclic guanosine 3′,5′-monophosphate; *cAMP* cyclic adenosine 3′,5′-monophosphate; *GPR3/12* G-protein-coupled receptor 3 and 12; *ADCY* adenylyl cyclase; *ODPF* oocyte-derived paracrine factors; *PDE3A* phosphodiesterase 3A; *FSH* follicle-stimulating hormone; *LH* luteinizing hormone; EGF-like factors, epidermal growth factor-like factors

specificity [12]. The inhibitory effect of OMI can be overcome by the addition of LH, supporting that OMI has a physiological role in the regulation of meiosis [12].

Cyclic Nucleotides

Meiotic arrest depends on a high level of cyclic adenosine 3′,5′-monophosphate (cAMP). Cyclic AMP is produced within oocytes by a constitutive activation of heterotrimeric G-protein (Gs)-coupled receptor GPR3 and GPR12 to stimulate adenylate cyclase (ADCY) [14–18] and is sustained by cyclic guanosine 3′,5′-monophosphate (cGMP) inhibiting cAMP-specific phosphodiesterase 3A (PDE3A) activity in oocytes [19, 20]. Inability to sustain oocyte cAMP concentrations leads to precocious gonadotropin-independent resumption of meiosis, which interrupts the synchrony between oocyte maturation and ovulation and compromises female fertility [15, 16, 21].

Cyclic AMP

Intraoocyte cAMP controls meiosis Intraoocyte cAMP plays a central role in the regulation of meiosis [22]. The sustained high levels of cAMP are essential for the maintenance of meiotic arrest of fully grown oocytes [1, 23, 24], and a drop in intraoocyte levels of this nucleotide is required for resumption of meiosis [1, 25, 26]. When the oocytes are released from the antral follicle, they resume meiosis spontaneously in parallel with decreases in cAMP levels [27–29]. The pharmacological increase of intraoocyte cAMP levels prevents LH-induced meiotic resumption in vivo [30] and spontaneous maturation in vitro [24, 31, 32].

High levels of cAMP within the oocyte activate protein kinase A (PKA), which in turn phosphorylates (and activates) the kinase WEE1B/myelin transcription factor 1 (MYT1). In addition, PKA-mediated phosphorylation of the phosphatase cell division cycle 25 (CDC25) results in its cytoplasmic retention. The combined action of these two PKA substrates, inhibiting CDC25B and activating WEE1B/MYT1, insures low levels of cyclin-dependent kinase 1 (CDK1) activity, rendering maturation-promoting factor (MPF, a complex of CDK1 and cyclin B) inactive such that the oocyte maintains meiotic arrest [2, 22, 32, 33]. The decrease in oocyte cAMP triggers maturation by alleviating the aforementioned phosphorylations of WEE1B/MYT1 and CDC25B [2]. Meiosis arrest female 1 (MARF1) expressed in oocytes is critical for the activation of MPF, possibly by downregulating the expression of protein phosphatase 2, catalytic subunit, betaisozyme (*Ppp2cb*) [34].

Cyclic AMP synthesis A long-standing hypothesis is that cAMP is generated by somatic cells and diffuses into oocytes through heterologous gap junctions between cumulus cells and oocytes [9, 35–37]. Recent studies using knockout mice and microinjection of inhibitory factors confirm that oocyte itself produces sufficient cAMP for meiotic arrest [14–18]. In mouse oocytes, cAMP is produced mainly by GPR3-Gs-ADCY3 pathway. Depletion of GPR3 and ADCY3, or microinjection of inhibitory Gs antibody into follicle-enclosed oocytes, results in precocious resumption of meiosis [14, 15, 38]. However, GPR3 knockout mice are fertile in young animals [21], indicating additional pathway(s) for generation and maintenance of a sufficient cAMP level. In human, GPR3 expressed in oocytes is the predominant receptor signaling for meiotic arrest [39], but in rat, GPR12 is the predominant receptor signaling for meiotic arrest for that downregulation of GPR12 causes meiotic resumption [16].

Taken together, oocyte cAMP is essential for maintaining meiotic arrest and is generated by oocyte ADCY, which is produced by the constitutive action of GPR3 and GPR12 via Gs protein [16, 32]. Although the transfer of cAMP from surrounding cumulus cells to oocytes is possible, it is not sufficient by itself to maintain meiotic arrest [32, 40]. Currently, the natural ligand(s) for GPR3 and GPR12 remain unknown. It has been reported that the GPR3 is most closely related to lipid and peptide receptors [41]. Furthermore, incubation of mouse oocytes with sphingosine 1-phosphate (S1P) and sphingosylphosphorylcholine (SPC) has been shown to delay spontaneous oocyte maturation [16], indicating that a lipid may stimulate GPR3 and GPR12.

Cyclic AMP degradation Intraoocyte cAMP levels depend on the synthesis by GPR3/12 and the degradation by an oocyte-specific phosphodiesterase (PDE) 3A [15, 30, 42–45]. Maintenance of meiotic arrest is associated with undetectable cAMP-PDE activity [31, 46, 47], and inhibition of PDE3 activity elevates intraoocyte cAMP and prevents the resumption of meiosis in many species including human [2, 48]. Moreover, genetic ablation of PDE3A causes complete meiotic arrest either after an LH surge or COCs culture in vitro and female sterility [47, 49]. Depleting both of GPR3 and PDE3A genes allows spontaneous meiosis resumption in vivo as depletion of GPR3 alone, and the increases in cAMP levels have not been detected in oocytes isolated from those double-knockout mice [40], suggesting that PDE3A is downstream of GPR3 in regulating intraoocyte cAMP levels. These

studies demonstrate that intraoocyte cAMP levels are regulated via control of PDE3A-mediated degradation, rather than endogenous synthesis. Inhibition of PDE3A activity is essential for sustaining elevated cAMP levels that maintain meiotic arrest, and activation of PDE3A is required to promote the cAMP degradation that initiates meiotic resumption.

Cyclic GMP

As early as 1980, it is found that cGMP levels are highest at diestrus but lowest during estrus or LH stimulation in hamster ovary [50], suggesting a functional relationship between cGMP levels and meiosis. Many studies show that cGMP is involved in the regulation of oocyte maturation. The blockade of inosine monophosphate dehydrogenase (needed for cGMP production) causes meiotic resumption in follicle-enclosed oocytes [51]. Intraoocyte cGMP levels decrease during oocyte spontaneous maturation [27], but increase of cGMP levels by 8-Br-cGMP or cGMP-specific PDE5 inhibitor suppresses meiotic resumption [28, 52, 53].

The microinjection of a cGMP-specific PDE5 into oocytes causes meiotic maturation of wild-type oocytes, but this effect is absent in PDE3A-deficient oocytes [20], suggesting the inhibitory effect of cGMP on oocyte maturation through the regulation of PDE3A activity. It is reported that cGMP has an inhibitory effect on PDE3A activity. Cyclic GMP and cAMP-binding regions in PDE3A are overlapping but not identical [54], and GMP inhibits PDE3A activity via completion with cAMP in the hydrolysis process [55]. The concentration of cGMP in GV-stage oocytes isolated from equine chorionic gonadotropin (eCG)-primed immature mice is sufficient to inhibit PDE3A activity [20, 27, 54]. Guanylyl cyclase agonists have inhibitory effects on spontaneous meiotic resumption in COCs, but not in isolated oocytes [28, 56], suggesting that the oocyte depends on the somatic cells for its supply of cGMP. Further studies showed that cGMP, produced by somatic cells, diffuses through the gap junction network to the oocyte and inhibits PDE3A activity [19, 20]. This inhibition sustains a high level of cAMP in the oocyte for meiotic arrest. Thus, the production of cGMP in the somatic cells is a critical component required to maintain prophase I arrest.

NPPC

Cyclic GMP can be produced by two distinct classes of guanylyl cyclases, soluble and particulate, activated by nitric oxide (NO) and natriuretic peptides, respectively [57–59]. NO is a chemical messenger enzymatically produced by three isoforms of nitric oxide synthases (NOS): endothelial (eNOS), neuronal NOS (nNOS) and inducible isoform (iNOS) [59]. Although these three isoforms of NOS have been detected in mammalian ovaries [60], a high concentration of NO donor sodium nitroprusside (1 mM) has an inhibitory effect on mouse oocyte spontaneous maturation [61]. However, knockouts of *Nos1* and *Nos2* affect ovulation [62, 63], and knockout of *Nos3* appears to impair oocyte development [64]. The role of NO appears to be mainly in the control of ovulation and not in the regulation of meiotic arrest.

The natriuretic peptide system forms a family of three structurally homologous but genetically distinct endogenous ligands: type A (NPPA, also known as ANP), type B (NPPB, also known as BNP) and type C (NPPC, also known as CNP) [57]. In general, NPPA and NPPB activate particulate guanylate cyclase natriuretic peptide receptor 1 (NPR1, also known as GC-A, NPRA), and NPPC activates receptor NPR2 (also known as GC-B, NPRB) [65]. NPPA and NPPB are cardiac hormones that are predominantly synthesized in atrial and ventricular cardiomyocytes, respectively, and play important roles in the regulation of cardiovascular homeostasis [65]. NPPA is reported to slightly inhibit spontaneous meiotic resumption of rat oocytes [28]. However, *Nppa* and *Nppb* mRNA could not be detected by in situ hybridization [66] and by specific riboprobes in mouse ovary [67]. Also, the expression of *Npr1* transcription in cumulus cells is very low by real-time PCR analysis [66]. All these results

indicate that NPPA, NPPB and their receptor NPR1 do not seem to be the crucial mechanism of meiotic arrest.

NPPC, on the contrary, is expressed in a wide variety of central and peripheral tissues and acts locally as autocrine and paracrine regulator but little natriuretic activity [68]. NPPC and its guanylyl cyclase receptor NPR2 are present in rat granulosa cells and show coordinate estrous cycle-dependent variation with maximal expression at proestrus [69]. Further studies show that *Nppc* mRNA is expressed predominantly by mouse MGCs, which line the inside of the follicular wall, and, in contrast, *Npr2* mRNA is expressed predominantly by cumulus cells surrounding the oocyte [70]. Application of NPPC to the culture medium prevents spontaneous meiotic resumption of oocytes that are surrounded by cumulus cells, and increases intraoocyte cGMP and cAMP levels [70]. NPPC has no inhibitory effect on denuded oocytes for the lack of NPR2 receptors. Importantly, meiosis resumes precociously in oocytes within antral follicles of *Nppc* and *Npr2* mutant mice [70, 71]. Disruption of gap junctions by isoform-specific connexin mimetic peptides indicates that both connexin-43 (GJA1) and connexin-37 (GJA4) gap junctions are required for NPPC-mediated meiotic arrest [72]. Thus, NPPC produced by follicular MGCs stimulates the generation of cGMP by cumulus cells NPR2, which diffuses into oocyte via gap junctions and maintains meiotic arrest by inhibiting PDE3A activity and cAMP hydrolysis [20, 70, 73, 74]. In mammals, meiotic maturation of oocytes must be coordinated precisely with ovulation to produce a developmentally competent egg at the right time for fertilization. Therefore, NPPC/NPR2 stimulates production of cGMP for preventing premature meiosis in oocytes, which is critical for maturation and ovulation synchrony and for normal female fertility. Inappropriate decrease of NPPC and NPR2 in the growing follicles reduces oocyte developmental capacity and so fertility [21, 75, 76].

For a long time, it has been suggested that MGCs original OMI, acting on cumulus, maintains meiotic arrest. It is interesting that MGC-derived NPPC has the character similar to the OMI: a 22 amino acid residues peptide with molecular weight of 2197.6, action via NPR2 by cumulus cells, and identical sequences among mouse, rat, pig and human [70, 77]. NPPC from human shows the inhibitory effect on the maturation of oocytes from mouse [70], rat (unpublished data), pig [78, 79] and cattle [76]. Furthermore, LH can overcome the inhibitory effect of NPPC [80], supporting the concept that NPPC may be the 'OMI' responsible for meiotic arrest in mammal oocytes. It is reported that porcine NPPB (pNPPB) shows a high affinity for NPR2 in the cells from pig, human, rat and mouse to produce the functional effect [65, 81]. pNPPB also shows a inhibitory effect on spontaneous oocyte maturation of pig and mouse [66, 78, 79]. Clearly, this relationship between pNPPB and native or non-native NPR2 activation needs further study. A better understanding of the factors that maintain an oocyte in meiotic arrest may help in the development of strategies to improve culture conditions with particular regard to the quality of cytoplasmic maturation [76, 82, 83].

FSH

As noted above, NPPC/NPR2-produced cGMP is essential for maintaining meiotic arrest of oocytes within antral follicles. Thus, stimulating the expression of NPPC/NPR2 during follicular development is required to maintain oocyte meiotic arrest. During each estrous cycle, some early antral follicles are 'recruited' by follicle-stimulating hormone (FSH) stimulation from the pituitary to continue growing and develop into preovulatory antral follicles (Graafian follicles) [84, 85]. Interestingly, NPPC/NPR2 levels in rat ovary vary during the estrous cycle and are maximal at proestrus [69]. The treatment of equine chorionic gonadotropin (eCG), a glycoprotein hormone that possesses primarily FSH activity, stimulates *Nppc* mRNA expression in mouse MGCs and increases NPPC content in the ovaries [80, 86–88]. The expression of *Npr2* mRNA in both MGCs and cumulus cells is also

increased after eCG treatment [86, 87]. However, FSH cannot stimulate the expression of *Nppc* and *Npr2* mRNA in MGCs and cumulus cells cultured in vitro [87, 88], suggesting that FSH activity of eCG induces the expression of NPPC/NPR2 indirectly. The distinct physiological action of FSH is to stimulate follicular growth [89] although FSH can stimulate oocyte maturation of COCs cultured in vitro [53, 90]. Thus, the increasing expression of NPPC/NPR2 insures their ability to prevent oocyte precocious maturation during FSH-stimulated antral follicular development. Inappropriate expression of NPPC/NPR2 in the growing follicles may disrupt meiotic arrest and normal follicular development [91, 92].

Estrogen

Mammalian follicular development is associated with increased production of estrogen by MGCs through FSH or eCG-stimulated aromatization of testosterone [89], which partially mediates FSH action [93–95]. The synthetic estrogen diethylstilbestrol (DES) stimulates *Nppc* and *Npr2* mRNA expression in rat ovary in vivo [96]. In vitro, 17β-estradiol (E2) raises the levels of *Nppc* and *Npr2* mRNA in cultured mouse MGCs [88]. E2 also promotes expression of *Npr2* mRNA by cumulus cells, thereby augmenting NPPC ability to produce cGMP and maintain meiotic arrest of COCs cultured in vitro [87]. E2-promoted *Nppc* mRNA expression can be enhanced by interaction with FSH [88]. Testosterone promotes *Npr2* mRNA expression by cumulus cells of cultured COCs possibly due to aromatization of testosterone to estrogens [87]. All these results implicate the physiological role of estrogens is involved in maintaining oocyte meiotic arrest through inducing the expression of NPC/NPR2. However, there is no indication from published reports that the oocytes within antral follicles show precocious resumption of meiosis after estrogen receptors or *Cyp19α1* (aromatase) deletions [97–101]. Thus, other pathways could participate in compensation for the absence of estrogens, in the mechanisms maintaining meiotic arrest in vivo. Optimal fertility requires synchrony in the regulation of oocyte meiotic events and ovulation [15, 16, 21]. It would not be surprising, therefore, if compensatory or redundant mechanisms evolved into interacting pathways that maintain meiotic arrest and assure this essential synchrony.

Oocyte-Derived Paracrine Factors

An increasing body of evidence indicates that MGCs have important endocrine functions, and oocyte-derived paracrine factors (ODPFs) profoundly affect the differentiation of cumulus cells [102]. Higher expression of *Npr2* mRNA in cumulus cells than in MGCs implies that ODPFs promote its expression [102]. Indeed, removing oocytes from follicles reduces *Npr2* mRNA expression in cumulus cells, but culturing these cumulus cells with denuded oocytes restores its expression [70]. Many studies have focused on oocyte-secreted transforming growth factor-β (TGF-β) superfamily members, in particular growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15; also called GDF9B) and fibroblast growth factor 8B (FGF8B) [103]. Each of these three ODPFs slightly promotes expression of *Npr2* mRNA by cumulus cells in vitro, and combinations of three proteins restore *Npr2* mRNA expression in isolated cumulus cells [18, 70]. It is surprised that ODPFs also stimulate *Nppc* mRNA expression in cumulus cells [88] although the levels of *Nppc* mRNA in cumulus cells are very lower compared with that in MGCs [70]. Although ODPFs can promote the production of estradiol by cumulus cells [104, 105], it is unlikely that ODPFs promote NPPC/NPR2 expression in cumulus cells by estradiol [87]. ODPFs are suggested to act on cumulus cells by the activation of Smad (Sma and Mad-related protein) signaling pathway [102, 106]. Knockout of Smad4, the central component of the canonical TGF-β signaling pathway, reduces *Nppc* and *Npr2* expression in both MGCs and cumulus cells, and maintaining oocyte meiotic arrest is weakened [92]. All these results suggest that ODPFs-induced NPPC/NPR2

expression plays an important role in maintaining meiotic arrest of oocytes within antral follicles, especially when the estradiol signal is absent or reduced.

NPPC/NPR2 system requires the activity of inosine monophosphate dehydrogenase (IMPDH), the rate-limiting enzyme required for the production of guanylyl metabolites and cGMP. ODPFs, particularly the GDF9-BMP15 heterodimer, also promote expression of IMPDH and elevate cGMP levels in cumulus cells that required for meiotic arrest [107]. Thus, oocytes themselves contribute to meiotic arrest by generating cAMP via GPR3/12 and maintaining cAMP levels via promoting expression of NPPC/NPR2 and IMPDH to elevating cGMP levels in cumulus cells. These results support the view that the signals originating from the oocytes play an essential role in orchestrating follicular growth and development [103].

Meiotic Resumption

LH

Fully grown mammalian oocytes within Graafian follicles are held in meiotic prophase arrest by NPPC/NPR2-produced cGMP. The preovulatory surge of LH from the pituitary gland triggers meiotic resumption during the estrous or menstrual cycle. The LH signal is amplified by promoting the production of epidermal growth factor (EGF)-like growth factors in MGCs and then the transactivation of the EGF receptor (EGFR) in cumulus cells, by which LH reduces NPPC content in the follicle and NPR2 activity in cumulus cells, and the resulting cGMP decrease and meiotic resumption.

Reduction of Intraoocyte cAMP Levels

In a normal reproductive cycle, the preovulatory surge of LH from the pituitary acts on the granulosa cells of Graafian follicles to cause oocyte maturation and ovulation [2]. However, the precise mechanisms underlying the LH-induced oocyte maturation are not completely understood [45, 108]. LH receptor activation stimulates Gs and activates adenylyl cyclase [109] and, as a consequence, elevates cAMP levels in the MGCs [110, 111]. Through a series of incompletely understood steps, LH ultimately causes a decrease in cAMP in the oocyte that is required for meiotic resumption [110, 112, 113]. LH induced the decrease of intraoocyte cAMP levels either by the reduction of cAMP synthesis or an increase in cAMP hydrolysis. LH-induced signaling does not terminate GPR3/12-Gs-ADCY signaling [73] or stimulate a Gi-mediated pathway in the oocyte [114]. On the contrary, the increase in oocyte-specific PDE3A activity after LH surge is likely sufficient to decrease cAMP levels in oocytes and thereby initiates pathways governing meiotic resumption [89].

Reduction of Intraoocyte cGMP Levels

PDE3A activity is regulated by intraoocyte cGMP levels. LH surge results in the decrease of intraoocyte cGMP levels [89]. The reduction of cGMP levels in oocytes relieves the inhibition of PDE3A, cAMP hydrolysis and meiotic resumption [19]. LH stimulation could not induce oocyte maturation under the conditions of elevated intraoocyte cGMP levels [20]. The reduction of intraoocyte cGMP levels is caused by lowering cGMP levels in the somatic cells and/or by closing the heterologous gap junctions coupling the somatic cells and oocytes [19, 20]. Gap junctions play an important role in signaling between somatic cells and oocyte. Connexin-43 is the predominant connexin present in granulosa cells, whereas connexin-37 is the major connexin present in junctions between the cumulus cells and oocyte [115, 116]. LH induces the phosphorylation of connexin proteins via the activation of extracellular signal-regulated kinases 1 and 2 [ERK1/2, also known as mitogen-activated protein kinases 3 and 1 (MAPK3/1)] [74, 111, 117, 118], by which LH decreases the permeability of gap junctions to reduce cGMP flux from somatic cells into oocytes [45]. Although the pharmacological closure of gap junctions is sufficient to initiate meiotic resumption of follicle-enclosed oocytes [113], studies in human, porcine, ovine and murine oocytes suggest meiotic resumption precedes the closure of gap junctions [18, 45,

119]. Furthermore, blocking the closure of gap junctions using the REK1/2 inhibitor U0126 (10 μM) does not prevent LH-mediated meiotic resumption [74]. These results indicate that the decrease of intraoocyte cGMP levels primarily contributes to LH-induced cGMP reduction in the somatic cells [80].

The decrease of cGMP levels in the somatic cells could result from the reduction in cGMP synthesis and an increase in cGMP degradation. There is some evidence for regulation of the guanylyl cyclase rather than the cGMP-PDEs [120]. The increased cGMP-PDEs activity has not been detected in mouse and rabbit ovaries during LH stimulation [20, 120], and human chorionic gonadotropin (hCG) has no effect on cGMP hydrolysis in rat granulosa cells in vitro [121]. In the presence of cGMP special PDE5 inhibitor, LH still decreases cGMP levels [20], suggesting that LH acts by decreasing cGMP synthesis in somatic cells rather than increasing cGMP-PDEs activity [120]. Recent study shows that EGF receptor-dependent events are involved in the short-term regulation of cGMP, whereas the long-term effects may involve regulation of the NPPC [122]. It is reported that LH-induced ERK1/2 activation could rapidly inhibit the expression of *Cyp19α1* mRNA, which encodes aromatase, and so decrease estradiol levels [123–125], by which LH may negatively regulate the expression of NPPC/NPR2 in somatic cells to decrease cGMP levels.

Reduction of NPPC/NPR2 Function

LH decreases cGMP levels in the somatic cells by decreasing the function of NPPC/NPR2 [80, 120]. The activation of LH receptors by hCG, a pregnancy hormone that exhibits LH activity with a long serum half-life, decreases *Nppc* mRNA levels in MGCs by approximately half of basal levels within 2 h before GVB occurs [80, 86, 88, 126]. This could result in a rapid decrease in the amount of NPPC, since NPPC has a half-life of approximately 3 min in plasma [127]. It is also possible that increased protease activity results in the degradation of NPPC [128]. Although LH receptor activation increases the expression of *Npr3* mRNA in MGCs [88] and NPR3 agonist enhances NPPC-mediated meiotic arrest of porcine oocyte cultured in vitro [129], an NPPC clearance receptor, NPR3, probably does not participate in regulation of ovarian NPPC levels for that there is no effect on hCG-induced NPPC decrease in *Npr3* mutant mice [88]. Nevertheless, NPPC peptide levels are completely decreased in mouse and human ovaries after the activation of LH receptors [80, 86], which occurs early enough to potentially contribute stimulation of nuclear envelope breakdown [80].

LH receptor signaling also decreases *Npr2* mRNA levels in cumulus cells [86]. However, this decrease occurs approximately 3 h after hCG stimulation when most oocytes have resumed meiosis [87]. On the other hand, LH could induce meiotic resumption of oocytes within cultured follicles even in the presence of 100 nM NPPC (unpublished data). It is suggested that NPR2 guanylyl cyclase activity can be decreased in a manner that is independent of its protein levels [130, 131]. Consistent with this, LH treatment for 20 min decreases NPR2 activity of approximately 50% in MGCs without the change of NPR2 protein levels, resulting in the rapid reduction in follicle cGMP levels [80]. The rapid decrease in NPR2 activity can be caused by dephosphorylation [128, 132].

Thus, LH decreases NPPC content and NPR2 activity, each of which is enough to induce oocyte maturation by decreasing cGMP levels in the somatic cells and then in oocytes [80, 133]. A decline in oocyte cGMP results in increased PDE3A activity, cAMP hydrolysis and meiotic resumption [49]. It has been long hypothesized that the action of LH could either relieve a maturation-arresting substance from the somatic cells or alternatively provide a positive maturation-promoting substance to override the follicular inhibition [1, 23]. Above data are consistent with a model in which LH removes the inhibitory function of NPPC/NPR2 to subsequently trigger oocyte maturation. It will be of interest to examine the exact mechanisms by which LH decreases NPPC content and NPR2 activity.

EGF-Like Growth Factors

LH receptor exists in MGCs, but not in cumulus cells [134]. LH stimulation triggers synthesis of EGF-like growth factors amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC) in MGCs [135], which is essential to transmit the LH signal from the MGCs to the cumulus cells to induce oocyte maturation and ovulation [111, 135–137]. AREG and EREG are the primary signaling molecules synthesized in response to LH to induce oocyte maturation [135]. Oocytes from both AREG and EREG knockout mice display a significant delay in the onset of meiotic maturation after the LH surge in vivo [111]. The disruption of ERK1/2 in mouse granulosa cells impairs hCG-induced generation of EGF-like growth factors [138], suggesting that the activation of ERK1/2 is involved in the production of these growth factors. EGF-like growth factors are produced as transmembrane precursors and release soluble growth factors after cleaved at cell surface by extracellular proteases. These factors trigger tyrosine-kinase EGF receptor (EGFR) signaling on the target cells leading resumption of meiosis [18, 45, 109, 135]. Activation of EGFR, as indicated by increased phosphorylation of the receptor protein, occurs as early as 30 min after LH treatment [137, 139], and LH-induced resumption of meiosis is strongly inhibited in $AREG^{-/-}$ $EGFR^{wa2/wa2}$ mice [111].

The activation of EGFR by amphiregulin and EGF rapidly suppresses *Nppc* mRNA levels within 2 h in cultured granulosa cells [71, 88]. However, this EGFR-mediated decrease of *Nppc* mRNA is about half of control. On the other hand, the EGFR inhibitor AG1478 incompletely inhibits LH-induced decrease of *Nppc* mRNA and cGMP levels [20, 117, 140]. These findings suggest that EGFR activity is not required for all of the LH-induced NPPC decrease, and two separate and partially redundant mechanisms contribute to the decrease of NPPC content in response to LH. The activation of the EGFR also decreases the levels of *Npr2* mRNA in cumulus cells cultured in vitro [133]. However, this decrease may not be involved in EGF-induced meiotic resumption, since the blockade of *Npr2* mRNA decrease by 10 μM U0126 could not reverse EGF-induced meiotic resumption [133]. The inhibition of NPR2 protein de novo synthesis by cycloheximide has also no effect on EGF-mediated oocyte maturation [133], suggesting that nascent gene transcription is not required in this process [141, 142]. EGF overcomes NPPC-mediated inhibition of maturation of oocytes in cultured COCs by decreasing cGMP levels, but has no effect on oocyte maturation when meiotic arrest is maintained in the presence of cGMP analog 8-bromoadenosine-cGMP [133]. Thus, LH stimulation of EGF-like growth factors in MGCs activates EGFR transactivation in cumulus cells, which is essential for meiotic resumption by the reduction of NPR2 activity of cumulus cells [22, 109, 135, 139]. The activation of EGFR in cumulus cells also causes phosphorylation of AKT and mTOR activation in oocytes, resulting in an increase in translation of a subset of maternal mRNAs. These mRNA translations are essential to reprogram the oocyte for embryo development [143].

The downstream target of EGFR is to activate ERK1/2 in cumulus cells, which is essential for LH-induced meiotic resumption [45, 138, 144–146]. The levels of LH-induced ERK1/2 phosphorylation are reduced in $Areg^{-/-}$ $Egfr^{wa2/wa2}$ follicles, and oocyte meiotic resumption is impaired [111]. The blockade of ERK1/2 activity by 100 μM U0126 could completely inhibit LH-induced meiotic resumption of oocytes in cultured follicles [145]. Furthermore, the activation of EGFR by AREG could not overcome hypoxanthine-mediated inhibition of maturation of oocytes in cultured COCs from $Erk1/2^{gc-/-}$ *mice* [138]. The remaining question is the nature of the link between EGFR and ERK1/2 activation in the cumulus cells and the meiotic resumption in oocytes.

Calcium Signaling

It is known that calcium signaling is required for gonadotropin-induced oocyte maturation in many species [22, 24, 45, 147, 148]. EGFR signaling can activate phospholipase Cγ [149], which may

increase calcium levels [133]. The elevation of calcium by hormones (such as arginine vasopressin, S1P, and lysophosphatidic acid) can lead to NPR2 inactivation through reducing the maximal velocity by NPR2 dephosphorylation [130–132, 150]. EGF overcomes NPPC-mediated inhibition of maturation of oocytes in cultured COCs by decreasing cGMP levels in both cumulus cells and oocytes [133]. The effects of EGF on oocyte maturation and cGMP levels could be mimicked by calcium-elevating reagents ionomycin and sphingosine-1-phosphate (S1P), but blocked by the calcium chelator bis-(o-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid, tetra(acetoxymethyl)-ester (BAPTA-AM) [133]. Thus, EGFR signaling-induced calcium elevation promotes meiotic resumption by decreasing NPR2 activity of cumulus cells. The elevation of intracellular calcium may activate protein kinase C (PKC) pathway. PKC can also cause inactivation of NPR2 with the different molecular basis from that of calcium: PKC primarily affects the affinity of NPR2 for NPPC and GTP by the reduction of NPR2 phosphorylation [132]. However, the PKC activator phorbol 12-myristate 13-acetate (PMA) could not induce meiotic resumption, and PKC inhibitors chelerythrine chloride and GF-109203X are without effect on EGF-induced meiotic resumption [133]. Moreover, the activation of PKC has not been detected during LH receptor activation [151]. All these results suggest that PKC is not involved in EGFR-induced oocyte maturation. PKC may amplify LH receptor signaling by activating metalloprotease to release a soluble EGF domain to activate the EGFR [18, 90, 140, 152].

Conclusion

By the regulation of oocyte meiotic prophase arrest and resumption, it may be summarized as follows: (1) Natriuretic peptide type C (NPPC) produced by mural granulosa cells stimulates the generation of cyclic guanosine 3′,5′-monophosphate (cGMP) by natriuretic peptide receptor 2 (NPR2) of cumulus cells; (2) the cGMP then diffuses into oocytes and arrests meiotic progression by inhibiting oocyte-specific phosphodiesterase 3A (PDE3A) activity and cyclic adenosine 3′,5′-monophosphate (cAMP) hydrolysis; (3) intraoocyte cAMP is produced by G-protein-coupled receptor GPR3/12 activation of adenylyl cyclase endogenous to the oocyte; (4) oocyte itself also promotes cumulus cell expression of NPR2 and inosine monophosphate dehydrogenase (IMPDH) to elevate cGMP levels for meiotic arrest; (5) FSH, through E2, enhances NPPC/NPR2 expression to ensure meiotic arrest during antral follicular development; (6) LH-induced EGF-like growth factors decrease NPPC content and NPR2 activity, resulting in cGMP decrease and meiotic resumption.

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Mitochondria of the Oocyte

4

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Introduction

Since in vitro fertilization and embryo transfer (IVF-ET) was clinically practiced for the first time, remarkable advances have been rapidly made in its in vivo and in vitro technology such as manipulation and culture methods for oocytes and spermatozoa. As a result, improved clinical outcomes have been achieved. However, some patients receiving IVF-ET still undergo repeated failure. These patients tend to have common features of embryonic dysfunction caused by the poor quality of their gametes. In this chapter, we have focused on oocyte quality. Although the precise mechanism for the decrease in the quality of oocytes remains obscure, their aging in the ovary has been postulated to be one of the major causes of infertility.

Although it has been well documented that the ovary in a new born female contains millions of oogonial cells, the number of surviving oocytes decreases rapidly with aging and reaches a few thousand by the age of 50 years [1]. By the marked changes in women's life style, their average age at marriage has recently increased significantly, particularly in advanced countries. Hence, female patients of advancing age face an uphill struggle to have their own babies. A further reproductive problem is the aging of oocytes after ovulation. Recent study revealed that aging of oocytes also occurs during their passage through the Fallopian tubes [2]. Thus, post-ovulatory oocyte aging has been proposed, at least in part, to be an important factor causing decreased fertilization rate, poor quality embryos, and abnormal offspring.

There are a variety of site-specific reactions that require large amounts of energy. Because oocyte cellular volume is significantly larger than that in somatic cells and their glycolysis activity is fairly low, appropriate amounts of ATP should be generated by mobilizing mitochondria to the subcellular sites for energy-dependent reactions. Thus, mitochondrial dynamics should be regulated appropriately to support the bioenergetics of maturing oocytes.

Aging-associated dysfunction of mitochondria such as decreased membrane potential and oxidative phosphorylation increases oxidative stress that perturbs redox-dependent metabolism in and around a wide variety of cells [3]. Hence, mitochondria have been suggested presumably to affect the quality of oocytes, thereby playing critical roles in their growth and maturation and in the development of embryos [4]. The quality of oocytes is a major factor that determines their maturation capacity. It should be noted that germinal vesicle (GV) stage oocytes undergo maturation followed by germinal vesicle break down (GVBD), a prerequisite step to enter the

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metaphase I (MI) stage. Up to MI, cumulus cells have been considered to support the growth and maturation of oocytes by supplying a variety of metabolites such as the pyruvate and/or lactate required for energy production [5].

In vitro maturation (IVM) is a useful method for preventing ovarian hyperstimulation syndrome that may be critical to the patients. IVM was examined for the first time in 1935 by Pincus and Enzmann [6] and subsequently used clinically by Edwards and Veeck [7–9]. Cha et al. [10] used immature oocytes from the unstimulated ovaries of patients in their donation program. This technique was improved to a more sophisticated method by Chian et al. [11] and applied widely to patients with polycystic ovary syndrome (PCO). However, it failed to become a leading method in ART because of its low success rate partly caused by PCO-induced high testosterone levels in ovarian follicles. As mitochondria play important roles in the maturation of oocytes, insufficient energy supply has also been considered to underlie the cause of poor quality oocytes and the low maturation rate of immature cells. GVBD followed by extrusion of the polar body are energy-dependent processes. Thus, appropriate numbers of mitochondria should be localized at the sites of GVBD and polar body extrusion. Mitochondrial distribution was proposed to determine the quality of oocytes [12, 13]. Furthermore, Van Blerkom [14] showed that mitochondria play an essential role in the regulation of calcium homeostasis in oocytes. In fact, mitochondria have been shown to change their subcellular localization during GVBD and the subsequent stages of oocyte maturation. Detailed properties and physiological importance of mitochondrial dynamics in maturing oocytes are described in this section.

Mitochondrial dysfunction could be induced by oxidative stress and mitochondria DNA (mtDNA) mutation associated with aging. Recent studies suggest that diabetes mellitus and heart failure could be classified as so-called "mitochondrial diseases" [15]. Thus, improvement of mitochondrial quality in oocytes may be clinically important in improving the outcome of ART in patients with infertility. In this context, cytoplasmic transfer was clinically applied to improve the quality of oocytes and several offspring were delivered successfully [16]. However, the use of this method in IVF has not been allowed by the Food and Drug Administration (FDA) in USA, predominantly due to a possible occurence of heteroplasmy. The FDA statement also virtually prohibited mitochondrial transfer of oocytes during IVF without showing any scientific evidence. Because healthy mitochondria and their dynamics play essential roles in the maturation and development of oocytes, mitochondrial transfer to oocytes with low competence may improve clinical ART outcome. This possibility is also discussed in future perspectives.

Structural Property of Mitochondria in Mammalian Oocytes

Mitochondria is important for mammalian oocytes if to provide sufficient ATP for their maturation; this is a prerequisite step to fertilization [17]. Dysfunction of mitochondria and subsequent low ATP production is one of the major factors that affects oocyte quality [18–20]. It has been suggested that impaired mitochondria may underlie the aging-related dysfunction of human oocytes [21]. Mammalian oocytes generally contain about 20,000–100,000 DNA copies in mitochondria that occupy about 2.5% of their cytoplasmic volume [22–24].

Mitochondria in fetal oogonia at 13–15 weeks of gestation have an oval and/or elongated structure with a dense matrix and tubular cristae resembling those of somatic cells secreting steroid hormones [25–28]. During the formation and growth of oocytes, mitochondria retain their spherical shape, whereas the structure of their cristae changes markedly. Mitochondrial size increases from that present in dividing oogonia to those of oocytes in primordial and primary follicles, reaching a diameter of 1–1.5 μm. Thereafter, mitochondrial diameter becomes slightly reduced during follicle development [29, 30]. The structure of inner membranes and cristae changes from the tubulo-vesicular form in

primordial germ cells and oogonia to the lamellar form observed during late leptotene, zygotene, and pachytene cells. Mitochondria proliferate particularly in the perinuclear region where their cristae become orientated parallel to the nuclear membrane [28, 31]. Mitochondrial cristae in diplotene oocytes show an arch-like structure representing a loose arrangement parallel to the outer membrane [29, 30].

Analysis using a confocal microscope and fluorescence dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide) revealed that mitochondria showed an aggregated structure and were localized in the cytoplasm of GV stage human oocytes with the exception of the periplasmic region. During the process, at the time of GVBD, the aggregated state of mitochondria disappeared and they became localized homogenously in the cytoplasm including the periplasmic region [13].

Analysis using transmission electron microscopy showed that mitochondria in maturing oocytes predominantly exhibited a spherical or oval structure. Mitochondria in GV stage oocytes have a structurally inert appearance with a dense matrix and a few arch-like or transverse cristae. At this stage of human oocytes, the subcortical region of their cytoplasm lacks mitochondria (Fig. 4.1a). Mitochondria localized homogenously in the ooplasm of MI, MII, and fertilized oocytes; they also localized in the subcortical region (Fig. 4.1b) [25]. Rosália et al. [32] reported that human MII stage oocytes sometimes showed aggregates of tubular smooth endoplasmic reticulum that were surrounded by abnormal mitochondria and clusters of small dense bodies. The rates of fertilization, cleavage, and blastocyst formation were significantly lower with these abnormal MII oocytes than those with normal oocytes [32].

In pronuclear stage of ova, mitochondria form an aggregated structure in the central region of the ooplasm around the pronuclei (PN). This occurs in normal and abnormal embryos. The structure and intracellular distribution of mitochondria in 2–8 cell embryos are similar to those in mature oocytes [26]. Although the density of mitochondria decreases slightly at the morula stage, the structure of their cristae remains unchanged as compared with those found in early stage embryos. Developing clear areas in mitochondrial matrices have been postulated to be the sites of localization of mitochondrial DNA and initiation of their transcription [19].

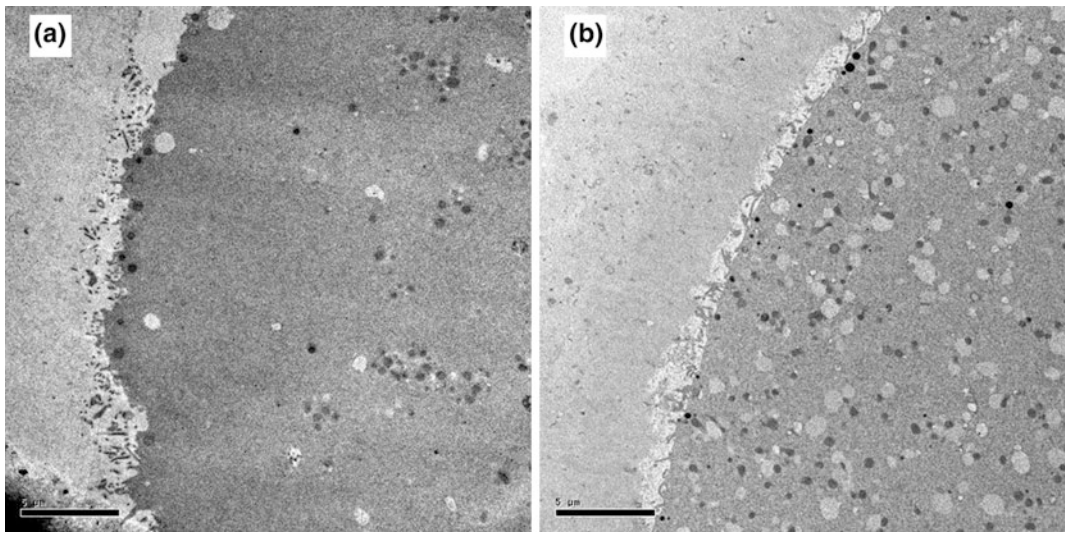

Fig. 4.1 Human germinal vesicle (**a**) and metaphase I (**b**) oocytes. Mitochondria are absent from the cortical region of the cytoplasm in germinal vesicle oocytes while they are dispersed in the ooplasm of metaphase I oocytes. Magnification × 5000; scale bar = 5 μm

The most striking change in mitochondrial morphology occurs during the expansion and hatching of blastocysts. Round- or oval-shaped mitochondria undergo transformation into elongated tubular forms. The inner membranes of these mitochondria are enriched with well-defined transverse cristae, a morphological sign of increased metabolic activity. Extremely elongated mitochondria align parallel to the surface in stretching trophoblast cells that appear during blastocyst expansion [26]. Taken together, mitochondrial structure (oval shape with minimum amounts of cristae), but not their subcellular localization, remains unchanged during the maturation of oocytes from the GV stage to MI, MII, pronuclear embryo, and to the morula stage.

Mechanism of Mitochondrial Traffic in Mammalian Oocytes

The intracellular localization of mitochondria changes significantly during meiotic maturation of mammalian oocytes. Although the mechanism and molecular properties of mitochondrial traffic have been energetically studied in somatic cells, limited information is available about the dynamic aspects of this organelle in mammalian oocytes. Among the various somatic cells, mitochondrial traffic has been studied most extensively using cultured neuronal cells [33]. Because neuronal cells have characteristic long axons and cell bodies, bidirectional movement of mitochondria (and other organelles) either from cell bodies (minus end of microtubules) toward free nerve endings (plus end of microtubules; anterograde traffic) or from nerve endings toward cell bodies (retrograde or centripetal traffic) in cultured live cells can be observed precisely using a light microscope equipped with time-laps cinematography. Kinetic analysis revealed that mitochondrial traffic is closely associated with the cytoskeleton, such as microtubules and microfilaments [33, 34]. Mitochondrial movement in axons depends predominantly on microtubules, kinesin and dynein, motor protein families [33, 35–41]. Kinesins transport mitochondria and other organelles from the minus end to the plus end of microtubules. Among various motor proteins, Kif5b and Kif1b (class-1 and 3 kinesins, respectively) play critical roles in the mechanism of mitochondrial traffic in neurons. Knockout of Kif5b and Kif1b markedly changed mitochondrial distribution and energy metabolism in neurons [36, 38, 41]. Dyneins transport mitochondria and other organelles in neurons from nerve endings to cell bodies via centripetal traffic. The dynein heavy chain and related proteins have been shown to interact with mitochondria and other organelles [39]. Inhibition of dynein significantly altered the movement and distribution of mitochondria in axons [35, 37, 40].

It should be noted that neuronal cells survive for a long time in vitro without undergoing mitosis while most cultured somatic cells show active mitosis. It has been well documented that the intracellular localization and metabolic status of mitochondria markedly change during cell cycles. Because subcellular sites and amounts of energy required for cells significantly differ during the cell cycle and maturation stages, intracellular localization and mitochondrial functions should also change depending on their status. In fact, Lee et al. [42] showed that the microtubular association of mitochondria was apparent in HeLa cells that grew rapidly, particularly at interphase of the cell cycle, while it disappeared during their mitotic phase. Thus, mitochondrial traffic changes markedly depending on the cell cycle.

Information about the structure, function, and intracellular traffic of mitochondria in mammalian oocytes is highly limited as compared with that of somatic cells. Changes in intracellular distribution of mitochondria and its relationship with the cytoskeleton during the meiotic maturation of oocytes have been reported in some mammals [43]. The GV stage of murine oocytes shows characteristic localization of mitochondria in the perinuclear region. Because the perinuclear accumulation of mitochondria is associated with the formation of a microtubule organizing center, this traffic has been postulated to depend on the microtubular network [44].

Calarco [45] showed that depolymerization of microfilaments by cytochalasin B failed to inhibit the perinuclear localization of mitochondria. In contrast, mitochondria preferentially localized in the periplasmic region of porcine GV oocytes while they moved to the central region of cells during GVBD and anaphase I. Mitochondria remained localized preferentially around the central region of mature oocytes. Because the presence of specific inhibitors of microtubules, but not of microfilaments, blocked mitochondrial movement, this translocation has been postulated to depend on the network of microtubules [46]. Similar changes in the localization of mitochondria during meiosis have also been observed with human oocytes through a mechanism that could be inhibited by colchicine but not by cytochalasin B [47]. However, Yu et al. [48] reported that a network of cortical microfilaments also underlies the mechanism of mitochondrial localization in murine oocytes. Furthermore, Duan et al. [49] showed that Rho-associated coiled coil-forming kinase played a role in the preferential distribution of mitochondria around the spindle of murine MI oocytes. Recent studies in our laboratory also showed that microfilaments rather than microtubules played a critical role in the vectorial transport of mitochondria in porcine GV oocytes [50].

Fluorescence-labeled oocyte mitochondria injected into the central region of donor oocytes preferentially moved to the subcortical region close to plasma membranes, whereas those injected into the subcortical region dispersed along plasma membranes. These mitochondrial vectorial movements were inhibited by specific microfilament inhibitors (such as cytochalasin B and D) but not by microtubule inhibitors (such as colcemid and nocodazole). These observations suggest that both microtubules and microfilaments underlie the mechanism of mitochondrial traffic in mammalian oocytes, though their dependency on the two cytoskeletons differs based on the species and/or the maturation stages of cells.

Although the possible involvement of motor protein families in the mechanism of mitochondrial traffic has also been suggested with mammalian oocytes, detailed information is lacking. Microfilament-associated mitochondrial traffic in neurons has been shown to be driven by the myosin motor protein family [33, 51, 52]; myosin transports various organelles from the minus end to the plus end of microfilaments. In cultured chicken neurons, myosin V has been shown to be associated with the movement of mitochondria in vitro at similar rates to those of axonal transport on microfilaments [34]. Thus, myosin V seems to play a major role in the actin-dependent mitochondrial traffic in axons [53, 54].

Dalton and Carroll [55] reported that preferential localization of mitochondria around the spindle of murine MI oocytes was suppressed by inhibiting dynein and accelerated by inhibiting kinesin. These observations suggest that both kinesin and dynein also play important roles in the regulation of mitochondrial traffic in mammalian oocytes. Possible roles for myosins in the regulation of mitochondrial traffic in mammalian oocytes remain unknown, and this requires further study.

Energy Metabolism in Mammalian Oocytes

A variety of physicochemical events occurring in actively metabolizing cells require large amounts of ATP synthesized by glycolysis in the cytoplasm and/or oxidative phosphorylation in the mitochondria. The former pathway depends on glucose uptake across plasma membranes, whereas the latter depends on the cellular availability of pyruvate and lactate, which are precursor substrates for acetyl-CoA used for the TCA cycle. Cellular uptake of glucose principally depends on the presence of Na^+-dependent and independent glucose transporters.

Fertilization of mammalian oocytes requires a wide variety of cellular events including their maturation from the GV stage to MII; this is associated with the accumulation of cortical granules at the subcortical region and formation of plasma membrane lipid rafts. Furthermore, fertilization induces striking changes in oocytes

such as excretion of cortical granules, Ca^{++}-oscillation to inhibit polyspermy, chromosomal rearrangement followed by spindle formation, polar body extrusion, cell cleavage, and formation of blastocysts. All these events require large amounts of energy. Hence, cellular ATP should be supplied in sufficient quantities through glycolysis and/or oxidative phosphorylation in mitochondria. In fact, energy metabolism has been suggested to play important roles in the maturation of human, murine, and bovine oocytes [18, 19, 56–60]. However, despite the presence of glucose transporter 1, 3, and 8 (SLC2A1, SLC2A3, and SLC2A8) in bovine, human, sheep, and rhesus monkey oocytes [61–64], their activity to uptake and use glucose is fairly low, presumably due to low activity of phosphofructokinase, a rate-limiting enzyme in glycolysis [65–67].

It should be noted that maturing oocytes, particularly those in the ovary (and just after ovulation), are surrounded by nursing granulosa cells that express SLC2A4, a functional high-affinity glucose transporter [68–70]; the rate of glucose uptake by SLC2A4 depends on the presence of insulin and/or insulin-like growth factors but not on extracellular glucose concentrations [71]. Tompson et al. [72] reported that cumulus cells metabolize glucose at a rate 23 times higher than that of oocytes, whereas the former cells consume oxygen at a rate of 3.2 times greater than the latter. Furthermore, even in the absence of glucose, oocytes denuded of their nursing cumulus cells underwent maturation in the presence of pyruvate [73]. Based on such observations, the surrounding cumulus cells have been postulated to supply pyruvate to oocytes as a substrate for mitochondrial electron transport.

The structure and function of mitochondria differ significantly depending on cell types and stages of cell differentiation. In fact, the structure of mitochondria in oocytes is characterized by their round shape with minimum amounts of cristae. Ovarian oocytes in newborn babies are arrested after their birth at prophase of the first meiotic division; then, on a daily basis, some are selected and gradually increase their cell size. Unlike in most somatic tissue stem cells, the mitochondria in oogonial stem cells show characteristic properties of being enriched with cristae that are normally observed in actively functioning somatic cells during maturation and differentiation. The cristae-like structure is not apparent with mitochondria observed in immature mammalian oocytes [59, 74–76]. This round shape of oocyte mitochondria is preserved even after their cleavage up to the 8–16 cell stage in cattle [75] and to the morula stage in human [75].

The relationship between cellular ATP levels and developmental competence of oocytes was studied in IVF procedure [18, 19, 56–60]. Assuming that most oxygen was utilized for oxidative phosphorylation during the pre-compaction stages of bovine oocytes, approximately 96% of cellular ATP would have been synthesized by mitochondria [77]. However, mitochondria in immature bovine oocytes utilized approximately 63% of oxygen consumed, whereas those in mature cells used only 43% [78]. Mitochondria in murine embryos consumed approximately 30% of oxygen [79]. Thus, the contribution of mitochondria to ATP synthesis in oocytes seems to differ with maturation stages and from one species to another. It should be noted that cellular ATP levels are appropriately regulated by a dynamic equilibrium between ATP synthesis and utilization by glycolysis and/or oxidative phosphorylation. Because mammalian oocytes have exceptionally large numbers of mitochondria (20,000–100,000/cell) [22–24], they seem to have sufficient capacity to synthesize the necessary amounts of ATP and maintain its steady-state level required for cell maturation, fertilization, and blastocyst formation despite the minimum enrichment of cristae-like structures in the mitochondria. Thus, it is not surprising that ATP levels remained unchanged during the maturation of oocytes. In fact, van Blerkom et al. [18] reported that ATP levels in murine oocytes remained unchanged during their maturation. In contrast, Brevini et al. [80] and Iwata et al. [60] reported that ATP levels changed significantly during the maturation of oocytes from pigs and cattle. Thus, the bioenergetic properties of maturing oocytes require further study.

Genetic and Epigenetic Control of Mitochondria

Mitochondria have their own DNA (mtDNA) and play important roles in a wide variety of cellular metabolism including ATP synthesis, maintenance of membrane potentials, induction of apoptosis, and regulation of aging. Subcellular localization of mitochondria and their activity to synthesize ATP differs significantly from one cell type to another depending on their role in supporting the survival of organisms. Thus, the cell-specific properties of mitochondria should be regulated appropriately by nuclear and/or mitochondrial genes. It should be noted that nuclear genes are principally responsible for the transcription of mRNAs followed by synthesis of the proteins required by mitochondria, whereas mtDNA is predominantly responsible for the synthesis of rRNAs and a small number of proteins in electron transport chains [81]. Accumulating evidence suggests that mitochondrial dysfunction and various diseases cause epigenetic modification of nuclear DNA (nDNA) [82–84].

Epigenetics is an important mechanism that regulates gene expression without changing the sequence of genomic DNA either in a permanent or transient manner [85]. Epigenetic modification of nDNA involves at least three systems including DNA methylation (5-mC) or hydroxymethylation (5-hmC) at the position of carbon five in cytosine residues juxtaposed to a guanine base (termed CpG dinucleotides), covalent modification of the N-terminal tails of histones (two H3, H4, H2A, and H2B), and non-coding RNA-associated regulation of gene expression [86–93]. These systems are currently considered to initiate and sustain epigenetic cell changes [94].

An early study conducted three decades ago reported that there was no methylation of mtDNA [95]. Subsequently, the presence of low levels of methylation restricted to CpG dinucleotides in mtDNA was reported with several species [96–98]. Methylation of CpG in mammalian mtDNA has been shown to suppress gene expression at similar levels to those of nDNA [99]. This observation suggests that 5-mC is susceptible to mutation in mtDNA, and its modification is important for the regulation of mitochondrial function. It has been demonstrated that murine embryonic cells have nDNA methyltransferase 1 (DNMT1), which is transferred to the mitochondrial matrix to modify mtDNA [83]. The enzyme translocation is regulated by the conserved mitochondrial-targeting sequence located at the upstream region of the transcription start site within the nuclear gene. These findings suggest the possibility that the epigenetic regulation of mtDNA by DNMT1 also occurs in mitochondria.

The mitochondrial genome in mammals encodes 13 proteins that constitute the respiratory chain complexes; two rRNAs and 22 tRNAs specific to this organelle. All other proteins in mitochondria, including those necessary for mtDNA replication and transcription, are encoded in the nDNA. The proteins synthesized in the cytoplasm are transferred into mitochondria via fully specialized import systems, some of which recognize N-terminal mitochondrial-targeting sequences [100]. Unlike nDNA, mtDNA is not associated with histones. However, mtDNA is associated with protein-containing nucleoids [101].

Transcription of mtDNA depends on nuclear-encoded gene products [102]. Oxidative stress has been shown to stabilize peroxisome proliferator-activated receptor γ-coactivator 1α (PGC1α), which activates the transcription of several nuclear-encoded transcription factors including nuclear respiratory factor 1 (NRF1). PGC1α and NRF1 form a complex that up-regulates transcription of transcription factor of activated mitochondria (TFAM) and multiple members of the mitochondrial respiratory chain complexes [102]. Several nuclear-encoded genes involved in mitochondrial function are regulated by DNA methylation. Inversely, it has been shown that mitochondria regulate epigenetic modification in the nucleus. This observation suggests that mitochondria affect the level of cytosine methylation in nDNA through changing the flux of one-carbon units for the generation of S-adenosylmethionine (SAM), a donor of the methyl group for DNA methylation [82]. DNA methyltransferase catalyzes the methylation of cytosine residues at their carbon five positions by

translocating the methyl group of SAM-CH_3 [103–105].

Mitochondrial dysfunction might affect cellular production of SAM-CH_3 and cause perturbation of the methylation of nDNA via cross talk between mitochondrial and nuclear genomes [106, 107]. Aberrant methylation of DNA at certain loci in the nuclear genome was associated with the deletion of mtDNA [82]. The study using mtDNA-depleted cells (ρ^0 cells) suggested that depletion of mtDNA resulted in the aberrant methylation of promoter CpG islands (high CG-rich regions). The 5′ UTR comprising a CpG island in genetically modified ρ^0 cells (143B ρ^0) was found to be hypomethylated as compared with that in the parental cells that was completely hypermethylated at this region [82]. The authors concluded that partial loss of genomic DNA methylation was associated with the loss of mtDNA and/or mitochondria [82]. Replenishment of mtDNA deficient cells with wild-type mitochondria partially restored the methylation profiles similar to their original state. This observation suggested that mitochondria controls nDNA methylation.

Cross talk between mitochondrial and nuclear genomes has been suggested to play important roles in the mechanism of aging and carcinogenesis in which DNMT1 activity was perturbed [108, 109]. Mitochondrial dysfunction has been shown to participate in the occurrence of certain types of cancers [82, 110, 111] and neurodegenerative disorders [112]. Accumulating evidence suggests that mitochondrial dysfunction and a variety of diseases cause epigenetic modification of nDNA. This possibility requires further study.

Future Perspective

Aging-associated decrease in the quality and developmental competence of oocytes and increase in chromosomal abnormality of newborns are critical problems in human reproduction. A similar decrease in the quality and developmental competence of oocytes is also observed in other mammals. In fact, spontaneous aging and repeated ovulation are known to decrease the number and quality of ovulated oocytes in rodents. Sato et al. [113] reported that oxidative stress plays important roles in the mechanism of LH surge-induced ovulation in mice and rats. Kinetic analysis revealed that the superoxide radical generated by NADPH oxidase in and around granulosa cells in the ovary induced their apoptosis, thereby allowing ovulation of matured oocytes into the peritoneal cavity by a mechanism that could be inhibited by superoxide dismutase (SOD) [114]. Miyamoto et al. [4] reported that superoxide generated by mitochondria selectively induced the apoptosis of maturing oocytes, thereby regulating the number of oocytes that finally undergo ovulation. These observations suggest that oxidative stress underlies the mechanism of both ovulation and aging of the ovary, and this decreases the quality of oocytes remaining in this organ. Kinetic analysis revealed that repeated ovulation increased the number of aggregated mitochondria in oocytes and decreased their developmental competence in mice [4]. Repeated ovulation caused perturbation of subcellular distribution of mitochondria and gene expression of NRF1 and TFAM.

It has been well documented that oxidative stress enhances the mutation of genes both in nuclei and mitochondria; the rate of mutation is 10 times faster with the latter than with the former [114]. In fact, mutation and deletion of mtDNA in somatic tissues accumulated fairly rapidly with aging as compared with those of nDNA [115–118]. Mutation and/or deletion of mtDNA decreased cellular ATP levels presumably due to perturbation of the mitochondrial electron transport system [119]. Obesity, insulin resistance, diabetes, and maternal aging have been shown to change the structure, distribution, membrane potential of mitochondria, and mtDNA abundance with a concomitant decrease in the developmental competence of oocytes [120–123]. Mitochondrial dysfunction may decrease the fertility and induce the developmental arrest and retardation of human embryos [124–126]. Because mitochondria in embryos are inherited exclusively from oocytes [127], mutation and/or deletion of mtDNA in oocytes and embryos may underlie the etiology of mitochondrial diseases [128–132].

To maintain and improve mitochondrial function in oocytes, various nutrients with antioxidant nature have been tested. L-Carnitine has been shown to decrease oxidative injury of mitochondria, cells, and tissues both in vitro and in vivo [3, 133]. Administration of L-carnitine successfully inhibited oxidative stress in various cells and tissues and decreased tissue injury in animals that had received anticancer agents [134]. L-Carnitine also suppressed the aging of senescence-accelerated mice and mice with amyotrophic lateral sclerosis (ALS) [3, 135]. Furthermore, Hino et al. [136] reported that oral administration of L-carnitine successfully inhibited hypoglycemia-induced brain damage in rats. These observations suggest that L-carnitine has beneficial effects in suppressing the oxidative injury of mitochondria, cells, and tissues in vivo. Thus, we have hypothesized that L-carnitine may also suppress the aging-enhanced pathologic events in ovarian cells and tissues. In fact, oral administration of L-carnitine successfully suppressed the pathological events induced by repeated ovulation and by natural aging in mice, such as aggregation of mitochondria and a decrease in the developmental competence of oocytes [4]. Furthermore, L-carnitine added to a culture medium alleviated abnormal distribution, decreased membrane potential of mitochondria, and normalized the spindle structure after in vitro maturation following vitrification [137].

Dichloroacetic acid, an inhibitor of pyruvate dehydrogenase kinase, added to the culture medium increased mitochondrial membrane potential and decreased oxidative stress of oocytes in aged mice, thereby enhancing their developmental competence in vitro [138]. Coenzyme Q^{10} added to a maturation medium also suppressed the abnormal distribution and decreased the membrane potential of mitochondria in bovine oocytes and improved their mtDNA expression and developmental competence [139]. Thus, the similar effects of L-carnitine and related compounds that protect mitochondrial functions to improve the quality and developmental competence of oocytes should be tested further with human oocytes, both in vivo and in vitro.

Another approach to improve mitochondrial function and the developmental competence of oocytes has been tested by directly microinjecting ooplasm and/or mitochondria into recipient oocytes. In this context, ooplasm has been transferred from healthy donor cells to recipient oocytes obtained from patients undergoing infertility treatment who showed recurrent implantation failure presumably due to poor embryonic development. Approximately, 30 healthy babies were born after injection of donor ooplasm derived from fresh MII oocytes, frozen-thawed MII oocytes, and 3-PN zygotes [140–147]. Furthermore, transfer of ooplasm using an electrofusion method was found to improve the developmental ability of pig and bovine oocytes [148, 149]. These results suggest that the transfer of cytoplasmic factors seems to confer beneficial effects on oocytes having poor developmental competence. It should be noted that mammalian ooplasm contains a variety of cytoplasmic factors such as mRNAs, proteins, and various organelles including mitochondria. Hence, factors other than the mitochondria may have produced the beneficial effects on maturation and development of oocytes with poor developmental competence.

Nuclear transfer could replace most ooplasmic components other than nuclear constituents. Thus, the effect of nuclear transfer has been studied to evaluate the efficacy of ooplasm to support oocytes without causing possible inheritance of injured or mutated mtDNA that otherwise induces mitochondrial diseases. In fact, nuclear transfer has been shown to prevent the transmission of mtDNA from donor oocytes to the reconstructed embryos, embryonic stem cells, and offspring in mice, humans, and other primates [150–153]. Thus, nuclear transfer may be a useful method to avoid the possible inheritance of mitochondrial diseases. Nuclear transfer has also been tested to rescue the genetic material of oocytes with low developmental competence.

Takeuchi et al. [20] reported that GVs obtained from oocytes with low maturation and developmental competence, and photo-oxidatively damaged mitochondria, could be rescued by nuclear transfer into an intact ooplasm. Similarly, the developmental competence of MII chromosomes

from oocytes challenged with in vitro aging was also rescued by transferring them into fresh MII ooplasm [154]. In contrast, Cui et al. [155] reported that the transfer of GVs from aged mouse oocytes into ooplasm from young animals failed to rescue aging-associated chromosomal misalignment during meiosis. Thus, the clinical impact of nuclear transfer to rescue oocytes with poor quality ooplasm requires further study.

The effects of the transfer of mitochondria to improve developmental competence of oocytes and embryos have also been studied. El Shourbagy et al. [156] reported that the transfer of mitochondria from good quality oocytes improved the fertilization rate of poor quality cells. Transfer of mitochondria isolated from granulosa cells inhibited the apoptosis of murine MII oocytes [157], whereas it improved the preimplantation development of poor quality bovine oocytes [158]. Similarly, Yi et al. [159] reported that the microinjection of liver mitochondria into 2-PN zygotes derived from both young and aged mice improved their preimplantation development. Furthermore, Tzeng et al. [160] reported that a successful pregnancy was achieved after transfer of mitochondria derived from granulosa cells to autologous oocytes from a patient with recurrent implantation failure. In contrast, Takeda et al. [161] reported that the microinjection of mitochondria obtained from cumulus cells suppressed the cleavage and development of oocytes. The reason for such discrepancies remains unclear.

It should be noted that the quality of the isolated mitochondria and/or donor cells used for the transfer would have affected these results. Furthermore, the properties and functions of mitochondria differ significantly among cells and tissues. In this context, typical mitochondria observed in healthy oocytes have a round shape with small amounts of cristae as described previously. Although the structure of mitochondria observed in most somatic cells changes dynamically, they generally have an elongated shape enriched with cristae (see Fig. 4.2). These structural and functional differences of donor

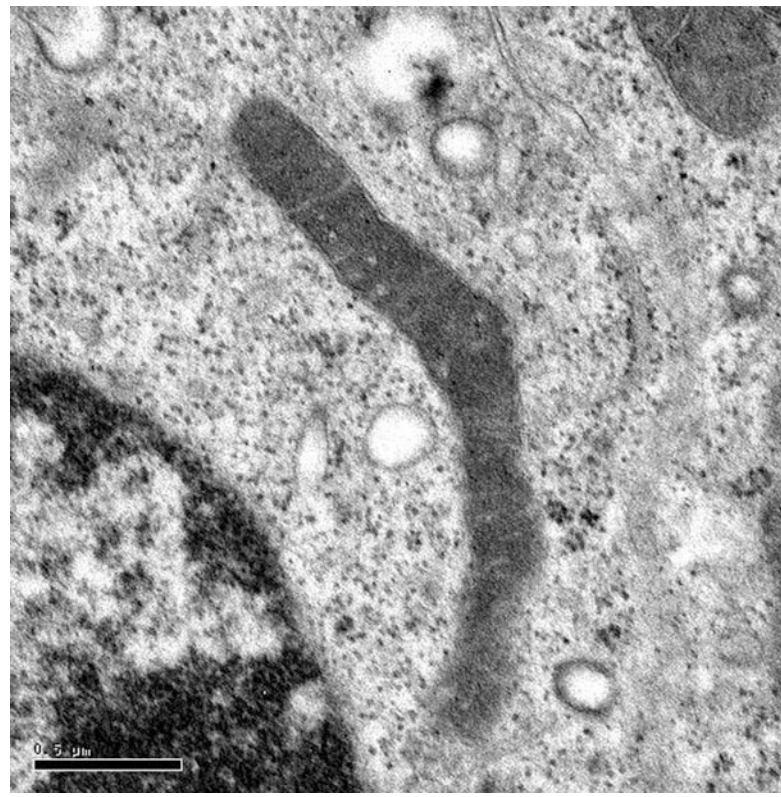

Fig. 4.2 A mitochondrion in a somatic cell. The mitochondrion has an elongated structure. Magnification × 50,000; scale bar = 0.5 μm

mitochondria may affect the outcome of mitochondrial transfer into recipient oocytes. Evaluation of a suitable source(s) and the properties of donor cells seem to be critical for improving the competence of oocytes by using mitochondrial transfer. Mitochondria in embryonic stem cells and induced pluripotent stem cells generally have a round shape with few cristae as observed with oocytes [162–164].

Transfer of either ooplasm or nuclei to improve the competence of poor quality oocytes requires healthy donor cells. Furthermore, transfer of heterologous ooplasm and/or mitochondria may result in the occurrence of heteroplasmy [146, 147]. Nuclear transfer would cause perturbation of the cross talk between mtDNA and nDNA [150]. Autologous transfer of mitochondria obtained from somatic cells would avoid unfavorable interaction of the two genomes in mtDNA and nDNA. Mitochondrial transfer would be effective in the elimination of pathologic events arising from endogenous mitochondria. Further studies on effective methods are necessary to increase the maturation and developmental competence of oocytes from patients with infertility caused by diabetes, mitochondrial disease, and aging.

We hypothesize that mitochondria from oogonial stem cells could be potential donor specimens for transfer into recipient oocytes. Clinical trials using donor mitochondria from oogonial stem cells from the ovaries of IVF patients for transfer into autologous oocytes with poor developmental competence are currently under investigation.

Conclusion

Mitochondria play an important role not only as cellular powerhouse, but also as potential sources for reactive oxygen species that impair a wide variety of biomolecules, thereby inducing cellular aging and apoptosis. Cytoplasmic factors including mitochondria have been known to regulate the expression of nuclear genes to induce physiological maturation of oocytes. Quality of cytoplasm and mitochondria in oocytes could be improved practically by dietary intake of foods and some supplements that work as scavengers against reactive oxygen species. Such scavengers have been used successfully to maintain or improve the quality of oocytes during IVF. In vitro handling of cytoplasmic factors including mitochondria could be performed safely without disturbing cell integrity. Hence, possible effect of mitochondrial transfer to improve the clinical outcome of in vitro maturation procedure needs to be evaluated carefully.

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Part II

Natural Cycle IVF and Stimulated Cycle IVF

Natural Cycle IVF: Summary of the Current Literature

5

Sabine Roesner, MD and Thomas Strowitzki, MD

Introduction

Since the first successful in vitro fertilization (IVF) by Steptoe and Edwards in 1978 [1] which was performed in a natural cycle, this way was abandoned in favour of controlled ovarian hyperstimulation to retrieve more oocytes and therefore to reach a better pregnancy rate and live birth rate.

In the last years, the natural cycle IVF received a renaissance and the in vivo-maturated oocyte without stimulation is assumed to have a better competence for fertilization and implantation. The most common problem in natural cycle IVF is still the spontaneous LH-surge as well as the premature ovulation. Furthermore, natural cycle IVF is the method of choice for women with low response in former controlled ovarian hyperstimulation cycles or with a low ovarian reserve, shown by a low antral follicle count and/or low anti-Muellerian hormone. Therefore, many studies deal with possibilities to enhance the success rates in natural cycle IVF.

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For this summary of the current literature dealing with natural cycle IVF/ICSI, a PubMed, Embase and Cochrane database search was performed with the key words "natural cycle IVF/ICSI", "fertilization rate" and "pregnancy rate". For the actuality, the search was limited to articles published in English in the period 2009 till 2014. This strategy yielded 144 articles, 28 of them were suitable for this summary (Table 5.1). The design of the studies was heterogeneous, most of them were retrospective studies, with study populations from 30 to 7244 patients undergoing 28–20,244 cycles with a mean age from 30.8 to 40.3 years.

Natural Cycle IVF

Many patients are asking for a "natural" IVF approach without any hormonal stimulation because of "fear of hormones", ethical or religious reasons or a history of hormone-dependent cancer in their own or family history [2]. A natural cycle IVF is emphasized to be more cost- and time-effective for the patients [3, 4]. Thus and for the scientific assumption that the unstimulated, in vivo maturated oocyte has a better competence for development, many IVF units established natural cycle IVF again.

However, difficulties such as premature LH-surge and ovulation, failure of retrieve an oocyte, and therefore lesser success rates still

Table 5.1 Overview on current studies on NC-IVF/ICSI

Study	Year	Method	Patients (n)	Age (years)	Cycles (n)	FR (%)	PR/ET (%)
Kedem et al. [8]	2014	Modified NC-IVF/ICSI in POR	111	39	111	0.67	0.9
Von Wolff et al. [21]	2014	NC-IVF	112	35.2	108	45.4	27.9
Von Wolff et al. [21]	2014	NC-IVF + Clomiphene	112	35.2	103	57.3	25.0
Bodri et al. [32]	2014	NC-IVF/ICSI + NSAID	365	40.3	1183	57.0	35.0
Roesner et al. [6]	2014	NC-IVF/ICSI	159	36.4	463	59.4	10.9
Rijken-Zijlstra et al. [20]	2013	Minimal stimulation-IVF + GnRH Antagonist + NSAID	60	30.9	250	67.1	Ongoing PR/Cycle 5.6
Rijken-Zijlstra et al. [20]	2013	Minimal stimulation-IVF + GnRH Antagonist	60	31.8	60	274	Ongoing PR/Cycle 8.4
Son et al. [29]	2013	NC-ICSI/IVM	28	33.3	28	73.4	20.8
Polyzos et al. [7]	2012	NC-IVF in POR	136	37.3	390	n.a.	LBR 7.4
Kawachiya et al. 19]	2012	NC-IVF/ICSI	n.a.	36.5	903	44.5	35.9
Kawachiya et al. [19]	2012	NC-IVF/ICSI + NSAID	n.a.	36.2	962	55.3	39.1
Kato et al. [26]	2012	Minimal NC-IVF	7244	39.4	20,244	77.0 IVF 83.2 ICSI	21.8
Gordon et al. [5]	2011	NC-IVF	n.a.	n.a.	795	n.a.	26.1
Papolu et al. [24]	2011	Mild stimulation IVF	150	n.a.	n.a.	n.a.	56.0
Lou and Huang [23]	2010	Modified NC-IVF	30	30.6	30	62.7	PR/Cycle 30.0
Xu et al. [28]	2010	NC-ICSI/IVM	323	31.5	364	86.3	35.9
Aanesen et al. [22]	2010	Modified NC-IVF	43	34.2	129	n.a.	26.7
Aanesen et al. [22]	2010	Mild NC-IVF	145	32.8	250	n.a.	27.2
Kim et al. [11]	2009	Minimal stimulation-IVF/ICSI in POR	90	n.a.	90	n.a.	LBR 13.5
Schimberni et al. [12]	2009	NC-ICSI	294	39.3	500	57.0	17.1
Lim et al. [27]	2009	NC-ICSI/IVM	140	31.4	153	81.9	40.4

FR Fertilization rate, *PR* Pregnancy rate, *ET* Embryo transfer, *LBR* Live birth rate, *n.a.* Not applicable

limit the use of natural cycle IVF. Gordon et al. [5] evaluated the SART Clinical Outcome Reporting System (CORS) database in the USA and found that unstimulated IVF cycles represent less than 1% of all IVF cycles. They could show that natural cycle IVF leads to significantly higher implantation rates in some age groups (35–37 years and 38–40 years) when compared to conventional IVF cycles (40.4% vs. 23.8% and 28.4% vs. 15.4%). In the authors' opinion, this may support the hypothesis that the endometrial receptivity is enhanced in natural cycles. A pregnancy rate of 35.9% in patients <35 years and live birth rate of 19.9% for all patients could be achieved. In conclusion, the authors would recommend natural cycle IVF especially to patients with good preconditions (e.g. <35 years, no poor ovarian response (POR)).

Another retrospective study by Roesner et al. [6] evaluated 463 cycles of natural cycle IVF and came to similar results. Because patients undergoing natural cycle IVF present often unfavourable preconditions (long duration of infertility, age >40 years, known history of low response in former conventional stimulated cycles), pregnancy rates still remain low. Further studies to optimize treatment strategies and to define patient groups suitable for natural cycle IVF were recommended.

Low Responders

In patients who are known to have a low ovarian reserve or who showed a low response in a former controlled ovarian hyperstimulation cycle, many teams prefer a natural cycle IVF instead of another controlled ovarian hyperstimulation attempt. The following studies focus on poor responder patients.

Only two studies [7, 8] followed the Bologna criteria of POR (at least two of the following): (1) advanced maternal age ($\geq$40 years) or any other risk factor for POR, (2) a previous POR ($\leq$3 oocytes with a conventional stimulation protocol) and (3) an abnormal ovarian reserve test [9]. Polyzos et al. [7] found a significant lower embryo transfer rate as well as live birth rate in the study group of low responders compared with the control group with normal responders. Acknowledging the limitations of their study—a retrospective design and a significant younger control group—they conclude that older patients with POR may be candidates for alternative therapies, e.g. such as oocyte donation programs.

Kedem et al. [8] support this thesis in their retrospective study with patients also fulfilling the Bologna criteria and undergoing a modified natural cycle with GnRH antagonist and human menopausal gonadotropin (hMG) stimulation after an IVF attempt with controlled ovarian hyperstimulation and poor response. The GnRH antagonist was started when the leading follicle had a diameter of 13 mm and two to three ampules of hMG were injected daily. Because of very poor pregnancy rates in this patient group, they conclude that genuine poor responders with a yield of only one oocyte in a previous conventional cycle did not benefit from a natural cycle program and should therefore not be offered a mild stimulation natural cycle IVF. The option of a controlled ovarian stimulation, egg donation or adoption should be discussed with these patients.

In contrast, Kadoch et al. [10] stated in a retrospective study that a modified natural cycle IVF with GnRH antagonists starting at a follicle diameter of 15 mm, mild human menopausal gonadotropin stimulation (150 IU/d) and 50 mg indomethacin three times a day to avoid a premature ovulation should be the first choice in young poor responders because it is a cheap and monthly repeatable option. They mentioned that a single oocyte of better quality as a consequence of natural selection and a better endometrial receptivity resulting in natural cycle IVF balances the low chance for an embryo transfer in these attempts.

In a prospective assessment, Kim et al. [11] reported a similar pregnancy rate and live birth rate in a minimal stimulation natural cycle IVF with FSH and GnRH antagonist compared with a conventional antagonist protocol in low responder patients. The GnRH antagonist was administered when the leading follicle reached 13–14 mm together with 150 IU/d FSH. The ovulation was triggered at a follicle diameter of 17–18 mm and the retrieval was performed 34–35 h later. Natural cycle IVF with minimal stimulation is considered to be a last chance for women who have failed to respond adequately to a conventional hyperstimulation IVF cycle before oocyte donation.

Another retrospective study by Schimberni et al. [12] reported about 500 consecutive cycles of natural cycle intracytoplasmic sperm injection (ICSI) in poor responders without any hormonal intervention. Similar rates of retrieved oocytes, embryo transfers and pregnancies per consecutive cycle but significantly different pregnancy rates in younger patients when analysing the date

depending on the patients age were found ($\leq$35 years PR = 29.2% vs. 36–39 years PR = 20.6%, vs. $\geq$40 years PR = 10.5%). In conclusion, natural cycle ICSI up to four attempts in younger patients ($\leq$40 years) was considered as a possible chance for patients with a low ovarian reserve.

Besides these studies, successful natural cycle IVF was also reported in case reports. Hyman et al. [13] described the live birth of twins after a modified natural cycle ICSI in a woman with decreased ovarian reserve. Despite of elevated FSH levels the patient present with regular monthly menstruation. After two IVF cycles with high dose controlled ovarian stimulation and poor response, it was decided to perform a natural cycle ICSI with early hCG administration. From three antral follicles with a maximal diameter of 12 mm at retrieval, three mature oocytes were collected. Two were fertilized, and a twin pregnancy was achieved.

Another team reported a successful pregnancy after a "double rescue" retrieval in a patient with low ovarian reserve following a natural cycle IVF [14]. The patient felt that she might have surged the previous evening when she attended the IVF unit. Because there was a leading follicle with good perifollicular blood flow as well as a triple-layer endometrium in the ultrasound present the decision for retrieval on the same day was made. No oocyte was identified. With the patients' consent, a second attempt after hCG injection for the following day was scheduled. A control on the next day showed a regular follicle with good ultrasound criteria (very good peri-follicular blood flow), so a further retrieval was attempted. At that time, a metaphase I oocyte was found which was matured after a few hours in in vitro maturation (IVM) medium. Fertilization could be achieved, and a pregnancy was induced.

Li et al. [15] described a series of three women with poor response in a former controlled ovarian stimulation cycle who got pregnant after a natural cycle IVF combined with IVM. In all three cases, immature and mature oocytes were collected. The immature oocytes were matured in IVM medium and fertilized by ICSI and transferred with the resulting embryos resulted after the fertilization of the mature oocytes.

In the last case, a pregnancy after modified natural cycle with GnRH antagonist started at a follicle diameter of 15 mm and 75 IU/d hMG began the same day in a poor follicular responding young (<35 years) patient with elevated FSH levels is reported [16].

All authors of the described case reports conclude that their attempt in a natural cycle IVF in low responders combined with new approaches such as early hCG administration, double retrieval, IVM or modified stimulation may be additional alternatives for poor responders as a last chance before, e.g. oocyte donation.

GnRH Antagonists

Of upmost importance in natural cycle IVF is the risk of spontaneous LH-surges and therefore premature ovulation. Many attempts are made to avoid this event. Meanwhile, the addition of GnRH antagonists became a standard in most natural cycle IVFs.

In a prospective randomized trial, Kim et al. [11] used a GnRH antagonist in combination with a low-dose FSH stimulation for natural cycle IVF in comparison with a conventional antagonist protocol. Monitored by ultrasound examinations the GnRH antagonist as well as the stimulation with 150 IU FSG daily were started in the natural cycle group when the leading follicle reached 13–14 mm. Ovulation was induced when the dominant follicle reached a diameter of 17–18 mm with 250 µg hCG. In this protocol, they achieved a similar cancellation rate, pregnancy rate and live birth rate as in the control group. Therefore, they conclude that a natural cycle protocol with the use of GnRH antagonists is a patient-friendly and cost-effective alternative especially in low responders.

Kadoch et al. [10] as well used GnRH antagonists for preventing the spontaneous LH-surge. When the dominant follicle reached 15 mm, the GnRH antagonist was started and indomethacin was also added to avoid a premature ovulation. At the same time, hMG was

administered to prevent a decrease in the estradiol concentration. hCG was given when the leading follicle had a diameter of 18 mm and the retrieval was performed 34 h later. Kadoch et al. consider better embryo quality and better endometrium receptivity as a result of the natural oocyte selection and thus a better chance for an embryo transfer.

NSAID

Another approach to prevent the premature ovulation is the administration of non-steroidal anti-inflammatory drugs (NSAID). They may delay the ovulation by inhibiting the production of cyclooxygenase-2 which is important for the ovulation process [17, 18].

Kawachiya et al. [19] proofed and confirmed this hypothesis in a large retrospective non-randomized study with 1865 natural cycles: 962 with NSAID use and 903 without. No other medication such as GnRH antagonists to prevent a spontaneous LH-surge nor other stimulation drugs (FSH, hMG) were used.

The NSAID (25 mg suppositories of diclofenac 8 and 14 h before oocyte retrieval) was added according to the level of serum LH: LH <10 IU/ml and progesterone <1.0 ng/ml no NSAID was given, triggering and oocyte retrieval as usual, LH 10–30 IU/ml, progesterone <1.0 ng/ml NSAID every 6 h, hCG immediately and oocyte retrieval the next morning, LH 30–110 IU/ml, progesterone <1.0 ng/ml, NSAID optional, hCG immediately, oocyte retrieval the next morning, LH 10–110 IU/ml, progesterone >1.0 ng/ml no NSAID, no triggering of ovulation, oocyte retrieval the same day.

A significant difference was found in the rate of premature ovulation in cycles using NSAID compared to cycles without administration of NSAID (3.6% vs. 6.8%). Therefore, the fertilization rate and the embryo transfer rate were also significantly lower in the group without NSAID (53.3% vs. 44.5% and 46.8% vs. 39.5%).

In contrast, a prospective randomized clinical trial by Rijken-Zijlstra et al. [20] analysing the effectiveness of indomethacin to prevent the ovulation in a natural cycle could not confirm these results. While monitoring the cycle per transvaginal ultrasound and serum estradiol measurements, the GnRH antagonist was started when the leading follicle reached a diameter of 14 mm, 150 IU gonadotrophins per day were also added. About 50 mg indomethacin or placebo capsules were administrated 3 times per day from the day of hCG injection till the morning of oocyte retrieval. No benefit could be shown in administration of NSAID to prevent premature ovulation in comparison with the placebo group (cancellation rate 6.4% vs. 10.6%).

Clomiphene Citrate

Another approach to prevent the premature ovulation is the administration of clomiphene citrate. Von Wolff et al. [21] demonstrated that the daily intake of 25 mg clomiphene citrate started at day 6 (in a 26–27 day menstrual cycle) or day 7 (28–30 days length of menstrual cycle) may reduce the premature ovulation rate significantly (6.8% vs. 27.8% without clomiphene citrate) and increases the embryo transfer rate significantly (54.4% vs. 39.8%) without enhancing side effects (e.g. hot flushes, headaches or ovarian cysts). The clinical pregnancy rate showed no significant difference. Just one or two consultations were necessary before the introduction of the ovulation. Clomiphene citrate consists of two isoforms: enclomiphene (trans form) with an estradiol antagonist effect and an elimination time of 24 h and zuclomiphen (cis form) with an estradiol agonist effect that may cause ovarian cysts and a much shorter elimination time. Therefore, clomiphene citrate must be administrated once a day until the introduction of ovulation. In the described low dose of 25 mg/d, just mild side effects such as mild headache and mild or moderate hot flushes were reported by the study patients. Because of fewer consultations, this protocol is considered as a patient-friendly approach. In addition, the authors conclude that the natural cycle protocol allows the oocyte to mature in vivo resulting in higher implantation rates.

Mild Stimulation

Despite the expected advantages of natural cycle IVF such as better competence for fertilization and implantation of in vivo-matured oocytes without any hormonal treatment, the disadvantages of low retrieval rates due to premature ovulation rates and only one retrieved oocyte leaded to the idea of mild stimulation for enhancing the outcome of natural cycle IVF.

Aanesen et al. [22] report on a ten-year experience with natural cycle IVF by a so-called modified natural cycle IVF or mild stimulation natural cycle IVF. Both varieties were offered to women with desire for low hormonal treatment as well as for women with former ovarian hyperstimulation syndrome, unexpected side effects in former controlled hyperstimulation IVF or women who are not allowed to get a hormonal treatment (e.g. history of breast cancer). For modified natural cycle, IVF monitoring includes ultrasound examinations and measurement of serum estradiol concentrations. When the dominant follicle reached 17 mm and the estradiol concentration was between 500 and 750 pmol/l, ovulation was induced with 5000 IE hCG and the retrieval was performed 37 h later. For minimal stimulation IVF, the patients were stimulated with 100 mg clomiphene citrate from day 3–7 of their menstrual cycle. The ovulation was triggered with hCG when the leading follicle reached a diameter of 18 mm and the retrieval was performed 37 h later. The cancellation rate was 13.6% in the modified natural cycles and 31% in the minimal stimulation cycles. The authors suppose a partial effect of clomiphene citrate in preventing a premature LH-surge because of its estradiol antagonist effect. No significant differences were found in implantation rates or pregnancy rates.

Lou and Huang [23] described in their study a mild stimulation natural cycle protocol where 150 IU/d hMG were administered beginning at day 2 or 3 of the menstrual cycle. When two or more follicles reached a diameter of 17 mm, hCG was given to introduce the ovulation. Oocyte retrieval was performed 32 h later. Results were compared to a group of patients undergoing a conventional-long agonist protocol. Except the number of retrieved oocytes (7.8 vs. 12.2), no significant differences were found nor in fertilization rates nor in pregnancy rates.

Papolu et al. [24] have used a protocol with 150 mg/d clomiphene citrate started between cycle days 5 and 7. Additionally, 300 IU hMG were injected on day 5 and 300 IU FSH on days 7 and 9 of the treatment cycle. At a diameter of the leading follicle of 17 mm, hCG was administered and oocyte retrieval was performed 30–35 h later. There were no significant differences shown between the study group and a control group undergoing a conventional long protocol.

Another mild/minimal stimulation protocol is described by Zarek and Muasher [25]. Patients started on day 3 of their menstrual cycle with 100 mg/d clomiphene citrate until day 7. One day after 150 IU/d, hMG were administered and from day 11 a GnRH antagonist was also added. When two follicles reached 17 mm, hCG for triggering the ovulation was injected. In this approach also, no differences were found in clinical pregnancy rates compared to the patients stimulated in a controlled ovarian hyperstimulation procedure.

In the largest study by Kato et al. [26], 7244 patients undergoing 20,244 natural cycles with minimal ovarian stimulation were included. In the majority of cycles (82%), 50–100 mg/d clomiphene citrate starting on day 3 until the day before oocyte retrieval were administered together with 50–150 IU/d of FSH or hMG. Ovulation was induced with a GnRH agonist. In 16.2% of the cycles, no hormonal interventions took place, and in 1.8% a letrozol stimulation was performed. When the leading follicle reached 18 mm and the estradiol level was more than 250 pg/ml, ovulation was triggered and the retrieval was scheduled 30–34 h later. A single embryo transfer to avoid multiple pregnancies was performed in all cases. If more than one embryo was obtained, the surplus embryos were vitrified and were transferred in a subsequent cycle if no pregnancy occurred. High fertilization rates were shown regardless the age of the

patients, but live birth rates showed a strong age-dependent decrease (>45 years <1% pregnancy rate). The authors registered also higher pregnancy rates in transfers with frozen-thawed embryos compared to fresh cycles and postulate also an anti-estrogenic effect of clomiphene citrate on the endometrium.

In Vitro Maturation

Besides the conventional ovarian hyperstimulation for IVF/ICSI, in vitro maturation was developed. This method was established especially for patients with a polycystic ovarian syndrome to avoid the risk of an ovarian hyperstimulation syndrome and for patients who have suffered for an ovarian hyperstimulation syndrome in a conventional stimulation protocol. In the attempts to enhance the success rates in natural cycle IVF, IVM was combined with the natural cycle. It is expected that besides the mature oocyte from the dominant follicle, immature oocytes could be collected and matured afterwards to yield more embryos for transfer. In cases where no oocyte could be obtained from the leading follicle, there could be also a chance to yield viable immature oocytes so that such cycles must not be cancelled.

In a large retrospective study, Lim et al. [27] combined natural cycle IVF with IVM. The first aim of this study was to identify the patients who would profit from this approach irrespectively their history of polycystic ovarian syndrome or not. The data from 410 cycles were analysed. In 63 cycles, an IVM protocol was used, in 196 cycles the patients were stimulated in a conventional ovarian hyperstimulation protocol. About 151 cycles were treated in a natural cycle protocol without any hormonal stimulation except triggering of the ovulation with hCG when the leading follicle reached a diameter of 12–14 mm. Oocyte retrieval was performed 36 h later. Mature oocytes were inseminated per ICSI the same day, and immature oocytes were cultured in a special IVM medium for 24 h. The oocytes which reached maturity were also inseminated per ICSI the next day. The resulting embryos of mature and immature oocytes were pooled and transferred together. Compared to the IVM and the conventionally stimulated group, there were no differences found neither in implantation rates nor in clinical pregnancy rates. Just the miscarriage rate was significant higher in the IVM group (38.5% vs. 27.9% in the NC-IVF/IVM group vs. 24.3% in the conventionally stimulated group). Therefore, the authors stated that more than the half of infertile women treated with IVF may profit from a natural cycle IVF combined with IVM.

Xu et al. [28] confirmed these findings in another large study with 323 cycles of natural cycle IVF combined with IVM. The patients in this trial were divided into five subgroups according to their infertility reasons (tubal factor, male factor, combined tubal and male factor, unexplained, other/mixed cases). IVM was carried out in the same way than in the study of Lim et al. [27]. They could also find no significant differences in pregnancy rates and live birth rates in the different subgroups they observed. Therefore, they consider the combination of natural cycle IVF with IVM as an efficient treatment for patients with various causes of infertility. It is patient friendly because of minimizing stress and costs for the patients.

In another study by Son et al. [29], natural cycle IVF in combination with IVM was analysed again. The induction of ovulation was also triggered when the leading follicle reached a diameter of 12 mm and oocyte retrieval was scheduled 36 h later. Acceptable pregnancy rates were found in total. However, the pregnancy rate was significantly better in cycles where at least one embryo obtained from an in vivo-maturated oocyte could be transferred (30.8% vs. 9.1% without in vivo-maturated oocytes). The authors conclude that further evaluations are needed to find out at which diameter of the leading follicle the ovulation should be induced to obtain viable immature oocytes also.

A case report by Yang et al. [30] described the first pregnancy after the transfer of vitrified blastocysts yielded from a natural cycle IVF combined with IVM. The patients' ovulation was induced when the leading follicle reached 13 mm and one mature oocyte from the dominant follicle

as well as five more mature oocytes and six immature oocytes were retrieved. All mature oocytes were fertilized, and four out of six immature oocytes matured after 24 h were also fertilized. Three of the embryos achieved from mature oocytes were transferred in the first embryo transfer, but no pregnancy could be achieved. The remaining embryos were cultivated and four reached the expanded blastocyst stage and were cryopreserved by vitrification. Six months later, two of them were transferred to the patient in a spontaneous cycle and a singleton pregnancy with the birth of a healthy girl was achieved.

Costs

In a clinical trial, Lou and Huang [23] described significant lower costs in natural cycle IVF compared with conventional stimulated IVF cycles with similar clinical pregnancy rates in both groups. Also Aanesen et al. [22] calculated the costs of modified and mild stimulated natural cycles. Modified natural cycle IVF would cost 2.5% and mild stimulated natural cycle IVF 3.7% of the costs for the least-expensive IVF cycle. Groen et al. [31] focussed on costs in a retrospective study with GnRH antagonists and 150 IU FSH started when the dominant follicle reached 14 mm. Ovulation was triggered with 10,000 IU hCG and follicle aspiration took place 34 h later. Despite the lower costs in each modified natural cycle IVF compared to a conventional IVF cycle, the cumulative costs to achieve a pregnancy were higher in modified natural cycles because of higher pregnancy rates in controlled hyperstimulation cycles and the need for multiple approaches in natural cycle IVF. On the other hand, multiple pregnancies and ovarian hyperstimulation syndromes were avoided and ensuing lower costs per live birth.

Additional Aspects

Some articles dealing with further interesting topics in natural cycle IVF were also found.

Timing of Oocyte Retrieval

Bodri et al. [32] described in a retrospective study the timing of the oocyte retrieval depending on the spontaneous LH-surge in a natural cycle IVF. The collective was divided into four groups: 1. LH <10 IU/l, 2. LH 10–30 IU/l, 3. LH 30–140 IU/l, 4. LH decreasing and progesterone >1.0 ng/ml. As in this department NSAIDs were routinely used, all patients belonging to the groups 1–3 achieved NSAIDs to prevent premature ovulation. The ovulation was induced immediately after the examination, and the oocyte retrieval was scheduled 1–2 days later. Patients of groups 3 and 4 did not get any triggering of the ovulation, and oocyte retrieval was performed in group 3 one day after the examination and in group 4 at the same day. The oocytes were fertilized with IVF or ICSI, and most of the achieved blastocysts were electively vitrified for a transfer in a subsequent cycle. No significant differences were found among the groups with regard to amount of retrieved oocytes, fertilized oocytes and live birth rate.

Analysis of Follicular Fluid

Since the beginning of hormonal stimulation for IVF, the impact of gonadotrophins is discussed to have an influence on the quality of the oocytes as well as on endometrial receptivity and therefore on the success rates of IVF.

Von Wolff et al. [33] analysed the concentrations of anti-Muellerian hormone, testosterone, androstenedione, DHEA, estradiol, FSH and LH in follicular fluid collected from patients undergoing natural cycle IVF and in comparison with conventional gonadotrophin-stimulated IVF cycles. Except DHEA concentrations, significant differences were found in all other hormonal analyses. However, no association between hormonal concentrations and implantation rates was found because the oocytes from stimulated cycles were cultured in groups in the IVF laboratory. The hypothesis was proposed that the endocrine follicular fluid profile could influence the outcome of an IVF attempt. Particularly the low Anti-Mullerian hormone in the follicular fluid of stimulated cycles, which is known as a marker for a high implantation potential of the

oocyte, may explain the unphysiological environment in conventional IVF cycles and therefore the difficulties to enhance the success rates in hormonal stimulated IVF cycles.

Immune cells are known to be increased during the growing of the follicle. The stimulation with gonadotrophins strengthens this effect and thus has a negative impact on oocyte quality. In regard to the concentration of cytokines in serum and follicular fluid in natural cycle IVF and gonadotrophin-stimulated IVF cycles, the team of Bersinger et al. [34] could not find any differences between the two groups in follicular fluid but in serum concentrations. In the authors' opinion, this may suggest that the gonadotrophin stimulation does not affect the follicular immune system.

Summary

Due to patients asking for a more natural IVF treatment option, natural cycle IVF relives a renaissance. Several studies deal with this renewed approach.

Completely non-stimulated cycles were reported as well as modified or mild stimulated natural cycles. A yet unsolved problem in natural cycle attempts is the spontaneous LH-surge and therefore the premature ovulation. Different therapy strategies are tested including GnRH antagonists as well as clomiphene citrate. In recent years also, NSAIDs attract notice, but divergent results were found.

For patients, the natural cycle IVF is a patient-friendly, cost-effective option which is monthly repeatable. The risk of multiple pregnancies is minimized, also the hazard of an ovarian hyperstimulation syndrome. Possible negative long term-side effects of hormonal stimulation, which are yet not fully ruled out, can be avoided.

From the scientific view, oocytes yielded from a natural cycle seem to be of better quality and might offer higher chances for fertilization and implantation than oocytes retrieved by conventionally stimulated cycles.

Natural cycle IVF seems to be also a last chance for patients with POR before alternatives such as adoption or oocyte donation must be considered.

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Follicular Fluid Hormone Profiles in Natural Cycle IVF Patients During Follicular Phase

6

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Introduction

Normally, the human ovary produces a single dominant follicle that ovulates in each menstrual cycle. Folliculogenesis begins with the recruitment of a primordial follicle into the pool of growing follicles and ends either with its selection, dominance, growth and ovulation or death by atresia, a process that can take up to a year in humans [1]. All primordial follicles develop between the sixth and ninth months of gestation in the human foetus, and their numbers constitute the reserve of oocytes for the entire reproductive life, commonly termed 'ovarian reserve'.

Folliculogenesis, occurring in the ovarian cortex, is divided into two phases. The first phase, termed 'pre-antral', is independent of gonadotrophin (Gn) and is characterized by the growth and differentiation of the oocyte. It is controlled mainly by locally produced growth factors operating through autocrine/paracrine mechanisms. In this phase, very limited amounts of ovarian steroids are synthesized by the granulosa cells (GCs). This phase includes the transition of the primordial follicle to the primary pre-antral follicle. By the conclusion of this phase, the theca interna layer has developed, and follicle-stimulating hormone (FSH) receptors have developed on the GCs. The second phase, antral (Graafian) or Gn-dependent phase, is characterized by the growth of the follicle and is regulated by FSH, luteinizing hormone (LH) and also several growth factors such as members of the transforming growth factor (TGF)-β family (e.g. activin) [2], growth differentiation factor-9 and bone morphogenic protein-15 [1, 3–5]. LH receptor expression in the TCs is accompanied by increased androgen substrate production and subsequent aromatization to oestrogen (E_2) by the GCs under the influence of FSH via the cytochrome P450 complex. The follicular mean maximal diameter increases due to cellular multiplication alongside accumulation of the hormone rich follicular fluid (FF), the composition of which changes as the follicle–oocyte complex matures.

It is thought that transition of the primordial follicle to a fully grown secondary follicle is a long process that may take approximately 290 days [1]. A further 60 days may be required for the Graafian follicle to develop, at which point it measures approximately 20 mm [1]. Atresia can occur in developing follicles after the secondary phase [3] (Fig. 6.1).

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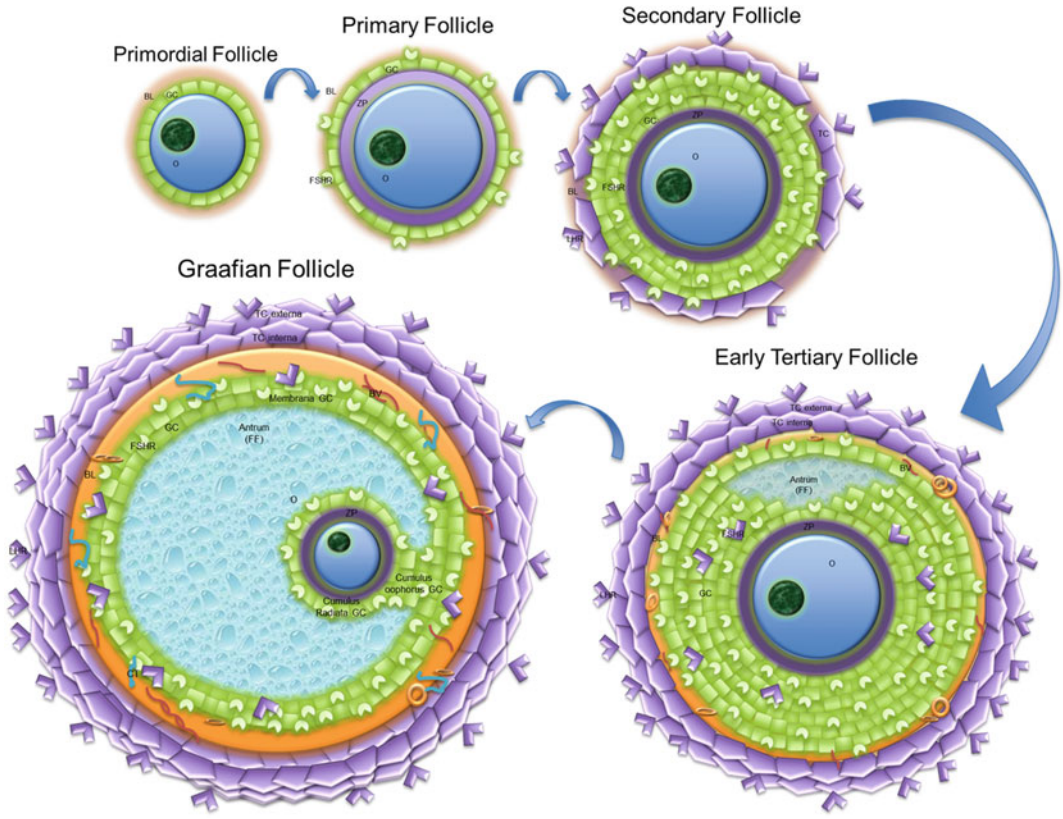

Fig. 6.1 Schematic representation of development of primordial follicle to pre-ovulatory Graafian follicle. *BL* Basement laminae, *O* Oocyte, *GC* Granulosa cell, *ZP* Zona pellucida, *TC* Theca cell, *FF* Follicular fluid, *BV* Blood vessel/capillary, *CT* Connective tissue (loose), *FSHR* FSH receptor, *LHR* LH receptor

Follicular Fluid Hormones in Folliculogenesis

In contrast to the previous belief, it has become apparent, over the past two decades that the oocyte is not a passive recipient of developmental signals from oocyte-associated GCs, termed the cumulus. It is now clear that a dialogue occurs between oocyte, GCs, adjacent theca/interstitial cells, and even with the surrounding follicles [4]. A reciprocal cooperation between the different cells is brought into play at all stages of growth and/or atresia of the follicle [6]. This complex interplay of regulatory factors governs the development of somatic cells and the oocyte and influences the follicular fluid (FF) composition. This interaction is essential for both the oocyte and the follicle's development, beginning with primordial follicle's transition to the primary follicle and then its continuation through to ovulation [7, 8]. The resultant oocyte's quality influences subsequent embryo viability [9] such that the regulatory factors communicating within the follicle, and which are potentially quantifiable in the FF, may in part be responsible for the cycle's outcome. Although such agents are largely unidentified, it is believed that they are produced locally within each follicle and include various cytokines/growth factors that are themselves influenced by the steroidal milieu [10]. Thus, it appears that the hormonal milieu in the FF is crucial in the oocyte's development, and its analysis may help us understand its prognostic value which may influence the selection of treatment regimen for a better outcome.

Composition of Follicular Fluid

The FF is initially derived from and is similar in composition to thecal capillary serum [11–13]. It contains a conglomeration of non-hormonal components including inorganic elements such as sodium and potassium, gases, carbohydrates, mucopolysaccharides, lipids and proteins [14]. However, as development progresses, in addition to the locally produced steroid hormones, pituitary-derived hormones, other systemic hormones such as insulin and cortisol, have been found to be present in the FF. The transformation growth factor anti-Müllerian hormone (AMH) that is produced by the GCs has been the focus of much clinically applicable research recently, and this too is detectible in the FF [15, 16].

Analysis of the FF at various time points throughout the follicular phase of the natural cycle suggests that a precise sequence of hormonal changes occurs within the microenvironment of the evolving Graafian follicle [17]. The order of these changes and the relative ratio of various hormones impacts follicle's growth and secretory activity of GCs [17]. Oocytes with the capacity to resume meiosis are more likely to originate from follicles with lower androgen: oestrogen ratio and converse is true for those that show degenerative changes [17]. Furthermore, recent significant developments in analytical techniques, specifically the multiplex immunofluorescence assay, have enabled the quantification of more than 40 cytokines and growth factors simultaneously within FF [18, 19]. The NMR spectroscopy has facilitated the identification of the FF metabolome [20, 21].

As the follicle develops, the GCs produce large polysaccharides, steroid hormones and growth factors, which cannot cross the 100-kDA follicle–blood barrier. This results in an osmotic gradient that leads to the FF accumulation and antrum formation and thus increases the FF volume [22, 23]. The FF from mature follicles in naturally cycling women is enriched with locally produced steroid hormones. Within the FF, as much as 1000 times higher concentration of oestrogen and progesterone (P) than that in the serum has been detected, whilst other pituitary-derived hormones, such as FSH, are not differentially concentrated [24].

Ovary-Derived Hormones

Steroid Hormones

There has been extensive work in the literature studying the steroid hormone composition of the FF. At the turn of the twentieth century, experiments in ovariectomized rodents and rabbits involved injection of ovarian extracts to prevent uterine atrophy. Subsequently steroid hormones, specifically oestrogen, were recognized in "liquor folliculi" [25]. In the past century, with the development of analytical methods more detailed classification of hormonal profile has emerged. In the mare, nine steroid hormones, including oestrogens, progestogens and androgens, were identified in the FF collected during oestrous and luteal phases of the cycle [26]. Oestradiol-17β was identified as the dominant steroid, and concentration of some, notably androstenedione (A4), was found to be higher in FF than in the plasma [26].

During the 1960s, it was found that the concentrations of oestrogens and progestogens in the FF of women with clinical disorders varied. For instance, follicles of women with Stein–Leventhal syndrome had relatively higher A4 and lower oestrogen concentration, probably due to a paucity of 19-hydroxylase activity [27–29]. In FF of women with dysfunctional uterine bleeding, higher levels of P when compared with normally cycling women were identified. This suggests that ovulatory dysfunction is the primary cause of dysfunctional uterine bleeding and that is more frequently seen in adolescent and climacteric period [30].

The level of steroid hormones in the FF also fluctuates throughout the natural cycle. Overall, more steroids, especially oestrogen, are found in the FF of growing follicles. The highest levels of

oestradiol-17β and P are reached at the mid-point of the cycle and their levels decline as the follicle enters the pre-ovulatory phase [31]. Studies in rabbits demonstrated a transient increase in FF steroid concentration in the pre-ovulatory follicle followed by a rapid decline, and this is thought to be secondary to an alteration in steroidogenesis by the follicles. This pattern is also replicated by a decline in plasma steroid hormone concentration just prior to ovulation [32]. Edwards concluded that FF must be a repository for the steroids produced by the GCs and TCs, and their concentrations are a reflection of their evolving pattern of synthesis [14].

The type of oestrogen synthesis varies during the cycle too. During the follicular phase of normally cycling women, more free compared with conjugated oestrogens are present in the FF, but during the luteal phase, the corpora lutea contain similar concentrations of free and conjugated oestrogens [33]. Incidentally the follicles of women with polycystic ovaries even during the follicular phase display a deranged steroid hormone profile that is similar to that of the luteal phase corpus luteum of normally cycling women.

Steroidogenic pathways have been comprehensively scrutinized during the twenty-first century, and the 'two-cell, two-Gn' model has been described. Produced de novo from cholesterol, progestogens, androgens and oestrogens are synthesized sequentially by the GCs and TCs within the ovary and are secreted into the follicular antrum and from there into the peripheral circulation [34]. All products of the steroidogenic pathway act via specific nuclear receptors to regulate reproductive function [34]. Since each steroid hormone serves as a substrate for the next, many of these roles are inextricably linked (Fig. 6.2). Within the systemic circulation, these steroids actively participate in the regulation of pituitary gonadotrophin secretion, and within the ovarian microenvironment, they act as important paracrine factors that qualitatively influences the development of follicle–oocyte complex.

Oestrogens

The receptors for FSH have developed by the time the primordial follicle transits to the primary pre-antral follicle stage. By the late pre-antral stage, follicles have developed the capability of synthesizing oestrogen. As a consequence of the high numbers of GCs, and capacity for androgen aromatization, the pre-ovulatory follicle exhibits the highest levels of oestrogen in its FF [35]. Local paracrine/autocrine effects of oestrogen are demonstrated by the hypophysectomized rat model, where oestrogen has been shown to promote follicle's growth, GC proliferation and development of the antrum even without the pituitary hormones [36, 37]. Furthermore, oestrogen in association with gonadotrophins also promotes the differentiation of GCs by induction of the FSH, LH, P and prolactin receptors and by stimulation of 3′,5′-monophosphatase (cAMP) accumulation. The increase in GC cAMP binding sites is also dependent upon oestrogen [38, 39].

There are two forms of oestrogen receptors (ER): ERα and ERβ [34], with the ERβ predominating in the ovary [40]. Both receptor forms have different roles; whilst ERα inhibits follicular rupture or ovulation, ERβ is responsible for follicle's growth, prevention of atresia and induction of specific gene expressions. Studies in ER knockout oestrogen-depleted mice have demonstrated that oestrogen is vital for follicular development beyond the antral stage. Furthermore, oestrogen is also essential to maintain the female phenotype of ovarian somatic cells [34]. During COH, the levels of AMH in FF decrease in growing follicles that predominantly express ERβ, and this is thought to be due to the rising E_2 in the FF [41].

Studies of FF and plasma concentrations of E_2 in various subfertility-related disorders have provided an understanding into their pathophysiology. For example, even though plasma level of E_2 was elevated, E_2 level in the FF of women with polycystic ovarian syndrome (PCOS) did

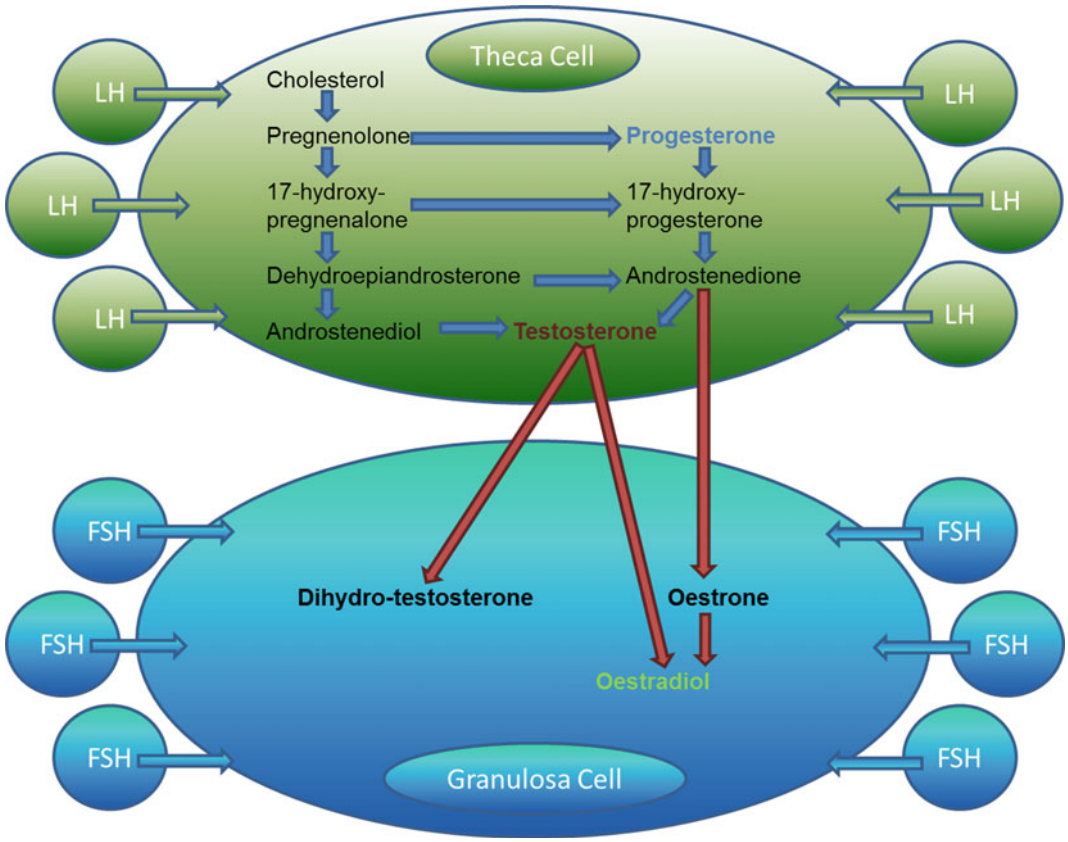

Fig. 6.2 The "two-cell, two-gonadotrophin" model. *FSH* Follicle-stimulating hormone, *LH* Luteinizing hormone

not show a corresponding rise unlike normal women undergoing IVF with male factor infertility (MFI) or controls [42]. Furthermore unlike normally cycling controls, the free and conjugated oestrogens had similar concentrations in the FF of women with PCOS [33]. On the other hand, similar E_2 levels were observed in the FF of women with endometriosis, normal women undergoing IVF with MFI or controls [43]. This exemplifies disordered steroidogenesis within even the pre-ovulatory follicles of women with PCOS which may impact the development of the follicle–oocyte complex.

Progestins

One of the most abundant hormonal components of FF, P, is critical for the end stages of follicle development and ovulation [34], and knockout mice lacking P fail to ovulate [44]. The role of P is inextricably linked with E_2 as increased levels of oestrogen induce the production of P receptors.

The cellular response to P is more disparate when compared with E_2, and therefore, it is harder to implicate, although it plays a key role in ovulation, implantation and maintenance of pregnancy [45, 46]. Within the follicle, progestins have been reported to have a direct effect on GCs. In rat GCs, P increases cAMP and consequently enhances the GCs response to FSH, but it also inhibits FSH-stimulated E_2 production by GCs, a function that may be of value when providing luteal support with high-dose parenterally administered progesterone in women at risk of OHSS after IVF.

The P levels in FF are significantly affected by age and rise with advancing age. They are also

increased with reduced ovarian reserve (ROR) [24]. Follicular P levels have been found decreased in women with PCOS and endometriosis [42, 43]. The precise cause or impact of this is not clear. In an IVF cycle, following COH, the relationship of serum P on the day of hCG and pregnancy rate has been debated extensively because of contradictory results [47, 48].

Androgens

Whilst predominantly involved in male development, androgens are also expressed in the ovary and the fallopian tube and are critical in the development of the early follicle [49]. Although in the female circulation adrenal cortex is the major source of androgens, ovarian follicles also contribute towards their FF and systemic levels. Androgens may affect folliculogenesis directly via androgen receptors (ARs) or indirectly through aromatization to oestrogen. Androgens, predominantly A4 and testosterone (T) are produced by the TCs in response to stimulation by LH (see Fig. 6.2). In vitro and in vivo studies have demonstrated that androgens can stimulate the growth and development of follicles in mammals [49, 50]. Female mice lacking a functional AR are less fertile and have a shorter reproductive life [49]. ARs are located on GCs, TCs and stromal cells [51]. The AR expression is more pronounced in the early and intermediate stages of folliculogenesis [52]. During this time, local androgen production facilitates the transcription of genes involved in the transition of pre-antral follicles from the reserve to the growth pool as well as the subsequent development of the more mature follicle [51]. Exposure to androgens augments FSH receptor expression in developing GCs, thus enhancing FSH-induced cAMP formation required for the transcription of genes that control GC proliferation and differentiation, as evidenced by an increase in E_2 and P production [53–56]. Activation of the AR in the oocytes of primordial follicles and in the GCs and TCs of growing follicles enhances the expression of insulin-like growth factor (IGF-1) and its receptor. This in turn leads to follicular recruitment and development [57]. Thus, androgens may play an important role in selection and transition of primordial follicles to antral stages.

However, in mature follicles, the AR expression is reduced and there is associated reduced androgenic activity, which indirectly implies that androgens at this stage may adversely influence the appropriate maturation of the follicle and may induce atresia [48]. In vitro mice oocytes arrest in meiosis after T exposure, and hence, in late stages of follicle development, T is thought to adversely influence oocyte maturation [58].

Age and ROR have not been shown to affect T levels in the FF [24]. However, the FF of mature follicles in women with PCOS has been found to contain significantly raised T levels [42]. Hyperandrogenism is a classic symptom of PCOS, and the finding of raised T level in the FF of women with PCOS provides further evidence that abnormal steroidogenesis by the ovary is responsible for the systemic excess of androgens and the finding of poor-quality oocytes. It has been proposed that in this condition, TCs fail to respond to Gn down-regulation and this associated with TC hypertrophy in PCOS leads to hyperandrogenism [59].

Anti-Müllerian Hormone

AMH is a TGF-β family growth factor, is secreted by pre-antral and antral follicle GCs and is present in both the FF and the circulation. In the past decade, it has also been implicated in folliculogenesis and is thought to play a role in both the growth and differentiation of the follicle [60]. One primary function of AMH is its inhibition of primordial follicle growth. Even though AMH knockout mice have normally developed ovaries and are fertile, they have a narrow window for fertility [61]. AMH serum levels vary throughout the female lifespan, with increased levels in early childhood, a peak in the early 20s, followed by a decline to undetectable levels in menopause [60, 62, 63]. AMH decreases sensitivity to FSH and is negatively correlated with FSH and E_2 [64–66]. AMH expression has been

found to be differentially regulated by E_2 and depends on the type of ER. During COH, its levels in FF decrease in growing follicles that predominantly express ERβ, and this is thought to be due to rising E_2 [41]. However, the growing follicle's AMH level is only a marker of its status, and its level thereafter is not affected by the fluctuations in other hormones during the menstrual cycle [65].

The levels of AMH in the FF also reflect serum concentrations, thus decreasing with age and ROR. The FF AMH levels are negatively correlated with FF FSH concentrations. AMH in both serum and FF predicts ovarian response to COH in an IVF cycle and this correlates with treatment outcome [16]. Higher AMH concentration in the FF has been correlated with higher fertilization, pregnancy and implantation rates in women undergoing COH [67, 68]. In mice, even the oocyte has been shown to influence AMH expression, thus suggesting a direct role for the developmentally competent oocyte. It is thought that this effect may be mediated by oocyte's activation of various physiological processes in the surrounding GCs [70].

Pituitary-Derived Hormones

Pituitary hormones, particularly Gn, play a critical role in folliculogenesis. Historically continuation of pre-antral follicular growth following hypophysectomy has been demonstrated, thus establishing the notion of the Gn-independent phase of folliculogenesis [71]. By quantifying the levels of Gn in the FF and plasma during the follicular phase of the menstrual cycle, their actions with respect to follicular steroidogenesis have been studied and their key functions have been established [17]. It has been found that in the FF, E_2, which has long been recognized to increase the sensitivity of the ovary to FSH, is highest in the mid-follicular phase, whereas LH significantly increases in the late follicular phase. Pre-ovulatory follicles have higher concentrations of E_2 and P even though the LH and FSH levels are only 30–60% of those found in serum [17]. FSH is essential for GC proliferation and development of the basement membrane, whilst both FSH and LH are responsible for the follicular antrum formation and development of the thecal vasculature [72]. Follicle's growth is accompanied by an increase in both the quantity and quality of Gn binding sites [14]. Follicles containing high levels of FSH in the early follicular phase have a higher mitotic index and a faster growth rate [17]. The GC LH receptor expression also correlates with increased mitotic activity. Subsequent to the entry of LH into the follicle, there is cessation of mitotic activity and associated P secretion increases. A correlation between the LH level in the FF and oocyte maturation has been demonstrated. Oocytes in metaphase I and II stages have been found to originate from follicles containing comparatively higher LH levels [17].

Follicle-Stimulating Hormone

The predominant function of FSH is to stimulate the development of the primordial follicles via a feedback loop enabling selection and maintenance of the dominant follicle [73]. FSH blocks apoptosis in pre-antral follicles, consequently FSH receptor (-R) knockout mice are anovulatory, lacking circulatory oestrogen, possess significantly less P, and serum T levels are elevated, thus implicating FSH firmly in the steroidogenic pathway [74]. Serum FSH levels rise at puberty and climax at the menopause. Higher levels of serum FSH signify ovulatory dysfunction and in younger women are indicative of a ROR [75, 76]. Conversely, despite ovulatory dysfunction, young women with PCOS exhibit lower levels of FSH [77]. There is a paucity of research investigating FSH levels in the FF during natural cycles. A study identified higher concentrations of FSH in the FF of older women with ROR, but younger women with ROR and healthy controls exhibited similar levels. The authors wondered whether the elevated intra-follicular FSH level in older women was simply due to higher serum FSH concentration or whether it was secondary to reduced FSH receptor expression [24]. In stimulated cycles of women with endometriosis,

FF concentration of FSH was not altered [43], but to date there are no studies that have investigated the FF FSH level in women with endometriosis or PCOS.

Luteinizing Hormone

LH is fundamental to supporting steroidogenesis via TC LH receptors (-R) [78]. LH-R knockout mice are infertile with atrophic ovaries and have low serum E_2 and P levels [79]. Whilst LH is essential for normal oocyte and embryo development, follicles exposed to high concentrations of LH may luteinize prematurely, which is thought to compromise normal oocyte development [73]. Basal LH levels in the serum rise during reproductive life and peak at the menopause [80, 81]. However, FF levels of LH remain consistent throughout reproductive life [24, 82] and are not affected by age, ROR and endometriosis [24, 43, 82].

Prolactin

Prolactin and E_2 levels in the FF of the maturing follicle are inversely related. Whilst E_2 levels in FF progressively increase throughout the follicular phase, FF prolactin levels decrease, but this reduction is restricted to the follicle destined to become the pre-ovulatory Graafian follicle [83]. This fall in prolactin is also inversely related to the progressive increase of P in FF [83]. A similar inverse correlation between prolactin levels and the number of LH receptors was demonstrated in the follicle of postpartum cows [84]. However, with regard to the relevance of prolactin levels following COH with gonadotrophins, the findings are conflicting [85–93].

Growth Hormone

Growth hormone (GH) is primarily produced by the pituitary but is also produced locally within the follicle itself. It acts on GH receptors of the GCs, TCs and luteal cells and enhances GC FSH-dependent E_2 secretion [94] directly or via an insulin growth factor (IGF)-1-mediated mechanism and perhaps also the expression of FSH and LH receptors in GCs [95]. GH also impacts follicular growth [96], has been shown to increase the sensitivity of ovaries to Gn stimulation [97] and hence has been suggested as an adjuvant therapy in assisted reproduction [98]. However, a clear association between intra-follicular GH and successful pregnancies has not been identified and results have been contradictory. Both lower [87] and higher [99] GH levels in the FF of follicles generating oocytes/embryos resulting in pregnancy after in vitro fertilization (IVF) have been reported.

Systemic Hormones

Various systemic hormones have been identified in the FF.

Insulin

Insulin stimulates GC aromatase activity as well as steroid production [23, 100]. Furthermore, it has been recognized as a regulating factor for oocyte maturation [101]. Insulin has been identified in FF following COH with gonadotrophins and clomiphene citrate (CC), and its concentrations have been found to correlate with P concentrations in the FF [102].

Cortisol

At the time of the LH surge, an increase in total and free cortisol is apparent in FF, and it is postulated that cortisol, together with its regulation by 11β-hydroxysteroid dehydrogenase, is involved in oocyte maturation and ovulation [103]. Similarly, following COH in IVF cycles, a high cortisol/cortisone ratio in FF has been associated with increased pregnancy rates, and it is thought to enhance final oocyte maturation as well as subsequent embryo implantation [104, 105]. Low FF cortisol levels in women with

endometriosis have been suggested to contribute to follicular dysfunction associated with subfertility secondary to this condition [106]. However, a trial involving administration of adjuvant corticoid therapy during IVF has failed to improve oocyte fertilization, embryo implantation and clinical pregnancies [107].

Renin

Renin levels have also been studied in FF throughout natural cycles [108]. Active renin levels increased in the FF during the follicular phase, peaking in the peri-ovulatory phase following the LH surge (in patients undergoing laparoscopic sterilization) or exogenous hCG administration (in modified natural IVF cycles), thus supporting the hypothesis that the ovarian renin–angiotensin system is also under Gn control [108].

Follicular Fluid Hormones in Natural Cycle IVF

Evidence for the Detrimental Impact of Exogenous Gonadotrophin Stimulation

Gonadotrophins with COH have improved success rate of IVF by increasing the number and availability of embryos for selection before transfer [109–111] and also by enabling the cryopreservation of supernumerary embryos [112, 113]. However, there is increasing evidence that COH may be detrimental to a variety of pathways involved in reproduction. The foundation of COH involves bypassing physiological regulatory mechanisms in order to support the growth of multiple follicles. This is achieved via the administration of exogenous gonadotrophins to achieve levels above the obligatory threshold for selection of the dominant follicle; hence, the entire cohort of recruited follicles is able to attain pre-ovulatory status [112]. An agent to desensitize the pituitary and prevent premature LH surge is also given in either a long protocol (Gn-releasing hormone [GnRH] analogue) or a short protocol (GnRH antagonist), thus preventing the adverse consequences of supra-physiological E_2 levels leading to premature LH surge and luteinization or ovulation. An ovulation trigger using either human chorionic gonadotrophin (hCG) or GnRH analogue (in short protocol) is finally administered.

There is growing evidence within the literature that indicates that COH has adverse consequences upon oogenesis, embryo quality and endometrial receptivity [114–120]. Obtaining >10 oocytes per woman has been shown to negatively affect their quality as measured by oocyte/embryo morphology, fertilization and implantation rates. [114, 121]. Others have determined that the optimum number of retrieved oocytes associated with a clinical pregnancy is 13 [122]. An excessively high level of E_2 is seen in ovarian hyperstimulation syndrome (OHSS); this is commonly associated with poor-quality oocytes and serves as an extreme example of the detrimental impact of COH [123]. There is evidence that COH may disturb oocyte maturation and completion of meiosis, thus increasing the likelihood of resultant aneuploid oocytes and/or embryos [117, 124]. In rodents, a delay in embryo development has been attributed to exogenous Gn administration [125, 126].

Suppression of LH levels approaching the peri-ovulatory phase has been associated with downstream disruption in follicular steroid synthesis. Accordingly, stimulation protocols incorporating exogenous LH were established and an improvement in the percentage of diploid and good-quality embryos was reported [127, 128]. However, such findings were not consistent, and other studies detected a decrease in pregnancy rates and increase in miscarriage rates following the incorporation of exogenous LH into protocols [129, 130]. This leads to the suggestion that there is an "LH window" below which E_2 production is inadequate and above which high levels of LH promote premature luteinization or degenerative changes [131]. Excessive LH may stimulate TCs, increase FF androgen levels, switch off E_2

synthesis and increase FF P levels prematurely all of which are known to have a detrimental effect on oocyte development.

Notably, in large clinical studies, comparing COH protocols with human menopausal gonadotrophin (HMG), recombinant (r)FSH and rFSH +LH, a definite difference in pregnancy rate per oocyte retrieved was identified [127, 132]. It is thought that low-dose exogenous LH during COH administered either as HMG or as rFSH +LH combination positively affects the quality of the embryos [127, 133], thus implying that type of exogenous Gn administered has a differential impact on follicular metabolism.

Natural Cycle IVF

Natural cycle IVF (NC-IVF) was initially proposed as an alternative treatment for older women and poor responders [134]. Given that the natural cycle that results in a pregnancy is the prototype that we aspire to reproduce in all women, with the emerging evidence of the detrimental effects of exogenous Gn stimulation, an interest in developing protocols avoiding COH has been gaining momentum. This is with a view to creating a cohort of better quality embryos as opposed to obtaining as many as is possible [135]. Other more recently realized benefits of NC-IVF include supporting the international drive to reduce multiple pregnancies with elective single embryo transfer (eSET) and avoiding the deleterious impact of COH induced OHSS. However, the success rates after NC-IVF are much lower than those achieved with COH-IVF cycles and have been reported at less than 10% per cycle [109] in one study and 15.2% live birth rate per initiated cycle in a more recent analysis [136]. Modified natural cycle IVF (MNC-IVF) is the term applied when drugs are administered in a spontaneous cycle to minimize the risk of cancellation due to spontaneous ovulation; hCG is given to induce final oocyte maturation [137] and for luteal support. The third alternative of Mild ovarian stimulation IVF (M-IVF) has also been proposed as a compromise utilizing low-dose Gn stimulation aimed at generating no more than eight oocytes per cycle [138]. A recent publication detailing the outcomes from a Swedish clinic offering MNC-IVF and M-IVF described high cycle cancellation rates of 53.5 and 39.6%, respectively, but ongoing pregnancy rates in women ending up with an embryo transfer (ET) were significantly superior to earlier publications with natural cycle IVF at 26.7 and 27.2%, respectively, per ET [139]. However, including the cancelled cycles, success rate per treatment attempt with MNC-IVF and M-IVF remains significantly lower than that with COH although cost and risk is also lower and more treatments can be performed repetitively in consecutive cycles than is possible with COH.

Follicular Fluid in Natural Cycles

As previously described, during normal folliculogenesis, FF composition exhibits dynamic fluctuations as a consequence of individual cell types responding to gonadotrophins by secreting various hormones, growth factors and cytokines which in turn influence the development/function of both the somatic cells and the oocyte [140, 141]. As oocyte quality impacts ensuing embryo viability [141], it has been postulated that the disruption in the delicate balance of these intra-follicular mediators as a consequence of exogenous Gn stimulation may impact the outcome of the treatment cycle [99, 142, 143]. Precise regulation of hormone pathways is a prerequisite to sustain healthy physiology, and this fundamental purpose is emphasized with a multitude of endocrine disorders being associated with dysfunctional follicles. Such hormonal dysregulation in FF also underpins several pathologies in the reproductive system [29, 43, 46, 50, 69, 106]. Study of the intra-follicular endocrine environment in natural cycles provides a greater understanding of the impact of pathological disorders and exogenous stimulation with Gn upon the developing follicle and oocyte. Such investigations will also help in establishing whether or not mild stimulation with lower doses or exogenous hCG in natural cycle IVF

minimizes such risks and whether in randomized studies better success rate per treatment attempt can be achieved.

Comparing Follicular Hormonal Milieu Between NC-IVF and COH-IVF Cycles

Subsequent to the introduction of IVF, a number of studies have analysed FF from women undergoing COH. Majority of these studies were instigated to identify prognostic variables for the likelihood of retrieving a viable oocyte, successful fertilization, high-quality embryo morphology and ultimately a clinical pregnancy [144–146]. There is a paucity of publications studying the intra-follicular endocrine profile of unstimulated ovaries [147–152].

Original studies included McNatty et al. [147] measuring hormones in follicles resected from ovaries removed following oophorectomy at various stages in the menstrual cycle using an immunoassay (IA) as well as quantifying GCs and assessing oocyte maturity. Their findings led them to postulate that follicles with the potential for further development had a high number of GCs relative to their diameter, contained higher concentrations of 17-β-oestradiol and/or GCs with the capacity to generate this hormone in response to FSH together with a germinal vesicle stage oocyte [147]. Subsequent to this fundamental work, intra-follicular E_2 concentrations were shown to rise in dominant follicles and correlate with the diameter of these follicles, implying that significant increase in aromatase activity is restricted to the dominant follicle. This research also only identified P production in the largest dominant follicles, and the authors speculated a limited role for P during follicle development [151].

One of the early studies investigated the impact of pituitary down-regulation and COH on the intra-follicular hormonal milieu and compared FF steroids and hCG levels in women undergoing either a long or short stimulated cycle, or MNC-IVF in which exogenous hCG was administered [140]. In this study, the first aspirate was the only sample analysed and only if an oocyte was retrieved. Both E_2 and T levels were significantly higher in the NMC FF when compared with COH FF [140]. Lin et al. [153] also demonstrated similar findings in the FF of naturally cycling women, who in this study had also not received the exogenous hCG. The FF P levels were equivalent between the COH and NC patients, and this led them to suggest only a minor role for P in follicular development [153]. This study utilized IA (solid-phase fluoroimmunoassay [140] and ^{125}I RadioImmunoAssay for hormone analysis [153]). The IA is limited in part because of the diminutive quantity of FF in individual follicles and the inability to quantify multiple analytes simultaneously in extremely small volume samples. Furthermore, there is a high potential for cross-reactivity and this raised doubt regarding the precision of results and hence the conclusions of these studies.

Subsequently, Kushnir et al. [154] attempted to improve the accuracy of quantification of the hormone concentration using liquid chromatography-tandem mass spectrometry and this also enabled simultaneous quantification of multiple steroids. Using this technique, levels of 17-hydroxyprogesterone (17-OHP), total androgens and oestrogens were found to be 200–1000-fold greater in the FF than in the serum confirming the Edwards conclusion of the FF being the repository for hormones produced by GCs and TCs [14]. In keeping with other studies [148], androgens were also the most abundant class of steroids in the FF of natural cycles. However, the absolute concentrations of T, A4 and E_2 were much lower than those previously measured by IA [154]. Furthermore, contrary to earlier work, it appears that FF as measured in young healthy egg donors has lower E_2 in FF after COH [155], and this finding has also been further verified in other studies [140, 153].

Contradictory results have also been reported in the FF samples from women undergoing COH where they not only had significantly higher levels of E_2 compared to NC FF but there were also higher levels of pregnenolone, 17-OHP and cortisol [154]. Notably, this study is limited by the low numbers of participants, and also the fact that the women in the NC arm underwent laparoscopic follicular aspiration prior to day 7 of

the follicular phase, with aspiration of all follicles measuring 5–8 mm, and pooling of follicular fluid; hence assessment of the features of a dominant follicle was not possible.

The Association Between FF Endocrine Profiles, Resultant Oocyte and Embryo Quality in NMC-IVF and COH-IVF

Even though there is increasing evidence supporting the deleterious effects of COH, the results per cycle in NC and MNC-IVF are poorer when compared to those in COH-IVF cycles. Jančar et al. [156] attempted to ascertain whether this is associated with a suboptimal intra-follicular environment of NC and MNC-IVF cycles. When a MNC-IVF cohort (hCG to induce ovulation) was compared with a COH cohort (short antagonist stimulation protocol utilizing rFSH) [156], there were no differences in embryo quality but the implantation rate per ET was 4.9% in the MNC cohort compared with 36.4% in the COH cohort ($p = 0.031$). The authors hypothesized that the administration of hCG (administered when the dominant follicle measured ≥ 16 mm in diameter and serum E_2 exceeded 0.39 nmol/L) in MNC-IVF cycles may have been too premature and may have had detrimental effect on the endometrium. Unlike what one would have expected, amongst the MNC cohort, follicular AMH, FSH, E_2 and P concentrations were not found to correlate with embryo quality even though the overall results were higher [67, 68]. By contrast, following COH FF AMH levels were significantly lower in empty follicles or those yielding degenerated oocytes. We think that one explanation for this discordant result could be the follicular heterogeneity seen following exogenous gonadotrophins, which is not a feature of MNC-IVF. When comparing LH in FF of COH group, its level was significantly lower in those follicles that yielded oocytes and ultimately embryos that resulted in an implantation after transfer when compared with follicles that generated embryos that were transferred but which did not implant [156].

Notably LH was significantly higher in the FF and serum of the MNC cohort when compared with the COH cohort, and the authors have attributed to a spontaneous LH surge on the day of oocyte retrieval, in spite of early hCG administration. A high level of LH would be expected to correlate with a high P level secondary to enhanced luteinization. By contrast, serum P concentration was significantly lower in the MNC cohort, implying that in fact premature luteinization had not actually occurred. These discordant results are difficult to explain and the authors have suggested that synchronization between the developing oocyte and receptivity of the endometrium may be affected in such circumstances. [156].

Recently, de los Santos et al. [155] conducted a study on healthy oocyte donors undergoing MNC-IVF (hCG administered when the dominant follicle reached 18 mm in diameter) and COH-IVF (GnRH agonist long protocol). The dominant FF in the MNC cohort and the FF from one of the larger follicles in the COH cohort were analysed. In contrast to the studies previously described, T was higher in the COH cohort. However, in agreement, E_2 was significantly higher in the MNC cohort together with A4 and LH. Interestingly, in the MNC cohort, in spite of these findings the E_2: T ratio was conserved. Previous work has shown that the E_2: T ratio correlates with fertilization and cleavage rates [157, 158]. In this study, in agreement with Jančar et al.'s findings [156], LH in the FF was higher by approximately 100-fold in the NMC cohort, but again this has been presumed secondary to an undesirable premature LH surge in this group. Once again there was no correlation between the FF LH and P levels in both cohorts. Follicular FSH and P were the only follicular hormones that did not appear to be affected by COH. In this study, regardless of intra-follicular hormone levels, there were no differences in embryo morphology between the two groups. However, in the same study the oocyte meiotic spindle and cumulus gene expression profiles were simultaneously studied in the two groups. COH was found to induce changes in gene expression, and together with the alteration in the FF hormone profile, it has been suggested that Gn stimulation may affect immune processes, meiosis and ovulation pathways.

Pursuing the notion that AMH might be a positive marker for oocyte viability following COH, in an attempt to identify the mechanism stimulating AMH production, Andersen and Lossl compared various COH regimens and proposed that a follicle rich in hCG and androgens promoted AMH secretion [159]. A hypothesis thus arose: suppression of endogenous LH in COH cycles may lead to a reduction in TC androgen production and in turn lower intra-follicular androgen concentrations and ultimately lower AMH levels with associated poorer oocyte quality. Whilst this had not been identified in previous studies [155, 156], von Wolff et al. investigated this hypothesis further and simultaneously defined the NC intra-follicular endocrine milieu. They studied the effects of gonadotrophins on follicular physiology by comparing FF hormones following COH with HMG in short cycles (FF analysed from first large follicle aspirated) and in NMC-IVF cycles (hCG administration to trigger ovulation) [160]. AMH, T, E_2, A4 and LH were all higher in the FF of the MNC cohort.

Conversely P concentrations were similar in both protocols in the FF, although the serum levels were significantly raised in the COH cohort. The authors demonstrated a positive correlation using regression analysis between AMH and T ($r = 0.34$, $p = 0.0002$). The increased T in the MNC cohort may be explained by the elevated LH activity as previously described and is supported in this study by a positive correlation between T and LH, thus supporting, although not proving, the supposition of a dependency of the AMH concentration on T levels. The authors conclude that in FF, LH plays an important role in indirectly contributing towards oocyte quality, and use this argument to favour protocols which enhance LH levels, including both MNC-IVF and also short GnRH antagonist cycles where LH concentrations are less suppressed when compared with long GnRH agonist protocols. Therefore, it has been suggested in studies comparing these stimulation protocols that implantation rates in short cycles are higher [161].

Pathology Related Follicular Hormone Profiles in NC-IVF

A prerequisite for NC-IVF is the ability to spontaneously ovulate. This therefore precludes women with ovulatory dysfunctional PCOS. For this reason, FF endocrine profiles in such cycles are absent from the literature. However, there are data pertaining to intra-follicular hormone levels in different cycle regimens. It is well established that the FF in women with PCOS contains high T concentrations, regardless of the stage of follicle development, as well as reduced P levels, implicating that paracrine factors may inhibit follicular cell P secretion. Such findings offer some understanding into the complex pathophysiology of this disorder [162]. Interestingly, in short cycles, rFSH was found to result in higher FF P concentrations in PCOS women when compared with HMG [163]. Thus, demonstrating how unravelling the intra-follicular milieu in subfertility-related pathologies may assist in designing individualized treatment protocols.

Whilst ovulation may occur spontaneously in women with other subfertility-related pathologies, there remains a paucity of research into the intra-follicular milieu of such patients in natural cycles. In one small study, both endometriosis and unexplained infertility patients demonstrated reduced LH concentrations in FF, implying that there may be a shared common pathophysiology of impaired LH secretion [164]. Furthermore, FF AMH concentrations have been found to be similar in women with mild endometriosis when compared with control subjects [165]. Further work in this area is warranted and in particular may assist in determining undiagnosed endocrine pathology in women considered to have unexplained infertility.

Weaknesses in Published Literature

An important discussion regarding the impact of exogenous Gn upon the ovarian follicle relates to the consequential follicular heterogeneity.

Numerous interdependent events affect oocyte maturation and the acquisition of developmental competence, including interactions between follicular somatic cells and the oocyte [4, 7], the composition of the FF and the vascularity of the follicle [166, 167]. Many of these mediators within the follicle including hormones alter with follicle size and oocyte growth in stimulated cycles [168].

Whilst McNatty et al. in their early work demonstrated an evolving endocrine profile throughout the follicular phase in natural cycles which related to follicular antral diameters [17], the studies described compared COH cycles with NMC cycles and generally have analysed the FF from the lead follicle only, or only from the first follicle aspirated in the COH cycles. Thus, the variation in the hormone profiles relative to the size of the pre-ovulatory follicle is inherently and by design not addressed in these studies. Ultimately, the quality of the oocyte is determined by its nuclear and cytoplasmic maturation [169], which have been shown in themselves to be asynchronous in stimulated cycles [170]. Our studies have also demonstrated asynchrony of follicular size and oocyte quality as well as heterogeneity between follicles of the same volume.

Furthermore, the authors have found that the lead follicle in stimulated cycles is not consistently the follicle that yields the oocyte with the greatest viability. Exogenous gonadotrophins accelerate the follicular phase by increasing the follicular growth rate [171], and this may also accelerate cytoplasmic maturation of oocytes in all follicles in both the leading and the secondary cohorts. Consequently, follicles from secondary and tertiary cohorts are also capable of yielding mature oocytes that have a good developmental potential. The authors think that failing to cater for follicular heterogeneity and asynchrony of follicular and oocyte development induces errors in assessments when correlating FF endocrine profiles and these errors could lead to lack of or incorrect conclusions.

A further concept worthy of consideration is the actual growth rate of the follicles. Several animal studies have investigated the rate of follicular growth in unstimulated cycles [172, 173]. Zegers-Hochschild et al. describe a distinctive "conceptual pattern" of growth whereby there is rapid early follicular growth that stabilizes in the 24-h preceding ovulation in human natural cycle conceptions [173]. Nayudu replicated Zegers-Hochschild et al.'s findings following COH [174]. Furthermore, it is thought that the rate of follicular growth immediately preceding hCG trigger has an influence on oocyte's competence [175].

Recently the authors have demonstrated differential growth rates following hCG administration. A slower growth rate correlated with improved oocyte viability. To date, no studies have measured follicular hormone levels in such circumstances, which could improve our understanding regarding the endocrine events following ovulation induction.

Attaining oocyte competence is dependent on a perfectly regulated process of folliculogenesis [4, 7, 176–178]. The FF from NC follicles that lead to a mature oocyte extraction and transfer of a competent embryo resulting in a pregnancy and birth can be considered as a model for the ideal follicle as evolution is likely to have perfected folliculogenesis. Even though results of COH-IVF remain substantially higher than NC and MNC-IVF, any endocrine intervention is thought to adversely disrupt the hormonal milieu. The growing interest in this area is important, in minimizing the risks of multiple pregnancy and OHSS and in understanding the impact of different regimens in different conditions so that individualization and tailored approach in treatment can be made.

Many studies aiming to characterize the follicular fluid endocrine milieu have had inconsistent results. The study design for many limits their value, and many are too small and unpowered. Contamination of FF with blood, and therefore inaccurate assessment of FF hormones, due to the presence of systemic levels has been rarely considered in analysis, and when addressed, the solution is to use the first aspirate, and to discard samples if the FF appears blood stained, thus only accounting for macroscopic contamination. When analysing FF from COH-IVF cycles, the first large follicle aspirated is most frequently used for analysis. Thus, the heterogeneity of individual follicles is not addressed.

Where multiple follicles are collected, these are often pooled, again disregarding the relevance of the heterogeneous nature of the follicular cohort following COH. Unless oocytes are cultured individually and tracked longitudinally to their fate, the FF profile cannot be correlated with individual oocyte outcomes, thus limiting many studies. Table 6.1 illustrates the findings of the main studies comparing NC FF with COH FF and demonstrates the inconsistencies between the studies, making the transfer of information between studies difficult to interpret. Furthermore, natural cycles in infertile women can hardly be considered as the normal prototype as the condition leading to infertility *per se* including PCOS, endometriosis and other pelvic

Table 6.1 Comparing follicular fluid concentration of hormones in NC-IVF and COH-IVF cycles as measured in various studies

FF hormone	Study	Measurement	NC-IVF		COH-IVF		*P* value
			Concentration	*n*	Concentration	*n*	
Ovarian-derived hormones							
T	von Wolff et al. [160]	Median (μmol/l) (range)	47.2 (1.5 ≥ 52)	36	18.8 (2.8 ≤ 52)	40	<0.0001
	Enien et al. [140]	Mean (nmol/l) (95% CI)	21.80 (15.85–29.99)	20	15.03 (45.14–56.90)	40	0.0034
	Lin et al. [153]	Mean (ng/dl) (±SEM)	1798.3 (±226.3)	3	593.5 (±51.6)	16	<0.0001
	de los Santos et al. [155]	Mean (nM) (±SD)	14.5 (±13.9)	42	34.2 (±44.8)	18	0.04
A4	von Wolff et al. [160]	Median (nmol/l) (range)	290 (8.0 ≥ 350)	36	206 (29 ≥ 350)	40	0.0035
	Lin et al. [153]	Mean (ng/dl) (±SEM)	171.0 (±51.6)	3	50.4 ± 3.9	14	<0.0001
	de los Santos et al. [155]	Mean (nM) (±SD)	368.8 (±441.4)	42	92.2 ± 196.5	18	0.02
P	Enien et al. [140]	Mean (nmol/l) (95% CI)	63,570 (53,380–73,760)	20	56,010 (51,580–60,440)	40	NS
	Lin et al. [153]	Mean (ng/ml) (±SEM)	9200.0 (±529.1)	3	10,263.6 (±1212.0)	11	NS
	de los Santos et al. [155]	Mean (nM) (±SD)	21245.9 (±11107.1)	40	24333.5 (±11063.5)	18	NS
	Jančar et al. [156]	Mean (nmol/l) (±SD)	26482.2 (±12942.7)	29	33276.8 (±15827.4)	30	NS
E_2	von Wolff et al. [160]	Median (nmol/l) (range)	3292 (369–7153)	36	1225 (105–5020)	40	<0.00001
	Enien et al. [140]	Mean (nmol/l) (95% CI)	3298 (Unknown)	20	3017 (2915–3119)	40	0.0032
	Lin et al. [153]	Mean (pg/ml) (±SEM)	730,933 (±153,260)	3	28,672 (±52,634)	11	<0.04
	de los Santos et al. [155]	Mean (nM) (±SD)	1711 (±1009)	40	824 (±591)	18	<0.001
	Jančar et al. [156]	Mean (nmol/l) (±SD)	7447.5 (±4401.4)	29	3356.7 (±2742.8)	30	<0.001

(continued)

Table 6.1 (continued)

FF hormone	Study	Measurement	NC-IVF		COH-IVF		*P* value
			Concentration	*n*	Concentration	*n*	
AMH	von Wolff et al. [160]	Median (pmol/l) (range)	32.8 (0.5–281)	36	10.7 (1.0–238)	40	<0.0001
	Jančar et al. [156]	Mean (ng/ml) (±SD)	6.1 (±5.5)	29	2.5 (±1.7)	30	<0.001
Pituitary-derived hormones							
FSH	von Wolff et al. [160]	Median (mIU/ml) (range)	4.9 (0.2–15.6)	36	7.2 (1.3–17.3)	40	0.04
	de los Santos et al. [155]	Mean (mIU/ml) (±SD)	3.6 (±1.7)	40	4.5 (±5.2)	18	NS
	Jančar et al. [156]	Mean (IU/l) (±SD)	5.9 (±3.0)	29	7.1 (±10.4)	30	NS
LH	von Wolff et al. [160]	Median (mIU/ml) (range)	14.4 (0.3–60.0)	36	0.9 (0.2–12.2)	40	<0.0001
	de los Santos et al. [155]	Mean (mIU/ml) (±SD)	13.7 (±8.4)	40	0.1 (±0.2)	18	<0.001
	Jančar et al. [156]	Mean (IUl/l) (±SD)	15.6 (±8.7)	29	2.0 (±4.6)	30	<0.001

FF Follicular fluid, *NC* Natural cycle, *COH* Controlled ovarian hyperstimulation, *T* Testosterone, *A4* Androstenedione, *P* Progesterone, E_2 Oestradiol, *AMH* Anti-Müllerian hormone, *FSH* Follicle-stimulating hormone, *LH* Luteinizing hormone, *SD* Standard deviation, *SEM* Standard error of mean

inflammatory disorders such a PID may have an impact on follicular hormonal milieu through paracrine and autocrine effects that cannot be easily excluded. Even in the so-called normal women there will be some dysfunctional cycles and these cannot be prospectively identified for treatment.

Impact of HCG in MNC Cycles

The MNC-IVF cycles commonly use hCG to trigger ovulation, and plan the oocyte retrieval; thus, the endocrine environment in the FF does not represent a pure physiological condition. Chorionic Gn stimulation has been shown to increase the blood–follicular permeability in animal models [179, 180]. Notably, however, a study comparing the FF/serum ratios of extra-ovarian hormones (cortisol and prolactin) in MNC and COH cycles failed to reveal a Gn-induced blood–follicular transportation capacity, thus implying that exogenous gonadotrophins of follicular hormones are not a consequence of increased ovarian permeability of extra-ovarian hormones [181]. Perhaps of significance in this study, hCG was administered in the MNC cohort, which may alter the interpretation of these findings. Interestingly, FF following hCG for ovulation trigger has been compared with GnRH agonists in COH cycles and significant differences were identified. FF levels of P, together with inhibin levels, were elevated in the hCG trigger cohort, indicating that hCG causes a prolonged luteotrophic effect well before ovulation, compared to the endogenous surge of gonadotrophins secondary to GnRHa agonists, suggesting that follicular maturation with an endogenous surge of gonadotrophins may be closer to the NC than when ovulation is induced with hCG [182]. In everyday practice, the option of omitting hCG in NC-IVF, and recreating the pure physiological cycle, is perhaps unrealistic, thus limiting the possibility of avoiding any effect of intervention on the follicular milieu [183].

Conclusion

This review highlights the complex interrelationships between hormones both locally produced within the follicular cells, and extra-ovarian sources, and that an exquisite balance exists, which if perturbed, either by pathological conditions or by intervention with exogenous hormones, there is a consequential disruption to the folliculogenesis processes, and in certain cases this may impact the oocyte viability. Whilst there are many identifiable detrimental effects on the reproductive tract as a consequence of COH, synchronizing the optimal oocyte maturation processes with endometrial receptivity in natural cycles remains a challenge and requires further exploration.

Important research is required in this field utilizing larger appropriately powered studies, to further determine the optimal FF endocrine environment in the natural cycle, and thus develop a treatment protocol with the capacity to replicate this. Studies exploring the degree of changes on the intra-follicular milieu with varying doses of exogenous Gn including minimal stimulation regimens to ascertain whether there is a level of stimulation that should not be exceeded should be pursued. Similarly, research utilizing larger studies would be useful to identify demographic and cycle predictors of FF hormone levels, and their effects on fertilization rates, embryo quality and pregnancy outcome.

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Standard Ovarian Stimulation Protocols and Their Outcomes

7

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Introduction

The principles of optimization of controlled ovarian stimulation for IVF can be divided into four concepts:

Concept 1: Assessment of ovarian reserve
Concept 2: Optimizing the ovarian stimulation by individualizing protocol

- 2.1. The choice of gonadotropin preparation, urine or recombinant FSH
- 2.2. LH supplementation
- 2.3. Ovarian stimulation protocol: GnRH agonists or antagonists
- 2.4. Dose of gonadotropins
- 2.5. Cycle scheduling for IVF treatment with oral contraceptive pills or estradiol
- 2.6. Treatment monitoring

Concept 3: Trigger of ovulation

- 3.1. HCG trigger
- 3.2. GnRH agonists trigger
- 3.3. Timing of HCG or GnRH agonists administration
- 3.4. Lag time from ovulation trigger to oocyte aspiration
- 3.5. Predicting successful induction of ovulation by HCG and GnRH agonist

Concept 4: Luteal phase support

Assessment of Ovarian Reserve

Women's natural fertility is optimal between the ages of 20 and 30 and starts to decline after the age of 30. The acceleration rate of oocyte degeneration is higher when women are in their mid-30s, which leads to a decline in fertility potential up to the age of 41 [1]. The declining of fertility in older age women has two causes: The first is the decreased probability of natural conception. The second cause is an increased likelihood of spontaneous miscarriage due to a rise in chromosome abnormalities as women age [2]. Some investigations have demonstrated that the majority of embryos originating from women over 37 years old are chromosomally abnormal, containing both chromosomal monosomies and trisomies [3].

A pretreatment evaluation before the process of in vitro fertilization (IVF) is an important step in identifying and classifying patients into the groups of high, normal, and low responders. The benefit of evaluation is that it allows patients to better understand the root causes of success or failure of the treatment, thereby lessening the

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disappointment of an unsuccessful process and helping couples to decide on alternative treatment. Likewise, in high responders, the evaluation allows the doctor to choose the treatment protocol while minimizing the risk of ovarian hyperstimulation syndrome (OHSS).

Ovarian reserve assessment is the first step in determining the functional potential of the ovary, reflecting both the number and quality of oocytes before the IVF treatment. Effective ovarian reserve tests should have a high prognostic value regarding pregnancy outcomes for individual patients, with or without treatment. Furthermore, ovarian reserve tests can help doctors determine which ovarian stimulation protocols work best for individual patients, thereby optimizing the chances of a successful outcome.

There are various tests and markers of ovarian reserve, which are described in the literature. The following are the three most common markers currently being used in ovarian reserve tests:

Antral Follicle Count

Initially, a simple antral follicle count is conducted via transvaginal ultrasound [4, 5]. Studies have confirmed that this method of assessing ovarian reserves is noninvasive and easy to perform [6, 7]. An assessment of the number of antral follicles during the early follicular period is usually performed with a two-dimensional ultrasound which is used to measure the two dimensions of follicles and calculated into the mean diameter of the follicles. The number of small antral follicles that are 2–6 mm is closely related to the ovarian function. This number declines with age; however, the larger follicle number is not shown to relate with age [8]. The predictive ability of the small antral follicle count for ovarian function was supported by a study group that the small follicles at 2.1–4.0 mm are the most significant predictive factor regarding the number of retrieved mature oocytes [9]. However, several studies have shown that the total number of antral follicles, regardless of size, is related to ovarian response as well [10, 11].

The ability of an antral follicle count to predict whether there will be a poor ovarian response in IVF was significantly better than that of a basal FSH [10]; however, the antral follicle count's ability to predict a pregnancy outcome is as poor as a basal FSH [10].

Anti-Mullerian Hormone

Anti-Mullerian hormone (AMH) is a dimeric glycoprotein hormone and a member of the transforming growth factor-β (TGF-β) superfamily. It is secreted by the granulosa cells of pre-antral small follicles. The AMH level is detected in follicles 4 mm or less in size, from the pre-antral stage through the small antral stage [12]. In larger follicles of 4–8 mm, the AMH level is gradually decreased in secretion until it disappears [12]. The AMH level significantly correlates with transvaginal ultrasonography for antral follicle count [13] without inter-cycle variability [14]. The level of this hormone does not fluctuate throughout the menstrual cycle and can be used to evaluate ovarian function on any day of the menstrual period [15, 16]. AMH is not just used for detecting the ovarian reserve; it is also used to predict an excessive ovarian response to medication [17]. The cutoff level of AMH for predicting which patients will be poor responders has not been determined. The controversial points that remain on the different assay to detect AMH can make a different AMH level, and a great variation of laboratories and samples are needed for further investigation [18].

Basal Serum FSH

The day 3 FSH level test is the most widely used for assessing ovarian function [19]; however, the level is variable for both inter- and intramenstrual cycle [20, 21]. There is no cutoff level to predict the ovarian reserve except when at a high FSH level, the ovarian response is poor [22]. The predictive ability of high FSH levels for IVF failure is unclear [23]. However, in moderately high FSH

levels (more than 11.4 mIU/ml), the pregnancy outcome is poor [24]. Anyway, the acceptable cutoff level for FSH in determining diminished ovarian reserve is 10 IU/L or more [18].

Mutlu et al. [25] studied the predictive value of AMH serum and an antral follicle count (AFC). The authors concluded that the AMH serum cutoff level between a poor and normal response is 0.94 mg/ml (with sensitivity of 70% and specificity of 86%). For AFC, the cutoff value is 5.5 (with sensitivity of 91% and specificity of 91%) [25]. The authors concluded that age is still the most predictive factor for determining the probability of pregnancy [25]. Currently, studies on finding the cutoff value of the tests to determine pregnancy outcomes are ongoing and not conclusive. We should not use the tests of ovarian reserve to eliminate the patient from the treatment program.

Optimizing Ovarian Stimulation by Individualizing the Protocol

The Choice of Gonadotropin Preparation, Urine, or Recombinant FSH

A large independent meta-analysis by the National Institute of Clinical Excellence of the UK [26] including a total of 21 randomized controlled trials compared recombinant FSH to all kinds of urine FSH in a GnRH agonist protocol. The study concluded that there were no significant differences between recombinant and urine FSH in terms of live births, ongoing pregnancy, and clinical pregnancy rates. However, the efficiency of recombinant FSH is greater than that of urine FSH in each unit, so the dose requirement of recombinant FSH is lower due to batch-to-batch consistency. A summary of a meta-analysis published by the Cochrane Library [27] concluded that there are no significant differences between recombinant and urine FSH in terms of pregnancy outcomes, live birth rates, and incidences of OHSS, both in fresh and frozen-thawed cycles. The authors concluded that all available FSH preparation could be used for an ovarian stimulation protocol in IVF without differences in pregnancy outcomes and incidences of OHSS.

LH Supplementation

Both follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are essential for ovarian follicular development, according to the two-cell, two-gonadotropin hypothesis. In the early follicular phase, FSH plays a crucial role in recruiting small antral follicles from apoptosis. In the late follicular phase, LH signals theca cells to produce androgens. FSH signals granulosa cells to increase the transcription of gene encoding enzymes for the conversion of androgens to estrogens [28, 29]. A study showed that only 1% of occupied endogenous LH receptors can be successfully stimulated with FSH alone [30].

The efficacy of recombinant FSH in ovarian stimulation has been established; however, the role of LH supplementation in ovarian stimulation to improve pregnancy outcomes remains inconclusive. In specific cases with hypogonadotropic hypogonadism or with endogenous LH production less than 1.2 mIU/ml, stimulation with FSH alone produced poor pregnancy outcomes [31, 32], while when both FSH and LH were supplemented, pregnancy rates improved [33–36]. In the poor responder subgroup, the strategies for improving pregnancy outcomes were limited [37–39] and increasing the dose of FSH did not increase the rate of success [40, 41]. A recent meta-analysis assessed the outcomes of r-FSH compared with r-FSH plus r-LH for ovarian stimulation in IVF [42]. The study concluded that there was no difference in the number of oocytes retrieved in the overall population; however, in poor responders, the number of oocytes retrieved in r-FSH plus r-LH was significantly greater than that in r-FSH alone. Furthermore, the clinical pregnancy rate increased by 30% in r-FSH plus r-LH, compared with r-LH alone in the overall population [42].

Ovarian Stimulation Protocols: GnRH Agonist or Antagonist

The goal of controlled ovarian stimulation in IVF is to produce multiple mature follicles for the process of IVF. There are several studies, which confirm that the number of oocytes and embryos is associated with the success of IVF treatment [43, 44]. During the process of multiple follicular developments, a premature LH surge can be prevented by the application of GnRH agonists [45]. There are two GnRH agonist regimens available for the prevention of an LH surge. The most frequently used regimen is the GnRH agonist long protocol [46]. The alternative regimen is the GnRH agonist short protocol, which is commonly used in poor responders [47]. By adding GnRH agonists on day 3 of the patients' cycle in short protocol, the regimen has two benefits: It causes an initial flare-up of gonadotropins to stimulate the follicles and avoids excessive pituitary suppression. Hence, the response in poor responders would be improved. The new GnRH antagonist is introduced to the ovarian stimulation program for the purpose of improving the response, especially in poor responders, by preventing a premature LH surge without excessive suppression of the level of endogenous gonadotropins [48]. GnRH antagonists' effect on pituitary suppression is immediate after injection, thus avoiding excessive suppression with a reduced duration of gonadotropins [49–51]. The additional benefit of GnRH antagonist protocol is that the protocol lowers the incidence of OHSS by its own protocol and by substituting the medicine for triggering ovulation from HCG to GnRH agonists [52].

The most recent Cochrane database systemic review showed no significant difference between GnRH long agonist protocol and GnRH antagonist protocol for ovarian stimulation in terms of live birth rates; however, the incidence of OHSS is significantly lower in the GnRH antagonist group [53]. In patients less than 35 years old with a positive prognosis, GnRH long agonist protocol was associated with a higher live birth rate per transfer and a lower rate of cancelation than GnRH antagonist protocol [54]. In addition, GnRH long agonist protocol was associated with a higher implantation rate per elective single embryo transfer than GnRH antagonist protocol. However, the incidence of OHSS in the GnRH antagonist group is lower than that in that agonist group [54].

In a specific group, three ovarian stimulation regimens (long agonist, short agonist, and antagonist protocol) were randomized for poor responders. These responders were defined as women who had had a previous IVF treatment with a gonadotropin dose ≥ 300 IU and had 3 oocyte retrieved or who had cycle cancelation due to <3 oocytes being stimulated [55]. The study demonstrated that the long GnRH agonist and antagonist regimens are comparable in efficacy regarding the number of oocytes yielded. The number of oocytes retrieved was lowest in the short agonist protocol. The total dose of gonadotropins was highest in the GnRH agonist group. However, there is no enough power of the study to detect the difference in pregnancy rates among groups.

Other than the pregnancy outcome concerns of GnRH antagonist compared with long GnRH agonist protocol, there are some beneficial concerns of using GnRH antagonists instead of agonists. GnRH antagonists are associated with a reduced duration of treatment and a reduced risk of OHSS [56, 57]. Furthermore, due to the initial flare-up effect of GnRH agonists, ovarian cysts can occur and interfere with the success of treatment by decreasing the quality of oocytes and embryos, hence compromising the implantation and pregnancy rate and increasing the cycle cancelation rate [58]. Lastly, distress and discontinuation of the treatment are two of the most important factors affecting the success of infertility treatment. After successful pituitary down-regulation from the GnRH agonist protocol, hormone profiles are in the hypo-estrogenic stage. Some patients who received the GnRH agonist regimen experienced physical discomfort after down-regulation that included hot flashes, night sweats, depression, anxiety, and mood disorder one week before ovarian stimulation. However, no study confirmed that distress from the effect of hypo-estrogen is the main cause of leaving from the IVF program before time.

GnRH agonist administration: The purpose of GnRH agonist application is to initiate pituitary down-regulation while simultaneously stimulating the ovary with exogenous gonadotropins. Usually, it takes at least two weeks on GnRH agonists for the pituitary suppression to begin prior to the commencement of exogenous gonadotropins. In order to achieve down-regulation, the estradiol level should be low enough but usually may vary from 20 to 100 pg/ml [59–61]. The incidence of inadequate down-regulation is about 15% of the usage, which should be considered carefully in cases of occult pregnancy or the presence of functional ovarian cysts [62, 63]. The onset of menstruation is usually the sign of adequate down-regulation. Menstruation will be delayed in cases of inadequate suppression. GnRH agonist administration can be done through the nasal, intramuscular, or subcutaneous route with no difference in effectiveness between routes.

The two most common protocols for down-regulation are the long agonist and short agonist protocols. GnRH agonist is administered on approximately day 21 of the preceding menstrual cycle and continued until the day of triggered ovulation in the long luteal protocol. In the short protocol, GnRH agonist is administered on day 2 or day 3 together with gonadotropins until the day of triggered ovulation. Due to the longer pituitary suppression and the initial flare-up effect of the short protocol, the long protocol usually requires more gonadotropins than the short protocol; however, a meta-analysis confirmed that the long protocol allowed more oocytes to be retrieved and produced a higher pregnancy rate compared to the short protocol [64].

GnRH antagonist administration: Several studies were conducted to determine the ideal dose of GnRH antagonist for producing the optimum clinical pregnancy rate with the lowest incidence of premature LH surge [65, 66]. They found that both single dose (3 mg) and multiple dose (0.25 mg) of Cetrorelix acetate are comparable in efficacy and safeness [65, 66]. Ganirelix is one of the medications in GnRH antagonists, which is only available in multiple doses. A study in different doses of Ganirelix [67] concluded that Ganirelix dose of 0.25 mg/day started from day 6 or 7 of stimulation is the lowest effective dose that can prevent LH surge and still maintain good pregnancy outcome. The following studies [68–70].

The GnRH antagonist protocol can be divided into two approaches, based on the day GnRH antagonist administration begins. In the fixed protocol, GnRH antagonist is started on day 5 or 6 of FSH administration regardless of the size of the follicles. In the flexible protocol, the GnRH antagonist is started when the size of the follicles is larger than 24 mm or the serum estradiol level is greater than 300 pg/ml. The GnRH antagonist protocol is continued until the day of triggered ovulation in both protocols, and the interval of the antagonist is not greater than 30 h.

Several randomized control trials have compared outcomes for the fixed and flexible GnRH antagonist protocols. The dose requirement of both GnRH antagonist and gonadotropins in the flexible protocol was less than that of the fixed protocol [71]; however, the pregnancy outcome could not be determined. In 2003, Kolibianakis et al. [72] demonstrated that there was no significant difference in pregnancy rates between the fixed and flexible GnRH antagonist protocols as well as in 2004 Escudero et al. [73] showed that there was no difference in cycle outcomes between the fixed and flexible protocols. However, the patients who are poor responders had a higher incidence of cancelation of treatment when the fixed regimen was applied compared with flexible regimen [73].

The Dutch Ganirelix Study Group conducted the first randomized study regarding GnRH antagonists in 2004 [74]. They reported that there was no difference in the mean number of oocytes retrieved between the fixed and flexible regimens. Likewise, the clinical and ongoing pregnancy rates were the same [74]. A meta-analysis in 2005 confirmed that there was no significant difference in pregnancy outcomes between the fixed and flexible protocols; however, the required dose of GnRH antagonists and gonadotropins was lower in the flexible protocol than in the fixed protocol [75]. The most recent study by Kolibianakis et al. [76] evaluated the incidence of a premature LH rise between the fixed

and flexible protocols, starting on day 3, and demonstrated that there were no difference in LH rise and pregnancy rates. The author suggested that the fixed protocol was preferable to the flexible protocol due to the simplicity of the regimen.

Dose of Gonadotropins

Gonadotropins are the principle medication for ovarian stimulation in IVF. The gonadotropin preparation can be in the form of human menopausal gonadotropin or HMG (containing both FSH and LH), highly purified urine FSH, or recombinant FSH and recombinant LH.

The starting doses of gonadotropin vary between 100–600 IU/day [77, 78]. There have been several randomized control trials showing that a higher dose of gonadotropin combined with GnRH agonist does not improve pregnancy outcomes, even in older patients [79–83]. Likewise, a high dose of gonadotropin does not ameliorate the pregnancy outcome in poor responders or in older patients [84, 85]. The standard dose for optimizing pregnancy outcomes in normal responders is still inconclusive; however, a meta-analysis suggests that the dose of recombinant FSH in normal responders younger than 39 years old should be started at 150 IU [86].

For high and low responders as determined by ovarian reserve testing, the starting dose and protocols should be different. The criteria for high responders include younger than 30 years old, having evidence of polycystic ovarian syndrome (PCOS), lean body status, and a previous history of high response. The lowest dose of gonadotropin should usually not be more than 150 IU/day. To date, the protocol that is most feasible for PCOS patients is the GnRH antagonist protocol with a GnRH agonist trigger combined with cryopreservation of the embryo. The GnRH antagonist protocol allows the administration of GnRH agonists in order to trigger ovulation with a reduction (but not virtual elimination) in the risk of OHSS. Thus, there are reports of incidences of severe OHSS in GnRH agonist trigger combined with low-dose HCG (1500 IU) [87] and GnRH agonist trigger without HCG [88].

Poor response to ovarian stimulation during the IVF treatment program is frustrating, especially when the patient is young. The factors that predispose patients to poor ovarian response are advanced age, a history of ovarian surgery, a history of ovarian disease, and poor ovarian reserve tests. There is no ideal protocol for improving pregnancy outcomes in poor responders, regardless of how many modified protocols are proposed in the literature. The treatment option of increasing the dose of gonadotropins does not improve pregnancy rates in poor responders [40, 41]. It is obvious that the dose of gonadotropins has a limited ability to improve the number as well as the quality of the oocytes in diminished ovarian reserve patients [89]. Two meta-analyses of randomized controlled trial have demonstrated that the GnRH antagonist protocol with gonadotropins has pregnancy outcomes that are comparable to those of the GnRH agonist long and short protocols [90, 91]. The antagonist protocol, however, has a significantly shortened treatment duration compared to the agonist protocols [91]. A very recent meta-analysis about the addition of recombinant LH to recombinant FSH showed that recombinant LH combined with recombinant FSH in poor responders improves the number of oocytes retrieved and the pregnancy rates by 30%, compared with recombinant FSH alone [42].

Cycle Scheduling for IVF Treatment with Oral Contraceptive Pills or Estradiol

The purpose of cycle scheduling before treatment is to avoid the medical personnel working on the weekend from oocyte retrieval process or an excessive workload of the personnel on a specific day by controlling the first day of the period. The efficacy of cycle scheduling program by pretreatment with oral contraceptive pill (OCP) has been postulated [92–95], and recently, estradiol has been advocated [96]. However, a meta-analysis summarized that OCP given pretreatment lowered ongoing pregnancy rates in normal responders

compared with non-OCP. The effect of OCP could be from the effect of the progesterone component on endometrial receptivity and estrogen on LH over suppression when using the FSH-only protocol [97]. In addition, the dose and duration requirements of gonadotropins were increased after OCP pretreatment [98]. In contrast, in a later randomized controlled trial that used only one type of OCP for a shortened duration of 12–16 days, the pregnancy outcomes were not affected (compared with non-OCP in the antagonist protocol) [99]. Recently, Fanchin et al. [100] studied the effects of administering oral estrogen beginning in the mid-luteal phase of the cycle preceding ovarian stimulation by GnRH antagonist protocol. The purpose of administering estrogen is to inhibit only endogenous FSH during the luteo-follicular transition period without suppressing LH, in order to promote coordinated follicular growth [101]. The authors found that the proportion of retrieved cycles on the weekend is significantly reduced by pretreatment with estradiol valerate without a negative effect on the pregnancy outcome [100].

Treatment Monitoring

A recent systemic review and meta-analysis concluded that ultrasonography alone is adequate for monitoring during controlled ovarian stimulation [102]. Adding the serum measurement of estradiol to ultrasonography did not make a difference in the number of mature oocytes retrieved [103]. Nonetheless, we need more data on pregnancy outcomes before we can conclude that ultrasonography is the only effective tool for monitoring during controlled ovarian stimulation.

Trigger of Ovulation

HCG Trigger

Exogenous HCG has been proven to be an effective hormone substitute for an endogenous LH surge. The dose of HCG at 5000–10,000 IU is adequate to induce final oocyte maturation. Due to the similarity between alpha subunit of HCG and LH and 81% of beta subunit sharing, HCG and LH can bind to the same receptor [104]. Recombinant LH has been investigated for use in triggering ovulation at a dose of between 15,000 and 30,000 IU, which is equivalent to 5000 IU of HCG [105]. However, recombinant LH is not routinely used at present because of its short half-life and because multiple doses are needed for luteal support, and it is not cost-effective compared with HCG combined with a vaginal progesterone suppository. For the specific ovulation trigger to the specific ovarian stimulation protocol, GnRH agonist long or short protocols only need HCG to trigger ovulation, while in GnRH antagonist protocol, both HCG and GnRH agonist can be used to induce the final maturation of oocytes.

Due to batch-to-batch variation of urine-derived hormone, recombinant HCG is currently being used as an alternative to urine HCG. A Cochrane review and meta-analysis concluded that no significant differences exist between recombinant HCG versus urine HCG in terms of ongoing pregnancy rates, miscarriage rates, and the incidence of OHSS in the GnRH agonist protocol [106]. In contrast with a later study that single blastocyst transfer with positive pregnancy outcomes was higher in favor of the recombinant HCG. The positive pregnancy outcome of recombinant HCG might be the direct effect of HCG on endometrial receptivity or HCG on better oocyte maturation [107]. However, a sufficient data are needed before drawing a final conclusion.

GnRH Agonist Trigger

Due to the long half-life of HCG compared with natural LH, the LH activity of HCG can prolong the luteotrophic effect that leads to the development of OHSS [108]. GnRH agonists, on the other hand, produce the flare-up effect of natural FSH and LH that lasts only 34 h. By application of GnRH agonists, a physiologic LH surge can be used to trigger ovulation followed by the pituitary

down-regulation effect of GnRH agonists that can reduce the risk of OHSS in the same time. Before the starting use of GnRH antagonists in ovarian stimulation protocol, the application of GnRH agonists for trigger of ovulation is not feasible because GnRH agonists cannot be used in a down-regulation ovarian stimulation protocol. Until the introduction of a GnRH antagonist protocol, the GnRH agonist trigger is becoming the promising method for the induction of ovulation, due to its minimized risk of OHSS. Unfortunately, in the early use of a GnRH agonist trigger, there was a high risk of early pregnancy losses and low ongoing pregnancy rates due to the luteal phase defect that cannot be corrected with routine luteal phase support [109–111]. Devroey et al. [111] proposed a strategy for IVF treatment that would be OHSS-free by using the antagonist protocol with a GnRH agonist trigger. Because of the defective luteal phase in GnRH down-regulation, the embryo transfer procedure is postponed to the following natural cycle with cryopreservation of all embryos (in IVF cycle segmentation). Owing to the widespread use of effective vitrification method for embryo cryopreservation, the method today can increase the chance of pregnancy due to higher embryo survival rates [112, 113] and higher ongoing pregnancy rates, as compared with traditional slow and ultrarapid freezing [113].

For the implantation effect of GnRH agonists, some authors have focused on the ways to improve the defective luteal phase for fresh embryo transfer. Modified luteal phase support has been created to improve pregnancy outcomes by adding a small dose of HCG on the day of oocyte retrieval [114–118] or on the same day of GnRH agonist administration (dual trigger) [119, 120]; however, the improvement of pregnancy outcomes by modified luteal phase supports is still controversial. Apart from the minimization of OHSS that occurs with a GnRH agonist trigger, some studies have shown that a GnRH agonist trigger also has a higher percentage of metaphase II oocytes than an HCG trigger [110, 121]. Likewise, a GnRH agonist could be beneficial for a specific group of patients with immature oocyte syndrome. Immature oocyte syndrome occurs when at least 25% of a patient's oocytes are immature after the process of ovarian stimulation without the evidences of imperfect administration of HCG and incorrect timing of retrieval. Due to the physiologic surge of both LH and FSH after GnRH agonist, FSH could be an important factor for promoting oocyte maturation in vivo.

Timing of HCG or GnRH Agonist Administration

The best timing for HCG or GnRH administration to induce oocyte maturation has not been determined. Usually, HCG is administered when the number of leading follicles (>17 mm) is more than 3 [104, 122]. HCG can also be administered when the number of leading follicles (>18 mm) is 2 or more [123]. Some studies have found that when gonadotropin administration is prolonged to duration of 13 days or more, the likelihood of a live birth is decreased compared with duration of 10–12 days [124, 125]. Delaying the administration of HCG for 24 h has no significant negative effect on pregnancy outcomes in the agonist protocol [126] and antagonist protocol [127]. However, there is evidence that although two-day delayed HCG administration did not affect the embryo quality, it did decrease ongoing pregnancy rates per embryo transfer by advanced endometrium of longer stimulation [128, 129]. Zhang et al. investigated whether early and late HCG administration can be performed in order to avoid working on the weekend. Delaying one to two days is better than early HCG administration in terms of the number of mature oocytes yielded, the fertilization rate, and the number of good quality embryos per cycle that could improve cumulative pregnancy rates [130].

Vandekerckhove et al. studied the relationship between serum progesterone and the maturation rate of oocytes. The authors found that delaying oocyte maturation for 24 h did not improve the number of mature oocytes in patients with high serum progesterone >1 ng/ml. In patients with low serum progesterone (<1 ng/ml), the number of mature oocytes increased significantly; however, there were no significant differences in the

number of fertilized oocytes and the number of good quality embryos between patients with high or low serum progesterone [131].

According to the results of Vandekerckhove et al.'s study, even in cases where the patients have rising progesterone, the oocyte trigger can be delayed for a few days to maximize the number of mature oocytes and the transfer of frozen embryos can be completed later. A recent RCT study concluded that delaying HCG or GnRH agonists by one or two days does not affect pregnancy outcomes, either in agonist and in antagonist protocols, which makes current ovarian stimulation protocols quite convenient for both patients and doctors [132].

Lag Time from Ovulation Trigger to Oocyte Aspiration

The optimal time from ovulation trigger to oocyte aspiration has not been studied adequately and varies between 32–38 h [133–138]. A study conducted to find the ideal time interval between ovulation trigger and aspiration concluded that it should be at least 35 h between ovulation trigger and oocyte aspiration to increase the number of mature oocytes, regardless of the different ovarian stimulation protocols or ovulation methods [139]. In PCO cases, a study [140] found that the lag time should be longer than 38 h to decrease the incidence of empty follicles. The study also showed the improved fertilization and high embryo quality rates when the lag time is 38 h compared with 34 h. A meta-analysis demonstrated that the oocyte maturation rate was higher when the interval between the time of ovulation trigger and the time of oocyte aspiration is longer; however, the fertilization, implantation, and pregnancy rates were not significantly different [141]. The study concluded that prolonging the interval between time of ovulation trigger and time of oocyte aspiration beyond 36 h could increase the number of mature oocytes without affecting the fertilization, implantation, and pregnancy rates [141].

Predicting Successful Induction of Ovulation with HCG or GnRH Agonist

Empty follicle syndrome (EFS) is a condition characterized by a failure to retrieve the oocytes after completing the process of ovarian stimulation and triggering ovulation. It is a frustrating condition for both patient and doctor [142]. There are many causes of EFS. Human error is the common and preventable cause of EFS related to false EFS [143]. Some authors have proposed a strategy for preventing this condition by administering the second rescue HCG with a batch different [144–148].

Stevenson et al. examined the possible treatment options for EFS [149]. In cases where the serum HCG level is less than 40 mIU/ml immediately before retrieval, the second rescue HCG is performed and retrieval is completed 36 h later. Likewise, Reichman et al. [150] proposed another strategy in which absorption of IM HCG is assured via early detection of the serum beta-HCG one day after HCG injection. If the serum HCG is negative, the second rescue HCG is administered and oocyte retrieval is done 35–37 h after the second HCG. By reassuring the bioavailability of serum HCG, the authors found that the incidence of genuine EFS is very low (0.25%) [150].

In cases where ovulation is triggered with GnRH agonists, the incidence of false EFS is not statistically different from cases where ovulation is triggered with HCG [151]. Kummer et al. [152] studied that after GnRH agonist administration for 8–12 h, EFS can be detected when the level of LH $\leq$ 15 IU/L or progesterone level 3.5 ng/ml. The results correspond with a study by Chen et al. [153]. When LH and progesterone levels are lower than the threshold, the second rescue HCG is performed to prevent EFS, followed by oocyte retrieval 35 h later. [153].

Shapiro et al. found that cycles with an LH level less than 52 IU/L 12 h after GnRH agonist administration are suboptimal and that cycles with an LH level less than 12 IU/L are clearly inadequate [154]. The solution for a suboptimal

or inadequate endogenous LH surge is administering the second dose of recombinant LH or HCG to improve the number of mature oocytes.

Luteal Phase Support

Luteal support is a necessary step after the process of ovarian stimulation with either GnRH agonist or antagonist protocol because of defective progesterone secretion that is the result of insufficient endogenous LH activity [155]. The timing of luteal phase support varies and can be done either the day of triggered ovulation, the day of oocyte retrieval, or the day of embryo transfer. The pregnancy outcomes are comparable [156]. The newest Cochrane review on luteal phase support confirmed that progesterone has a beneficial effect on luteal phase support, while other medications for cotreatments such as HCG or estrogen did not help to improve pregnancy outcomes. HCG is the cause of OHSS; hence, there is no indication for the usage except as a dual trigger with GnRH agonist in the antagonist protocol [157]. Synthetic progesterone is preferable to micronized progesterone in terms of better pregnancy results [157].

Regarding the route of progesterone administration, studies have shown that the intramuscular and intravaginal routes have comparable results in terms of clinical and ongoing pregnancy rates [158, 159]. Intramuscular progesterone administration is not commonly used today because of the ease of vaginal progesterone administration and the side effects associated with the intramuscular route such as inflammation, pain, and local abscess on the injection site [160].

The duration of progesterone administration beyond 6–7 weeks of gestation (at the time of the first ultrasound) is not beneficial [161]. A Web-based survey showed that more than 70% of doctors still continue prescribing progesterone for patients until 8–10 weeks of gestation or beyond [162]. The optimal dose of progesterone has not been determined. The dose most commonly used is 600 mg/day vaginally [163].

Andersen et al. [164] reviewed on the luteal phase support and concluded that determining

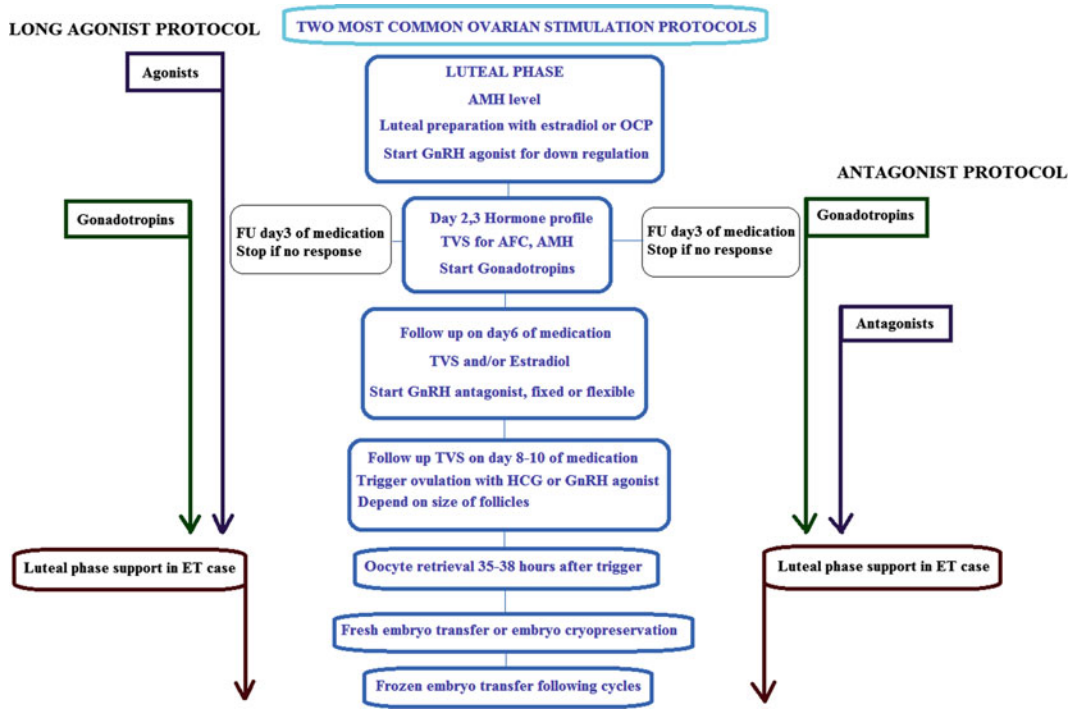

Fig. 7.1 The overall picture of ovarian stimulation protocol: two most commons

progesterone level during mid-luteal phase of ovarian stimulation could be the method to improve ongoing pregnancy rate and reduce early pregnancy loss. The threshold of the progesterone level should be at least 80–100 nmol/L in the ovarian stimulation cycle. In GnRH antagonist protocol with GnRH agonist trigger, luteal phase support could be optimized by dual triggering of ovulation with GnRH agonist plus a low dose of HCG and continue with a low dose of HCG or recombinant LH, in order to avoid the chance of OHSS but still maintain the pregnancy outcomes [115–118] (Fig. 7.1).

Conclusion

Controlled ovarian stimulation is the fundamental program in IVF treatment. Fine-tuning of ovarian stimulation for the purpose of pregnancy with less complication is the main goal of treatment. However, the controversies in the management still exist in the practice today. Randomized controlled studies with enough sample sizes are needed to answer the points that have no conclusions. Poor ovarian response is one of the most challenging tasks in reproductive medicine. Finally, no one ovarian stimulation program for all but each patient needs individualized approach for optimizing the best pregnancy outcomes with the maximum safeness.

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Which Women Are Suitable for Natural and Modified Natural Cycle IVF?

8

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What Is Natural Cycle IVF?

The term natural cycle in-vitro fertilisation (NC-IVF) applies 'when IVF is carried out with oocytes collected from a woman's ovary or ovaries in a spontaneous menstrual cycle without administration of any medication at any time during the cycle' [1]. The aim is to retrieve a single oocyte originating from a naturally selected follicle. Various modifications of the NC-IVF are possible to minimise the risk of premature ovulation and individualise the treatment protocol based on clinical needs and patient's choice.

The first IVF baby was born from an oocyte collected in a completely natural cycle [2]. Subsequently, multi-follicular development to retrieve maximum number of oocytes, with concomitant suppression of premature luteinising hormone (LH) surge by gonadotropin-releasing hormone (GnRH) analogues, became the target of any IVF programme. Thus, pituitary 'downregulation' with GnRH agonists and ovarian stimulation with gonadotropins (the so-called long protocol) evolved as the standard protocol of today's conventional IVF (C-IVF). However, the side effects of intense ovarian stimulation and multiple embryo transfer (ET) were also being appreciated: ovarian hyper-stimulation syndrome (OHSS), twin or higher order births, or menopausal symptoms due to downregulation can rendered IVF a risky procedure. With the increasing complexity of administering multiple injections for prolonged period, along with significant hormonal changes often make the patients systemically unwell and emotionally stressed [3]. The use of medications for a longer period and at a higher intensity in C-IVF raises the overall treatment cost. In contrast, NC-IVF, being conducted on a spontaneous natural menstrual cycle with no or minimum medication(s), is usually well tolerated by the patients and is less expensive and almost devoid of the above risks associated with C-IVF.

Why Natural IVF?

Presently, there is a drive in making IVF safer, more patient-centred and accessible worldwide [4]. Many IVF clinics around the world have appreciated the concept of NC-IVF and have reported their success stories [5–7]. It is increasingly being realised that quality, not quantity is the desirable goal of an IVF programme. By allowing a physiological approach to follicular recruitment, usually only the healthiest and most competent follicle(s) develop in NC-IVF and mild stimulation IVF (MS-IVF).

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Indeed, a randomised controlled trial (RCT) showed same number of euploid embryos, whether created from a small oocyte cohort of MS-IVF cycles or from a larger pool of oocytes in C-IVF cycles [8]. Another RCT found significantly higher proportion of good quality embryo from MS-IVF cycles, compared to that following standard long protocol (61% vs. 29%, $p = 0.008$) [9]. A more recent RCT concluded that the number of available blastocyst did not correlate with the gonadotropin dose [10]. Rather, an inverse relationship has been depicted between increasing gonadotropin doses and blastocyst–oocytes ratio [10] or live birth rates (LBRs) [11]. To try to find an explanation of these findings at biochemical level, the follicular fluid hormonal milieu has been shown to be disturbed by high level of ovarian stimulation [12]. Follicular fluid Anti-Mullerian Hormone (AMH), which is believed to be a marker of successful fertilisation and implantation, is maintained at a higher level in NC-IVF cycles compared to that of conventional stimulation [12].

In addition to possible direct influence of high ovarian stimulation on the oocytes or embryos [13], there is a number evidence of detrimental effect of very high oestrogen (and progesterone) levels on endometrial receptivity [13–17]. Supra-physiological hormone levels and high oocyte numbers have also shown to be associated with adverse perinatal outcomes including prematurity, low-birth weight [18, 19], intrauterine growth restriction [20] and cardiovascular disturbance in the neonates [21].

Despite obvious advantages mentioned above, NC-IVF remains under-utilised, mainly due to its alleged low success rates (average ongoing pregnancy rates: 7.2% per cycle and 15.8% per ET from a review of 20 studies) [22] and high risk of premature ovulation. Less flexibility in cycle scheduling resulting in 7-days-a-week service is not an acceptable option for most of the IVF clinics. However, the review by Pelinck et al. found only 3 small-scale RCTs, 2 of which compared pure NC-IVF with clomiphene citrate (CC)-stimulated IVF and the other with long GnRH agonist protocol; the bulk of evidence was derived from case series or retrospective studies [22]. The real effectiveness of NC-IVF is judged in its cumulative birth rates. Data since the early days of NC-IVF in unselected patients showed a 3–5 cycle cumulative pregnancy rates (PRs) of 41.7–46% [7, 23, 24] and a 32% cumulative LBRs [7]. A widely quoted non-inferiority RCT found no difference in cumulative 1-year LBRs, when a day 5 commencement of low-dose follicle stimulation hormone (FSH) regimen was assessed against C-IVF (43.4% vs. 44.7%) [25]. More recent studies comparing cumulative fresh and frozen single embryo transfer (SET) in C-IVF with multiple natural IVF cycles also demonstrated that NC-IVF could be a cost-effective alternative [26, 27]. The advent of GnRH antagonists made certain modification of NC-IVF possible that potentially has reduced the chance of premature LH surge [28, 29]. One of the largest series of modified natural cycle (MNC) ($n = 1503$ cycles) in recent time reported 14.5% PRs per cycle and 34.5% per ET for normal-responder women under 35 years of age, with 5.7% cycle cancellation rate due to premature ovulation [6]. Considering NC-IVF and MS-IVF safer, cheaper and more patient-friendly, a need for revival of this approach has long been voiced [29, 30].

What Are the Types of Natural IVF?

To streamline the use of various terminologies to describe different ways of ovarian stimulation, the International Society of Mild Approach Assisted Reproduction (ISMAAR), a consensus paper was published in Human Reproduction [1]. A brief protocol for each type of Natural- IVF has been described in Table 8.1.

Other than the follicular size and serum estradiol (E2) levels, assessment of perifollicular blood flow by Doppler ultrasound also aids in managing a natural cycle IVF (Fig. 8.1). Good peak systolic blood flow in the perifollicular blood vessels in the pre-ovulatory period has been shown to be associated with probability of retrieving an oocyte and development of high-quality embryo [31]. Perifollicular blood

Table 8.1 Brief description of various natural IVF protocols

Types	Definitions	Conduction of cycles (protocol)
Natural cycle	When IVF is carried out with oocytes collected from a woman's ovary or ovaries in a spontaneous menstrual cycle without administration of any medication at any time during the cycle	The cycle is monitored by serial ultrasound scans ± serum LH and E2, usually starting from day 4–6 onwards. Urine LH test is commenced once the dominant follicle reaches ≥12 mm in diameter. Optimal timing of OC is determined by hormone levels and follicular diameter. Occurrence of endogenous LH surge necessitates OC within 24 h of the surge to prevent ovulation. Indomethacin may be added if there is a risk of premature ovulation. Luteal support is not necessary
Modified natural with hCG	"The use of hCG to induce final oocyte maturation" in a natural cycle	Elective 'trigger' of final oocyte maturation by hCG, once the dominant follicle reaches ≥15 mm average diameter with satisfactory serum E2 levels. OC scheduled 35–36 h later. Triggering before endogenous LH surge reduces the need for emergency OC and the incidence of premature ovulation. Luteal support is optional
Modified natural cycles with addition of GnRH antagonist	'The administration of GnRH antagonist to block the spontaneous LH surge with or without FSH or HMG as add-back therapy'	The cycle starts with natural selection of the dominant follicle. Low-dose FSH or HMG at 150 IU/day is started along with Cetrorelix (antagonist), once the leading follicle is 13–14 mm size and serum E2 is >500 pmol/l. The hCG trigger is planned when the follicle reaches >16 mm in average diameter with a satisfactory serum E2 level and OC follows 35–36 h later. The luteal phase support is administered

LH Luteinising hormone. *E2* Estradiol. *OC* Oocyte collection. *hCG* Human chorionic gonadotrophin. *GnRH* Gonadotrophin-releasing hormone. *FSH* Follicle-stimulating hormone. *hMG* Human menopausal gonadotrophin. *MNC* Modified natural cycle

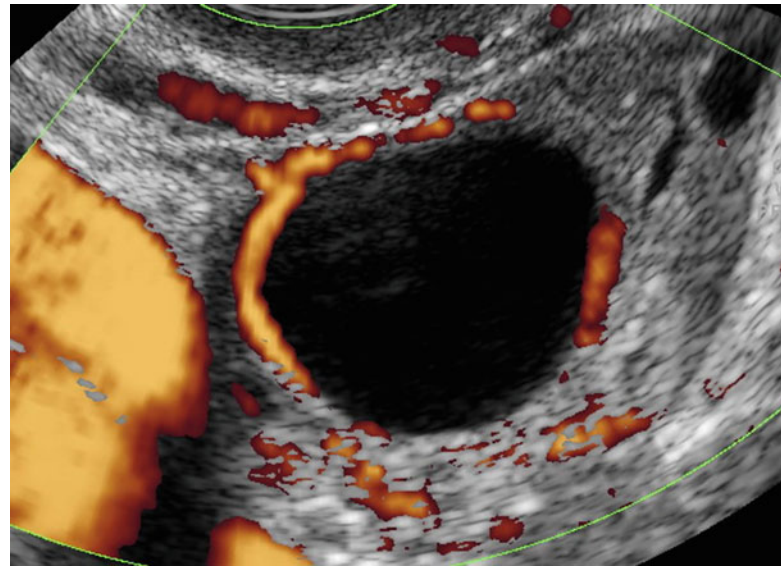

Fig. 8.1 Image by Doppler ultrasound for perifollicular blood flow

flow velocity of $\geq$10 cm/second gave rise to 70% high-grade embryos, as opposed to only 14% when the flow was below 10 cm/s [32].

To increase efficiency, NC-IVF is usually offered as a multiple-cycle package. While availability of oocytes or embryo per OC is less, accumulating oocytes from multiple cycles and subsequent transfer of fresh and frozen embryos have been shown to improve the treatment outcome, when compared with multiple individual cycles (PRs: 34.4% vs. 16%) [33]. The opportunity of selecting the best embryo(s) and double ET, as opposed to usually SET in repeated fresh cycles, may explain this observation.

What Are the Indications of Natural IVF?

NC-IVF can be considered for anybody with regular menstrual cycle, whether on medical ground or on patient's request. However, there are certain situations where it appears to be particularly useful. The following are the most common ones.

Women with Poor Ovarian Reserve (POR)

Women classified of having POR based on low antral follicle count (AFC) and/or low AMH, with or without elevated baseline FSH usually, have a poor prognosis in C-IVF, despite having high dose of ovarian stimulation. Traditionally, a day 3 FSH level is regarded as predictor of ovarian response, oocyte quality and IVF outcome, irrespective of women's age [34]. In 2011, the European Society of Human Reproduction and Embryology (ESHRE) working group on 'poor ovarian response' organised a meeting at Bologna to form consensus on universally acceptable definitions of POR, which are now regarded as 'Bologna criteria' [35]. However, many IVF centres decline treatment to women over 40 years of age or with high day 3 FSH values. Recovery of fewer oocytes is recognised as an under-response for C-IVF, whereas it is normal and a very intended response in NC-IVF. Low oocyte yield following high gonadotropin stimulation is believed to be a result of follicular dysfunction or ageing ovary. In contrast, NC-IVF encourages only the most competent follicle(s) to develop, and therefore, its outcome is not much dependent on oocyte yield. Being naturally selected, quality of oocytes and embryo in NC-IVF is expected to be better [36]. On this theoretical background, NC-IVF could be a cost-effective solution for those who had failed treatment with high-stimulation dose C-IVF.

Earlier, a small uncontrolled case series of 32 women with POR (defined as basal FSH >12 iu/l) and $\geq$1 previous failed IVF with <6 retrieved oocytes reported poor treatment outcomes with MNC [37]. A cohort study (n = 164) found application of MN protocol in women with POR, as defined by Bologna criteria, resulted in low LBRs (7.4% per patient); the outcome was assessed against that of normal responders which was not a like-to-like comparison [38]. In contrast, another larger cohort study found no such difference in women $\leq$35 years age group (LBRs-normal responders: 35.05% vs. poor responders: 29.63% per ET) [6]. However, in women older than 35 years who were poor responders as well, NC-IVF led to inferior outcome compared to normal responders of the same age group. This finding was similar to that by Kedem et al. who found no benefit from NC-IVF among women aged between 35 and 43 years and also classified as 'genuine' poor responders (n = 111) according to Bologna criteria [39]. In contrast, a study of women aged 37–43 years and elevated serum FSH achieved 11.5% PR per cycle and 20.0% PR per ET by pure NC-intracytoplasmic sperm injection (ICSI) [40]. A more recent cohort control study that included women with POR according to Bologna criteria (n = 242) found significantly higher adjusted LBRs with MNC (7.5% vs. 3.1%; OR 4.01, 95% CI: 1.14–14.09) as compared to high-stimulation GnRH antagonist cycles [41]. Interestingly, more cycles were cancelled in the high-stimulation group mainly due to inadequate follicular growth (13.4% vs. 5.0%; p = 0.02) in this study, with no difference in cancellation rates due to premature

ovulation. The reliability of these findings has been questioned (mainly on the principle of applying multivariate analysis), and re-emphasis was on the need for well-designed RCT [42].

Whether or not NC-IVF works better in women with POR in comparison with those with normal reserve, there is yet no evidence of superiority of C-IVF in this clinical setting, and rather, high stimulation is suggested to yield poorer outcomes [41]. Moreover, women often find intense ovarian stimulation regimen of C-IVF to be physically daunting, stressful and unrewardingly expensive [3]. NC-IVF being more patient-friendly and cost-effective, it could be a better option for these women.

Previous Poor Responders

There is no single strategy which is unquestionably beneficial after a suboptimal response with standard IVF treatment [43]. Earlier, several case reports, case series or small prospective trials on application of NC-IVF on previous poor responders revealed encouraging results [44–47]. As described above, the studies on women who had previous failed treatment and also had POR showed inconsistent results [37, 41]. The RCT (n = 215) that compared NC-IVF with 'micro-dose flare' protocol recruited women aged <43 years, with <4 dominant follicles in the previous treatment C-IVF cycle(s) [48]. It found similar PRs (per cycle: 6.1 vs. 6.9%; per ET: 14.9 vs. 10.1%) and a trend of higher implantation rates (14.9 vs. 5,5%; p = 0.05) with NC-IVF. A 3-cycle cumulative PRs/ET of 37.5% was achieved with negligible expense on medications [48]. Women dropped out from repeated C-IVF cycles more likely to find NC-IVF the way forward [3].

Advanced Women's Age

The role of NC-IVF in treating women of advanced age has not yet been fully evaluated. Theoretically, older women are more likely to produce poor quality oocytes or aneuploid embryos and therefore may benefit from 'natural selection'. As mentioned earlier, Shaulov et al. in their large uncontrolled study (n = 782 couples) found no significant decline in PR per ET (26.6% vs. 35.0%) in the older age group (>35 years) who had normal ovarian reserve and/or adequate response in the previous treatment cycle [6]. Cycle cancellation rates were also not significantly different. In this study, the PR in poor responder women of >35 years however was 6.25% per ET which was similar to those of Polyzos et al. (6.8% LBRs in women >40 years) [38]. Data are insufficient to compare relative effectiveness between NC-IVF and C-IVF in older age group. To date, the only RCT that compared NC-IVF (hCG only regimen) with one of GnRH agonist protocols (micro-dose flare) reported similar PRs per ET in 36–39-year age group (10% vs. 4%) and 40–43-year age group (8.0% vs. 9.7%) with a trend of higher implantation rates in favour of NC-IVF and minimal cost on medication [48]. Overall, the results are encouraging. Further work is needed to find the place of NC-IVF in women with advanced age.

Previous Conventional Stimulation Cycles with Poor Quality Embryos

High gonadotropin stimulation has been shown to generate higher proportion of poor quality of aneuploidy embryos [8, 9]. In the study by Arce et al., the number of blastocysts did not correlate with the FSH dose; however, the blastocyst–oocyte ratio and fertilisation rates declined significantly with the escalating gonadotropin dose [10]. By maintaining the follicular fluid hormone milieu (AMH, E2, androstenedione and LH) close to the physiological levels, NC-IVF improves fertilisation [12]. There was a paucity of comparative data between NC-IVF and failed C-IVF due to poor quality of embryos.

Contraindications to Ovarian Stimulation

Conventional ovarian stimulation possesses considerable risk in certain medical conditions

including estrogen receptor-positive breast cancer, endometrial cancer or acute intermittent porphyria. Selective estrogen receptor modulators, particularly tamoxifen and aromatase inhibitors (e.g. letrozole), are being widely used in women with breast cancer requiring fertility preservation [49, 50]. In women with acute intermittent porphyria, even anti-estrogen may trigger disease flare up [51]. Pure NC-IVF could be an option for these patients undergoing fertility treatment. While multiple cycles of NC-IVF increase the number of oocytes or embryos to be cryo-preserved in cancer patients, very often the urgency of commencement of gonadotoxic chemotherapy or radiation does not allow the time for having repeated cycles: in vitro maturation (IVM) of oocytes from non-dominant follicles of a natural cycle could increase the number of available embryos [52]. Lim et al. reported a PR of 40.4% in a combined natural IVF+IVM cycles and 41.3% with IVM alone, as opposed to 37.8% with C-IVF among infertile couples undergoing treatment [53].

Women at Significant Risk of OHSS

Women with polycystic ovary are at risk of developing OHSS. Treatment of women who have already had severe OHSS is always a challenge. A number of very effective strategies to prevent OHSS have been described in recent years. GnRH agonist as an ovulation trigger followed by intense luteal phase support (by high dose of E2 and progesterone or low-dose luteal hCG) or freezing all embryos has made OHSS a rare event [54]. However, OHSS has recently been reported with agonist trigger and subsequent luteal-phase hCG [55] or even with freezing all embryos [56, 57]. NC-IVF with or without IVM may be an alternative option for high responder women who are at considerable risk of OHSS. Successful pregnancies can be achieved with this policy of NC-IVF and IVM [58]. However, a regular menstrual cycle is a prerequisite for NC-IVF. This option is not suitable for women with oligomenorrhoeic polycystic ovarian syndrome (PCOS). There is a large section of ovulatory PCO patients or high responders with regular periods who may benefit from NC-IVF-IVM treatment. Although theoretically reassuring, existing data are too small to determine the risk of OHSS and the cycle outcome with this strategy. At present, most of the IVF centres do not practice IVM routinely. With further experience of NC-IVF-IVM, it may come up as a safe and effective treatment for high responders.

Patient's Choice

Finally, honouring patient's choice is a basic principle of any medical treatment. Mild/natural IVF has been regarded as more patient-centred and 'tailor-made' approach [4]. 'Natural' IVF appeals many women, particularly those who had multiple failed cycles with C-IVF [3].

Conclusion

NC-IVF provides a safe, low-cost and a patient-centred option for women wishing to avoid ovarian stimulation or where stimulation is medically contraindicated. NC-IVF should be regarded as a multiple-cycle approach, and cumulative success rates over 3 cycles (which can be done in successive cycles) are promising. The disadvantage of NC-IVF is that only one oocyte is obtained and that spontaneous ovulation can occur before the oocyte can be obtained. MNC-IVF overcomes this problem and with add-back FSH from the day of antagonist commencement can result in more than one oocytes being obtained. This strategy is particularly beneficial in poor prognosis patients with low ovarian reserve in whom standard IVF only adds cost to the treatment with no clear advantage in the final outcome. A simplified way of conducting the treatment cycles is physically and mentally less distressing to the patients and therefore appears to be more acceptable option to them. A 7-days-a-week dedicated service and expertise in advanced ultrasound assessment, e.g. follicular blood flow, are essential prerequisites to achieving an optimum outcome.

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Natural Cycle IVF with Spontaneous LH Surge

9

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Revival of Natural Cycle and Mild IVF

Although the first IVF pregnancies by Edwards et al. were established in an unstimulated natural cycle, this approach was quickly abandoned in favor of more effective (but also more complex) ovarian stimulation regimens. From the early 2000s, however, after gradually recognizing the deleterious effects of conventional stimulation, an opposite trend has emerged and mild and unstimulated IVF approaches were "rediscovered" [1, 2]. The current paradigm of in vitro fertilization (IVF) treatment involves controlled ovarian hyperstimulation (COH) with high-dose gonadotropins and the implantation of multiple embryos which largely contribute to the most important complications of IVF: ovarian hyperstimulation syndrome (OHSS) and the risks of multiple pregnancies. In contrast, the concept of mild ovarian stimulation consists in obtaining a milder ovarian response (ideally fewer than eight oocytes) often coupled with a single embryo transfer policy [3]. There is increasing evidence which suggests that mild IVF protocols are associated with decreased physical/psychological burden [4], lower drop-out [5] and diminished costs [6–8]. Other potential advantages of mild IVF include increased endometrial receptivity [9], fewer embryo aneuploidies [10] and better neonatal outcome in singleton pregnancies [11] From the side of the embryological laboratory, workload of the staff might be greatly diminished even if the need for excellent laboratory performance would become a must (due to less available oocytes) [2]. Also, there would be fewer embryos needed to be cryopreserved.

The main drawback of mild IVF approaches is a decreased per cycle success rate that could limit their acceptance and spread to everyday clinical practice. Currently, there is a lack of well-designed clinical trials comparing the efficacy of mild and conventional IVF approaches. In a seminal study Heijnen et al. compared the performance of a mild stimulation protocol based on GnRH antagonist co-treatment, low-dose gonadotropins and single embryo transfer with the classical long agonist protocol combined with the transfer of two embryos over a 1-year period [4]. The authors have concluded that cumulative live births were not significantly different (43.4 vs. 44.7%) after performing 4 mild versus 3 conventional IVF attempts. More recently, Zhang et al. reported the outcomes of a large 4-year clinical trial involving 564 patients who were randomized to receive mild ovarian stimulation (mini-IVF) with clomiphene and gonadotropins coupled with single embryo transfer versus conventional ovarian stimulation with the long agonist protocol coupled with (mainly) double embryo transfer [12]. Although cumulative live birth rates were in favor of the conventional approach (63 vs. 49%), mini-IVF was also associated with the absence of OHSS, reduced rate of multiples and reduced gonadotropin

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Table 9.1 Challenges of natural cycle IVF

Unsolved problems	Possible solutions	Advantages/drawbacks
Uncontrolled LH surge	Flexible scheduling	Very challenging
	GnRH antagonists	Additional cost, inefficient?
	Low-dose clomiphene	Endometrial effect?
Premature ovulation	NSAIDs	Cheap, efficient
Empty follicle	Follicular flushing	Time consuming
Immature oocyte	In vitro maturation	Low efficiency
Mandatory single embryo transfer	Optimized ultrasound-guided embryo transfer technique	Requires training
Low per cycle efficiency	Repeated cycles	Risk of drop-out
	Patient selection	Limits patients access

consumption. The authors have concluded that the lower success rate should be weighed against the advantages of mini-IVF and that it could be offset by performing a series of lower-cost cycles. In the context of natural cycle IVF, Pelinck et al. from the Netherlands have also suggested that 4 cycles of ncIVF are comparable to 1 attempt of conventional IVF [13]. A robust cost-effectiveness analysis from the same Dutch group have investigated three different scenarios concluding that 3–6 mncIVF treatments with minimized medication is cost-effective alternative for one cycle of controlled ovarian hyperstimulation with strict application of single embryo transfer [14]. Natural cycle IVF protocols are especially fraught with additional challenges that are related to procedural and biological losses at each step of the IVF process (Table 9.1). Several groups have tried to develop different approaches to tackle some of these problems and increase the overall efficiency of ncIVF protocols.

Pioneering Work at the Beginning of the IVF Era

Edwards and Steptoe's initial attempts to obtain eggs for in vitro fertilization involved stimulation by hMG (150–225 IU every 2–3 day) and hCG to induce ovulation (between 1000 and 12,000 IU on days 9–11 of the menstrual cycle). Oocytes obtained this way were capable of fertilization but none of the first 77 patients who reached embryo transfer conceived successfully (although an ectopic pregnancy was obtained in 1976) [15]. Subsequently, the strategy has changed and Steptoe and Edwards had to convert to IVF in an unstimulated cycle mainly because they have noticed that the luteal phase was considerably shortened following ovarian stimulation [16]. The intensive monitoring of the natural cycle was cumbersome and involved the measuring urine excretion of oestrone glucoronide and LH levels (3 hourly), although later it was replaced by a more specific radioimmunoassay determination. They had to perform laparoscopy more than 24 h after the accumulation of LH in urine at detectable levels. Thus, the first IVF pregnancy in the world was obtained using the completely natural cycle approach [17]. Despite this seminal achievement, natural cycle IVF was technically and logistically too demanding and also inefficient to be widely adapted as a routine infertility treatment. Subsequently, it became evident that working with multiple oocyte and embryos would increase substantially the likelihood of clinical success. The next wave of practitioners developed different ovarian stimulation protocols involving clomiphene, gonadotropins, the combination of these two and with the advent of GnRH analogues the now well-established agonist (long, short and ultrashort) and antagonist protocols

(multiple or single dose) were added to the armamentarium of controlled ovarian hyperstimulation [15].

Early Development of ncIVF Protocols

During the late 1980s and early 1990s, several groups tried to repeat the experience of early pioneers and establish natural cycle IVF programs but with limited success [18–20]. The Sheffield group from the UK reported extensively on its experience and outlined many of the challenges and difficulties (some of which still persist today) related to development of successful ncIVF protocols [20, 21]. The center's protocol was a completely natural, drug-free approach without administering any stimulatory drug such as clomiphene or gonadotropins. Oocyte retrieval scheduling was based on the spontaneous LH surge only and triggering with exogenous hCG was not applied. Understandably, intensive cycle monitoring was performed requiring a lot of flexibility both from patients and the clinic's staff. After initial attempts with semi-quantitative urine LH monitoring which was shown to be unreliable (the onset of LH surge was frequently misinterpreted due to uncontrollable changes in individual urine excretion) and also uncomfortable to patients (collecting 24 h urine samples was needed), the clinic switched to twice-daily (morning and evening sample) serum LH monitoring. For convenience, the evening (20 h) blood sample was self-obtained from capillary blood thus the patients were not required to come twice to the clinic. Hormonal monitoring usually started from cycle day 9; first with E2 determinations only, and afterward continued with measuring LH levels only in the morning samples. The onset of the LH surge was established at the first sign of increasing LH levels (>10 IU/ml), and the exact time of the oocyte retrieval was confirmed later by looking at the pattern of the last 3–4 consecutive samples. Oocyte retrievals could be scheduled between 09:00 and 17:00 daily (afternoon retrievals were needed to accommodate cycles where LH surge started in the early morning hours), and services were provided on a 7 days a week basis. Despite all these efforts in their study from the early 1990s, the authors have reported 162 treatment attempts (performed in 117 couples) of which 89 (55%) reached embryo transfer and only 9 (5.6% per started cycle) resulted in live births. The authors have argued that this relatively low success rate could be probably increased by applying more careful patient selection criteria (excluding patients >40 years and male infertility cases).

Spontaneous LH Surge Versus hCG Triggering: Which Is Better?

Whereas the risk of premature LH rise and ovulations might be diminished by GnRH antagonist co-treatment or NSAID use, successful oocyte retrieval is greatly influenced by the way of its timing. The efficiency of different oocyte retrieval scheduling strategies could be compared by looking at the "oocyte retrieval rate per started cycle" which is also influenced by cancellation rates occurring before oocyte retrieval (depending on cancellation criteria and premature ovulation rate) and the efficiency of oocyte retrieval itself (depending on center and operator-specific oocyte retrieval technique, the use of follicular flushing). An extensive review which analyzed the efficiency of ncIVF treatment in 20 studies published between 1989 and 2001 involving 1800 cycles showed that successful oocyte recovery rate varied greatly between studies (30–96%) [22]. The timing of oocyte retrieval was mostly done with exogenous hCG but with cancellation of the cycle if LH surge occurred prematurely. In the absence of an LH rise, triggering usually is performed by administering exogenous hCG at a fixed interval between 31 and 36 h. However, in some centers, oocyte retrieval scheduling timing is based on the occurrence of a spontaneous LH surge which is a far more challenging approach. In normally cycling women, ovulation is usually expected to occur 24–36 h after the onset of the LH surge (the onset of LH surge often starts in the early morning hours). However, a comprehensive

study also suggested great individual variations and have found that ovulation occurred on average 41 h (range: 24–56 h) after the onset of LH surge and 18.4 h (range: 8–40 h) after the LH peak [23]. This makes the estimation of the onset of LH surge very challenging, thus only few groups planned the oocyte retrieval based on this. In the previously cited retrospective study of Zayed et al. involving 162 cycles, the oocyte retrieval rate was per planned cycle was quite acceptable with 89% [21]. A subsequent retrospective review from the same group compared the outcome of ncIVF treatments based on whether scheduling was performed according to the occurrence of spontaneous LH surge or with the use of terminal hCG injection. In the LH surge group (534 cycles), eggs were collected in 81% of the scheduled cycles whereas in the hCG group (241 cycles) this rate was slightly lower 76%. The authors concluded that compared to twice-daily LH monitoring (as described in the previous section) the administration of hCG did not have any benefit with respect of the eggs collected or pregnancies obtained [24]. In our experience (see large cohort study discussed in a later section), we routinely use LH surge-based oocyte retrieval scheduling and oocyte retrieval rates are satisfactory with 78%. Therefore, in this author's opinion, there is currently no robust evidence which could suggest that hCG triggering is in fact superior to the spontaneous LH surge-based approach. Most clinics prefer the former approach because it permits the exact timing of oocyte retrievals (or at least gives the impression) and is convenient both for the patients and the clinic's staff.

Recent evidence from animal models has even suggested that exposure to even small doses of hCG might have deleterious effect on endometrial receptivity [25, 26]. This is especially an issue when successive natural cycle IVF or minimal ovarian stimulation cycles are performed in a back-to-back manner or if cycles are initiated immediately after an early pregnancy loss or miscarriage. This notion was also supported by clinical studies in the context of (donor) intrauterine insemination and natural-cycle frozen–thawed embryo transfer treatment. The randomized clinical trial of Kyrou et al. on intrauterine insemination treatment showed that ongoing pregnancy rates were significantly higher (23 vs. 11%) in spontaneously versus hCG-triggered cycles [27]. Similarly the RCT of Fatemi el was stopped prematurely due to a considerable difference (31.1 vs. 14.3%) in favor of LH-triggered frozen–thawed embryo transfer cycles [28].

Modified Natural Cycle IVF Protocol: A Step Forward?

Since the advent of GnRH antagonists—beginning from the early 2000s—these drugs were also applied in the setting of ncIVF treatments, thus establishing a novel "modified" natural cycle IVF protocol. The ISMAAR classification of mild IVF approaches labeled this protocol as "semi-natural" or "controlled" cycle compared to IVF in an unstimulated or spontaneous cycle [3]. The use of GnRH antagonists in the late follicular phase was thought to be beneficial by avoiding unwanted LH surges, diminishing the risk of premature ovulation and permitting the triggering by exogenous hCG at any convenient time which could greatly simplify oocyte retrieval scheduling. In a first French pilot study, daily GnRH antagonists were administered when a leading follicle reached 12–14 mm (with corresponding E2 levels of >200 pg/ml) concomitantly with 150 IU of hMG to avoid any decline in E2 levels [29]. In a selected group of <37-year-old patients, 44 cycles were performed leading to 40 (91%) oocyte retrievals, 22 (50%) embryo transfers and five (11.4%) ongoing pregnancies. Altogether these results were encouraging, and the new protocol became the treatment of choice for many centers around the world that tried to perform IVF in a natural cycle.

In 2007, a Canadian group reported its age-specific success rates with modified ncIVF compared with conventional IVF [30]. In patients under 35 years of age, 134 cycles were performed resulting in 75 (56%) embryo transfers and 20 (14.9%) clinical pregnancies. In older patients (between 35 and 38 years), the corresponding rates were much lower 44.4 and 3.7%, respectively. In contrast with conventional IVF,

success rates were considerably higher: in younger patients a 45.4% clinical pregnancy rate per started cycle was reached and in older ones it still remained quite acceptable with 33.8%. The authors concluded that although with modified ncIVF, the cancellation rate is very high it might be offset by the fact that the mild treatment option is less hard on patients, could be repeated in each month and costs less. The largest published series on modified ncIVF by Pelinck et al. reported the outcome of 1048 cycles on a cumulative basis in patients who were offered up to nine treatment cycles [13]. Per cycle ongoing pregnancy rates were 7.9% but reached 44.4% cumulatively.

Although up to date there were no direct comparisons between a completely drug-free and a GnRH antagonist-based "modified" natural cycle IVF protocol, the above studies suggest that the overall cycle outcome is basically the same as with simpler protocols that use no drugs during the follicular phase and only include the use of exogenous hCG (or GnRH agonist) for final oocyte maturation. The concept of using GnRH antagonists is intuitively appealing, but there is some evidence which suggest that antagonists cannot entirely prevent the occurrence of premature LH surges especially in the context of a non-stimulated cycles with relatively low steroid levels. Some groups even suggested that an increased (double) GnRH antagonist dose might be required to completely prevent the occurrence of LH surges even if the benefits of this approach are largely unclear, and this strategy would only contribute to increased drug costs [31]. In an innovative experimental study, Messinis et al. studies 8 women volunteers who were monitored during two menstrual cycles and submitted to transdermal E2 substitution with our without daily GnRH antagonist co-treatment [32]. The authors have found that GnRH antagonists were unable to block the occurrence of LH surge induced by supra-physiologic E2 levels. They have also suggested that GnRH antagonists behave differently during ovarian stimulation and non-stimulated cycles with an additional role of other yet unknown endocrine factors (such as the gonadotropin-surge attenuating factor).

How Efficient Is Natural Cycle IVF?

A systematic review on the efficacy of natural cycle IVF [22] was performed in 2002 summarizing the findings of 20 studies (published between 1989 and 2001) comprising a total of 1800 initiated ncIVF cycles. This review included studies where HCG was the only drug used for induction of oocyte maturation and where it was possible to calculate ongoing pregnancy rates per started cycle from published data. The review has concluded that on average, only 45.5% of initiated cycles reached embryo transfer resulting in a 7.2% ongoing pregnancy rate per initiated cycle which may partly explain the low clinical acceptance of this treatment option. However, there was a great variation among studies and embryo transfer and ongoing pregnancy rates per started cycle varied between 22.7–80% and 0–16.3%, respectively. The authors argued that (at the time of writing) future developments such as the use of GnRH antagonists for controlling LH surges or NSAID to prevent premature ovulation might further increase the efficiency of ncIVF treatments.

More recently, Roesner et al. reviewed the 5-year experience of their center in Germany involving a completely drug-free natural cycle IVF approach (hCG only) and compared it to the literature review of 28 ncIVF studies published between 1995 and 2012 [33]. In this review, they have found that clinical pregnancy rates per embryo transfer varied widely between 10.2 and 56% (unfortunately they were unable to present any data on ongoing pregnancy rate per started cycle which is a much more meaningful outcome). In comparison, this was comparable (9.8–50%) with the results published by Pelinck et al. (involving some of the same studies). Thus, it seems that during the 10-year period that separates the publication of the above-mentioned two literature reviews, there has not been any considerable improvement in the overall efficiency of natural cycle IVF treatments.

The overall low per cycle efficiency of natural cycle IVF approach can be offset by repeating successive treatment attempts. There are only two studies to date that have presented data on

cumulative success rates following (modified) natural cycle approach. The earlier UK study reported the outcome 181 cycles performed in 52 infertile women [6]. Although per cycle live birth rate was only 8.8%, by performing 4 treatment attempts a cumulative live births rate of 32% was reached. The authors concluded that four cycles of ncIVF are comparable to one attempt of conventional IVF. A large-scale report form a Dutch specialist center reported the cumulative outcome of 1048 modified natural cycle IVF (mncIVF) cycles performed in 256 patients [13]. The participants (who were all <37 years of age) were offered a maximum of nine cycles (even if finally completed on average "only" 4.1 attempts). Per cycle ongoing pregnancy rates were 7.9% but reached 44.4% cumulatively. The authors concluded that modified ncIVF represents a valuable treatment alternative to conventional ovarian stimulation.

A recently published 3-year cohort study from our group also attempted to calculate cumulative success from a program based uniquely on ncIVF and minimal ovarian stimulation [34]. Although this cohort was a mixture of different mild treatment approaches, natural cycle IVF still represented 57% of all treatment attempts. Crude cumulative live birth rates were favorable in young patients (65 and 60% in <35 and <38-year-old patients, respectively), still acceptable at intermediate age (39% for patients between 38 and 40 years of age) and declined gradually in >40-year-old infertile patients. Moreover, a plateau was also detected after approximately 4–6 treatment cycles.

A recent registry-based study from the US yielded interesting insights into the efficiency of ncIVF treatment in different age groups. The review presented data on all 795 unstimulated IVF cycles that were registered in the SART (Society for Assisted Reproductive Technologies) database during 2006 and 2007 [35]. Although the total number of presented treatment cycles is significant, an important drawback of the dataset is that the average number of unstimulated cycles were <10 per year and only represented a tiny fraction (1.5%) of all treatment cycles performed at each center (therefore hardly representing the experience of specialized centers in mild IVF approaches). Natural cycle IVF was used by many clinics primarily in poor prognosis older patients with diminished ovarian reserve and poor egg quality for whom stimulated IVF was no longer an option. The authors have found that embryo transfer and live birth rates per started cycle were excellent in <35 years patients (53.8 and 15.2%, respectively), slightly lower in 35–37-year-old patients (41.6 and 14.9%, respectively), declining in 38–40-year-old patients (41.6 and 8.7%) and became considerably lower in >40-year-old patients. However, compared to stimulated IVF embryonic implantation rates were statistically superior (up until 42 years of age), suggesting improved endometrial receptivity with ncIVF treatment protocols. Thus, the authors concluded that ncIVF should be considered only in younger patients (<38 years) instead of relegating this treatment option to older patients in whom all other treatment options have failed. They have also suggested that changing the current practice of SART reporting (by reporting the outcome of unstimulated cycles separately instead of grouping them together with conventional stimulated treatment cycles) would encourage US centers to offer this treatment option more frequently.

The above findings of the US registry were also corroborated by a recent retrospective review from the Brussels group that analyzed the outcome of 469 natural cycle IVF cycles performed in 164 Bologna poor responder patients [36]. The authors have found that embryo transfer (42 vs. 59%, $p = 0.011$) and live birth rates (2.6 vs. 8.9%, $p = 0.006$) in Bologna poor responder patients were significantly lower than in controls concluding that natural cycle IVF does not represent any substantial benefit to them.

Mild IVF Approaches in Japan

There is an increasing trend in using mild IVF approaches worldwide, but these treatment modalities have become especially widespread in Japan. Data from the official Japanese ART

registry shows that between 2007 and 2011 almost half (47%) of the started cycles involved unstimulated natural cycle IVF (10%) or clomiphene-based minimal ovarian stimulation (37%). This trend is probably due to a high proportion of advanced aged infertile women, the push of Japanese professional societies for the increased use of single embryo transfer and the efforts of specialist centers which have been developing these innovative treatment protocols for more than two decades [37]. One of these centers—which is also the largest single IVF unit in Japan, Kato Ladies Clinic (KLC) in Tokyo—has pioneered the development of mild IVF approaches since 1994. At KLC and at its other affiliate branches—including our center—ncIVF treatments represented a significant proportion of all cycles and considerable experience was gathered in their optimal management [38–40].

LH Surge-Based Scheduling: A Challenging Approach

In 2012, we have performed a retrospective review involving large 3-year cohort from our center with the aim of analyzing the effectiveness of oocyte retrieval scheduling based on the occurrence of spontaneous LH surge during natural cycle IVF treatment [40]. Our large retrospective study showed that in natural cycle IVF treatment oocyte retrieval timing based on the occurrence of spontaneous LH surge is feasible and permits the management of a large natural cycle IVF program on a 7-day/week basis within working hours. Moreover, acceptable oocyte recovery, fertilization and embryo cleavage rates were reached in concordance with previously published results on the efficacy of ncIVF treatment. The study included all 365 consecutive infertile patients who underwent 1138 ncIVF treatment cycles during 2008–2011 at our center (Kobe Motomachi Yume Clinic, Kobe, Japan). After obtaining informed consent, ncIVF was routinely offered to normally cycling (26–35 days) infertile women who ovulated according to their basal body temperature charts. Natural cycle IVF was usually proposed as a first, drug-free and cost-effective treatment option before starting a series of clomiphene-based minimal stimulation cycles in case no pregnancy was achieved. Patients were not selected and this treatment option was offered over a wide age range.

Natural Cycle IVF Protocol at KMYC (Kobe, Japan)

After obtaining a normal baseline ultrasound scan and hormonal profile on cycle day 3 monitoring usually started on day 8–10. Every other day follicular size was measured by two-dimensional transvaginal ultrasound scan together with serum hormonal level determinations (E2, LH and progesterone) with results available in-house within an hour. In the natural cycle IVF protocol, no GnRH antagonists were used to block the spontaneous LH surge. The center's opening times were such that patient examination and blood collection could be performed between 08:00 and 18:30 and oocyte retrievals could be scheduled between 08:00 and 17:00 on any day of the week (the entire staff followed a 6-day/week working schedule with a variable free day). The scheduling strategy during ncIVF treatment is summarized in Table 9.2. and depicted in Fig. 9.1. When the leading follicle reached 16–20 mm with a concomitant E2 level of approximately 200–250 pg/ml oocyte retrieval was scheduled according to the presumed stage of the spontaneous LH surge. For groups 1A (pre-surge: LH <10 IU/ml) and 1B (surge start: LH between 10 and 30 IU/ml group) triggering was performed around 23:00–24:00, and OR was scheduled 2 days later in the morning (with a 30–36 h interval between triggering and OR). For triggering, a GnRH agonist was used exclusively in form of a nasal spray (busereline 600 μg) and hCG was avoided completely. With ascending LH levels (30–140 IU/ml) OR was anticipated (for the next day performed during morning or afternoon working hours) and scheduled between 15 and 31 h after the examination. The GnRH agonist triggering dose was either administered

Table 9.2 Oocyte retrieval scheduling according to spontaneous LH surge

Group	Presumed stage of LH surge	Hormonal status on scheduling day	GnRHa triggering	Time of oocyte retrieval	Timing ranges
1A-1B	Pre-surge or surge start	LH < 10 or LH: 10–30, P4 < 1.0	23–24:00	2 days later in the morning (between 08:00 and 10:00)	30–36 h*
2	Ascending slope	LH: 30–53, P4 < 1.0	Triggering immediately	1 day later in the morning/afternoon (until 17:00)	17–31 h**
3	Peak	LH: 30–140, P4 < 1.0	Nothing	1 day later in the morning/afternoon (until 17:00)	15–28 h**
4	Descending slope	E2 and LH decreasing, P4 $\geq$ 1.0	Nothing	Same day (until 17:00)	1–2 h**

With permission from Bodri et al. [40]
*Between triggering and oocyte retrieval
**Between blood drawing and oocyte retrieval

immediately after the examination (group 2: ascending slope), or in case of even higher LH levels (group 3: peak), it was omitted. In very few cases, if the spontaneous LH surge was already on its descending side by detecting increased LH with a marked decline in E2 and rising progesterone (group 4) oocyte retrieval could even be scheduled for the same day of the examination (1–2 h later). With the exception of group 4 in all subgroups low-dose, NSAIDs were systematically used every 6 h before oocyte retrieval to diminish the risk of premature ovulation. The retrospective cohort was divided in five subgroups according to the presumed stage of spontaneous LH surge on scheduling day (1A: before onset, 1B: surge start 2: ascending slope, 3: peak and 4: descending slope). In our center, oocyte retrievals could be scheduled for any day of the week between 08:00 and 17:00 h and the whole procedure usually only took 5–6 min. Transvaginal ultrasound-guided oocyte retrieval was performed without anesthesia using a very thin 21–22G needle (Kitazato Medical Co., Ltd., Tokyo, Japan)—with has virtually no dead space—hence follicular flushing was not considered useful. After oocyte retrieval, any immature (MI or GV) oocytes were observed during maximum 12 h until most of them matured spontaneously. Mature (MII) oocytes were inseminated by conventional IVF or ICSI. Normally fertilized 2PN zygotes were cultured individually in 20 μl of cleavage-stage medium until day 2 or 3 and in a majority of cases subsequently cultured from day 4–6 until blastocyst stage in water jacket small multigas incubators (Astec, Japan). Most blastocysts were vitrified electively for subsequent use in frozen–thawed blastocyst transfer cycles. Details of the vitrification method using the Cryotop® (Kitazato, Japan) were described previously [41]. Single embryo transfer was performed in all IVF treatment cycles. The procedure was performed using transvaginal ultrasound guidance by precisely placing a single embryo to the mid-uterine cavity [42]. In fresh cycles, luteal support in form of oral dydrogesterone tablets (30 mg/day) was administered during two weeks after embryo transfer and continued in case of pregnancy. Frozen–thawed embryo transfers were performed in spontaneous natural or hormonal replacement cycles [43]. Main outcome measures of this retrospective review were the rate of cycles with successful oocyte recovery, fertilized oocytes and cleaved embryos; these were compared between subgroups (1A, 1B, 2, 3 and 4). The effect of female age, infertility type, cycle rank, follicular size, serum E2 level and the presumed stage of LH surge on oocyte recovery rate was evaluated by multivariate logistic regression analysis. Additionally, overall and age-specific live birth rates were also presented. Metric variables were analyzed by one-way ANOVA test and nominal variables were analyzed by the Chi-squared test. $P < 0.05$ was considered statistically significant.

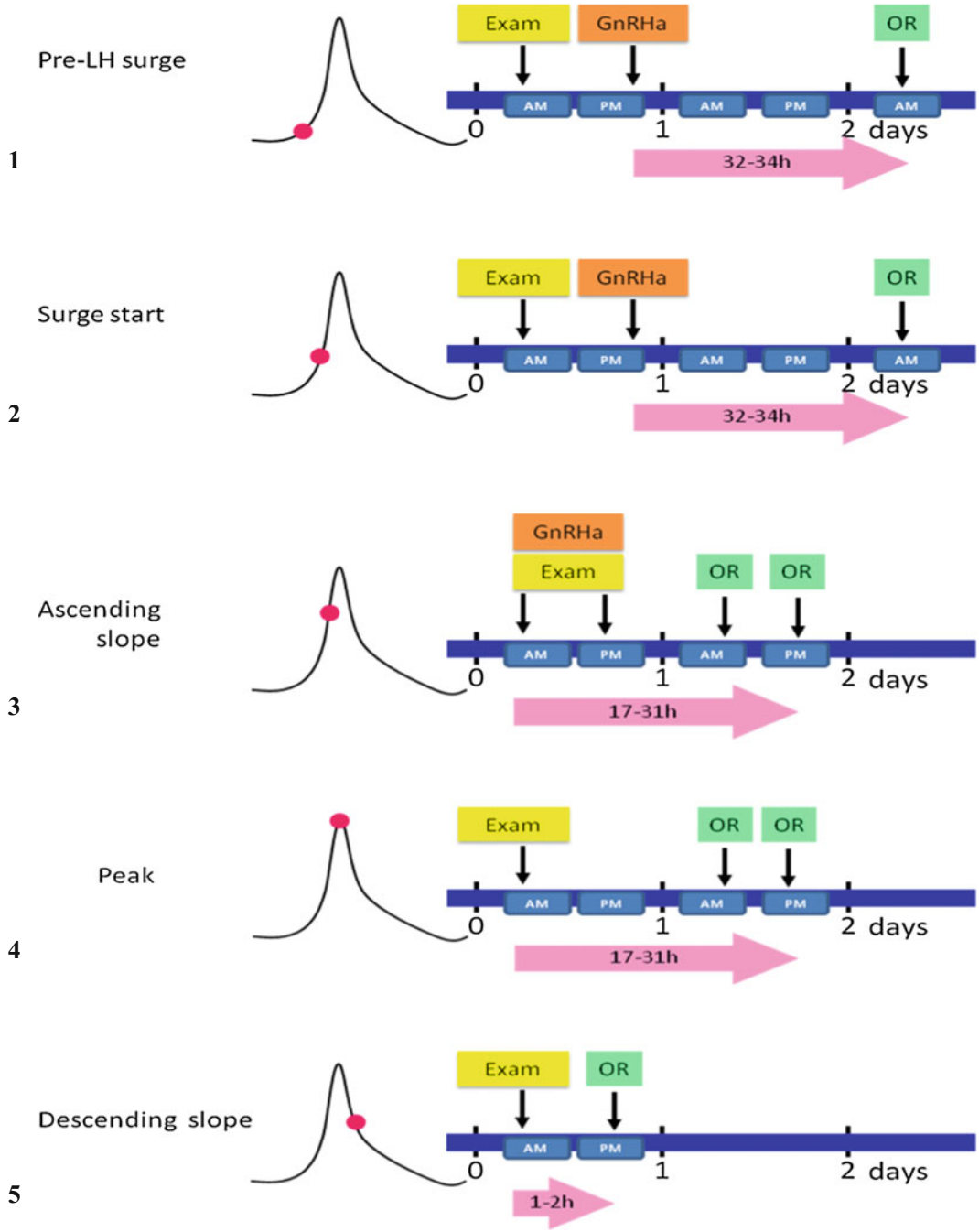

Fig. 9.1 Oocyte retrieval scheduling according to spontaneous LH surge. With permission from Bodri et al. [40]

Insights from Large Japanese Cohort Study

About 58% of our patients were older than 38 years at starting treatment. Mean BMI was low (20.8 ± 2.3) as expected in the Japanese population. Most patients had primary infertility (67%) and were nulliparous (87%). Among infertility causes Bologna poor responders (29%) were overrepresented. Only few patients (20%) did not have any previous fertility treatment and most of them (55%) had already undergone conventional and/or minimal IVF treatment cycles at other centers. Follicular size and hormonal levels (E2, LH and P4) on scheduling day as well as timing intervals according to different surge subgroups are represented in Table 9.3. Differences were statistically significant for each variable which was especially marked for LH levels and timing intervals. In 61% of the cycles, oocyte retrieval was schedule before or just at the start of the LH surge (groups 1 and 2), whereas in the remaining cases (39%) it was scheduled after LH surge has already started. Overall premature ovulation and successful oocyte recovery rates were 4% (45/1138) and 78% (887/1138), respectively. The proportion of cycles with an inseminated, fertilized oocyte and cleaved embryo was 67, 57 and 56%, respectively. Main outcomes according to LH surge subgroups are presented in Table 9.4. The proportion of cycles with successfully recovered (range: 71–86%), inseminated (range: 61–78%), fertilized oocytes (range: 47–68%) and cleaved embryos (range: 45–66%) was not significantly different between subgroups. Cleavage-stage embryos were transferred in 109 cycles which resulted in 23 (21%) live births. In the remaining 502 cycles, cleavage-stage embryos were submitted to prolonged culture yielding a good quality blastocyst in 177 (35%) treatment cycles. Out of these—to date—146 blastocysts were used (mostly frozen–thawed embryo transfer) resulting in 51 (35%) live births. For the entire cohort, live birth rate per embryo transfer and scheduled oocyte retrieval was 29% (74/256) and 6.5% (74/1138), respectively. Age-specific live birth rates per scheduled oocyte retrieval were 22% (29/134), 10.5% (16/153), 7.7% (18/233) and 4.2% (10/326) for <35, 35–37, 38–40, 41–43-old patients, respectively. No live births were obtained in ≥44 years old infertile women. In a multivariate analysis, successful oocyte retrieval was not associated with female age (adjusted OR: 0.98, 95%CI: 0.94–1.03, $p = 0.48$), follicular size (aOR: 0.96, 95%CI: 0.88–1.04, $p = 0.27$) and serum estradiol level on scheduling day (adjusted OR: 1.0, 95%CI: 1.0–1.0, $p = 0.90$) or cycle rank (adjusted OR: 1.03, 95% CI: 0.99–1.07, $p = 0.20$). On the other hand, patients with endometriosis had a lower chance of successful oocyte retrieval (aOR: 0.40 95%CI:

Table 9.3 Baseline cycle characteristics according to LH surge stage

Study groups	1A	1B	2	3	4	P^*
Scheduled ORs, n (%)	347 (31)	347 (30)	343 (30)	98 (8.6)	3 (0.3)	–
Leading follicle (mm)	18 (17–19)	17 (16–19)	18 (16–19)	17 (16–19)	17	0.001
E2 (pg/ml)	308 (270–341)	291 (256–334)	300 (250–358)	217 (171–279)	154	<0.0001
LH (IU/ml)*	8 (6–9)	13 (12–15)	25 (20–33)	68 (58–78)	30	<0.0001
P4 (ng/ml)	0.4 (0.3–0.4)	0.4 (0.4–0.5)	0.5 (0.4–0.6)	0.8 (0.7–0–9)	1.0	<0.0001
Timing interval (hours)	33.5 (33.3–33.6)**	33.5 (33.3–33.6)**	27 (23.9–28.7)***	22.8 (21.5–23.7)***	1.8***	<0.0001

With permission from Bodri et al. [40]
Values are medians and quartiles
*One-way ANOVA test
**Between triggering and oocyte retrieval
***Between blood drawing and oocyte retrieval

Table 9.4 Outcomes according to LH surge stage

Study groups	Total	1A	1B	2	3	4	*p*
Scheduled ORs, n (%)	1138	347 (31)	347 (30)	343 (30)	98 (8.6)	3 (0.3)	–
Patient age, years	40.3 ± 4.3	40.7 ± 4.4	40.2 ± 4	40.1 ± 4.2	40.2 ± 5	43 ± 3.5	0.224[b]
Cycle rank	3.4 ± 4.4	3.7 ± 4.6	3.4 ± 4.5	3.2 ± 4.1	2.8 ± 3.6	1.7 ± 2.1	0.264[b]
Ovulated cycles, n (%)[a]	45 (4)	10 (2.9)	18 (5.2)	12 (3.5)	5 (5.1)	0 (0)	0.58[c]
Cycles with retrieved oocyte, n (%)[a]	887 (78)	265 (76)	292 (84)	243 (71)	84 (86)	3 (100)	0.59[c]
Cycles with inseminated oocyte, n (%)[a]	768 (67)	226 (65)	255 (74)	209 (61)	76 (78)	2 (67)	0.49[c]
Fertilization rate	83%	85%	87%	77%	88%	100%	0.9[c]
Cycles with fertilized (2PN) oocyte, n (%)[a]	644 (57)	193 (55)	222 (64)	160 (47)	67 (68)	2 (67)	0.11[c]
Cycles with cleaved embryo, n (%)[a]	633 (56)	192 (55)	219 (63)	155 (45)	65 (66)	2 (67)	0.09[c]
Cycles with embryo transfer, n (%)[a]	256 (22)	79 (23)	88 (25)	64 (19)	24 (24)	0 (0)	0.46[c]
Cycles with live birth, n (%)[a]	74 (6.5)	24 (6.9)	27 (7.8)	14 (4.1)	9 (9.2)	–	0.25[c]

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Values are median ± SD
[a]Per scheduled oocyte retrieval
[b]One-way ANOVA test
[c]Chi-squared test

0.17–0.94, $p = 0.04$). In Bologna poor ovarian responders, a similar almost significant trend was observed (aOR: 0.51 95%CI: 0.26–1.02, $p = 0.06$). After adjusting for the above variables compared to the group 1A (pre-LH surge) group 1B (LH surge start) was associated (aOR: 1.64 95%CI: 1.10–2.44, $p = 0.02$) with a significantly higher whereas group 3 (peak) (aOR: 1.90 95% CI: 0.96–3.75, $p = 0.07$) with an almost significantly higher chance of retrieving an oocyte.

In our study groups, 1A (pre-surge) and 1B (surge start) were scheduled using the same strategy involving GnRH agonist triggering around midnight and OR scheduled at a fixed 33–34 interval two days later. Outcomes seemed to be somewhat better for subgroup 1B where the LH surge has already started (84% vs. 78% oocyte retrieval rate). In both of these subgroups, the triggering agent was administered several hours after the last examination; however, this did not seem to affect outcome negatively. Probably in those cycles where spontaneous LH surge started during this time period the protective effect of NSAIDs prevented the follicle from rupturing and an oocyte could be still retrieved. Group 2 (ascending slope) performed the worst with the lowest oocyte retrieval rate of 61% in the cohort. This might indicate that some cycles were timed too early (at 24–29 h). In contrast, the highest oocyte retrieval rate of 86% was reached for group 3 (peak) which suggest that with more advanced LH levels OR was scheduled (anticipated) correctly. Finally, only very few cases were in group 4 (descending slope) indicating that with frequent monitoring every other day (and corresponding center's opening hours 7 days/week) most ncIVF cycles (61%) could be scheduled before or at the start of the spontaneous LH surge. A multivariate analysis in our study did not show any significant impact of female age or cycle rank on the chance of retrieving an egg following ncIVF. On the other hand, among infertility diagnoses patients with endometriosis had a significantly lower (OR:

0.40, 95%CI: 0.17–0.94, $p = 0.04$) possibility of retrieving an oocyte which might be related to destruction of ovarian tissue by endometriomata and/or previous surgery. Similarly, Bologna poor ovarian responders had a marginally lower chance (OR: 0.51, 95%CI: 0.26–1.02, $p = 0.06$) of successful oocyte retrieval. This is in contrast with a recent retrospective review from the Brussels group where oocyte retrieval rates between Bologna patients and controls were not significantly different (75% vs. 78%) [36]. However, in their study, live birth rates per started cycle were still much lower among Bologna poor responders (2.6% vs. 8.9% in controls) and the authors concluded that these patients are not benefitting from a ncIVF approach. Similarly to a previous UK study, the large ncIVF program managed at our center implicated a 7-day/week working schedule, and the oocyte retrievals were fit in a 9 h interval (between 08:00 and 17:00) within the day [24]. However, most of the procedures were performed in the morning hours (08:00–10:00) by establishing a very rapid turnover of procedures (every 5–6 min). This was only feasible by an extremely well-organized flow of patients and by using a very thin (21–22G) retrieval needle that permitted the avoidance of any anesthesia.

Although overall live birth rates per scheduled OR were low (6.5%) in our series in younger age groups (<38 years), they were more acceptable. Success rates in our series they were greatly affected by the fact that blastocyst culture was performed in most (79%) treatment cycles. Probably due to a high proportion of >38-year-old patients, this turned out to be counterproductive due to a high proportion of cycles (65%) that did not reach the blastocyst stage. On the other hand, in another paper from our group with a large series of 1865 ncIVF cycles where embryos were transferred earlier at day 2–3 the rate of cancelled cycles was much lower, more cycles reached embryo transfer (43% vs. 22%) with acceptable live birth rates (14.7% vs. 6.5%) per scheduled cycles underlining the increased efficiency of cleavage-stage embryo transfer compared to blastocyst-stage transfer [39]. Another main limitation of our study is related to its retrospective nature; however, we included only consecutive patients treated with the same protocol, therefore any selection bias might be limited considerably.

Although with our current approach to natural cycle IVF overall results were acceptable more intensive endocrine monitoring (twice-daily) and further fine tuning might still be needed to obtain uniformly high oocyte retrieval rates in all LH surge subgroups.

Adjuvants to Boost ncIVF

The overall efficiency of natural cycle IVF protocols is greatly hampered by the fact that losses occur at each stage of the IVF procedure (from oocyte retrieval until the embryo implantation). Therefore, even in the "best-case" scenario, the expected rate of embryo transfers and live births per started cycle is only around 50 and 10%, respectively. The main reasons for this phenomenon are: difficulties in controlling the spontaneous LH surge, unwanted premature ovulations detected at oocyte retrieval, failure of retrieving an oocyte, immature oocytes and others. Several groups have tried to develop different approaches to tackle some of these problems and increase the overall efficiency of ncIVF protocols.

NSAID Use to Prevent Ovulation

Non-steroidal anti-inflammatory drugs (NSAID) may efficiently delay or even prevent follicular rupture by blocking the cyclooxygenase-2 enzyme which has a key role in the ovulatory process [44, 45]. High-dose NSAID administration in humans during the late follicular phase was recently explored as an effective method for emergency contraception [46, 47] and a way to control spontaneous ovulations during natural cycle IVF [48, 49]. However, concerns were raised because of potential deleterious effects of high-dose NSAID administration which was shown to cause infertility and adversely affect embryonic implantation in animal studies [50, 51].

Prostaglandin, which is an important mediator of the ovulation process in humans, is produced within the ovulatory follicle by the COX-2 enzyme [44, 45]. The inhibition of the COX-2 enzyme, limits prostaglandin production, prevents follicular rupture and prevents oocyte release [52].

In the context of natural cycle, IVF Nargund et al. were among the first to describe the use of NSAIDs to delay premature ovulation [48]. At a relatively high-dose (50 mg tablets three times a day), indomethacin was successfully used in patients with a beginning LH surge to delay follicular rupture for up to one week (usually from Friday evening until Monday morning to avoid weekend oocyte retrievals). NSAIDs were also successfully applied by a Canadian group to diminish the rate of unwanted premature ovulations. In their retrospective, observational study an NSAID was non-randomly used in one-third of 255 nIVF cycles and it was associated with a significantly diminished rate of premature ovulation (6% vs. 16%) and a non-significantly higher clinical pregnancy rate per initiated cycle (13% vs. 6%) [49].

As a part of our center's natural cycle IVF protocol short-term, low-dose non-steroidal anti-inflammatory drugs (two separate 25 mg diclofenac rectal suppositories at 8 and 14 h before oocyte retrieval at a 6 h interval) are routinely used to reduce the rate of premature ovulations. This is in contrast with the previously mentioned studies where NSAIDs were used in a high-dose regimen (150 mg indomethacin/day) over a course of several days. In 2012, we have conducted a large-scale retrospective analysis using data from the Tokyo branch of our group involving 1865 first-rank cycles with an imminent LH surge on triggering day (10–30 IU/ml). The study has shown that NSAID use was associated with a significantly lower risk of premature ovulation (3.6% vs. 6.8%, adjusted OR: 0.24 95%CI 0.15–0.39, $p < 0.0001$) and higher embryo transfer rate (46.8% vs. 39.5%, adjusted OR: 1.38 95%CI: 1.06–1.61, $p = 0.01$) per scheduled cycle. This persisted even after adjusting for several confounders (patient age, LH, E2, follicular size on triggering day) by a multivariate logistic regression analysis. Clinical pregnancy (39.1% vs. 35.9%) and live birth rates per embryo transfer (31.3% vs. 31.4%) were comparable in both groups. However, it could also be appreciated that the reduction in premature ovulation rates had a relatively small impact on overall success rates per started (14.7 vs. 12.4% live birth rate per started cycle) (Fig. 9.2). In summary, retrospective series short-term, low-dose NSAID application positively influenced ncIVF cycles by diminishing the rate of unwanted premature ovulations and increasing the proportion of cycles reaching embryo transfer [39].

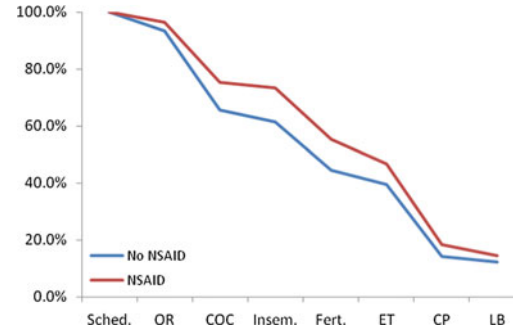

Fig. 9.2 Natural IVF cycle outcome per scheduled cycle according to NSAID use. With permission from Kawachiya et al. [39]

In a recently published paper using a methodologically more powerful double-blind placebo-controlled randomized clinical trial, similar encouraging results were found by an experienced Dutch specialist center. This RCT was performed using a modified natural cycle IVF approach by randomizing 120 infertile women to series of maximum six treatment cycles with our without oral indomethacin treatment (at a 150 mg/day dosage from the day of hCG triggering until the morning of oocyte retrieval). Although the authors did not find any significant influence of indomethacin treatment on the probability of premature ovulation in the entire patient group (OR: 2.38, 95%CI: 0.94–6.04), this might be simply related to their relatively limited sample size. However, in a subgroup analysis of patients without an LH surge, they did find a very significant association (aOR: 8.29 95% CI: 1.63–42.3, $p = 0.009$) and

suggested that this patient group could have a significant benefit from the NSAID treatment. Per cycle success rates were fairly low (ongoing pregnancy rate per started cycle of 5.6 and 8.4% in the treated and placebo-controlled groups, respectively) but cumulative ongoing pregnancy after a maximum of 6 cycles (on average 4.1–4.6 performed treatment cycles) were more favorable (23.7 and 38.3%, respectively) [53]. In our practice, we do encourage the routine use of NSAIDs in all stages of natural cycle IVF treatment (before and after spontaneous LH surge has occurred) due to a good safety profile and potential beneficial effects. In conclusion, all retrospective and prospective studies point in the same direction and suggest beneficial effect of NSAID use on preventing premature ovulations during ncIVF treatment.

Adjuvants Drugs—Low-Dose Clomiphene

In a recent prospective study from a Swiss group, low-dose clomiphene citrate (25 mg/day) was tested in the late follicular phase on 112 infertile patients [54]. Study patients first started with a ncIVF protocol (without GnRH antagonists and coupled with hCG triggering) and continued with a second treatment by adding clomiphene citrate if no conception occurred. Their mean age was 35 ± 14.5 years and almost half of them showed signs of low ovarian reserve. The authors have found that the rate of premature ovulations was significantly decreased (27.8 vs. 6.8%, $p < 0.001$) and embryo transfer rate increased (39.8 vs. 54.4%, $p = 0.039$). Overall clinical pregnancy rates per embryo transfer and started cycles were 27.9 versus 25% and 11.1 versus 13.6%, respectively. The reduction in the rate of premature ovulation was attributed to one of the isomers of clomiphene citrate that diminished the risk of premature LH surges during the late follicular phase (no other adjuvants such as NSAIDs were used in this study). In fact, a similar beneficial effect was also demonstrated in a randomized clinical trial by an Egyptian group in the context of intrauterine insemination treatment [55]. On the other hand, differences in outcome might also be related to a mild stimulatory effect because both the number of follicles >15 mm (0.9 ± 0.4 vs. 1.4 ± 0.6, $p < 0.001$) and the number of retrieved eggs (0.6 ± 0.5 vs. 0.8 ± 0.7, $p = 0.004$) were in favor of the clomiphene group. Probably due to a low-dosage used only few, mild side-effects were seen and no deleterious endometrial effects were observed in the clomiphene-treated group.

Follicular Flushing

In the setting of IVF treatment performed in normal or and poor responder patients, follicular flushing was not shown to be beneficial and could even have deleterious effects by extending the duration of oocyte retrieval and increasing the quantity of anesthetic drugs used [56]. However, as non-randomized data suggests patients undergoing natural cycle IVF or minimal stimulation might benefit from it resulting in a larger oocyte yield and more embryos.

A recent study from a Swiss specialist group involving 164 mono-follicular IVF cycles (natural cycle IVF treatment with/without using a low-dose clomiphene adjuvant) showed that multiple flushing (up to 3 times) was found to be beneficial by increasing the oocyte yield from 45 to 81% and the embryo transfer rate from 20.1 to 38.4% [57]. Oocyte and embryo transfer rate per successive aspiration dropped gradually; therefore, the authors concluded that a maximum of three flushing steps would be sufficient. The authors have used a thin 19G needle without applying any anesthesia. The aspiration of a single follicle took around 30 s, and the flushing extended it by approximately 1 min. Moreover, there was no difference in the rate of mature and fertilized oocytes between successive aspirations suggesting no deleterious effect of repeated follicular flushing.

Similar findings were also found by a French study where laboratory outcomes were compared between eggs originating from the follicular fluid or the flushing liquid (up to 4 flushings) from a total of 146 oocyte retrievals following a

modified natural cycle IVF treatment [58]. The authors have found no significant difference in fertilization rates (79.7% vs. 88.1%), the proportion of top-quality embryos (28.8% and 37.8%) and clinical pregnancy rates (16.5% vs. 44.1%).

In our center, follicular flushing is currently not used due to specifics of the very fine (22G) needle used for oocyte retrieval (which has an extremely small dead space) but also for practical reasons (to avoid anesthesia and to ensure the rapid turnover of procedures).

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Risk Factors and Preventive Measures of Ovarian Hyperstimulation Syndrome

10

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Introduction

Ovarian hyperstimulation syndrome (OHSS) is an iatrogenic complication of exogenous ovarian stimulation used in assisted conception technique. The pathophysiology of OHSS is characterized by increased capillary permeability leading to vascular fluid leakage, third space fluid accumulation, and intravascular dehydration [1]. OHSS is classified according to the clinical features and laboratory findings as mild, moderate, or severe. In severe cases, the process can result in multiple organ dysfunctions, including a tendency for thrombosis, renal and hepatic dysfunction, and pulmonary edema. Such patients can develop critical disease, which may involve cerebral infarction, adult respiratory distress syndrome, hepatorenal failure, and even death [2].

OHSS develops as a result of mediators released from hyperstimulated ovaries. The nature of these vasoactive substances remains unclear, but vascular endothelial growth factor (VEGF) and a variety of pro-inflammatory cytokines may be involved [3]. The background changes that lead to OHSS occur during the phase of ovarian stimulation with follicle-stimulating hormone (FSH), but full manifestation of the clinical syndrome requires exposure to luteinizing hormone (LH) or human chorionic gonadotropin (hCG).

The onset of OHSS reflects the effect of hCG exposure at different stages of the condition. 'Early' OHSS occurs within 9 days of hCG triggering and reflects the effect of exogenous hCG, resulting in an excessive ovarian response. 'Late' OHSS occurs 10 or more days after the ovulatory dose of hCG and reflects the effect of endogenous hCG from an early pregnancy. Although early and late OHSS have distinct clinical presentations [4], they appear to be similar in terms of disease severity [5].

It is difficult to estimate accurately the incidence of OHSS, owing to the use of a variety of classification schemes, and the varying incidence between treatments and patient groups. Mild OHSS may be common in stimulated in vitro fertilization (IVF) cycles. Moderate-to-severe OHSS has been reported to occur in 2–8% of IVF cycles [6, 7]. In a report from Finland, the rate of hospitalization for OHSS following ovulation induction was 0.04% per cycle, whereas 0.9% was observed following IVF [8].

Risk Factors for OHSS

Pretreatment Patient Characteristics

Young patients are at a greater risk of developing OHSS [9, 10]; this may be related to the greater probability of pregnancy in younger women, and a greater sensitivity of their ovaries to exogenous gonadotropins. Navot et al. [6] observed a lower

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mean body weight among women with OHSS, but this was not confirmed in a larger study [9]. A previous history of OHSS is a risk factor for developing OHSS in subsequent ovarian stimulation cycles [9].

OHSS is more frequent in women with polycystic ovaries (PCO) or polycystic ovary syndrome (PCOS) [6, 9, 11]. Patients with PCO were twice as likely to develop OHSS, compared with patients without PCO, despite receiving lower dose of gonadotropins [11]. The presence of isolated characteristics of PCOS, such as hyperandrogenism and anovulation, may also be risk factors for OHSS, even if the patient does not fulfill all of the criteria for PCOS [9]. Patients with PCOS display a higher sensitivity to gonadotropins, due to the larger size of the FSH-sensitive cohort of small antral follicles. If the gonadotropin dose oversteps the threshold FSH level needed for ovarian stimulation, multifollicular development ensues.

High pretreatment serum level of anti-Müllerian hormone (AMH) and a high pretreatment antral follicle count (AFC) are known to be associated with an excessive response to ovarian stimulation [12]. Serum AMH level is usually elevated in women with PCOS and a higher level of serum AMH is associated with a greater risk of OHSS [13–15]. A cutoff value of AMH >3.5 ng/ml and AFC >10 is shown to be highly and equally predictive of ovarian hyperstimulation [13].

Ovarian Response Parameters

A high serum estradiol concentration during ovarian hyperstimulation, a large number of follicles, and a large number of oocytes retrieved have all been reported to be associated with an increased risk of OHSS [16, 17]. However, the value of these parameters in predicting the risk of OHSS is poor, and the cutoff values are often arbitrary. A peak serum estradiol level of 3000–6000 pg/ml has been suggested for predicting high risk for OHSS [18]. Further, no agreement is reached regarding the size and number of the follicles. Unlike late OHSS, early OHSS can be predicted to some extent by the serum estradiol concentration even though its predictive value is modest [4].

Preventative Measures

Starting Dose and the Type of FSH

Factors that may increase the risk of excessive ovarian response, such as PCO/PCOS, previous history of OHSS and young age, should be taken into account before choosing the starting dose of FSH. In those situations, a lower starting dose is usually recommended. The starting dose of FSH can be tailored according to the woman's age or serum level of AMH [15].

No significant difference in the occurrence of OHSS was noted between recombinant FSH and urinary FSH, either in patients with clomiphene-resistant PCOS [19] or in general IVF candidates [20]. Also, two meta-analyses showed similar incidence of OHSS between recombinant FSH and human menopausal gonadotropin (hMG) [21, 22]. A recent meta-analysis demonstrated that the incidence of OHSS was similar between recombinant FSH and highly purified hMG [23].

GnRH Agonist Versus Antagonist

Since the introduction of therapeutic gonadotropin-releasing hormone (GnRH) agonists in 1986, the incidence of severe OHSS has increased sixfold, compared with IVF cycles stimulated by clomiphene/hMG only [7]. A Cochrane review found a significantly lower incidence of OHSS in GnRH antagonist cycles compared with GnRH agonist cycles (relative risk, RR, 0.61) [24]. A meta-analysis by Ludwig et al. [25] reported no significant reduction in the risk of OHSS with the antagonist protocol, compared with the agonist protocol. However, a subgroup analysis indicated that there was a significant reduction in the incidence of OHSS in cycles using the GnRH antagonist cetrorelix (odds ratio OR, 0.23), but no such reduction in

cycles using another GnRH antagonist ganirelix (OR, 1.13). In two recent meta-analyses that included women with PCOS, the GnRH antagonist protocol showed either a statistically significant (OR, 0.36) or nonsignificant (OR, 0.64) reduction in severe OHSS, compared with a long agonist protocol [26, 27]. Thus, a GnRH antagonist protocol may be preferentially considered in patients at high risk for OHSS.

Dose of hCG for Triggering

Given the positive association between the dose of hCG and the risk of OHSS [28], the lowest effective dose of hCG should be used to minimize the risk of OHSS. Urinary hCG of less than 5000 IU for triggering would appear to be reasonable in high-risk patients.

Urinary hCG is used to mimic the endogenous LH surge due to its structural similarity with human LH. Nowadays, recombinant LH may be used for the same purpose. The luteotropic effect of hCG is more prolonged than that of endogenous LH; hence, it has been suggested that triggering ovulation using recombinant LH, instead of hCG, may reduce the risk of OHSS. However, a meta-analysis of two trials comparing the incidence of OHSS between cycles using recombinant LH and cycles using urinary hCG for triggering did not show any difference [29]. Moreover, the incidence of severe OHSS was quite high in both groups (recombinant LH, 10.3%; urinary hCG, 12.4%). Further trials are needed to assess the efficacy of use of recombinant LH in the prevention of OHSS.

A meta-analysis comprising 747 subjects reported no significant difference in the incidence of OHSS between those receiving recombinant hCG and those receiving urinary hCG [29]. A recent Cochrane review also supports this finding [30].

Choice of Luteal Support

Luteal support with hCG should be avoided in patients with a high risk of OHSS, because hCG has a critical role in precipitating and worsening established OHSS. Progesterone is as effective as hCG for luteal support and is associated with a lower risk of OHSS [31].

Metformin Co-treatment During Ovarian Stimulation

In an RCT in 2006, Tang et al. [32] administered placebo or metformin 850 mg, twice daily, from the first day of down-regulation to oocyte pickup (OPU) day, to women with PCOS undergoing IVF; the incidence of severe OHSS was significantly reduced in the metformin group (OR, 0.15). A systematic review showed a significant reduction in the incidence of OHSS with the use of metformin (OR, 0.21) in women with PCOS undergoing IVF [33]. A recent meta-analysis demonstrated significant reduction in the risk of OHSS (OR, 0.27) and the risk of miscarriage (OR, 0.50) after metformin administration [34]. Therefore, current evidence supports the concept that metformin co-treatment during ovarian stimulation may reduce the risk of OHSS in women with PCOS.

Coasting

Coasting refers to method where exogenous gonadotropin administration and hCG trigger are withheld (while maintains pituitary suppression) until serum level of estradiol declines to a 'safer' predefined level. After daily measurement of serum estradiol and follicular tracking, hCG trigger can be done, followed by OPU and embryo transfer (ET). The rationale for coasting is that larger follicles have a lower dependence on FSH than smaller follicles and are capable of continuing their growth and maturation in the face of depleted gonadotropin stimulation, while small and intermediate follicles undergo atresia. Declining concentrations of vasoactive mediators from the ovary, such as VEGF, may contribute to the efficacy of coasting [35].

There are several retrospective studies examining the efficacy of coasting in the prevention of

OHSS. In a qualitative systematic review of 12 studies including 493 patients, fertilization and pregnancy rates after coasting were acceptable, and 2.5% of the patients required hospitalization for OHSS [36]. Although coasting does not abolish the risk of OHSS, it appears to lower the incidence of OHSS [37]. A survey of 573 members of the European Society of Human Reproduction and Embryology (ESHRE) found that coasting is the most common strategy used to prevent OHSS [38].

The typical serum estradiol level used to initiate coasting is >3000 pg/ml [39] and the criterion for cessation (and hCG triggering) is below 3000 pg/ml or 3500 pg/ml [37, 40]. An abrupt drop in serum estradiol level, or a drop less than 1000 pg/ml, may be associated with a low fertilization rate and poor outcome [37]. The duration of coasting depends on the initial estradiol level, with higher estradiol levels requiring more prolonged coasting, before it drops to the designated 'safer' level. Coasting for more than 3–4 days may be associated with poorer cycle outcomes [37].

In a retrospective analysis of 1223 coasted cycles, Mansour et al. [40] found a lower clinical pregnancy rate following intracytoplasmic sperm injection (ICSI) cycles with coasting for >3 days, compared with cycles with ≤ 3 days of coasting (36% vs. 52%). Garcia-Velasco et al. [37] found a lower implantation rate in patients who had been coasted for >4 days (15.5% in >4 days group vs. 28.5% in ≤ 4 days group). Based on these data, cycle cancellation may be the preferred alternative if hCG triggering does not look feasible on the 4th day of coasting.

Follicle Aspiration Prior to hCG Administration

In an attempt to reduce the risk of OHSS by reducing the total pool of luteinized granulosa cells in the ovary, aspiration of some follicles prior to hCG triggering has been suggested. This approach produced beneficial results in 14 patients returning for further IVF cycles after cycles complicated by OHSS [41]. In contrast, Schroder et al. reported that 80% of the subjects developed severe OHSS following this procedure [42]. No benefit was seen for aspiration of all follicles from one ovary 6–8 h prior to hCG triggering in 16 women at high risk of OHSS compared with 15 matched control subjects in whom aspiration was not performed [43]. In patients at high risk of OHSS, 3 and 4 cases of OHSS out of 15 patients undergoing coasting and 15 patients undergoing early follicle aspiration were revealed, respectively [44]. Thus, the evidence regarding timed follicular aspiration prior to hCG triggering remains uncertain.

Cycle Cancellation

OHSS develops only in cycles with an exposure to endogenous or exogenous hCG. Consequently, the most effective method of prevention is to withhold hCG, and in turn, cancel the treatment cycle. Despite the fact that the emotional and financial costs of the cycle cancellation would be significant, cycle cancellation can be an alternative option when the ovarian response is excessive, and embryo cryopreservation is not a viable option.

GnRH Agonist Triggering in GnRH Antagonist Cycles

In GnRH antagonist cycles, a GnRH agonist can be used to induce an endogenous LH surge, leading to final follicular maturation. Endogenous LH has a shorter half-life than hCG; thus, it is less likely to cause OHSS compared with hCG. From a systematic review of 3 RCTs that compared the use of a GnRH agonist with hCG triggering in GnRH antagonist cycles in normal responders, GnRH agonist triggering was associated with a significant reduction of the clinical pregnancy (OR, 0.21) without a prevention of OHSS [45]. This discouraging pregnancy result might be associated with a frequent occurrence of luteal phase deficiency in GnRH agonist-triggered cycles. Therefore, Kol [46], Kol and Solt [47] mentioned adequate luteal support to

compensate luteolysis in GnRH agonist-triggered cycles for good clinical outcome. A recent meta-analysis indicated that 'GnRH agonist triggering combined with aggressive or modified luteal phase support' can provide a safe and OHSS-free clinical environment, with an acceptable rate of clinical pregnancy [48]. Currently, this strategy may be helpful in patients at high risk for OHSS, but not in normal responders. Modified luteal phase support includes daily intramuscular progesterone and oral estrogen [49] or low-dose hCG only (250 or 500 IU at OPU + 1, OPU + 4, and OPU + 7) [50]. GnRH agonist triggering in GnRH antagonist cycles may also be a useful method in oocyte donors, and cancer patients who pursue fertility preservation.

Intravenous Albumin Versus Hydroxyethyl Starch

Administration of intravenous (IV) albumin around the time of OPU has been proposed as a measure to prevent OHSS [51]. A number of RCTs that included small numbers of patients at high risk of OHSS showed a reduced incidence of severe OHSS in the group receiving IV albumin. In 2002, a Cochrane meta-analysis of 5 RCTs showed a significant reduction in the incidence of severe OHSS in women receiving IV albumin (OR, 0.28) [52].

However, a systematic review by Delvigne and Rozenberg [7], which included data from prospective randomized studies and a single retrospective study, reported no significant difference in the incidence of OHSS between patients receiving IV albumin and controls. A subsequent single-center RCT revealed that the incidence of moderate-to-severe or severe OHSS did not differ between albumin treatment and no treatment groups (40 g IV albumin group, 7.1% moderate to severe, 5% severe; no treatment group, 6.7% moderate to severe, 4.7% severe) [53]. In addition, a recent meta-analysis of data including 1613 women at high risk of OHSS demonstrated that the incidence of severe OHSS was 6.0% in the IV albumin group, and 7.9% in saline or no treatment groups, which was not statistically different [54]. Moreover, treatment with IV albumin was associated with a significant reduction in pregnancy rates (RR, 0.85). Thus, the currently available data do not support administration of IV albumin around the time of OPU in the prevention of OHSS.

Hydroxyethyl starch (HES) is a synthetic high molecular weight compound, used as a plasma volume expander, as an alternative to albumin. A group of investigators reported the use of 1000 ml of 6% HES at the time of OPU and 500 ml at the time of ET in 100 high-risk women [55]. Even though the incidence of moderate OHSS was reduced, the incidence of severe OHSS between the treatment and historical control group did not differ significantly.

A subsequent RCT assessed the effectiveness of 1000 ml of 6% HES, administered at the time of ET, in women at high risk of OHSS [56]. The incidence of moderate-to-severe OHSS was significantly lower in the treatment group than that in the placebo group (2% vs. 13%). Gokmen et al. [57] found both albumin and HES to be superior to placebo in preventing OHSS in women with a serum estradiol >3000 pg/ml, and >20 follicles on the day of hCG triggering. Although, in this study, there was no significant difference in the incidence of OHSS between the albumin and HES groups, the authors showed a preference for HES on the grounds of cost and safety. A recent Cochrane review demonstrated that HES significantly decreases the incidence of severe OHSS (OR, 0.12), without affecting the pregnancy rate [58]. This finding also supports the use of HES over albumin in the prevention of OHSS.

Administration of Dopamine Agonist

OHSS is the result of ovarian hypersecretion of VEGF, which activates the VEGF receptor-2 (VEGFR2). VEGF/VEGFR2 binding disrupts cellular junctions and increases vascular permeability, which is characteristic of OHSS; VEGF/VEGFR2 binding also enhances angiogenesis, which is a fundamental step in implantation. Several in vitro studies have demonstrated

that the dopamine receptor 2 agonist, cabergoline, inhibits VEGF secretion by luteinized granulosa cells, and inactivates VEGFR2 [59, 60].

Alvarez et al. [61] performed a RCT investigating oocyte donors at high risk of OHSS. Cabergoline, a dopamine agonist, 0.5 mg/d or a placebo was administered for 8 days from the day of hCG triggering. The incidence of moderate OHSS was significantly lower in the cabergoline treated group, compared with placebo (20.0% vs. 43.8%). Low-dose cabergoline (0.25 mg/d) was also reported to be a useful preventive measure for OHSS [62]. Regarding the initiation of cabergoline treatment, there is only one RCT, which showed a similar incidence of OHSS between the group starting cabergoline on the day of hCG triggering and those starting cabergoline on the day of OPU [63].

There are several reports about the impact of the use of cabergoline on pregnancy outcomes. A pilot study confirmed that administration of cabergoline in order to prevent OHSS is safe and does not affect pregnancy outcome [64]. A recent meta-analysis also showed that cabergoline significantly reduces the risk of moderate-to-severe OHSS (RR, 0.38) and has no clinically relevant negative impact on clinical pregnancy [65].

Another dopamine agonist, quinagolide, has been also investigated as a possible drug to prevent OHSS. A multicenter RCT demonstrated that 3 oral doses (50, 100, and 200 mg/d) of quinagolide are equally effective for preventing moderate-to-severe early OHSS [66]. Administration of quinagolide was done at least 2 h before hCG triggering, in high-risk patients, and was continued for 17–21 days, until the day before pregnancy testing. The 200 mg/d group, and all quinagolide groups combined, had a significant reduction in the frequency of moderate-to-severe early OHSS. None of the quinagolide doses tested had a detrimental effect on clinical pregnancy.

Although administration of cabergoline was observed to reduce the incidence of early OHSS in high-risk patients, it failed to prevent late OHSS even after a 3-week course of cabergoline [67]. In addition, further research is needed to validate the most effective dosage of various types of dopamine agonists and the proper timing and duration of administration [62, 68, 69]. A comparison of the risk reduction between cabergoline and other preventive measures should also be performed [70].

Cryopreservation of All Embryos

Avoiding fresh ET eliminates exposure to endogenous hCG from pregnancy and should thereby eliminate the possibility of late OHSS. Despite its theoretical value and widespread use in clinical practice, the efficacy of cryopreservation of all embryos as a method of preventing OHSS has been poorly studied. A Cochrane review [71] found only 2 studies of which one [72] compared embryo cryopreservation with IV albumin (20 g) and subsequent fresh ET. The other trial found a lower incidence of OHSS in the group where all embryos were cryopreserved, compared with the fresh ET group (0/58 vs. 4/67, $P > 0.05$) [73].

The efficacy of continuation of a GnRH agonist after hCG triggering in women undergoing elective cryopreservation of all embryos has been investigated. In a nonrandomized controlled trial, Endo et al. [74] administered a GnRH agonist for 7 days after hCG triggering in women undergoing elective cryopreservation of all embryos due to a high risk of OHSS (serum estradiol 43,000 pg/ml and >20 follicles >12 mm in diameter). The incidence of severe OHSS was significantly lower in the group in which GnRH agonist was continued, compared with the group in which GnRH agonist was discontinued (0/70 vs. 7/68).

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Prevention and Management of OHSS

11

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Introduction

Ovarian hyperstimulation syndrome (OHSS) is one of the most frequent and life-threatening complications of controlled ovarian hyperstimulation (COH). OHSS is characterized by increased capillary permeability and resulting fluid shifts into the abdominal cavity that is in large part mediated by the overexpression of vascular endothelial factor (VEGF) in the overstimulated ovary. Severe cases can result in electrolyte derangement, haemoconcentration, and renal and hepatic dysfunction, requiring hospitalization and close inpatient monitoring. The incidence of OHSS has been reported to be as high as 20–33% for mild cases, 3–6% for moderate cases, and 0.1–2% for severe cases [1]. Several strategies have been proposed to prevent OHSS. These include individualizing ovarian stimulation protocols, coasting, cycle cancellation, and the use of GnRH agonist and/or low-dose hCG triggers.

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Identifying Patients at Risk

Women at risk of developing OHSS can be targeted prior to ovarian stimulation to employ strategies to decrease the likelihood of developing the syndrome. Several factors, including history of prior OHSS, patient demographics (i.e. young age and low body weight), and polycystic ovary syndrome (PCOS), can predict the risk of OHSS. One meta-analysis demonstrated a 6.8-fold increased risk of OHSS in PCOS patients compared to women with other infertility diagnoses [2].

Serum markers may be used to predict the risk of OHSS. These include day 3 FSH, inhibin B, and anti-Mullerian hormone (AMH). Lee et al. [3] reported AMH and serum E2 as the most reliable predictors of OHSS in a study in patients undergoing agonist IVF protocols. They showed basal serum AMH level >3.36 ng/mL predicted OHSS with a sensitivity of 90.5% and specificity of 81.3%.

Secondary risk factors related to ovarian response include ultrasound and serum measures during ovarian stimulation. These include high number of follicles (greater than 20 follicles measuring over 10 mm), high or rapidly rising serum E2 (>3000 pg/mL), and number of oocytes retrieved [4]. Ho et al. [5] reported that high mid-follicular levels of E2 (>800 pg/mL on day 6 of gonadotropin stimulation) were associated with an increased risk of OHSS. Identification of risk factors is essential for primary prevention of OHSS. Patients at risk are the

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candidates for preventive measures which can decrease the risk of OHSS, as outlined below.

Individualizing Gonadotropin Dosing

With the introduction of gonadotropin-releasing hormone agonists (GnRHa) to IVF protocols in the late 1980s, higher doses of gonadotropins were used to yield more mature oocytes and lower cancellation rates [6]. However, higher doses of gonadotropins were associated with an increased risk of OHSS. In response to this, protocols were individualized with the goal to use minimal gonadotropin dosing to achieve the best oocyte quantity and quality while avoiding risks of OHSS. The CONSORT trial utilized an algorithm designed to individualize dosing of recombinant human FSH in increments of 37.5 IU according to basal FSH, body mass index, age, and antral follicle count [7, 8]. The results of this study demonstrated similar clinical pregnancy rates compared to that of a standard approach.

The use of GnRH antagonists to prevent the endogenous luteinizing surge has also been shown to reduce the risk of OHSS [9]. This allows for shorter duration of stimulation without compromising oocyte yield or overall pregnancy rates [10, 11]. GnRH antagonists are usually introduced into the IVF cycle once the leading follicle is 12–14 mm while E2 levels exceed 300 pg/mL. Using a GnRH antagonist protocol also provides the option to use a GnRH agonist trigger to induce final oocyte maturation, thus avoiding the use of hCG. Two meta-analysis reported a lower incidence of OHSS with the use of a GnRH antagonist, including women with PCOS [12, 13]. However, a Cochrane review in 2007 corroborating the above findings revealed a lower pregnancy rate when compared to GnRH agonist treatment [14]. Thus, GnRH antagonist protocols are effective in reducing the risk of OHSS; however, it may compromise overall pregnancy rates.

Coasting/Cycle Cancellation

Coasting can be applied to cycles in which there are multiple immature follicles (>20) or high/rapidly rising serum E2 levels to reduce the risk of OHSS. This strategy refers to withholding gonadotropin therapy until serum E2 levels fall within acceptable range to proceed. Coasting is usually applied when the dominant follicle is >16 mm and the serum E2 > 3500. When E2 levels fall below 3500, controlled ovarian hyperstimulation can be started safely again. During this time, GnRH antagonists are continued to prevent premature ovulation. This method allows for larger follicles which are less FSH-dependent to continue development, while the smaller more FSH-dependent follicles undergo atresia. By decreasing the follicle count and therefore the number of granulosa cells in the smaller follicles, the risk of OHSS is lowered by reducing the factors that contribute to the development of OHSS.

The use of coasting can be safely applied to controlled hyperstimulation cycles without compromising fertilization rates, implantation rates (IR), or pregnancy rates (PR) [15]. However, Ulug et al. [16] showed lower IR and PR in patients who were coasted for 4 or more days compared with patients who were coasted for 1–3 days. Therefore, a longer duration of coasting appears to negatively impact the outcome of IVF. One disadvantage of coasting is the possibility of cycle cancellation if E2 levels do not drop after 4 days or if E2 levels drop more than 30%, due to the association with poor oocyte quality.

Ovulation Triggers

One of the major contributors to the development of OHSS is the use of hCG as a trigger to induce final in vivo oocyte maturation prior to oocyte harvesting. hCG acts by activating the LH receptor, therefore mimicking the endogenous LH surge. An important difference is the half-life

of <60 min versus >24 h for LH and hCG, respectively. The prolonged half-life of hCG results in sustained VEGF activity, thus acting as an important stimulus for OHSS. The standard dose used in the most practices is 10,000 IU; however, in patients at risk of OHSS, a reduced dose, such as 3000–5000 IU, can be used, particularly in patients with a serum E2 > 3000 pg/mL [17]. Several studies, including a randomized study by Kolibianakis et al. [18], demonstrated similar pregnancy rates using 2500 and 5000 IU compared to that of standard 10,000 IU dosing in a population of women with PCOS. Schmidt et al. [19] also found a similar proportion of mature eggs, fertilization rates, and pregnancy rates in the group of high responders using a reduced hCG dose (3300 IU vs. 5000 IU).

Another strategy that aimed at reducing the risk of OHSS is the use of gonadotropin-releasing hormone agonist to trigger the final oocyte maturation. GnRH agonists can only be employed when using a GnRH antagonist protocol due to resulting pituitary suppression seen in agonist protocols. Its mechanism is related to the more physiologic surge of gonadotropins which mimic the endogenous levels of hormones. It also serves as a luteolytic agent due to the decreased circulating half-life of the induced LH/FSH surge. This in turn prevents the secretion of vasoactive substances, such as VEGF, from the corpora lutea and reduces the risk of OHSS development.

Due to the luteolytic properties of the GnRH trigger, it is prudent to use intensified luteal support in patients anticipating a fresh embryo transfer. A Cochrane review demonstrated a lower ongoing pregnancy rate and live birth rate as well as a high rate of early miscarriage in patients using a GnRH agonist trigger [10]. However, caution must be noted in interpreting these conclusions as each of the studies used very different luteal support methods. Thus, differences in protocols may have contributed to these findings. Nevertheless, aggressive luteal support should be used when using a GnRHa trigger.

All of the aforementioned studies have demonstrated nearly complete elimination of OHSS using the GnRH agonists. A small risk remains, however, particularly in patients who are pregnant following a fresh transfer.

Calcium Infusion

Plasma renin level and renin activity have been shown to be increased in OHSS [20]. One study showed that angiotensin II levels were 100 times higher in OHSS ascites fluid compared with non-OHSS ascites fluid [21]. Higher circulating levels of angiotensin II were found to directly increase the VEGF secretion, which has emerged as one of the factors most likely involved in the pathophysiology of OHSS. Calcium infusion is thought to inhibit the renin–angiotensin system, thereby decreasing the VEGF levels. El-Khayat and Elsadek [22] recently published the results from a randomized control trial demonstrating lower rates of OHSS in high-risk women treated with calcium infusion (7% vs. 23%). Women in this study received calcium gluconate in 100 mL 0.9% saline solution on the day of oocyte retrieval and for three consecutive days after the procedure. The treatment did not appear to affect pregnancy rates.

In Vitro Maturation

In vitro maturation (IVM) is a technique involving retrieval of immature, germinal vesicle-stage oocytes in an unstimulated or minimally stimulated cycle with subsequent conversion to the metaphase II stage in vitro. The benefit of this treatment is the avoidance of a rise in serum E2 which therefore eliminates the risk of OHSS [23]. Women with an increased risk of developing OHSS, particularly those with PCOS, may benefit from this treatment option. In a retrospective study comparing 61 IVM cycles to 53 IVF-GnRH antagonist cycles, fertilization rates and embryo quality were higher among the GnRH antagonist group; however, pregnancy and delivery rates were comparable [24]. In another study comparing 107 IVM patients to 107 IVF cycles, the risk of OHSS was eliminated

with the use of IVM (compared to 11.2% in the control group) [25]. Similarly, this study did not showed the differences in PR or LBR. Ortega et al. [26] conducted a retrospective series to assess the efficiency of embryo cryopreservation after IVM in patients with PCOS. LBR per ET was 16.2%, and the cumulative LBR per patient was 21.8%.

Concerns regarding the outcomes of pregnancy achieved using IVM were addressed in a study published by Cha and colleagues [27]. One hundred and thirty-nine pregnancies using IVM from the patients with a history of PCOS were followed in a prospective observational study. The gestational age and birthweight at delivery, as well as obstetric complications, were similar to those of women treatment with conventional IVF protocol. A larger study conducted in 2012 demonstrated similar results [28]. To date, there have been several hundred births without any apparent increase in congenital anomalies.

IVM has also been studied in a non-PCOS population. In one study with 56 patients undergoing controlled ovarian stimulation, hCG was administered when the leading follicle was 12–14 mm. Approximately 76% of oocytes were mature following IVM, and patients undergoing a fresh embryo transfer carried a 46% clinical pregnancy rate [29]. IVM is an effective method to prevent OHSS, and its use has become more globally recognized, although still not adopted worldwide.

Embryo Cryopreservation

Pregnancy can often exacerbate OHSS or leads to late-onset OHSS due to the higher levels of endogenous hCG. Therefore, embryo cryopreservation allows for the resolution of supraphysiologic levels of circulating hormones, thus eliminating the risk of pregnancy associated with OHSS. Advancements in blastocyst culture have allowed time to monitor patients with developing symptoms to decide whether a "freeze-all" approach is recommended.

Fitzmaurice et al. [30] compared pregnancy outcomes with fresh and embryo transfer in patients admitted with OHSS and found no statistically significant difference between the two groups (56.5% vs. 50%, respectively). They concluded that embryo cryopreservation does not compromise the outcome of women at risk of OHSS.

A Cochrane meta-analysis included only one randomized controlled trial comparing embryo cryopreservation with IV albumin infusion and subsequent fresh embryo transfer for at-risk women, defined as E2 > 10,000 pmol/L (2724 pg/ml) and >15 oocytes or E2 > 13,000 pmol/L (3541 pg/ml) [31, 32]. This study reported no reduction in the incidence of moderate and/or severe OHSS in the group undergoing embryo cryopreservation. Further research is needed to determine whether using elective cryopreservation of embryos can reduce the risk of OHSS.

Albumin

The pathophysiology of OHSS involves third-space fluid loss due to decreased intravascular oncotic pressure. Administration of volume expanders including intravenous albumin has been postulated to maintain intravascular volume, thus preventing the downstream cascade of OHSS. In a meta-analysis including eight randomized trials comparing intravenous albumin to placebo, they showed only a marginal statistically significant decrease in the incidence of severe OHSS (OR 0.67, 95% CI 0.45–0.99) [33–38]. The same study showed there was a statistically significant decrease in severe OHSS incidence with the administration of hydroxyethyl starch (OR 0.12, 95% CI 0.04–0.40). However, safety of this substance has not been well established. Overall, no difference was seen in pregnancy rates between the groups.

Dopamine Agonists

Dopamine agonists, such as cabergoline and quinagolide, have been shown to be an effective prophylactic agent for patients receiving hCG as

a trigger for final oocyte maturation. It acts by inhibiting the VEGF receptor phosphorylation, thus decreasing the capillary permeability. In a Cochrane analysis including data from 230 women, oral cabergoline administered as 0.5 mg daily starting on the day of hCG administration or the day of oocyte retrieval and decreased the risk of OHSS development by 60% [39]. Similar pregnancy rates were seen between the groups, with no increased risk of adverse events.

GnRH Antagonist

GnRH antagonist treatment in patients down-regulated with a GnRH agonist has been proposed to reduce the risk of OHSS by decreasing the circulating E2 levels. A retrospective study by Gustofson et al. [40] found a 36% drop in E2 levels 24 h after GnRH antagonist administration from 4219.8 to 2613.7 pg/ml. This protocol involved discontinuation of the GnRH agonist and addition of HMG (75 IU) at the time of antagonist initiation. This protocol did not appear to affect oocyte recovery, oocyte maturity, or pregnancy. The use of GnRH antagonists may also be used following oocyte retrieval in patients with early-onset OHSS. This results in a decrease in E2, progesterone, as well as markers of OHSS such as haematocrit, white blood cell count, and ovarian volume.

Metformin

Metformin is an insulin-sensitizing agent that suppresses the insulin levels and decreases the excessive ovarian production of androgens in women with PCOS. By decreasing the ovarian theca cell production, it is thought to improve ovulation and pregnancy rates in this patient population [41–43]. A recent summary of the previous Cochrane review pooled data from 9 randomized controlled studies and found that metformin increased clinical pregnancy rates (OR 1.52, 95% CI 1.07–2.15), while significantly decreasing the risk of OHSS (OR 0.29, 95% CI 0.18–0.49) [44]. However, there was no clear benefit in live birth rates, warranting further investigation.

Conclusion

In conclusion, several strategies have proven successful in reducing the risk of OHSS. Beginning with identification of at-risk patients, interventions such as the use of antagonist protocols, agonist triggers, and dopamine agonists can be used to prevent or reduce the risk of severe OHSS. It is important to note that none of these strategies can completely eliminate the risk of OHSS, and thus, it is the responsibility of the clinician to take a multifaceted approach to prevent this iatrogenic complication of gonadotropin ovarian stimulation.

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Part III
Minimal or Mild Stimulation for IVF

Minimal or Mild Ovarian Stimulation for IVF: Overview

12

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Introduction

The International Society of Mild Approach Assisted Reproduction (ISMAAR) defined Mild Stimulation *in-vitro* fertilization (MS-IVF) as 'a method when follicle stimulating hormone (FSH) or human menopausal gonadotropin (hMG) is administered at a lower dose and or for a shorter duration in a Gonadotropin realizing hormone (GnRH)-antagonist co-treated cycle, or when oral compounds, anti-estrogens or aromatase inhibitors (AIs) are used either alone or in combination with gonadotropins (Gn) with an aim to collect a fewer number of oocytes' [1]. Typically, the aim of a MS-IVF is to retrieve between 2 to 7 oocytes through a treatment cycle with minimum deviation from normal human physiology. Sequential clomiphene citrate (CC) and low-dose Gn in an antagonist cycle was introduced by the name of 'minimal stimulation' IVF [2]; many authors continue using this term to denote IVF cycles with CC or AIs with or without Gn or GnRH antagonist co-treatment [3, 4]. MS-IVF has also been variously termed as 'low-intensity' IVF, 'low intervention' IVF or 'mini' IVF. It is to be noted that MS-IVF is not synonymous with 'natural IVF' which is defined as: '..IVF is carried out with oocytes collected from a woman's ovary or ovaries in a spontaneous menstrual cycle without administration of any medication at any time during the cycle' or 'administration of GnRH antagonist to block the spontaneous luteinizing hormone (LH) surge with or without FSH or hMG as add-back therapy' [1].

The concept of 'mild' ovarian stimulation is not new. Following the birth of the first IVF baby from a natural cycle, low dose of Gn with or without oral anti-estrogens were used in the early days of IVF, in order to achieve multi-follicular growth [5]. Subsequently, controlled ovarian hyperstimulation (COH) with higher dose of Gn prevailed in the IVF world, as pituitary down-regulation (desensitisation) by GnRH agonist was introduced to prevent premature ovulation in late 1980s. The so-called long downregulation protocol soon became the mainstay of IVF treatment; today this is usually regarded as 'conventional' IVF (C-IVF). The aim of C-IVF is to produce as many oocytes as possible, to allow selection of one or more embryos for transfer from a decent cohort of embryos, and also to enable the remaining suitable embryos to be cryo-preserved [6]. The downregulation protocol thus requires administration of Gn, often for long duration and at a high dose, to develop follicles from completely shut-down ovaries [7]. The side

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effects and risks of intense ovarian stimulation and multiple embryo transfer (ET) in a C-IVF have been well recognized. Ovarian hyperstimulation syndrome (OHSS) and twin or higher-order birth are the two major concerns in relation to C-IVF programmes. In addition, prolonged treatment cycles with intense hormonal manipulation often causes physical discomfort and frequently appears as an emotional burden to the women undergoing treatment [8]. Not least, the cost of the treatment soars with the increasing requirement for medications. As a consequence, toward mid-90s, the need for a safer and more 'patient-friendly' ovarian stimulation protocol was called for [9, 10].

The advent of GnRH antagonist as an agent to suppress a premature LH surge and its increasing clinical use made the milder ovarian stimulation in IVF cycles possible. In contrast to GnRH agonists, the antagonists directly compete for the GnRH receptors in the pituitary and suppress the LH secretion within few hours of administration. Through a process of 'topping-up' the physiological follicular stimulation, only the healthier and more competent follicle(s) tend to develop [11], increasing the probability of obtaining better quality, euploid embryos [12]. Although fewer oocytes are obtained, comparable clinical outcomes are achieved in a treatment cycle which is less intense, less costly, safer, and more patient-friendly (see below) [13]. Compared to a C-IVF treatment, women find MS-IVF a less stressful experience and they are more likely to undergo repeat MS-IVF following a failed cycle [8, 14]. Today many centers around the world are actively involved in the practice of MS-IVF and its research.

MS Protocols

(1) *Low-dose Gn:* A fixed low dose of Gn (usually 150 i.u./day of FSH), titrated with body mass index (BMI), is started in early follicular phase (either on 2nd day or day 5 of a natural menstrual cycle). GnRH antagonist (e.g., Cetrorelix) is commenced when the leading follicle is around 14 mm in diameter or if indicated by serum estradiol level (usually >800 pmol/l). Ovulation is triggered when 3 follicles reaches a diameter of 17 mm or greater. Oocyte retrieval is performed 35–36 h after the ovulation trigger.

(2) *Oral anti-estrogens-selective estrogen receptor modulator (SERMs) or AIs with or without Gn top-up:* The SERM, commonly used is either CC (usually 100 mg/day) or tamoxifen (40 mg/day). Letrozole (2.5 mg/day) is the most commonly administered AI. There are two different regimens for anti-estrogens:

   (a) Anti-estrogens are administered for 5 days—starting from 2nd or 3rd day of a natural menstrual cycle and low dose (usually 150 iu) of FSH (±LH) is added from 5th day on either daily or alternate day, depending on the initial ovarian response. GnRH antagonist is commenced when the follicles are around 14 mm or as indicated by serum estradiol and LH levels.

   (b) CC or tamoxifen is commenced from 2nd or 3rd day of the cycle and continued until the day of ovulation trigger. FSH at a 150 iu daily dose may be added from 3rd–5th day and continued on alternate days or on a daily basis. SERMS, when administered in this way, effectively block the positive feedback action of estradiol on the initiation of LH surge; therefore, no antagonist is usually required to suppress the surge [15].

Advantages of MS-IVF

Since the inception of MS-IVF in clinical practice, there has been an increasing volume of evidence in the literature, describing its advantages over C-IVF. A number of randomised controlled trials (RCTs) and subsequent meta-analyses have also been published, comparing the effectiveness of the mild approach with the conventional ones. A review of the evidence is as follows:

Reduced Complexity, Less Medication, Fewer side Effects, and Better Tolerance

Prolonged suppression of the ovaries over a period of 2 weeks or more by GnRH agonist in conventional long downregulation protocol often gives rise to menopausal symptoms including hot flushes, night sweats, or mood swings. An initial flare reaction of the agonist is also responsible for ovarian cyst formation. These symptoms are absent when GnRH antagonists are administered in combination with low-dose Gn in MS-IVF cycles. A Cochrane review recognized significantly lower requirement for Gn in GnRH antagonist protocol, compared to that of agonist [7]. RCTs found total Gn doses were even lower, when Gn was commenced on cycle day 5 instead of day 2 of an antagonist cycle [16] or when co-treated with CC [17].

In the RCT by Heijnen et al. the depression, anxiety, and discomfort scores were not significantly different between MS-IVF and C-IVF [18]. On the other hand, a later report of the same RCT found significantly more symptoms of depression following a failed C-IVF cycle, compared to MS-IVF [19]. A study (n = 183) that specifically assessed patients' attitude toward the acceptability of two different treatment regimens found more treatment-related stress with conventional downregulated protocol compared to that of MS-IVF [14]. Patients felt the stress of cycle cancellation more acceptable following MS-IVF in this study. Psychological burden, mainly stress from a treatment cycle has been shown to be the leading cause of drop out from IVF programme [20]. The anxiety scores and drop out rates were found to be >50% lower than those of C-IVF, when a mild approach IVF was undertaken [8]. MS-IVF treatment cycles also involve fewer injections and are overall less painful [17]. Not a single drop out was reported in the recent prospect cohort study of 163 women undergoing up to 3 cycles of CC+Gn mild IVF regimen [21]. Intense hormonal manipulation along the course of COH has been postulated to have a significant impact on the physical and mental health of the women, which may get worse in the event of cycle cancellation or treatment failure. The blood estrogen levels being closer to physiological concentrations, MS-IVF appeared to be better tolerated by women undergoing IVF treatment.

Fewer Risks

The Cochrane review cited earlier demonstrated a statistically significant reduction of the incidence of OHSS when GnRH antagonist protocol was used in place of agonist downregulation (OR 0.43, 95% CI 0.33–0.57) [7]. With a lower stimulation dose in MS-IVF, another meta-analysis found the OHSS risk even lesser (OR 0.27, 95% CI 0.11–0.66) [22]. RCTs and systematic reviews found significantly reduced incidence of OHSS in a treatment protocol combining CC with Gn [23–25]. MS-IVF allows administration of lower dose hCG for ovulation trigger which further lessens the risk of OHSS [26]. GnRH agonist trigger for final oocyte maturation has recently been shown to be extremely effective in prevention of OHSS. Not a single OHSS occurred in the largest cohort study (44, 468 IVF cycles) to date, by using a protocol comprising of CC plus Gn till the day of GnRH agonist trigger [4]. MS-IVF with agonist trigger would therefore boost the currently drive toward an 'OHSS-free' clinic. Trigger of ovulation with GnRH agonist is not possible in a downregulated cycle. One of the risks of generating a very high estrogen levels in hyper-stimulated cycles is venous thromboembolism [27]. An increased incidence of deep vein thrombosis or pulmonary embolism has been reported in pregnancies following IVF treatment [28]. Pulmonary embolism could be lethal. Although comparative data are lacking, at least theoretically, there would be a reduced risk of this complication in MS-IVF. Thus, from all respect, MS-IVF has been proved to be a safer treatment option.

Elective single embryo transfer (SET) is recognized as one of the most effective strategies in reducing the chance of multiple births within an IVF programme. One large RCT found significantly lower incidence of multiple pregnancy

with similar cumulative livebirth rates (LBRs) when a mild regimen with SET was compared with C-IVF with double embryo transfer (DET) [18]. By applying strict SET policy in both mild approach and C-IVF cycles, one recent retrospective analysis of a large data base reported better cumulative LBRs (24% vs. 17.5%) with significantly fewer occurrences of OHSS and multiple pregnancy [29]. The aforementioned retrospective study by Kato et al. found very low incidence of ectopic pregnancy (0.36%, 9 out of 2523) and twin pregnancy (0.9%) by transferring single blastocyst following MS-IVF cycles [4].

High Proportion of Good Quality Oocyte/Embryo

Significantly fewer oocytes are retrieved in MS-IVF cycles [17, 22]. Analysis revealed that an optimum recovery of 13–15 oocytes maximized the LBR in C-IVF cycles [30, 31]. However, the same is not applicable for MS-IVF. An earlier RCT found 6 out of 10 women conceived when 4 or less oocytes were retrieved by MS-IVF, compared to none, when the similar numbers of oocytes were retrieved with long downregulation protocol [32]. A more recent RCT on good prognosis patients showed 46.7% top-grade embryos from mild IVF, compared to 42.1% from long protocol; however, the difference was not statistically significant [33]. It has been shown that 6 retrieved oocytes optimize LBR in mild approach IVF [11]. Intense downregulation regimens, by generating high number of oocytes also produce higher proportion of morphologically as well as chromosomally abnormal embryos [34]. One of the landmark RCTs (n = 111) found equal number of euploid embryos, whether mild or conventional IVF was undertaken, despite twice the number of embryos created in the latter [12]. Another more recent RCT (n = 265) found the doses of recombinant FSH strongly correlated with the number of recovered oocytes, but not with the number of blastocysts created [35]. Indeed, the blastocyst–oocyte ratio and fertilization rate (FR) declined with the increasing exogenous FSH dose in this study. Both the RCTs recruited women below 38 years. The emerging concept is that, due to low grade of stimulation, only healthier follicles with more competent eggs are encouraged to develop in MS-IVF cycles [11].

Improved Endometrial Receptivity

There is a large volume of evidence showing that very high, supra-physiological level of estrogen levels in the blood may adversely affect implantation. A basic science study demonstrated progressively less adhesiveness of mouse embryos with human endometrium from fertile oocyte donors, as they were exposed to increasing concentrations estrogen [36]. Very high serum estrogen levels at the time of ovulation trigger have been shown to be detrimental to the endometrial receptivity, regardless of the embryo quality [37–39]. Endometrial gene expression is altered in COH cycles with high circulating estrogen as well as progesterone levels, when compared with those in natural cycles or treatment cycles with low progesterone levels [40–42]. Gene expressions during receptive phase of the endometrium in antagonist co-treated mild stimulation cycles appeared to be closer to that found in physiological menstrual cycle than that of a long protocol [43, 44]. In practice, the implantation rates have been shown to be significantly lowered by high estradiol levels in normal or high responders [39]; milder stimulation in IVF cycles appeared to improve implantation [11, 45].

Comparable Treatment Success

Pregnancy outcomes including clinical pregnancy rates (CPRs), ongoing pregnancy rates (OPRs), and LBRs of MS-IVF have been compared with those of C-IVF in women with normal, low, and high ovarian reserve. The majority of RCTs that compared OPRs between low-dose Gn in an antagonist cycle and the long agonist protocol in good prognosis groups found no

significant difference [12, 32]. The RCT by Hohmann et al. reported pregnancy rates of 20% per cycle and 38% per ET with MS-IVF (n = 49) compared to 22% per started cycle and 39% per ET with long protocol(n = 45) [32]. The other RCT by Baarts et al. demonstrated similar OPRs of 19% per started cycle and 34% per ET from MS-IVF (n = 55) as opposed to 17% per started cycle and 23% per ET with C-IVF [12]. The quality of both the trials was affected by their small sample sizes. In contrast, larger (n = 404) non-inferiority RCT, mentioned earlier, found significantly better LBRs in favour of long downregulation protocol (conventional: 24% vs. mild: 15.8% per ET, OR 0.59, CI 0.41–0.85); however, the cumulative LBRs after 1 year, which was the primary outcome in this study, remained the same between the two strategies [18]. It is interesting to note that this RCT by Heijnen et al. actually employed a SET strategy following MS-IVF, whereas DET in C-IVF. SET is known to reduce the chance of conceiving in any type of assisted conception programme [46]. This may explain why LBR per ET was lower in MS-IVF than that of C-IVF, even though cumulative LBRs were similar in that trial (MS-IVF: 43.4% vs. standard IVF: 44.7%). More recent retrospective analysis of large data base that applied strict SET policy in both mild (natural modified IVF) and conventional long downregulation approach, reported similar, if not better LBRs with the former [29]. Meta-analysis of these 3 RCTs found 22% OPRs/ET with MS-IVF and 26% OPR/ET with downregulation protocol; the difference was statistically significant [11]. Another more recent meta-analysis, that included the above 3 RCTs as well as 2 other RCTs revealed similar findings, with OPR per started cycles were 20 and 26% for MS-IVF and C-IVF, respectively, (OR 0.72, CI 0.55–0.93) [22]. The results of the aforementioned RCT by Heijnen et al. had large influence on the pooled data in these meta-analyses [11, 22]. A subsequent larger RCT (n = 412) on normal as well as ovulatory high-responders reported comparable LBRs between low-dose antagonist (150 daily FSH) and long agonist protocol—overall LBRs: 24.9% per started cycle and 28.6% per ET, versus 26.6% per started cycle and 28.6% per ET. LBRs from frozen-thawed ET cycles (21.4% per ET vs. 21.0%) as well as cumulative LBRs from fresh and subsequent frozen ETs together were also similar (42.7% vs. 41.7%) [33]. A report analyzing large volume of data (650,000 cycles) from the registry of the Society for Assisted Reproduction Technology (SART) in the United States identified an inverse relationship between the Gn doses and LBRs, independent of age, prognosis and retrieved oocyte number [47]. A combination of factors including embryo aneuploidy and reduced endometrial receptivity due to supra-physiological level of estrogen or premature progesterone rise has been speculated as possible explanations for this finding.

Treatment outcome are generally encouraging when oral agents, SERMS (usually CC) or AIs (letrozole) are used in MS-IVF protocols. A RCT (n = 100) with sequential CC and Gn protocol in normal responders found OPRs per started cycles similar to that with long downregulation protocol (32% vs. 26%) [48]. Two other RCTs using CC +Gn reported comparable pregnancy rates per ET, when judged against long downregulation protocol: 41.7% versus 40.0% in the RCT that used hMG in combination with CC (n = 120) [17] and 42.9% versus 36.6% in the other larger trial (n = 294) using CC + FSH regimen [24]. In contrast, another earlier RCT found better CPRs with the long protocol [49]. A Cochrane review of RCTs found no difference in LBRs, OPRs, and CPRs when cycles with CC + Gn were compared with long downregulation or antagonist protocol [23]. Later, another systematic review and meta-analysis of 7 trials (702 participants) comparing between CC+ antagonist and antagonist protocol in unselected population also found similar LBRs (CC+ antagonist: 30.2% vs. Antagonist: 26.0%) and miscarriage rates with significantly less risk of OHSS (0.5% vs. 4.1%) [25]. A RCT (n = 167) reported comparable CPR when CC was replaced by letrozole in the sequential regimen, with significantly higher implantation rate in women who received letrozole [50]. Evidence from retrospective studies comparing sequential CC+Gn and C-IVF in

young women with normal ovarian reserve were conflicting; some clinical outcomes were in favour of 'Mini-IVF' [51], while others supported long downregulation protocol [52]. The MS-IVF protocol comprising of continuous administration of CC till the day of ovulation trigger +/-Gn has emerged as an effective low-cost IVF protocol without the need for GnRH-antagonist for suppression of premature LH surge [53]. Data from the largest retrospective cohort study to date, comprising of 20,244 cycles in 7244 Japanese women showed age-specific LBRs per fresh ET ranging from 30.0% in $\leq$29 years age-group to around 10% in women aged 40–45 years with the stimulation protocol as above, along with GnRH agonist as an ovulation trigger [4]. The authors reported age-matched LBRs of 37.7% and 44.5% following vitrified–thawed cleavage-stage embryo and blastocyst transfers, respectively, in the best prognosis patients. The figures were higher than those registered in the SART database in the United States [4]. Another large uncontrolled cohort study using a similar protocol found 20% CPRs per fresh SET and 41% by vitrified–thawed SET [54]. Both the studies demonstrated a better LBR per ET in vitrified–thawed cycles relative to fresh ET cycles. A negative impact of CC on the endometrial receptivity has been implicated for this discrepancy. More recently, a prospective cohort study on CC+Gn regimen among good responders reported an impressive cumulative LBRs of 70% when followed up to 2.4 months (3 fresh or frozen ET cycles) [21]. The effectiveness of sequential anti-estrogens and Gn in the perspective of low ovarian reserve has been described under the section of poor responders.

In brief, the current evidence on treatment outcomes of MS-IVF appear to be comparable to those of C-IVF in good prognosis populations. Although OPRs per cycles with low Gn regimen were generally reported to be inferior to those with long downregulation protocol among good prognosis women, cumulative LBRs have consistently been demonstrated to be at per with C-IVF in RCTs and large prospective trials [18, 21, 33]. Further data from well-designed RCTs, using different mild stimulation protocols in different age-groups and ovarian reserves would confirm if MS-IVF can be regarded as standard practice of IVF/ICSI treatment in all clinical scenarios.

Better Maternal and Perinatal Outcome

LBRs have been considered as the benchmark of success in assisted conception. However, a singleton, appropriately grown healthy baby at term is now proposed to be a better marker of a success in an IVF programme [55]. A retrospective study showed higher mean birthweight of the babies born out of MS-IVF [56]. Recently, an analysis of the a massive data set of 63,686 singleton birth found an association between high number of recovered oocytes and higher incidence of perinatal complications including preterm birth and low birth weight babies [57]. Very high estrogen levels at the end of treatment cycles and early pregnancy have also been linked with intra-uterine growth restrictions [58] and cardio-vascular dysfunctions in the neonates [59].

Reduced Treatment Cost

There is a paucity of well-conducted studies on the health–economy of MS-IVF in comparison to C-IVF. Admittedly, cost-effectiveness assessment varies in different clinical settings: SET versus DET (and resultant multiple pregnancy), fresh versus cumulative fresh plus frozen ETs, use of Gn versus oral agents or populations of normal versus low responders, and so on. Earlier studies on normal responders with varied study designs and outcome end-points found MS-IVF not a cost-effective option, even though consumption of medication was less [52, 60]. In the RCT by Heijnen et al., however, the overall cost of MS-IVF cycles up to 1 year with SET was reported to be lower than that of conventional IVF with DET [18]. The main reasons of this difference were lower rates of multiple pregnancy in the former group. Subsequent further cost-effectiveness analysis of the same RCT

revealed higher incidence of OHSS, multiple pregnancy and preterm birth as well as neonatal care resulted in significantly increased total expenditure in the long downregulation group [61]. More recent RCTs that compared cost-effectiveness of CC-Gn-antagonist protocol with GnRH agonist downregulation protocols in poor responders identified clear economic advantage of MS protocols [25, 62, 63]. Indeed, extended course of CC till the trigger day has emerged as a potential option for 'low-budget' IVF, as this regimen suppress LH surge without the use of the expensive GnRH antagonist. Further, well-designed health–economic evaluation including the treatment cost and subsequent expenditure in pregnancy and delivery may confirm the overall cost-benefit of MS-IVF using both oral and injectable agents.

Limitations of MS-IVF

Potential Cycle Cancellation

A treatment cycle is usually canceled as a consequence of inadequate response or premature ovulation. A meta-analysis of 3 RCTs conducted on women with normal ovarian reserve found an increased incidence of cycle cancellation before oocytes retrieval in MS-IVF, as compared to conventional IVF (16% vs. 9%; OR 2.55, CI 1.62–4.02) [22]. In the large series with extended CC+Gn protocol, oocytes were obtained from around 80% of cycles in women below 44 years of age, despite only 2.1% cycles abandoned due to premature ovulation [4]. Similar inference was dawn in a RCT on women with poor ovarian reserve: CC+Gn in antagonist protocol was found to be associated with less chance of progression to ET in comparison to high-dose antagonist in a RCT (39% vs. 54%), although the incidence of cycle cancellation due to premature ovulation remained the same between the protocols (14% on each side) [62]. Another large RCT on poor responders reported higher cycle cancellation with CC+Gn cycles, as compared to downregulated cycles (13% vs. 2.7%) [64]. Ovarian aging and high BMI have been reported to be associated with cycle cancellation due to under-response [65].

In contrast, several other studies failed to find an increased risk of cycle cancellation by using MS-IVF. A prospective study with CC+Gn protocol in normal responders reported less chance of cycle cancellation (4.7% vs. 34.0%) [66]. Only 1% of the cycles were abandoned out of 205 women recruited in the MS-IVF arm in a RCT involving normal or high responders [33]. Two other RCTs mentioned earlier, that used CC+Gn as mild stimulation protocol in good prognosis patients, found similar cycle cancellation when compared with long protocol: Karimzadeh et al. reported 4% cancellation rate, while it was 16.9% in the trial by Weigert et al. [24, 48]. No cycle was canceled due to premature ovulation in the RCT comprising of 60 normo-responders in the MS-IVF group with CC+Gn protocol [17]. The Cochrane review on COH with CC+Gn regimen found an overall increased incidence of cycle cancellation when compared with downregulation protocol, but there was no difference in the sub-group where mid-cycle GnRH antagonist was used [23]. Low cancellation rates have also been reported in retrospective studies on poor responders applying CC+Gn protocol (11.7%) [67], or latrozole+Gn protocol (4.2%) [63]. Cancellation rates due to premature ovulation were between 2.1–2.8% by administration of CC up till ovulation trigger [4]. From the current evidence, it appears that the risk of cycle cancellation in MS-IVF is low and comparable to that of C-IVF, at least among good prognosis patients and when one of the oral agents were used in mild approach cycles.

The criteria for cycle cancellation due to under-response need to be set differently in MSIVF. The aim of MS-IVF is to collect higher quality and lower number of eggs. If this is taken into account, cycle cancellations would be rare and successful cycle outcomes are likely in the presence of less than 3 mature follicles at the time of trigger.

Fewer Embryos for Cryo-Preservation

The intention of MS-IVF is to generate fewer oocytes and embryos; so there should be less availability of excess embryo(s) for cryo-preservation [68]. A number of trials reported availability of fewer embryos for transfer or fewer cycles with embryo freezing when one of MS-IVF strategies was employed in good prognosis women [48, 69]. However, the higher probability of obtaining good quality embryos from MS-IVF cycles may somewhat offset this disadvantage in achieving successful outcome [32]. A number of studies including large RCTs demonstrated satisfactory cumulative OPRs/LBRs despite fewer oocytes being recovered and fewer embryos being created in MS-IVF [18, 21, 32, 33].

Less Flexible Scheduling for the Clinic and Need for High Quality Laboratory

Extended service in the weekends and a high standard of embryology laboratory are prerequisites for MS-IVF. Judicious monitoring by ultrasound and hormone levels and timely intervention may avoid premature ovulation and resultant cycle cancellation. A 7-day service and increased need for intense monitoring are some of the factors which make MS-IVF less acceptable to many clinicians involved in the practice of assisted reproduction.

Place of Mild Stimulation IVF in Current Practice

Poor Responders in Assisted Conception

MS-IVF protocols have probably been most extensively tested and applied in the treatment of poor responders. After an era of intensive COH with very high doses of Gn for poor responder women, it is now widely believed that increasing the stimulation does not translate in to better outcome [35, 70]. No improvement in the CPRs per stated cycle and per ET were found by doubling daily FSH dose from 150 iu to 300 iu in an antagonist cycle designed for poor responders [3]. There was some suggestion that GnRH antagonist protocol improved blastocyst quality and pregnancy outcome following multiple failed cycles due to poor response with GnRH agonist [71]. A recent large RCT (n = 695) that included women with poor ovarian reserve and or previous poor response found a higher incidence of cycle cancellation (13% vs. 2.7%) and fewer metaphase II oocytes, but similar implantation rates, CPRs per cycle (13.2% vs. 15.3%), per ET (23.2% vs. 19.9%) and OPRs per ET (17.8% vs. 16.8%) with CC +FSH-antagonist group, compared to those of long downregulation regimen [64]. The CC+Gn mild stimulation protocol has also been assessed against 'micro-dose flare' protocol [72] and 'short agonist flare' protocol [62] in RCTs involving women with poor ovarian reserve and reported no clear advantage of one way of management over the other. The latter study, which was a non-inferiority RCT of 304 subjects, inferred that the LBRs of either regimen were low (CC: 3% vs. high-dose 'short' protocol: 2% per started cycle; 9% per ET on either group); nevertheless, CC+Gn+antagonist conferred more cost savings [62]. Evidence is limited in relation to effectiveness of MS-IVF in older women [73]. In the large cohort series by Kato et al., CC+Gn and agonist trigger regimen resulted in 12.5% CPR and 7.4% LBR per cleavage-stage ET, while 31.6% CPR and 17.7% LBR per vitrified–thawed ET in women's age-group between 40 and 43 years—these outcomes were claimed to be comparable with age-matched SART registry outcome data; however, the CPRs and LBRs fell significantly in women $\geq$45-year age [4, 54]. In the sub-group of women between >37 years and 41 years (n = 180), a retrospective study reported non-significantly higher CPR and LBRs per ET with CC+Gn-antagonist protocol, compared to those of long downregulation (12.1% vs. 8.1% and 6.1% vs. 2.7%, respectively) [74]. The clinical outcomes have also been linked with basal serum FSH levels. Sub-group analysis of the RCT by Ragni et al. found that CC

+Gn-antagonist protocol did better in women with previous poor response but not in women with serum FSH levels >12 iu [62]. Low LBRs have also been observed when FSH was >15 iu in a cohort study [54]. AI, letrozole was used as an alternative to CC in poor responders. A retrospective study (n = 141) reported significantly higher CPRs (31.4% vs. 12.7%) and LBRs (21.4% vs. 7%) in MS-IVF comprising of letrozole + Gn-antagonist combination as opposed to high Gn dose ($\geq$300 iu/day) in an antagonist protocol [63]. Other retrospective studies found similar LBRs or CPRs between letrozole and conventional antagonist protocol, despite higher oocyte yield and increased number of available embryos [67, 75]. When compared between CC and letrozole as mild stimulation agents, a recent RCT (n = 391) found significantly thicker endometrium and better implantation rate with letrozole; however, the CPRs were similar [50].

The impact of age and ovarian reserve on the outcome of MS-IVF in poor responders requires further evaluation in large prospective trials. Even though the final outcomes appear to be similar, substantially reduced use of medication, shorter duration of treatment, and treatment cost ultimately lessen the physical and psychological and economic burden associated with aggressive IVF treatment among the poor responders [25, 62, 63].

Fertility Preservation for Cancer Patients

A very special indication of MS-IVF is in the field of fertility preservation through oocyte/embryo freezing for women with estrogen sensitive malignancies. Since the inception of natural cycle IVF, it has drawn attention as a potential 'no stimulation' assisted conception for fertility preservation of young women with breast cancer. Anti-estrogens, tamoxifen, and AIs (e.g., Letrozole) have been widely used in an ovarian stimulation protocol for women known to have estrogen receptor-positive breast cancer to prevent the risk of cancer recurrence from high estrogenic state in this process [76]. Tamoxifen, by its anti-estrogenic property protects the breast tissue from high levels of circulating estrogen, while letrozole, by inhibiting aromatase enzyme in the granulosa cells limits serum estradiol levels and also reduces Gn requirement in antagonist cycles [77, 78]. In a prospective study of 215 patients with breast cancer, the hazard ratio of cancer recurrence 0.56 (95% CI, 0.17–1.9) was not increased and the survival was not compromised following letrozole stimulation [79]. Letrozole has also been successfully used for young women with a history of endometrial cancer [80]. The scope of MS-IVF expanded further with the introduction of in-vitro maturation (IVM) following recovery of oocyte from both dominant and non-dominant follicles in natural or modified natural cycle IVF [81]. More oocytes/embryos for transfer or cryo-preservation can be made available in this way [81]. Initial reports of IVM in a pure natural cycle were satisfactory [82]. One study found a maturation rate of 77.4% and CPR of 29.9% in cycles with low-dose FSH priming and HCG, without increase in the risk of cancer recurrence [83]. The use of mild stimulation with anti-estrogen co-treatment or application of IVM needs to be explored further through prospective research.

Low-cost IVF in Low-Resourced Condition

As the cost of medications is less, MS-IVF is considered in low-resourced countries. Inexpensive oral agents—anti-estrogens are widely used for low-cost IVF treatment. When administration of CC or tamoxifen is extended beyond the usual 5-day course, right up to the day of ovulation trigger, it has been shown to suppress LH surge (and thereby ovulation) [15, 84]. This obviates the need for expensive GnRH antagonists and frequent ultrasound monitoring. The Gn, if needed in addition, is kept at a very low dose: typically, on alternate days for a short period. Reports of this regimen are encouraging [4, 53]; large prospective controlled trials are required to establish its effectiveness. Along with the use of 'simplified culture media' and minimal luteal

phase support, promotion of this low-cost IVF programme is one of the leading global agendas of ISMAAR today. The objective is to make IVF treatment more accessible and affordable in developing countries [85].

Patient-Centerd Assisted Conception

There has been a move from a 'clinician-centerd' to 'patient-centerd' approach in assisted conception service. Many clinics are more comfortable with the downregulation protocol that helps in better cycle scheduling and avoidance of weekend work. MS-IVF is considered 'patient-centerd' as it reduces patient's physical discomfort and distress pertaining to intense ovarian stimulation. It does not compromise patient's safety, while maintaining a comparable treatment outcome. Not the least, it is more favorable to patients' budget. Getting the treatment 'fitted' in to women's natural cycle, it causes less disruption to their working life [61, 86]. Women are more likely to seek MS-IVF after having gone through failed C-IVF with aggressive ovarian stimulation [8]. Flexibility in the protocol in MS-IVF has made the treatment more 'tailor-made' to patients' clinical characteristics, emotional and financial need. Again, ISMAAR has taken an important role in propagating the concept of patient-centerd IVF worldwide [85].

Future Prospect

Progress in the field of MS-IVF has raised the hope that it could replace C-IVF in the future under all clinical circumstances [87, 88]. The major deterrents in achieving this goal are as follows: lack of robust RCT data on the efficacy of MS-IVF in good prognosis patients, the clinicians' attitude of adhering to convenient cycle scheduling, satisfaction in retrieving as many oocytes as possible, and variation in the standard of embryology laboratories and the public funding policies in some part of the world [73].

The future lies in the hands of researchers and proactive clinicians to take MS-IVF further. Well-designed clinical trials are required to evaluate MS-IVF more analytically. Ideally, an adequately powered RCT should compare the cost and treatment outcomes, particularly in terms of cumulative LBRs of combined fresh and vitrified–thawed SET cycles between MS-IVF and C-IVF, with the analysis in different clinical settings. The final goal of any assisted conception treatment would be a 'singleton healthy livebirth at term'. Allegedly high cancellation rates, which somewhat lower the per-cycle CPRs or LBRs, may be minimized by the use of anti-estrogens throughout the proliferative phase until the trigger day [4], careful monitoring of cycles by ultrasound scans along with knowledge of serum estradiol and LH levels and the use of indomethacin in selected cases [89, 90] with rescue oocyte retrieval in the event of premature LH surge. An efficient vitrification programme would be an essential prerequisite to improve cumulative pregnancies [73]. Further advancement in IVM may make more embryos available for cryo-preservation [68]. Equally important is to work toward building up robust protocols taking account of the patient's characteristics and develop clear criteria of choosing between different mild stimulation protocols [73]. Women's age and BMI may have influence on the ovarian response [65]; these factors need to be taken into account while customising the protocol for individual patients. Further promotion of MS-IVF may be possible through education, training, and research [88]. Communication between the investigators and publishing research data on the progress would increase clinicians' acceptance of the mild approach.

Conclusion

Mild stimulation IVF has emerged as a safer, cheaper, and more patient-friendly alternative to conventional IVF. Although fewer oocytes are released, equivalent numbers of high quality euploid embryos can be obtained with this

approach. Available data from several RCTs and large retrospective studies on MS-IVF versus conventional regimens depicted comparable treatment outcomes. The use of oral agents in a MS-IVF protocol has been shown to be advantageous, particularly in poor responders, as it seems at least as effective as C-IVF with considerable cost saving. Further research may prove mild approach to be a cost-effective and acceptable treatment option for all women undergoing IVF treatment.

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Cumulative Pregnancy Rates After Six Cycles of Modified Natural Cycle IVF

13

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Introduction

In modified natural cycle (MNC) IVF, treatment is aimed at the use of the one follicle that naturally develops to dominance. In this treatment modality, medication can be used for the prevention of untimely ovulation (GnRH antagonist or indometacin) and for ovulation triggering (hCG). FSH or HMG can be added as add-back for the fall in gonadothrophins caused by the GnRH antagonist, and luteal support may or may not be administered [1]. Medication is explicitly not administered in order to induce multiple follicle development.

Compared to conventional IVF treatment with ovarian stimulation, MNC is a low-risk treatment modality, since the risk of ovarian hyperstimulation syndrome is negligible. Besides this, it offers a patient-friendly modality, since medication is used in low dose and for a few days only, thus causing few side effects. Furthermore, oocyte retrieval is less painful since usually only one follicle is aspirated. A treatment cycle is short and treatments are easily repeated, with no need for a resting cycle in between treatment cycles.

In our centre, a research project on MNC-IVF was started in 2001. Several cohort studies were done, in which patients were offered to undergo MNC-IVF for a maximum of three [2] or nine [3] consecutive cycles, preceding the start of conventional IVF. Treatments were offered for free. Patients requiring ICSI were not included in these studies. Maximum female patient age at inclusion was 36 years.

The clinical protocol used had some small adjustments over time but basically consisted of ultrasound monitoring from cycle day 3 or 8, repeated daily or every other day, according to the size of the leading follicle. When the leading follicle had a diameter of at least 14 mm (measured in three perpendicular planes), daily injections of a GnRH antagonist (0.25 mg) were started combined with 150 IU recombinant FSH. Blood samples were taken to measure LH and E2 levels. Cycles were cancelled when an LH level >20 IU/L was noticed at a follicle size of <15 mm. In cases where an LH level of 20–30 IU/L was noticed at a follicle >16 mm (after medication was started), the oocyte retrieval was planned, since the GnRH antagonist should be capable of

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blunting the LH surge enough. In cases where an LH level of >30 IU/L was noticed, the oocyte retrieval was cancelled. Ovulation triggering was done with 10,000 IU hCG when a follicle with a diameter of at least 18 mm was observed. When at the time of the planned oocyte retrieval unexpected ovulation had occurred, tubes were patent and semen of sufficient quality was available, an intra-uterine insemination was performed. Transvaginal ultrasound guided follicle aspiration was performed 34 h after hCG injection. No standard analgesia was given. Fertilization of oocytes was assessed 17–20 h after insemination. Embryo transfer was performed 72–76 h after oocyte retrieval. When additional embryos were available, these were cryopreserved. Luteal support consisted of hCG 1500 IU on day 5, 8 and 11 after oocyte retrieval.

After this research project, MNC was implemented as a standard treatment. MNC was offered to patients for a maximum of 6 cycles to be performed preceding IVF with ovarian stimulation. In the Netherlands, conventional IVF treatments with controlled ovarian hyperstimulation (COH) are refunded for a maximum of three cycles. A contract was made with insurance companies, in which the 6 cycles of MNC substitute the first COH-IVF cycle. A full IVF treatment 'package' thus consisted of up to 6 cycles of MNC, followed by two COH-IVF cycles.

The yearly number of cycles and patients from 2004 onwards are displayed in Fig. 13.1. In the decade that followed, some adjustments have been made to the protocol. Patients requiring ICSI were offered MNC from 2004 onwards. Maximum female age at the start of treatment was changed from 36 to 34 years (December 2007) and embryo transfer day was changed from day 3 to day 2 (January 2009). In cases where more than one embryo was available, double embryo transfer was no longer done, but in all these cases single embryo transfer was performed. Indometacin was added to the protocol in a small number of patients participating in a study [4]. Over time, culture medium and embryo transfer catheters were changed. Cumulative pregnancy rates per patient according to year of start of treatment are shown in Fig. 13.2.

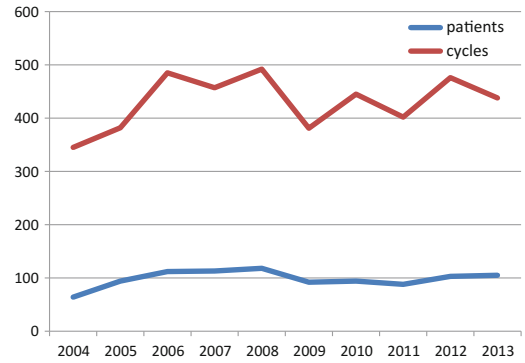

Fig. 13.1 Number of patients undergoing MNC-IVF and number of cycles performed

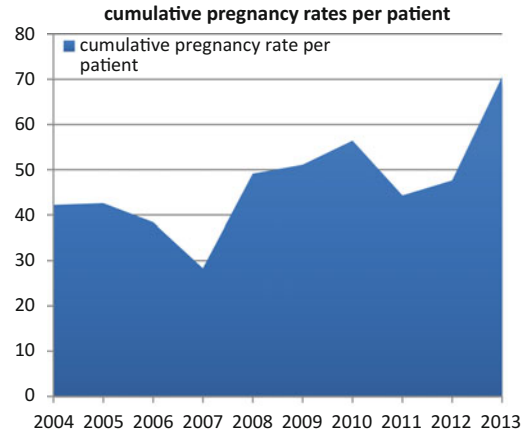

Fig. 13.2 Cumulative pregnancy rates per patient

In this chapter, results of our original studies and cumulative pregnancy rates after 6 cycles of MNC in a larger series of patients are discussed.

What Would Be the Optimal Number of MNC Cycles?

In comparing the results of modified natural cycle IVF to other treatment modalities, it is important to consider that although the pregnancy rate per cycle in general is rather low, duration of treatment is short and treatment can be easily repeated in consecutive cycles. Obviously, compared to standard IVF with ovarian stimulation, a higher number of cycles is necessary to obtain similar pregnancy rates. In our experience, patients are

quite willing to accept the necessity of a higher number of cycles, mainly based on anxiety for hormonal stimulation.

The additional value of an increase in the number of MNC-IVF cycles to be offered to patients will depend mainly on the willingness of patients to undergo these cycles. In order to determine the optimal number of cycles per patient, it is also important to evaluate whether or not the pregnancy rate per cycle decreases in higher cycle numbers.

In order to evaluate the optimal number of MNC-IVF cycles, a study was done in our centre in which a maximum of nine cycles of MNC-IVF was offered to 268 patients. Dropout rates after unsuccessful treatment cycles and pregnancy rates according to cycle number were evaluated [3]. This study is discussed in detail in the following section.

Cumulative Pregnancy Rates After Nine Cycles of MNC-IVF and Analysis of Patient Dropout

For details on methodology, we refer to the original publication [3]. In short, patients aged 18–36 years with an indication for conventional IVF and proven ovulatory cycles were included in this study. Only conventional IVF was performed, and no ICSI was done. All embryo transfers were done on day 3 after oocyte retrieval.

Patients were offered a maximum of nine treatment cycles. Treatments were performed in consecutive menstrual cycles (unless patients requested otherwise) and took place between March 2001 and September 2005. All treatments were offered for free.

End point in this study was pregnancy. Results according to cycle number and actual observed cumulative pregnancy rates per patient were calculated and life table analysis was done.

Patient Characteristics and Results of Treatment Cycles

Patient characteristics are shown in Table 13.1. Of 268 included patients, twelve withdrew from the study before starting treatment, in five of these because of the occurrence of a spontaneous pregnancy. Results according to cycle number are shown in Table 13.2. Overall, 256 patients started 1048 treatment cycles (4.1 per patient). Median duration of treatment was 5.0 months (range 1–24). Ninety-four cycles (9.0%) were cancelled before the planning of oocyte retrieval. Reasons for cancellation were LH rise or ovulation before or during cetrorelix administration (46 cycles), lack of follicular development or problems with monitoring due to difficult visualization of the ovary (28 cycles), or other reasons (28 cycles).

Table 13.1 Patient characteristics

No. of patients	268
Female patient age (years)[a]	33.3 (23–36)
BMI (kg/m^2)[a]	23.0 (16–34)
Duration of subfertility (months)[a]	46.0 (0–121)
Subfertility (%)	
Primary	164 (61.2)
Secondary	104 (38.8)
Indication (%)	
Tubal	82 (30.6)
Unexplained	106 (39.6)
Male factor	41 (15.3)
Endometriosis	22 (8.2)
Cervical factor	8 (3.0)
Failed AID	9 (3.4)

[a]Values are median (range)

Further 98 cycles (10.3% per planned oocyte retrieval) were cancelled at the time of planned oocyte retrieval, in one case because of inaccessibility of the ovary and in 97 cases because unexpected ovulation had occurred. Out of 856 oocyte retrievals, 625 were successful (73.0% per attempt). In most cases, one or two oocytes were obtained (576 and 44 cycles, respectively). In five cycles, three or more oocytes were obtained (three, three, six, nine and twenty oocytes, respectively).

In 453 cycles, fertilization occurred (72.5% per successful oocyte retrieval). Due to aberrant fertilization or defective embryo development, no embryo transfer was done in 71 of these. In 382

Table 13.2 Results according to cycle number of modified natural cycle IVF

Cycle number	1	2	3	4	5	6	7	8	9	Total
Cycles started	256	217	181	127	92	69	51	32	23	1048
OR not planned (%/cycle)	23 (9.0)	21 (9.7)	21 (11.6)	12 (9.4)	5 (5.4)	8 (11.6)	4 (7.8)	–	–	94 (9.0)
Planned OR cancelled (%/planned OR)	23 (9.9)	18 (9.2)	13 (8.1)	17 (14.8)	10 (11.5)	5 (8.2)	6 (12.8)	4 (12.5)	2 (8.7)	98 (10.3)
OR performed (%/cycle)	210 (82.0)	178 (82.0)	147 (81.2)	98 (77.2)	77 (83.7)	56 (81.2)	41 (80.4)	28 (87.5)	21 (91.3)	856 (81.7)
OR successful (%/attempt)	152 (72.4)	134 (75.3)	111 (75.5)	70 (71.4)	56 (72.7)	36 (64.3)	32 (78.0)	18 (64.3)	16 (76.2)	625 (73.0)
Cycles with fertilization (%/successful OR)	116 (76.3)	93 (69.4)	73 (65.8)	52 (74.3)	42 (75.0)	29 (80.6)	21 (65.6)	13 (72.2)	14 (87.5)	453 (72.5)
Embryo transfer (%/cycle)	99 (38.7)	76 (35.0)	60 (33.1)	43 (33.9)	37 (40.2)	25 (36.2)	19 (37.7)	11 (34.4)	12 (52.2)	382 (36.5)
Single ET	94	73	57	43	35	23	16	9	12	362
Double ET	5	3	3	–	2	2	3	2	–	20
Pregnancy rate (%/cycle)	27 (10.5)	20 (9.2)[a]	19 (10.5)	12 (9.4)[ab]	11 (12.0)[a]	5 (7.2)[a]	5 (9.8)[a]	3 (9.4)[a]	2 (8.7)	104 (9.9)
Ongoing pregnancy rate (%/cycle)	25 (9.8)	12 (5.5)	16 (8.8)	11 (8.7)[ab]	10 (10.9)[a]	5 (7.2)[a]	3 (5.9)	–	1 (4.3)	83 (7.9)
Live birth (%/cycle)	24 (9.4)	12 (5.5)	15 (8.3)	11 (8.7)	10 (10.9)	5 (7.2)	3 (5.9)	–	1 (4.3)	81 (7.7)

OR—oocyte retrieval; *ET*—embryo transfer
[a]Pregnancy after cancelled oocyte retrieval and IUI
[b]Spontaneous conception during cycle that was cancelled because of LH surge

cycles, embryo transfer was done (36.5% per started cycle; 61.1% per successful oocyte retrieval). In 20 cycles, two or more embryos were available for transfer and in all of these, double embryo transfer (DET) was done. In all other cycles, one single embryo was transferred (SET).

In 104 cycles, a pregnancy was obtained. One of these occurred spontaneously during a treatment cycle that was cancelled because of an LH surge, six occurred after IUI in cases where oocyte retrieval was cancelled because of unexpected ovulation, and 97 pregnancies occurred after embryo transfer (91 after SET and six after DET). The pregnancy rate was 9.9% (95% CI: 8.1–11.8) per started cycle. Three out of 104 pregnancies were twins (2.9%), of which one occurred after the transfer of one single embryo and two occurred after DET. Ongoing pregnancy rate was 7.9% (95% CI: 6.3–9.6) per started cycle. One pregnancy was interrupted because of severe congenital abnormalities. One pregnancy ended in foetal death at 17 weeks' gestation. Live birth was thus 7.7% (95% CI: 6.1–9.4) per cycle. OHSS did not occur after any of the cycles. Results according to cycle number were not significantly different.

Dropout Rates and Cumulative Pregnancy Rates

Dropout rates and cumulative pregnancy rates are specified in Table 13.3 and Fig. 13.3.

Out of 268 included patients, 102 (38.1%) left the study before completing nine cycles because a pregnancy was obtained. Fifteen (5.6%) left the study because of a treatment-independent pregnancy. Of the remaining 151, 128 (84.8%) dropped out of the study after 0-8 unsuccessful cycles. Of these, 86 (67.2%) proceeded with

Table 13.3 Dropout rates and cumulative pregnancy rates

Cycle number	Patients	Pregnancy	CPR[a]	TIP	CPR including TIP[b]	DO	CPR life table[c]	CPR life table[d]
0	268	–	–	5 (1.9)	5 (1.9)	7 (2.6)	–	1.9
1	256	27 (10.5)	27 (10.5)	3 (1.2)	35 (13.1)	9 (3.5)	10.5	13.4
2	217	20 (9.2)	47 (18.4)	2 (0.09)	57 (21.3)	14 (6.5)	18.8	22.1
3	181	19 (10.5)	66 (25.8)	1 (0.06)	77 (28.7)	34 (18.8)	27.3	30.8
4	127	12 (9.4)	78 (30.5)	2 (1.6)	91 (34.0)	21 (16.5)	34.2	38.4
5	92	11 (12.0)	89 (34.8)	–	102 (38.1)	12 (13.0)	42.1	45.8
6	69	5 (7.2)	94 (36.7)	1 (1.4)	108 (40.3)	12 (17.4)	46.3	50.5
7	51	5 (9.8)	99 (38.7)	1 (2.0)	114 (42.5)	13 (25.5)	51.5	56.3
8	32	3 (9.4)	102 (39.8)	–	117 (43.7)	6 (18.8)	56.1	60.4
9	23	2 (8.7)	104 (40.6)	–	119 (44.4)	na	59.9	63.8

Numbers in parentheses are percentages
CPR—cumulative pregnancy rate; *TIP*—treatment-independent pregnancy; *DO*—dropout; *na*—not applicable
[a]CPR calculated over patients starting treatment (n = 256)
[b]CPR calculated over patients included in the study (n = 268)
[c]Life table analysis, treatment-independent pregnancies censored
[d]Life table analysis, treatment-independent pregnancies not censored

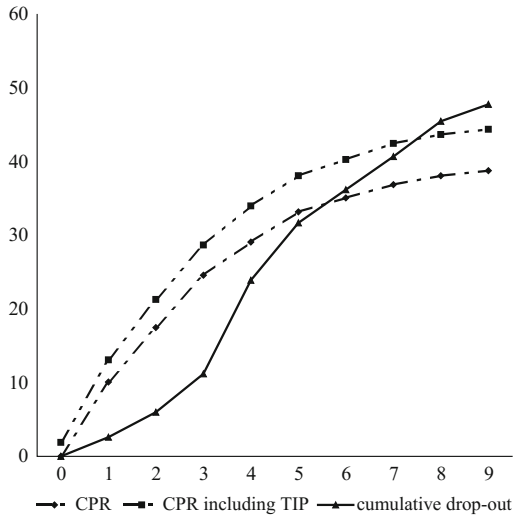

Fig. 13.3 Cumulative pregnancy rates and cumulative dropout rates. *CPR* cumulative pregnancy rate. *TIP*—treatment-independent pregnancy

standard IVF treatment and 42 (32.8%) stopped treatment altogether.

The dropout rate (not including those who stopped treatment because of treatment-independent pregnancy) was low after the first and second cycles (3.5 and 6.5%, respectively) and rose sharply thereafter to 13.0–25.5% in further cycles. Cumulative pregnancy rate per patient starting treatment was 40.6% (95% CI: 34.5–46.8). Including treatment-independent pregnancies, cumulative pregnancy and ongoing pregnancy rate per patient included in the study was 44.4% (95% CI: 35.2–53.6) and 34.7% (95% CI: 28.9–40.5) per patient.

Cumulative pregnancy rates were calculated with life table analysis according to two methods. In the first method, all patients who stopped treatment were censored, leading to a cumulative pregnancy rate of 59.9% (95% CI: 53.9–65.9). In the second method, patients who stopped treatment because of a spontaneous pregnancy were not censored and considered pregnant in the calculation. All other patients who stopped treatment were censored. Cumulative pregnancy rate according to this method was 63.8% (95% CI: 57.9–69.7).

Analysis of Dropout

To analyse whether selective dropout occurred, patients were divided in four groups (patients where a treatment-independent pregnancy occurred excluded): A. patients dropping out after completing 1–4 unsuccessful cycles; B. patients dropping out after completing 5–8

Table 13.4 Patient and cycle characteristics of dropouts, non-dropouts and pregnant patients

Group[a]	A	B	C	D	*P*
No. of patients	78	43	21	77	
Age (mean ± SD)	32.6 (3.2)	33.0 (2.6)	33.4 (2.3)	32.1 (3.0)	0.20[b]
Subfertility primary (%)	50 (64.1)	27 (62.8)	15 (71.4)	47 (61.0)	0.85[c]
Duration subfertility (mean ± SD)	51.6 (23.6)	46.8 (19.6)	45.8 (20.8)	43.7 (20.6)	0.16[b]
No of cycles	223	271	189	230	
OR performed (%/cycle)	162 (72.6; 66.7–78.6)	211 (77.9; 72.8–82.9)	160 (84.7; 79.4–89.9)	199 (86.5; 82.0–91.0)	
OR successful (%/attempt)	123 (75.9; 69.2–82.6)	148 (70.1; 63.8–76.4)	112 (70.0; 62.8–77.2)	129 (64.8; 58.1–71.6)	
Fertilization (%/successful OR)	64 (52.0; 43.0–61.0)	90 (60.8; 52.8–68.8)	90 (80.4; 72.8–87.9)	101 (78.3; 71.0–85.6)	
ET (%/cycle)	44 (19.7; 14.4–25.1)	72 (26.6; 21.2–31.9)	73 (38.6; 31.5–45.7)	89 (38.7; 32.3–45.1)	

[a]Patients with treatment-independent pregnancies excluded from analysis
A: Dropout after 1–4 unsuccessful modified natural cycles
B: Dropout after 5–8 unsuccessful modified natural cycles
C: 9 unsuccessful modified natural cycles completed
D: Pregnant (cycle in which pregnancy occurred not included)
[b]ANOVA
[c]Chi square
OR—oocyte retrieval; *ET*—embryo transfer

unsuccessful cycles; C. patients who completed nine unsuccessful cycles and D. patients whose treatment led to pregnancy (cycles in which the pregnancy occurred excluded).

Patient and cycle characteristics of these four groups are presented in Table 13.4. Age, percentage of primary subfertility and duration of subfertility were not significantly different between groups. The number of oocyte retrievals performed per cycle was significantly lower in group A compared to groups C and D. Fertilization rate and embryo transfer rate both were significantly lower in group A compared to groups C and D. When comparing group B to groups C and D, the same trend was seen for a number of oocyte retrievals and embryo transfer but differences were not significant. Fertilization rate was significantly lower in group B compared to groups C and D.

In order to analyse whether cancellation of oocyte retrieval, fertilization failure or failure to reach embryo transfer are repeating phenomena in further cycles, results of cycles 2–9 of patients where these events occurred were compared to those of patients where they did not. Results of this analysis are shown in Table 13.5. The number of performed oocyte retrievals as well as the embryo transfer rate was significantly lower in cycles 2–9 in the group where no oocyte retrieval was performed in the first cycle as compared to the group where oocyte retrieval was performed in the first cycle. Patients where fertilization failure occurred in the first cycle showed significantly lower fertilization rate and embryo transfer rate in cycles 2–9 as compared to those where fertilization did occur in the first cycle. In patients who failed to reach embryo transfer in the first cycle, fertilization rate and embryo transfer rate were significantly lower in subsequent cycles compared to patients where embryo transfer was done in the first cycle.

Conclusions from This Study

In this study, a cumulative pregnancy rate of 40.6% was found after nine cycles of MNC-IVF. Including treatment-independent pregnancies, the cumulative pregnancy rate was 44.4%.

Table 13.5 Results of subsequent cycles after cancellation of oocyte retrieval, fertilization failure or no embryo transfer in the first cycle

Results of first cycle	OR not performed	OR performed	Fertilization failure	Fertilization	No ET performed	ET performed
No. of patients	46	210	36	116	157	99
Results of cycles 2–9						
No of cycles	138	654	109	333	520	272
OR performed (%/cycle)	97 (70.3; 62.5–78.1)	549 (83.9; 81.1–86.8)	92 (84.4; 77.5–91.4)	281 (84.4; 80.4–88.4)	418 (80.4; 76.9–83.9)	228 (83.8; 79.4–88.3)
OR successful (%/attempt)	72 (74.2; 65.3–83.1)	401 (73.0; 69.3–76.8)	67 (72.8; 63.6–82.1)	218 (77.6; 72.6–82.6)	301 (72.0; 67.6–76.4)	172 (75.4; 69.7–81.1)
Fertilization (%/successful OR)	45 (62.5; 51.1–73.9)	292 (72.8; 68.4–77.3)	31 (46.3; 34.1–58.5)	178 (81.7; 76.4–86.9)	198 (65.8; 60.3–71.3)	139 (80.8; 74.8–86.8)
ET (%/cycle)	35 (25.4; 18.0–32.8)	248 (37.9; 34.1–41.7)	29 (26.6; 18.1–35.1)	149 (44.7; 39.3–50.2)	161 (31.0; 26.9–35.0)	122 (44.9; 38.8–50.9)
Pregnancy (%/cycle)	13 (9.4; 4.4–14.4)	64 (9.8; 7.5–12.1)	7 (6.4; 1.7–11.1)	33 (9.9; 6.6–13.2)	49 (9.4; 6.9–12.0)	28 (10.3; 6.6–14.0)

OR—oocyte retrieval; *ET*—embryo transfer
Numbers in parentheses are percentages; 95% confidence interval

The actual observed cumulative pregnancy rate in our study represents an underestimation of the cumulative pregnancy rate that could be reached in a cohort of patients, since the chance of pregnancy in patients dropping out of the study would not have been zero if they had continued treatment. The cumulative pregnancy rate found with life table analysis represents an overestimation, since it assumes that the chance of pregnancy is the same in dropouts and those who continue treatment while in our study we found that this seems not to be the case. A realistic estimate of the cumulative pregnancy rate, corrected for dropout, will be somewhere in between the actual observed CPR and the life table estimation.

It is rather artificial to correct for dropouts, since in the analysis of IVF results, dropouts are in most cases not lost to follow-up but rather patients deciding to stop treatment for various reasons. Therefore, dropout is an inherent part of IVF performance. Corrected estimations are, however, useful in counselling patients when deciding whether or not to continue treatment. In this study, dropout rates were high, especially in higher cycle numbers.

In patients dropping out of the study, general patient characteristics were not different from those not dropping out. However, we found that cycle cancellation, fertilization failure and failure to reach embryo transfer predispose for dropout of patients in subsequent cycles and also are repeating phenomena in subsequent cycles. We therefore concluded that dropout of patients is probably selective, in the sense that patients with a poorer chance for pregnancy tend to dropout.

Furthermore, we concluded from this study that the optimal number of treatment cycles per patients remains unclear. The pregnancy rate per cycle appears to remain constant throughout higher cycle numbers, and the decline in steepness of the cumulative pregnancy curve is mainly caused by dropout of patients during the study, suggesting that patients should be advised to undergo at least nine cycles of minimal stimulation before starting standard IVF with ovarian stimulation. However, due to selective dropout of patients with a possible poor prognosis the steady pregnancy rate in all cycle numbers may be only apparent and therefore nine cycles will not be the suitable number for all patients.

Since the occurrence of a cancellation of oocyte retrieval, fertilization failure and failure to reach embryo transfer all seem to be repeating phenomena in further cycles, patient counselling

on the number of cycles to be performed should be individualized, taking into account the results of previous cycles.

Cumulative Pregnancy Rates in a Larger Series of Patients

As mentioned before, after completion of our studies on MNC-IVF, it was introduced as a standard treatment modality in our clinic, to be offered to all suitable new patients for a maximum of 6 cycles. In our original studies, only conventional IVF was performed. After the introduction of MNC as a standard treatment modality, also patients requiring ICSI were offered MNC and over the years, male factor subfertility became the predominant indication for MNC.

In the following section, results of MNC according to age, indication for ART and BMI, are discussed. In our original studies, clinical pregnancy was chosen as end point. In the present analysis, ongoing pregnancy was chosen as end point. So, patients conceiving with MNC but with a not-ongoing pregnancy, who returned for further MNC, were not considered new cases and cycle numbering was continued.

For simplicity's sake, only the three main indications (tubal factor, unexplained subfertility and male factor) are shown. Since the majority of patients in this series were offered a maximum of 6 cycles, only cycles 1–6 were included. For this series, we do not have data on intercurrent spontaneous pregnancies.

Cumulative ongoing pregnancy rates are shown in Fig. 13.4. This graph shows that, also in this larger series, ongoing pregnancy rate does not decline in higher cycles numbers.

Overall, 1744 patients started 7097 cycles and 643 (36.9% per patient) ongoing pregnancies followed. In 49 of cycles, supernumerary embryos were cryopreserved. Out of these, so far, 36 embryo transfers were performed, leading to 6 ongoing pregnancies. Data on cryopreserved embryos are not included in the following analyses.

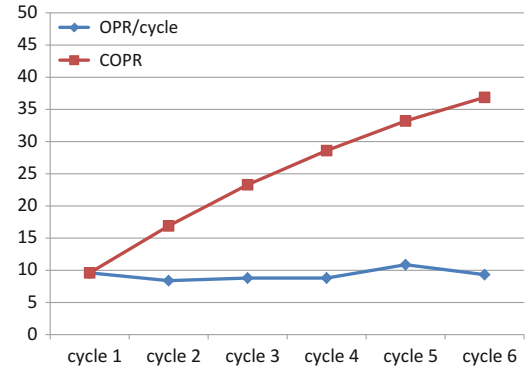

Fig. 13.4 Ongoing pregnancy rate (OPR) and cumulative ongoing pregnancy rate (COPR) according to cycle number

Cumulative Pregnancy Rates According to Patient Age

Results according to female patient age are shown in Table 13.6 and Figs. 13.5 and 13.6. Figure 13.5 shows the number of cycles according to age. The relatively low number of cycles in patients over 34 years is a reflection of the fact that from 2007 onwards, MNC was no longer offered to patients aged over 34 years. Figure 13.6 shows the pregnancy rate and ongoing pregnancy rate according to age. This figure shows that with increasing age there is a gradual decline in pregnancy rates per cycle.

Patients were grouped according to age at first cycle, and results per patient were calculated (Table 13.6). We found no apparent difference in performance in each step of the procedure (number of oocyte retrievals and successful oocyte retrievals, fertilization rate and embryo transfer per cycle all not significantly different), but ongoing implantation rate declines with age, which is of course not surprising.

The difference in pregnancy rates among the groups was not statistically significant. The cumulative ongoing pregnancy rate in patients aged 32–35 and 36–39 was significantly lower than in patients aged 28–31 years. The number of cycles per patient was not different between groups.

Table 13.6 Results according to female patient age

Age	20–23	24–27	28–31	32–35	36–39
No. of patients	43	233	530	792	146
Indication					
Tubal factor	5	40	76	132	33
Unexplained	5	24	79	182	52
Male	33	169	375	478	61
No. of cycles (No/pt)	168 (3.9)	890 (3.8)	2145 (4.0)	3340 (4.2)	554 (3.8)
Oocyte retrieval (%/cycle)	135 (80.4)	778 (87.4)	1880 (87.6)	2879 (86.2)	466 (84.0)
Oocyte retrieval successful (%/attempt)	109 (80.7)	620 (79.7)	1487 (79.1)	2258 (78.4)	357 (76.6)
Fertilization (%/successful oocyte retrieval)	73 (67.0)	408 (65.8)	1024 (68.9)	1529 (67.7)	281 (78.7)
Embryo transfer (%/cycle)	68 (40.5)[a]	374 (42.0)[a]	934 (43.5)[a]	1376 (41.2)[a]	250 (45.1)[a]
Pregnancy (%/cycle)	19 (11.3)[b]	117 (13.2)[c]	269 (12.5)[b,c]	361 (10.8)[c]	56 (10.1)
Ongoing pregnancy (%/cycle) *95% CI*	13 (7.7) *3.6–11.9*	92 (10.3)[c] *8.3–12.4*	223 (10.4)[c] *9.1–11.7*	271 (8.1)[c] *7.2–9.1*	44 (7.9) *5.6–10.2*
Ongoing implantation rate (%/embryo) *95% CI*	18.9 *9.3–27.9*	24.3 *21.8–26.2*	23.6 *20.8–26.4*	19.3 *17.2–21.4*	17.5 *12.7–22.2*
COPR (%/patient) *95% CI*	30.2 *16.2–44.2*	39.5 *33.1–45.9*	42.1 *37.8–42.1*	34.2 *30.8–37.6*	30.1 *22.5–37.7*

[a]In age categories 20–23, 24–27, 28–31, 32–35 and 36–39: 2, 5, 6, 13 and 2 DET
[b]In age categories 24–27 and 28–31: one and two twin pregnancies after DET
[c]In age categories 24–27, 28–31 and 32–35: 1, 3 and 4 pregnancies after IUI or coitus, of which 1, 3 and 3 ongoing

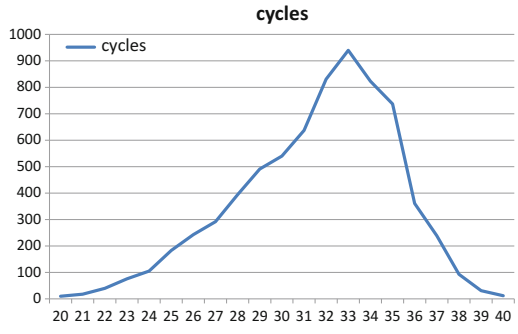

Fig. 13.5 Number of cycles according to female patient age

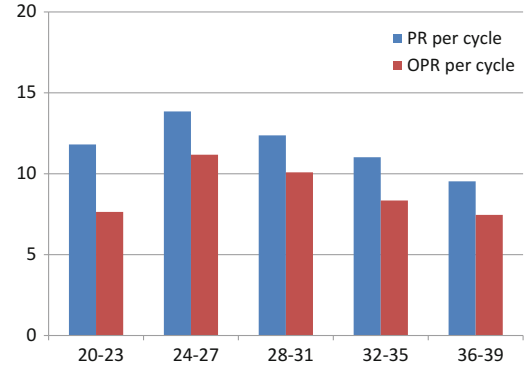

Fig. 13.6 Pregnancy and ongoing pregnancy rates according to female patient age categories

Cumulative Pregnancy Rates According to Indication for ART

Results according to indication for ART are shown in Table 13.7. These data show that the number of oocyte retrievals and the number of successful oocyte retrievals per patient are not different according to indication, but fertilization rate is. Not surprisingly, the lowest fertilization rate was found in male factor and the highest in tubal factor subfertility.

The high fertilization rate in tubal factor subfertility translates into a higher number of embryo transfers per cycle, while in unexplained subfertility, the fertilization rate was higher compared to male factor but the number of embryo transfers was not. Pregnancy rates per

Table 13.7 Results according to indication for ART

Indication for ART	Tubal factor	Unexplained	Male factor
No. of patients	286	342	1116
Mean age (range)	32 (20–38)	32 (20–39)	32 (20–39)
No. of cycles (No/pt)	1143 (4.0)	1329 (3.9)	4625 (4.1)
Oocyte retrieval (%/cycle)	989 (86.5)	1150 (86.5)	3999 (86.5)
Oocyte retrieval successful (%/attempt)	786 (79.5)	891 (77.5)	3154 (78.9)
Fertilization (%/successful oocyte retrieval) *95% CI*	655 (83.3) *80.7–86.0*	625 (70.2) *67.1–73.2*	2035 (64.5) *62.8–66.2*
Embryo transfer (%/cycle) *95% CI*	560 (49.0) *46.0–51.9*	538 (40.5) *37.8–43.2*	1904 (41.2) *39.7–42.6*
Pregnancy (%/cycle)	137 (12.0)	147 (11.1)[a]	538 (11.6)[b]
Ongoing pregnancy (%/cycle) *95% CI*	105 (9.2) *7.5–10.9*	112 (8.4)[a] *6.9–10.0*	426 (9.2)[b] *8.4–10.1*
COPR (%/patient) *95% CI*	36.8 *31.0–42.4*	32.8 *27.7–37.8*	38.2 *35.3–41.1*

[a] Three pregnancies (of which two ongoing) in cycles where oocyte retrieval was cancelled and IUI was done
[b] Five pregnancies (all ongoing) in cycles where oocyte retrieval was cancelled and IUI was done

cycle and cumulative ongoing pregnancy rates per patient were not different between groups. The number of cycles per patient was not different between groups.

Cumulative Pregnancy Rates According to BMI

Results according to BMI are shown in Table 13.8. Unfortunately, we have no data on BMI from patients treated before 2011. The table shows results from patients starting treatment in 2011, 2012, and 2013. Most of the BMIs in this table are self-reported. The low number of patients with BMI $\geq$ 35 is a reflection of the fact that in our centre, these patients are normally not admitted to the IVF program, except in research settings.

The number of (successful) oocyte retrievals, fertilization rate and embryo transfer rate was not different among BMI categories. There is an obvious trend towards a decrease in pregnancy and implantation rate with increasing BMI. The cumulative ongoing pregnancy rate in patients with BMI 30–34 was significantly lower than in patients with BMI 18–24. The number of cycles per patient was not different between groups.

It seems from these results that the lower success rate in obese women is due to a decreased implantation rate and not caused by lower success rate of oocyte retrieval or lower fertilization or embryo transfer rate. This finding is in analogy with what is found in COH-IVF [5].

Performance in First Cycle as a Predictor of Performance in Subsequent Cycles

As discussed before, in our earlier work we found cancellation of oocyte retrieval, fertilization failure and failure to reach embryo transfer to be repeating phenomena in subsequent cycles. In our larger series, we did a similar analysis, results of which are shown in Tables 13.9 and 13.10.

Cancellation of Oocyte Retrieval and Unsuccessful Oocyte Retrieval

Table 13.9 shows the results of subsequent cycles in three groups of patients: oocyte retrieval cancelled [either not planned or planned oocyte retrieval cancelled because of unexpected ovulation] (A), oocyte retrieval unsuccessful in

Table 13.8 Results according to BMI

BMI	18–24	25–29	30–34	35–40
No. of patients	182	100	39	4
Indication				
Tubal factor	25	11	4	–
Unexplained	26	7	2	–
Male	131	82	33	4
No. of cycles (No/pt)	764 (4.2)	417 (4.2)	165 (4.2)	17 (4.3)
Oocyte retrieval (%/cycle)	721 (94.4)	372 (89.2)	146 (88.5)	15 (88.2)
Oocyte retrieval successful (%/attempt)	604 (83.8)	314 (84.4)	123 (84.3)	11 (73.3)
Fertilization (%)	413 (68.4)	186 (59.2)	76 (61.8)	8 (72.7)
Embryo transfer (%/cycle)	382 (50.0)	176 (42.2)	69 (41.8)	8 (47.1)
Pregnancy (%/cycle)	111 (14.5)	54 (13.0)	11 (6.7)	2 (11.8)
Ongoing pregnancy (%/cycle) *95% CI*	91 (11.9) *9.6–14.3*	39 (9.4) *6.5–12.2*	9 (5.5) *1.9–9.0*	1 (5.9) *0–17.3*
Ongoing implantation rate (%/embryo) *95% CI*	91 (23.8) *19.5–28.2*	39 (22.2) *15.9–28.4*	9 (13.0) *4.9–21.2*	1 (12.5) *0–35.9*
COPR (%/patient) *95% CI*	91 (50.0) *42.6–57.4*	39 (39.0) *29.2–48.8*	9 (23.1) *13.5–36.6*	1 (25.0) *0–68.3*

Table 13.9 Results of subsequent cycles after cancellation of oocyte retrieval, unsuccessful oocyte retrieval and successful oocyte retrieval in the first cycle

Results of first cycle	A: OR not performed	B: OR unsuccessful	C: OR successful
No. of patients	242	304	1030
Mean age (range)	31 (20–39)	31 (20–39)	31 (20–39)
Indication			
Tubal factor	46	48	164
Unexplained	40	74	195
Male	156	182	671
Cycles 2-6 of same patients			
No. of cycles (No/pt)	752 (3.1)	1008 (3.3)	3593 (3.5)
Oocyte retrieval (%/cycle) *95% CI*	566 (75.3) *72.1–78.5*	864 (85.7) *83.5–87.9*	3206 (89.2) *88.1–90.3*
Oocyte retrieval successful (%/attempt) *95% CI*	437 (77.2) *73.7–80.7*	629 (72.8) *69.8–75.8*	2567 (80.1) *78.7–81.4*
Fertilization (%/successful oocyte retrieval) *95% CI*	275 (62.9) *58.3–67.6*	427 (67.9) *64.2–71.6*	1763 (68.7) *66.8–70.5*
Embryo transfer (%/cycle) *95% CI*	246 (32.7) *29.3–36.1*	384 (38.1) *35.0–41.2*	1590 (44.3) *42.6–45.9*
Pregnancy (%/cycle) *95% CI*	76 (10.1) *7.9–12.3*	110 (10.9) *9.0–12.9*	417 (11.6) *10.5–12.7*
Ongoing pregnancy (%/cycle) *95% CI*	57 (7.6) *5.6–9.5*	85 (8.4) *6.7–10.2*	333 (9.3) *8.3–10.2*
COPR (%/patient) *95% CI*	23.6 *18.1–29.0*	28.0 *22.8–33.1*	32.3 *29.4–35.2*

[a]First cycles with embryo transfer leading to an ongoing pregnancy excluded

Table 13.10 Results of subsequent cycles after fertilization failure, no embryo transfer and embryo transfer not leading to ongoing pregnancy in the first cycle

Results of first cycle	D: fertilization failure	E: no ET other reason[a]	F: ET, no ongoing pregnancy
No. of patients	331	91	608
Mean age (range)	31 (21–39)	31(22–38)	31 (20–39)
Indication			
Tubal	32	19	113
unexplained	58	27	110
Male	241	45	385
Cycles 2–6 of same patients			
No. of cycles (No/patient)	1234 (3.7)	234 (2.6)	2125 (3.5)
Oocyte retrieval (%/cycle) *95% CI*	1096 (88.8) *87.0–90.6*	211 (90.2) *86.3–94.1*	1899 (89.4) *88.0–90.7*
Oocyte retrieval successful (%/attempt) *95% CI*	892 (81.4) *79.0–83.7*	174 (82.5) *77.2–87.7*	1501 (79.0) *77.2–80.9*
Fertilization (%/successful oocyte retrieval) *95% CI*	532 (59.6) *56.4–62.9*	132 (75.9) *69.4–82.4*	1099 (73.2) *70.9–75.5*
Embryo transfer (%/cycle) *95% CI*	478 (38.7) *35.9–41.5*	108 (46.2) *39.6–52.7*	1004 (47.3) *45.1–49.4*
Pregnancy (%/cycle) *95% CI*	126 (10.2) *8.5–11.9*	30 (12.8) *8.4–17.2*	261 (12.3) *10.9–13.7*
Ongoing pregnancy (%/cycle) *95% CI*	104 (8.4) *6.8–10.0*	23 (9.8) *5.9–13.7*	206 (9.7) *8.4–11.0*
COPR (%/patient) *95% CI*	31.4 *26.3–36.5*	25.3 *16.2–34.4*	33.9 *30.0–37.7*

[a]GV or MI oocyte, >2 pronuclei at fertilization check or >50% fragmentation on day 2 or 3

the first cycle (B), and oocyte retrieval successful in the first cycle (C). This table shows that if oocyte retrieval was cancelled in the first cycle, the number of oocyte retrievals performed in subsequent cycles is lower, as compared to the other two groups of patients. Fertilization rate and embryo transfer rate in this group were lower compared to group C but not B. In group B, the proportion of successful oocyte retrievals was lower than in group C but not A.

Pregnancy rates and ongoing pregnancy rates were not different among groups. The cumulative ongoing pregnancy rate after six cycles was lower in group A compared to group C but not B. The number of cycles per patient was not different between groups. Overall, 480 (30.4%) of patients stopped MNC without completing 6 (unsuccessful) cycles. We do not have data on the number of patients that stopped treatment because of intercurrent spontaneous pregnancies, so the actual cumulative dropout rate is probably a bit lower. The cumulative dropout rates in groups A, B and C, respectively, were 42.8, 34.5 and 26.3%, suggesting that, as we found in our earlier study, dropout is selective in the sense that patients with poor prognosis tend to drop out.

Fertilization and Embryo Transfer

In order to evaluate fertilization failure as a repeating phenomenon, group C from Table 13.9 was subdivided in three groups: fertilization failure (D), no embryo transfer (immature oocyte, >2 pronuclei at fertilization check or >50% fragmentation on day 2 or 3; E) and

embryo transfer performed (first cycles with embryo transfer leading to an ongoing pregnancy excluded; F). Results are shown in Table 13.10. Fertilization rate was lower in group D versus E and F. Embryo transfer rate was lower in group D versus F but not E. Pregnancy and ongoing pregnancy rates as well as cumulative ongoing pregnancy rates were not different between groups. Comparison of results from Tables 13.9 and 13.10 show that embryo transfer rate was significantly lower in group A versus E and F, and cumulative ongoing pregnancy rate was lower in group A than in group F. The number of cycles per patient in group E was lower than in the other groups.

Discussion

In modified natural cycle IVF, treatment is aimed at the use of the one oocyte that is naturally selected. In our opinion, this is a major advantage of the MNC approach, since this one naturally selected oocyte probably represents the best one from a cohort of oocytes. This thought is supported by the fact that a large proportion of embryos arising from MNC are of good quality. Also, the overall implantation rate of embryos from MNC, although unselected, compares favourably to implantation rates of COH embryos [6].

The high implantation rates found in MNC may also be due to better endometrial quality compared to standard IVF. Ovarian stimulation is often reported to be detrimental to endometrial receptivity, especially with high numbers of oocytes and high estradiol levels.

A drawback of MNC-IVF, however, is the considerable loss in every step of the procedure. Cancellation of oocyte retrieval, whether because of lack of follicle development or because of LH rise or unexpected ovulation, unsuccessful oocyte retrieval, fertilization failure and abnormal fertilization frequently occur. In the large series described in this chapter, the embryo transfer rate was 42.3% per started cycle.

We routinely use a GnRH antagonist to prevent untimely ovulation, but still about 10% of planned oocyte retrievals are cancelled because of LH rise or unexpected ovulation. In natural cycle IVF (without the use of a GnRH antagonist), the range in reported cancellation rates is quite wide (3.8–53.1% per started cycle) [7–10]. The lowest cancellation rates are found in studies where quite intensive monitoring was applied, as well as great flexibility regarding planning of oocyte retrieval. Still, one could wonder whether the routine use of a GnRH antagonist in all cycles is justified. It would seem from the data that in a certain amount of cycles, a GnRH antagonist would not have been necessary, but it is unknown which are the patients that do or do not benefit from the GnRH antagonist. Omitting the GnRH antagonist from the protocol will reduce costs related to medication but on the other hand will require more intensive monitoring to obtain reasonable results.

Another way to prevent untimely ovulations is the use of indometacin. Indometacin as a cyclooxygenase inhibitor inhibits the production of prostaglandins, which are needed for follicular rupture and ovulation. Several studies reported on low ovulation rates (0–10%) in cycles where indometacin was added to a (modified) natural cycle protocol [9, 11–13]. In our centre, a randomized study was done in which results were compared in MNC cycles using GnRH antagonist with and without addition of indometacin. The number of patients with at least one ovulation after a maximum of six cycles was not different between groups, and no difference in terms of embryo transfer and pregnancy rate were found. In cycles where an LH surge was observed, the premature ovulation rate was not different between groups. In cycles without LH surge, however, the ovulation rate was significantly lower in the group where indometacin was used [4].

Other adjustments to the MNC protocol, such as changes in timing of ovulation triggering or changes in dosage of GnRH antagonist or gonadotrophins, are imaginable, in order to either raise success rates or reduce costs of medication. In order to improve the effectiveness of the oocyte retrieval procedure, flushing of the follicle is often proposed. So far, no studies comparing flushing to no flushing in (modified) natural cycle

are available, but data from studies on this subject in standard IVF suggest that it is of no benefit [14]. Flushing of the follicle will make the oocyte retrieval procedure more painful and time-consuming. There are no studies available on the necessity of luteal phase support in MNC-IVF. In theory, depletion of granulosa cells from the follicle at oocyte retrieval, as well as the use of GnRH antagonist, gonadotrophins and hCG may influence corpus luteum function. In a small series of cycles where no embryo transfer was performed (n = 24), we found shortened luteal phase and low mid-luteal progesterone levels in about one-third of cases (*unpublished data)*.

Modified natural cycle IVF is a low-risk treatment modality, with a close to zero risk of OHSS and a very low multiple pregnancy rate. Sporadically multiple follicles develop in a modified natural cycle, due to co-dominance of two or three follicles, or due to unintentional ovarian stimulation, when GnRH antagonist and gonadotrophins are started too early in the cycle. In our extended series, in only 5.5% of cycles more than one oocyte was obtained, and in only 9 of these cases (0.13%) $\geq$ 10 oocytes were retrieved. In our original studies and in the extended series described in this chapter, no OHSS occurred.

In 150 out of 7097 cycles (2.1%), 2 or more embryos were available for transfer. When we first started with MNC, double embryo transfer was done in cases where more than one embryo was available, and a twin pregnancy rate of about 2% (monozygous twins after transfer of a single embryo not included) followed. We then changed the transfer policy, and in all cases single embryo transfer was done. The twinning rate is now close to zero, with sporadically occurring monozygous twins. The very low multiple pregnancy rate in MNC is advantageous given the obstetrical risks involved, but in the current era with increasing application of single embryo transfer in COH-IVF, this is becoming a less-relevant argument *pro* MNC in comparison with COH-IVF.

Modified natural cycle IVF is a patient-friendly treatment modality, due to short duration and low dose of hormonal medication, easy oocyte retrieval (analgesia in most cases not required) and easy repeatability in consecutive cycles. On the other hand, frequent visits to the clinic may be burdensome for patients and disappointments due to cancellation of oocyte retrieval, unsuccessful oocyte retrieval, fertilization failure and failure to reach embryo transfer often occur. The few studies on patient perceptions on (modified) natural cycle or mild IVF that are available, all report the low dose and short duration of hormonal medication use to be an important positive aspect of these treatments [15–17]. In our cohort studies, we did a small questionnaire study. Patients who had completed a series of MNC-IVF cycles were asked, among other things, whether, looking back on things, they would make the same choice of participating in the study, and 80% of respondents answered positively. Injections of medication and oocyte retrieval were reported burdensome by 5 and 23% of respondents, respectively, whereas cancellation of the cycle (no embryo transfer possible) was reported as burdensome by 83% of respondents (*unpublished data*).

Judging from our data, it does not seem possible to select patients who are likely to do well with MNC-IVF based on patient characteristics such as age, indication for ART and BMI. However, it does seem possible to differentiate patients with relatively poor prognosis for success from those with better chances based on their performance in the first MNC cycle. In particular, cancellation of oocyte retrieval in the first cycle seems to predict poor overall outcome.

In selecting patients for MNC-IVF, it is very important not to consider the expected success rate per se, but to consider what would be the expected success rate after MNC in relation to that of other treatment modalities. For instance, our data show that success rates of MNC decrease with increasing age and higher BMI, but this is due to reduced implantation rates, which would also be expected in these patients when applying COH-IVF.

In specific situations, such as patients with a history of severe OHSS or those opposing to the creation of supernumerary embryos, MNC may

be preferred over COH-IVF. For poor responders to COH, MNC seems an attractive option since with COH these patients will have only few oocytes with low embryo transfer rate. Thanks to the easy repeatability in consecutive months, comparable numbers of oocytes could be obtained with MNC. Whether or not MNC should be the treatment of choice for poor responders to COH remains to be a subject to debate [18–21].

Only few comparative studies on MNC versus COH-IVF are available [22]. Preliminary results from a large randomized controlled trial comparing MNC to COH-IVF in unexplained and mild male factor subfertility suggested equal effectiveness in terms of ongoing pregnancy rates per patient [23]. Cost-effectiveness analysis in this study (only direct costs per pregnancy reported) seems unfavourable for MNC versus COH-IVF [24].

In conclusion, MNC-IVF is a low-risk and patient-friendly treatment modality and in our opinion is a feasible alternative to COH-IVF. The major drawback of MNC is the considerable loss in every step of the procedure, leading to rather low embryo transfer and pregnancy rates per cycle, but thanks to the short duration of a treatment cycle and easy repeatability in consecutive months, results in terms of time to pregnancy are favourable. Results regarding pregnancy rates or cost-effectiveness may be improved by adjustments to the protocol, or by more specific selection of patients.

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Minimal Stimulation for IVF with Clomiphene Citrate

14

Keiichi Kato, MD, PhD

Introduction

In order to improve the treatment outcomes for natural cycles and minimum stimulation cycle with clomiphene citrate for in vitro fertilization (IVF), the most important thing is to determine the optimal timing to retrieve the oocytes. Therefore, it is necessary to measure follicle numbers and check serum hormone levels on a daily basis. It is also important to improve the ability to judge the optimal timing of oocyte retrieval based on the above through the accumulation of data. The basic principle of minimum stimulation cycle is to effectively utilize endogenous hormones to minimize the use of exogenous agents. Minimum stimulation cycle for IVF would enable the reduction in the physical burden (including complications) as well as the financial burden. Furthermore, in minimum stimulation cycle, good quality oocytes can be recovered from the dominant follicles; therefore, the increasing likelihood of successful treatment outcomes would be considered. This chapter outlines the principles of clomiphene cycles for IVF and points of caution in real-world treatment.

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Characteristics of Minimal Stimulation Cycle Oocyte Retrieval

In natural cycles and minimal stimulation cycles (including clomiphene cycles), unlike in controlled ovarian hyperstimulation (COH) cycles, the function of pituitary gland is not inhibited. This enables endogenous hormones to stimulate ovaries and can minimize the use of the drugs. On the other hand, ovulation may occur because the luteinizing hormone (LH) surge occurs naturally. Therefore, strict monitoring of follicular growth and serum hormone levels is necessary to determine the optimal timing for oocyte retrieval.

Characteristics of Clomiphene

Clomiphene citrate (hereafter "clomiphene") is an estrogenic partial agonist (estradiol: 2–3% activation of E2 receptors) which demonstrates an anti-estrogenic effect in vivo. Its mechanism of action is to competitively bind with estrogen receptors in the hypothalamus, thereby inhibiting the binding of estrogen and blocking negative feedback from the hypothalamus and pituitary gland. This promotes the secretion of GnRH from the hypothalamus and of FSH and LH from the pituitary gland, thereby stimulating the ovaries and promoting ovulation. Therefore, onset of this action requires the secretion of at least a certain level of endogenous hormones. Therefore, clomiphene is ineffective for women with ovulation disorders associated with

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hypothalamic dysfunction, or hypopituitarism and ovarian failure (WHO group I). Clomiphene is, however, suitable for women with WHO group II ovulation disorders. Clomiphene citrate tablet (CLOMID®) is a mixture of two geometric isomers: cis (zuclomiphene) and trans (enclomiphene) and contains between 30 and 50% of cis-isomers. These two clomiphene isomers have mixed estrogenic and anti-estrogenic effects.

Protocol for Minimal Stimulation Cycles with Clomiphene

As previously stated, clomiphene cycles are indicated for WHO group I ovulation disorders; thus, they are suitable for a large number of patients of different ages [1–3]. Clomiphene alone or small doses of clomiphene used concomitantly with gonadotropins are used in minimal stimulation cycles at our hospital. One difference in the clomiphene cycle protocol at our hospital is that whereas clomiphene is normally administered only for five days beginning on day five of menstruation, we begin administration from day three and continue until right before the triggering ovulation. Because the pituitary gland is not inhibited, follicular maturation is induced prior to retrieval by triggering an endogenous LH surge with a nasal spray of a GnRH agonist (600 μg buserelin acetate). hCG is not administered in our protocol.

Patients with 28-day menstrual cycle as an example, we will explain the basic protocol for minimal stimulation with clomiphene. On day three of menstruation, serum hormone levels (E2, LH, and FSH) are measured. Upon confirming the levels of these hormones, the treatment regimen begins. Clomiphene administration in this technique differs from the typical method, as administration begins on day three of menstruation and is continued until just before the nasal spray for triggering ovulation. Clomiphene is administered for two purposes: for its main effect to promote follicular maturation and to the inhibition of the LH surge, which is achieved by its anti-estrogenic effect [4]. The dose of CLOMID® is set at one tablet (50 mg) per day. In the case of minimal stimulation with clomiphene only, the first hospital visit is scheduled on the third day of menstruation and the next visit is set on day 12. In each examination, ultrasonography, follicular maturation monitoring, and serum hormone tests are performed. Hormone tests involve measuring the levels of estradiol (E2), LH, FSH, and progesterone (P4). Once follicular growth is confirmed, GnRH agonist nasal spray is administered. The criteria for confirming follicular maturation are a dominant follicle diameter of $\geq$18 mm and an E2 level of $\geq$250 pg/mL. Oocytes are normally retrieved 32–34 h following GnRH agonist administration in accordance with hormone levels.

The desired outcome is the growth of multiple follicles. For patients with low levels of FSH, an appropriate dose of gonadotropins is administered. Gonadotropins are also administered when follicular growth is delayed. Although the anti-estrogenic effect of clomiphene limits the negative feedback of FSH reduction, FSH is replenished as appropriate when a decrease in FSH is observed as a result of elevation in E2 associated with follicular growth. The dosage of gonadotropins is 75–150 IU. Considering the half-life of gonadotropins, we consider alternate-day administration (rather than daily administration) is sufficient (Fig. 14.1).

Method for Determining the Oocyte Retrieval Period

After confirming follicular growth, the date and time of oocyte retrieval is determined. The ovulation period is estimated from hormone levels; oocytes must be retrieved immediately before ovulation. However, doing so requires an understanding of physiological hormone dynamics and the mechanisms of ovulation (Fig. 14.2). The most important point is the LH level, which varies greatly depending on whether the LH surge has begun. Determining whether the LH surge has begun is the most important

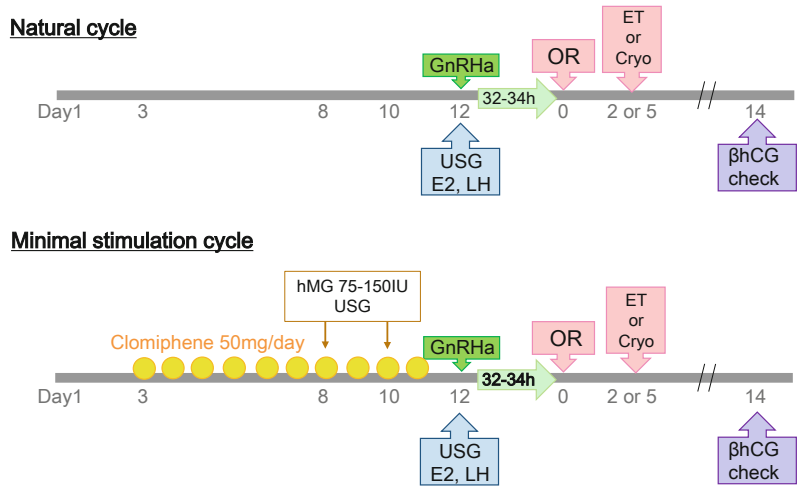

Fig. 14.1 Protocol for minimal stimulation and natural cycle IVF. *GnRHa* GnRH agonist, *OR* oocyte retrieval, *ET* embryo transfer, *Cryo* cryopreservation

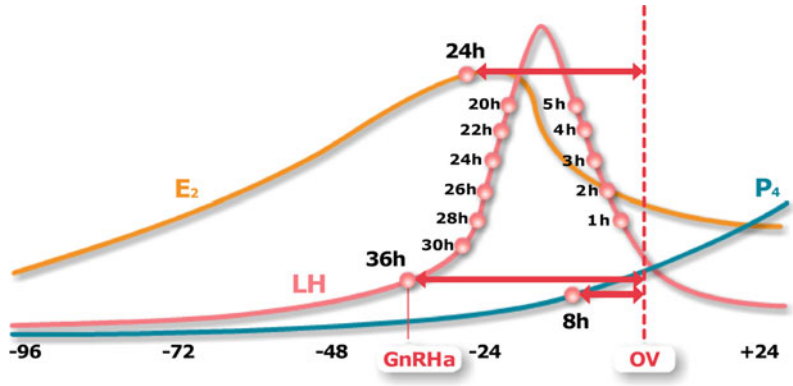

Fig. 14.2 Diagram of *LH* surge and timing of ovulation. *OV* ovulation

point in oocyte retrieval in natural and minimal stimulation cycles. This is done by an appropriate combination of the following methods: (1) direct assessment based on LH levels on the day of examination; (2) assessing whether LH levels have increased based on previous LH levels (including basic values) from the same cycle; and (3) the assessment based on hormone dynamics from the previous cycle. If the LH surge has not begun, GnRH agonist nasal spray is administered between 11:00 PM and 2:00 AM on the day of examination. In accordance with hormone levels, oocytes are retrieved 32–34 h following nasal spray administration (in the morning on day two following administration). If the LH surge has already begun, one of the two courses of action is taken. If the LH surge has only just begun, GnRH agonist nasal spray is administered immediately following examination; in accordance with hormone levels, oocytes are then retrieved 24–30 h following nasal spray administration (in the daytime on the day following administration). In this instance, if the examination is in the afternoon, it is not possible to secure sufficient time from nasal spray administration to oocyte retrieval; this situation results in an inability to retrieve oocytes at the ideal time. If the time has passed since the beginning of the LH surge, oocytes are retrieved within 24 h following nasal spray administration (the morning of the following day). There are also some cases in which the LH surge has already ended by the time of examination. In such cases, ovulation is predicted to occur that same day, making it

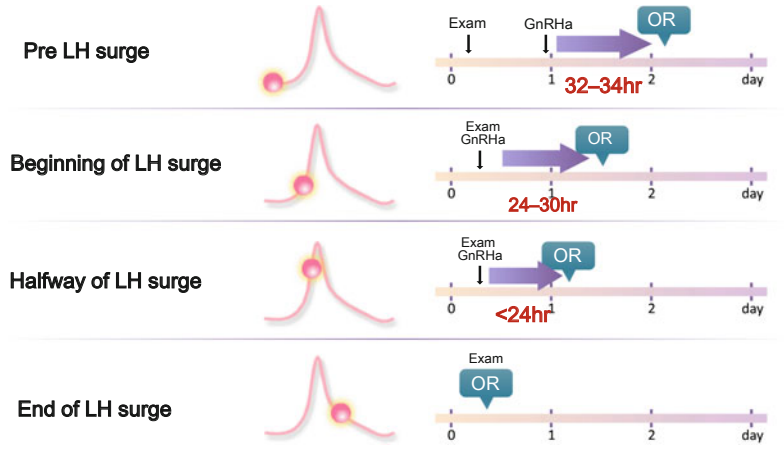

Fig. 14.3 LH surge and the appropriate timing of oocyte retrieval

necessary to retrieve oocytes that day (the day of examination) (Fig. 14.3).

Attempts to Prevent Ovulation

As already stated, the most important thing in natural cycles and minimal stimulation cycles is determining an appropriate timing of oocyte retrieval. If the timing is too late, ovulation will have already occurred; if the timing is too early, only vacuoles or immature oocytes will be collected. Therefore, it is necessary to determine how to adapt to the contradiction of securing time until the oocyte maturation while ensuring retrieval before ovulation. Factors related to the mechanism of ovulation include Cox-1 and Cox-2; the administration of nonsteroidal anti-inflammatory drugs (NSAIDs) inhibits Cox-1 and Cox-2 activity, thereby making it possible to delay and prevent ovulation. At eight hours and 16 h before the scheduled oocyte retrieval time, 25 mg diclofenac sodium (Voltaren®) is administered as a suppository. Although this cannot be used for patients with bronchial asthma or gastric ulcers, it can be used without side effects in a wide range of cases. Indeed, we confirmed that Voltaren® reduced the rate of natural ovulation and increased the likelihood of obtaining matured oocytes, and consequently, pregnancy rates were improved [5].

Dealing with Polycystic Ovary Syndrome

Polycystic ovary syndrome (PCOS) presents with ovulation disorders; therefore, in many cases, clomiphene dose not work. The simple administration of gonadotropin agents in these patients can easily result in ovarian hyperstimulation syndrome (OHSS), while administration of hCG often leads to even more serious symptoms. This makes the induction of ovulation difficult. For patients with PCOS, particularly patients with younger age, our clinic uses the aromatase inhibitor letrozole as the first choice in order to avoid OHSS. In doing so, we have achieved favorable results in terms of ovulation induction and pregnancy outcomes. Although not possible in principle in natural cycles, alternatively, clomiphene can also be applied. Specifically, a small dose (50–70 IU) of FSH (rFSH) is used concomitantly with clomiphene and administered on alternate days. The dose is then gradually increased as appropriate while confirming follicular growth. Although basic ovulation induction methods are not much different with those for non-PCOS patients, the risk of severe OHSS can be reduced by avoiding hMG administration; determining the timing of oocyte retrieval earlier even follicle size is smaller than normal in case of the large number of follicles that are

developed; not administering hCG; and considering cryopreservation of all embryos.

Oocyte Retrieval Techniques

Usually, oocyte retrieval does not require the use of anesthetic. At our clinic, oocytes are retrieved using a 21G (Kitazato, Japan) fine needle, and pain is normally not a problem. The use of a fine needle also reduces invasion into tissue, thereby reducing the risk of hemorrhage and infection following oocyte retrieval.

Protocol for Frozen Embryo Transfer

Fresh embryo transfer is performed on day 2 or 3 after oocyte retrieval at cleavage stage or on day 5 at blastocyst stage. Because transfers are performed following oocyte retrieval, protocol conforms to oocyte retrieval cycles.

When transferring frozen embryos, the protocol differs based on whether natural ovulation is observed. When natural ovulation occurs, thawed embryos are transferred on day 2 at cleavage stage or on day 5 at blastocyst stage following confirmation of natural ovulation. On the scheduled transfer day, hormone testing is performed for all patients, and the transfers are suspended for patients with abnormal hormone levels. Normally, strong progesterone replacement is unnecessary; rather, 30 mg/day dydrogesterone (Duphaston® tablets) is administered for 12 days in case of transfer at cleavage stage or for 7 days in case of transfer at blastocyst stage. When natural ovulation is not observed, a hormone replacement cycle is introduced. The precycle entails the use of norgestrel/ethinyl estradiol (Planovar® combination tablets); the cycle is then begun on day 2 following withdrawal bleeding. Administration of estradiol (0.72 mg Estrana® tape) is begun on day 2. On day 10, the dosage of Estrana® tape is reduced in accordance with hormone levels and endometrial thickness. Concomitant use of Duphaston® and Planovar® is begun on days 11 and 14, respectively. In the case of blastocysts, embryo transfer is normally performed on day 18 (Fig. 14.4).

Embryo Transfer Techniques

Embryo transfer for all patients consists of single embryo transfer guided by transvaginal ultrasound. When performing selective single embryo transfer, blastocyst culture is performed for surplus embryos; after embryo selection, favorable blastocyst development is confirmed, and the embryos are cryopreserved. Unlike transabdominal ultrasound guidance, the use of transvaginal ultrasound does not require filling of the bladder, thus allowing patients greater comfort. Another merit is that ultrasound and the embryo transfer

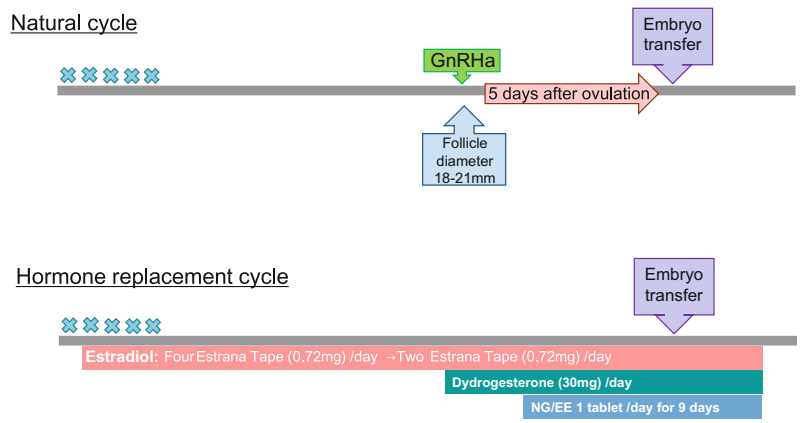

Fig. 14.4 Protocol for embryo transfer. *NG/EE* Norgestrel/Ethinyl estradiol

catheter can be operated by a single operator. The use of transvaginal ultrasound also enables a more detailed observation of the endometrium, thus allowing the embryo to be implanted in a more appropriate location [6].

Comparison of Natural Cycles and Clomiphene Cycles

Clomiphene cycles differ from natural cycles in two major ways: Due to the anti-estrogenic effect of clomiphene, the LH surge does not occur readily, and thinning of the endometrium occurs. Table 14.1 shows LH values at the onset of LH surge in clomiphene cycles and natural cycles at our clinic. For age-groups of <35 years, 35–37 years, 38–39 years, 40–41 years, and 42–45 years, clomiphene cycle LH values were 9.34 ± 0.12, 9.01 ± 0.12, 8.84 ± 0.13, 8.81 ± 0.13, and 9.33 ± 0.11 mIU/mL, respectively, while natural cycle LH values were 19.19 ± 0.86, 18.57 ± 0.93, 14.68 ± 0.90, 13.78 ± 0.94, and 14.31 ± 0.79 mIU/mL, respectively (Table 14.1). Regardless of patients' ages, clomiphene cycles demonstrated inhibition of the LH surge compared to natural cycles.

Comparisons of endometrial thickness at the time of fresh embryo transfer between clomiphene cycles and natural cycles are shown in Table 14.2. For age-groups of <35 years, 35–37 years, 38–39 years, 40–41 years, and 42–45 years, clomiphene cycle endometrial thickness was 10.15 ± 0.06, 10.06 ± 0.06, 9.74 ± 0.05, 9.51 ± 0.05, and 9.27 ± 0.05 mm, respectively, while natural cycle endometrial thickness was 10.52 ± 0.06, 10.23 ± 0.05, 9.84 ± 0.08, 9.72 ± 0.10, and 9.34 ± 0.08 mm, respectively (Table 14.2). Clomiphene cycles demonstrated a tendency toward endometrial thinning compared to natural cycles, with a significant difference observed in the age-group of <35 years. In both types of cycles, the endometrium tended to become thinner with advancing age.

Table 14.3 shows oocyte retrieval rates and mean number of ova in clomiphene cycles and natural cycles. For age-groups of <35 years, 35–37 years, 38–39 years, 40–41 years, and 42–45 years, oocyte retrieval rates in clomiphene

Table 14.1 Mean serum LH at the onset of preovulatory LH surge in clomiphene cycles and natural cycles

Age (years)	LH level (mIU/ml)	
	Clomiphene	Natural cycle
<35	9.34 ± 0.12^{a} ($n = 887$)	19.19 ± 0.86^{b} ($n = 233$)
35–37	9.01 ± 0.12^{a} ($n = 1293$)	18.57 ± 0.93^{b} ($n = 268$)
38–39	8.84 ± 0.13^{a} ($n = 1203$)	14.68 ± 0.90^{c} ($n = 131$)
40–41	8.81 ± 0.13^{a} ($n = 1409$)	13.78 ± 0.94^{c} ($n = 137$)
42–45	9.33 ± 0.11^{a} ($n = 2287$)	14.31 ± 0.79^{c} ($n = 216$)

Different letters indicate significant differences ($p < 0.05$)

Table 14.2 Mean endometrial thickness on the day of fresh day 2 embryo transfer in clomiphene cycles and natural cycles

Age (years)	Endometrial thickness (mm)	
	Clomiphene	Natural cycle
<35	10.15 ± 0.06^{ab} ($n = 1298$)	10.52 ± 0.06^{e} ($n = 971$)
35–37	10.06 ± 0.06^{ab} ($n = 1494$)	10.23 ± 0.05^{ae} ($n = 1151$)
38–39	9.74 ± 0.05^{c} ($n = 1547$)	9.84 ± 0.08^{ac} ($n = 462$)
40–41	9.51 ± 0.05^{cd} ($n = 1413$)	9.72 ± 0.10^{bcd} ($n = 369$)
42–45	9.27 ± 0.05^{d} ($n = 1622$)	9.34 ± 0.08^{cd} ($n = 509$)

Different letters indicate significant differences ($p < 0.05$)

cycles were 87.1, 85.7, 84.1, 82.2, and 78.6%, respectively. Although these oocyte retrieval rates tended to decrease with advancing age, they were significantly higher than oocyte retrieval rates in natural cycles (natural cycles: <35 years: 73.0%, 35–37 years: 71.2%, 38–39 years: 69.2%, 40–41 years: 67.5%, and 42–45 years: 64.5%; Table 14.3). Multiple oocytes were obtained from clomiphene cycle patients from whom oocytes were successfully retrieved.

Table 14.3 Mean oocyte retrieval rate in clomiphene cycles and natural cycles

Age (years)	Oocyte retrieval rate (%)	
	Clomiphene	Natural cycle
<35	87.1[a] (n = 4021)	73.0[e] (n = 2571)
35–37	85.7[a] (n = 5358)	71.2[ef] (n = 3090)
38–39	84.1[b] (n = 5778)	69.2[fg] (n = 1703)
40–41	82.2[c] (n = 6649)	67.5[g] (n = 1931)
42–45	78.6[d] (n = 11,085)	64.5[h] (n = 4146)

Different letters indicate significant differences ($p < 0.05$)

Table 14.4 Oocyte retrieval rate in minimal stimulation and natural cycle IVF (2008.1.–2008.12)

	Age (years)					Total	P value
	≤ 29	30–34	35–39	40–44	≥ 45		
OPU cycles	448	2751	6595	7600	2850	20,244	–
Ovulated before OPU (%)	10 (2.23)	65 2.36)	160 (2.43)	172 (2.26)	60 (2.11)	476 (2.35)	0.46
Cycles retrieved oocytes (%)	372 (83.0)	2243 (81.5)	5378 (76.8)	5838 (76.8)	1951 (68.5)	15,782 (78.0)	<0.0001
Cycles obtained matured oocytes (%)	314 (70.1)	1957 (71.1)	4787 (72.6)	5171 (68.0)	1698 (59.6)	13,927 (68.8)	<0.0001

Cochran–Armitage test for trend

Table 14.5 Fertilization, cleavage, and blastocyst formation rates in minimal stimulation and natural cycle IVF (2008.1–2008.12)

	Age (years)					Mean	P value
	≤ 29	30–34	35–39	40–44	≥ 45		
Fertilization rate (%)							
IVF	76.6	73.6	76.1	79.9	77.7	77.0	0.07
ICSI	85.4	83.7	83.1	83.1	83.0	83.2	0.75
Cleavage rate (%)							
IVF	92.4	89.2	87.5	88.5	87.7	88.3	0.70
ICSI	93.9	95.0	94.7	92.7	90.2	93.4	0.24
Blastocyst formation rate (%)							
IVF	70.5	67.0	64.4	49.7	25.9	57.3	<0.0001
ICSI	69.8	59.5	56.0	40.1	21.7	46.6	<0.0001

Cochran–Armitage test for trend

Table 14.6 Clinical pregnancy rate (CPR) and delivery rate (DR) followed by fresh or frozen embryo transfer in minimal stimulation and natural cycle IVF (2008.1–2008.12)

	Age (years)					Total	*P* value
	≤29	30–34	35–39	40–44	≥45		
Fresh cleavage stage embryo transfer							
Cycles	159	931	1972	1428	412	4902	
CPR (%)	33.3 (53/159)	34.8 (324/931)	25.7 (507/1972)	12.5 (179/1428)	1 (4/412)	21.8 (1067/4902)	<0.0001
DR (%)	30.2 (48/159)	29.5 (275/931)	19.1 (376/1972)	7.4 (106/1428)	0.5 (2/412)	16.5 (807/4902)	<0.0001
Fresh blastocyst transfer							
Cycles	17	29	25	73	5	149	
CPR (%)	35.3 (6/17)	51.7 (15/29)	36.0 (9/25)	27.4 (20/73)	40 (2/5)	34.9 (52/149)	0.29
DR (%)	35.3 (6/17)	37.9 (11/29)	28.0 (7/25)	13.7 (10/73)	20 (1/5)	23.5 (35/149)	0.036
Frozen cleavage stage embryo transfer							
Cycles	13	64	125	153	61	416	
CPR (%)	38.5 (5/13)	51.6 (33/64)	30.4 (38/125)	11.8 (18/153)	3.3 (2/61)	22.8 (95/416)	<0.0001
DR (%)	30.8 (4/13)	37.5 (24/64)	24.0 (30/125)	7.8 (12/153)	0 (0/61)	16.8 (70/416)	<0.0001
Frozen blastocyst transfer							
Cycles	184	1024	2072	1489	165	4934	
CPR (%)	50.5 (93/184)	54.0 (553/1024)	48.2 (998/2072)	31.6 (471/1489)	20 (33/165)	43.6 (2149/4934)	<0.0001
DR (%)	41.3 (76/184)	44.5 (456/1024)	36.5 (757/2072)	17.7 (263/1489)	6.1 (10/165)	31.7 (1562/4934)	<0.0001

Cochran–Armitage test for trend

Treatment Outcomes in Natural and Minimal Stimulation Cycle for IVF

For the treatment outcomes among patients aged ≤29 years to ≥45 years at our clinic for natural cycle and minimal stimulation cycle oocyte retrieval, the oocyte retrieval rate per oocyte retrieval cycle was 78%. The oocyte retrieval rate was constant regardless of age up to age 45 years (Table 14.4). The fertilization rate was 80.3%, while the cleavage rate was 91.1%; no age-based differences were observed. The blastocyst development rate decreased with advancing age from 70.1% to 22.8%. The live birth rate per transfer decreased with advancing age. Of all transfer methods, frozen blastocyst transfer yielded the highest live birth rate (Tables 14.5 and 14.6).

Conclusion

We have explained the characteristics and points of caution in natural cycles and minimal stimulation cycles for IVF, with a focus on methods for determining an appropriate timing of oocyte retrieval. The basic idea enables patients to treat their infertility as natural a way as possible. In doing so, a sufficient understanding of physiological hormone dynamics is required. In principle, it is necessary to establish a system with which oocytes can be retrieved 365 days a year.

Although doing so may be difficult due to the scales of infertility treatment centers and personnel issues, there is great potential benefit for patients. We hope natural cycles and minimal stimulation cycles for IVF will be introduced as a basic protocol in a greater number of institutions in the future.

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Mild Stimulation Protocols: Combination of Clomiphene Citrate and Recombinant FSH or HMG

15

Jiayin Liu, MD, PhD and Wei Wu, MD

Introduction

Individualized IVF is to offer every single woman the best treatment tailored to her own unique characteristics, thus maximizing the chances of pregnancy and eliminating the complication and avoidable risks resulting from ovarian stimulation. Individualized ovulation program has always been our goal in IVF treatment. Conventional control ovarian stimulation protocols aim to get many oocytes to compensate for inefficiencies in laboratory procedures and to generate several embryos for transfer. In recent years, calls for milder forms of ovarian stimulation in IVF have led to a revival of the use of clomiphene citrate [1]. As a classical effective ovulation drugs, clomiphene citrate is cheap, readily available, and safe and can be orally administered. Clomiphene citrate in combination with FSH or HMG is consistent with the concept of "mild and friendly IVF" [2, 3].

The aim of ovarian stimulation for IVF is the recruitment of acceptable fertilizable oocytes. At present, the common ovulation protocol in IVF includes the following: the conventional agonist long protocol, antagonist protocol, mild stimulation, and minimal stimulation/natural cycle IVF. The selection of the program for ovulation stimulation in IVF decides on "the aim number of oocytes" which you plan. If the aim number of oocytes is 8–15, then you should choose conventional superovulation programs; if the aim number of oocytes is 5–8, a mild stimulation protocol is recommended; and if the aim number of oocytes is 3–5, the minimal stimulation cycle should be chosen.

Clomiphene Citrate

Clomiphene citrate is still used as first-line drugs to induce ovulation for inexpensiveness, safeness, and convenience. Chemically, clomiphene is a nonsteroidal triphenylethylene derivative that exhibits both estrogenic agonist and antagonist properties [4]. Clomiphene has two isomeric forms: zuclomiphene and enclomiphene. The commercial preparation is a racemic mixture that contains 40% zuclomiphene and 60% enclomiphene. The action of it is mainly anti-estrogenic. Enclomiphene is the more potent isomer and the one primarily responsible for the ovulation-inducing actions of clomiphene. Approximately 85% of an administered dose is eliminated after approximately 6 days. Enclomiphene levels rise rapidly after administration and fall to undetectable concentrations soon thereafter. Zuclomiphene is cleared far more slowly, although traces may remain in the circulation for much longer (about six weeks) [5] clomiphene binds to

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the estrogen receptors (ERs) throughout the reproductive, and its binding is more prolonged and results in a decrease of the estrogenic effect.

The drug's effectiveness in ovulation induction can be attributed to actions at the hypothalamic level. Depletion of hypothalamic ER prevents correct interpretation of circulating estrogen levels. Reduced levels of estrogen feedback trigger normal compensatory mechanisms that alter pulsatile hypothalamic gonadotropin-releasing hormone (GnRH) secretion to stimulate increased pituitary gonadotropin releasing [6]. Clomiphene also has an effect on the ovary by sensitizing granulosa cells in the follicles to the action of gonadotropins and up-regulates aromatase activity. At the same time, clomiphene has a certain agonistic effect, especially in hypo-estrogenic states. Clomiphene induced an increase in gonadotropin on days 5–9 of the cycle. Clomiphene-induced ovulation rate was about 70–80%, and pregnancy rate was 30–40%. The main side effect of clomiphene is the effect of the thickness of the endometrium, cervical mucus secretion reduction, and a plurality of follicular development.

Clomiphene citrate in combination with FSH or HMG is consistent with the concept of "mild and friendly IVF" for cheap, readily available, safe, and less injection. The ovarian stimulation protocol combining clomiphene with gonadotropins could lead to a reduction in the dosage of gonadotropins required due to the combined synergistic effects. Clomiphene citrate increases pulsatile secretion of gonadotropin-releasing hormone(GnRH) by the hypothalamus. It also has an effect on the ovary by sensitizing granulosa cells in the follicles to the action of gonadotropins and up-regulates aromatase activity. Additionally, because gonadotropins may counterbalance the undesired anti-estrogenic effects of the clomiphene on the endometrium [7] which has been held responsible for the relatively low embryo implantation rates observed, this combination might lead to improved pregnancy rates compared with clomiphene alone.

The report from the Cochrane database is to determine whether clomiphene citrate with gonadotropins (with or without mid-cycle antagonist) is more effective than gonadotropins with gonadotropin-releasing hormone (GnRH) agonists for controlled ovarian stimulation in IVF or intracytoplasmic sperm injection (ICSI) treatment [5]. Fourteen studies were included in the review. Meta-analysis could be performed with the data of 12 included studies, with a total of 2536 participants. Main result of the review is that there was no evidence that clomiphene along with gonadotropins for IVF differed from gonadotropins alone in GnRH agonist protocol in terms of live births (5 RCTs, 1079 women; OR 0.93, 95% CI 0.69–1.24) or clinical pregnancy (11 RCTs, 1864 women; OR 1.07, 95% CI 0.85–1.33) (Fig. 15.1).

There was a significant reduction in the incidence of OHSS (5 RCTs, 1559 women; OR 0.23, 95% CI 0.10–0.52) Compared to a typical clinic with 3.5% prevalence of OHSS using a GnRH agonist regimen, clomiphene citrate protocols would be expected to reduce the incidence between 0.8 and 1.8% (Fig. 15.1) [5]. The evidence from this review suggests that the use of clomiphene along with gonadotropins leads to similar pregnancy rates as those occurring after the use of gonadotropins alone, and there was a significant reduction in the incidence of OHSS (Fig. 15.2).

Indication for Mild Stimulation Protocols: Combination of Clomiphene Citrate and Recombinant FSH or HMG

Patients Who Are at High Risk of Hyper-response

Personalization of treatment in IVF should be based on the prediction of ovarian response for every individual AFC (antral follicle count) and AMH (anti-Mullerian hormone) the most

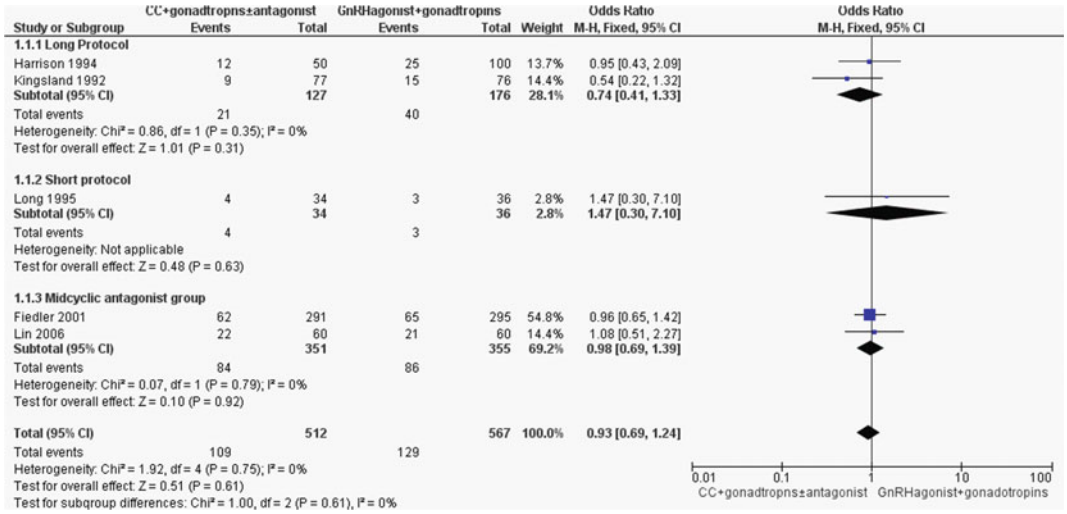

Fig. 15.1 Forest plot of comparison: 1 Clomiphene citrate with gonadotropins (with or without mid-cycle antagonist) versus gonadotropins with GnRH agonists protocols in IVF and ICSI cycles, outcome: 1.1 live birth

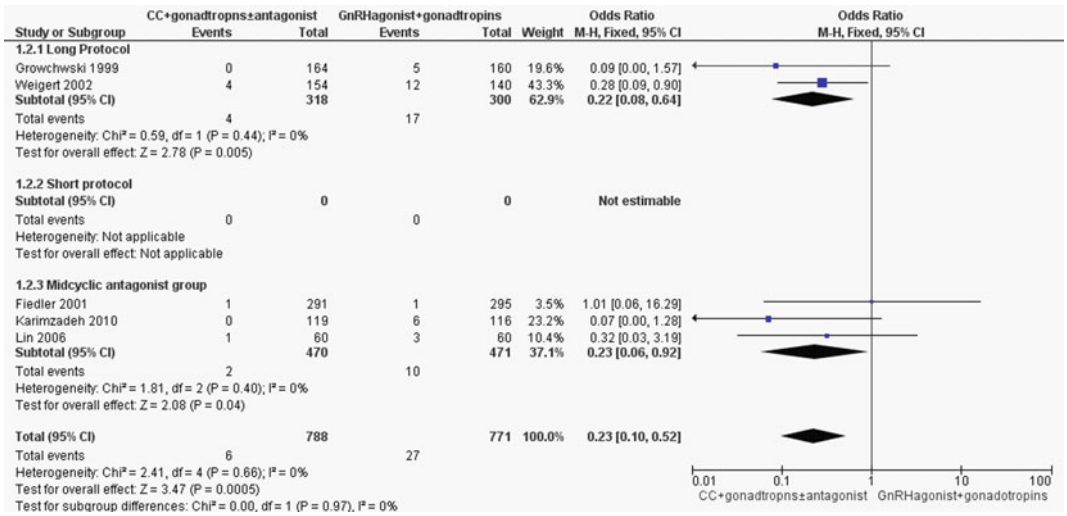

Fig. 15.2 Forest plot of comparison: clomiphene citrate with gonadotropins (with or without mid-cycle antagonist) versus gonadotropins with GnRH agonist protocols in IVF and ICSI cycles; outcome: ovarian hyperstimulation syndrome

sensitive markers of ovarian [8] (Fig. 15.3), reserve identified to date, are ideal in planning personalized COS protocols. AFC was found to be significantly associated with AMH levels and the number of retrieved oocytes, with the number of follicles between 5 and 6 mm having the highest correlation to both endpoints. AMH (as a paracrine product of immature follicles) is a more direct measure of ovarian status compared with other endocrine reproductive hormones. AMH is primarily produced by the pre-antral and small antral follicles and correlates with the number of primordial follicles at the gonadotropin-independent stage of follicular development (Fig. 15.4) [8].

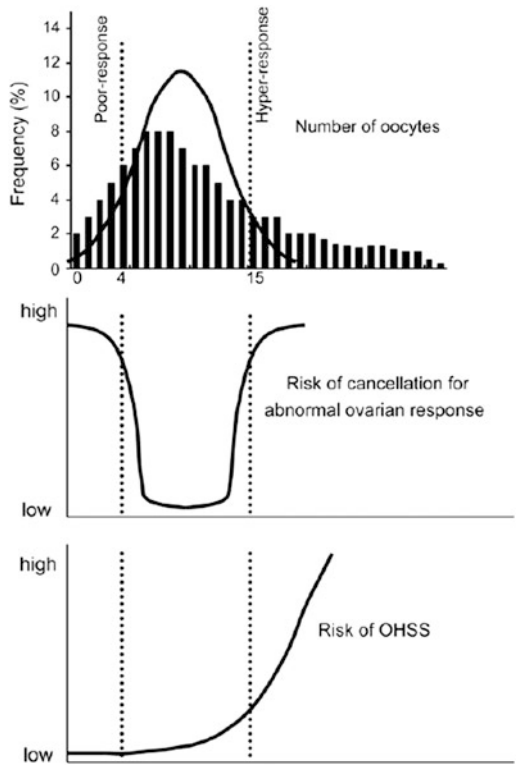

Fig. 15.3 Objective of the individualization of the treatment strategy would be possible to increase the percentage of patients with a number of retrieved oocytes considered appropriate, hence reducing the number of women at high risk of cycle cancellation and ovarian hyperstimulation syndrome (OHSS). Top of the figure: Bars indicate the actual frequency of retrieved oocytes as derived by La Marcal and Sunkara [8]. The line indicates the ideal frequency of retrieved oocytes, characterized by a very high percentage of women with an appropriate oocyte yield

High risk of hyper-response means high risk of ovarian hyperstimulation syndrome (OHSS) or had preliminary OHSS history. It is of great importance to accurately predict women who are likely to have a high response to COS as it is the main risk factor for OHSS [9]. Initial follicular recruitment and selection are undertaken by endogenous endocrine factors prior to starting the exogenous gonadotropin administration. Compared with the standard long GnRH agonist protocol, mild stimulation leads to a smaller number of growing follicles and this is undoubtedly an advantage in women with a high ovarian reserve and hence at risk of OHSS. In the study, recently [10], a modified therapeutic protocol with low gonadotropin doses and GnRH antagonist or clomiphene seems to be ideal for women at high risk of OHSS. [8] (Fig. 15.4). Mild/minimal stimulation offers an attractive option for patients who have experienced this complication in a previous treatment cycle, and it can reduce the incidence of OHSS in high-responder patients [11].

Polycystic Ovary Syndrome (PCOS)

Severe OHSS may occur particularly when the classical "long" protocol is applied to young, highly responsive women, and its incidence ranges between 2 and 6% of the IVF cycles in this kind of women [12]. PCOS patients usually have high number of AFC and high AMH level, and most of them are high risk of OHSS, especially severe OHSS.

Anticipated Poor Response

For anticipated poor response patients, treatment with mild stimulation protocol as likely to be beneficial reduced the treatment burden than the conventional GnRH agonist long protocol. According to published data, a cut-off value of AMH ranging between 0.7 and 1.3 ng/ml may be considered acceptable for the prediction of poor response in IVF. The recent European Society of Human Reproduction and Embryology Consensus Conference established a standardized definition of poor ovarian response as the retrieval of 4 oocytes following a standard IVF protocol, i.e., following maximal stimulation [13]. The most frequently reported cut-off values of AFC for prediction of poor response ranged between 5 and 7 [14, 15]. The advantages of mild stimulation protocols particularly apply to low-response patients regarding cost, convenience, and success rates. High-dose gonadotropin is unused for the poor response, and mild/minimal stimulation

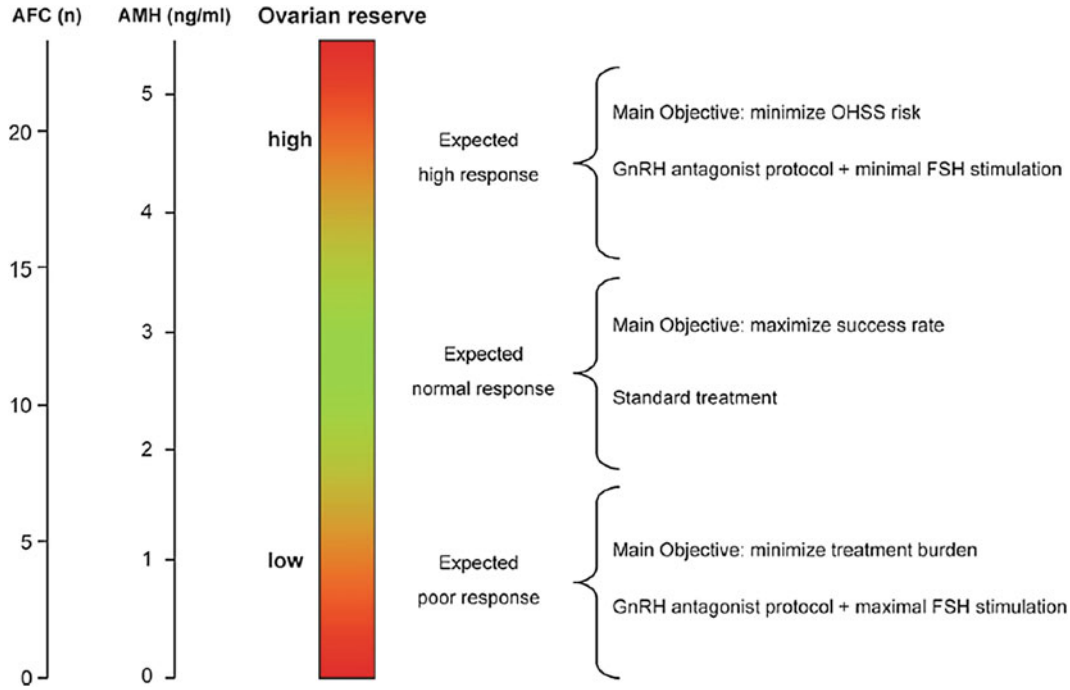

Fig. 15.4 Ovarian reserve testing before the first IVF cycle would be permit to categorize patients as expected poor, normal, or hyper-responders. Since there is no evidence of superiority of one approach over another in the treatment of poor responders, the protocol associated with reduced discomfort and treatment durden should be preferred. In hyper-responder patients, one of the most important objectives of medical counseling is to prevent OHSS. Hence, the first-line protocol would be based on administration of low doses of FSH in a GnRH antagonist-based scheme. AFC, antral follicle count; AMH, anti-Mullerian hormone

may be an optional protocol. Anticipated poor response would never be compensated by an increase in the exogenous FSH over the maximal dose. Accordingly, different studies performed on women anticipated to be poor response on the basis of low AMH or AFC showed that increasing the FSH dose was ineffective for preventing a negative ovarian response in those women [16–18].

Target Number of Oocyte Is 5–8

Conventional control ovarian stimulation protocols aim to get many oocytes to compensate for inefficiencies in laboratory procedures and to generate several embryos for transfer. With the development of the laboratory techniques, less oocytes were needed for IVF treatment and less embryos were transferred. Five to eight oocytes will be enough for most people.

Patients Who Are Afraid of Long Time of Injection

The injection duration of mild stimulation time usually is 7–10 days, much less than conventional protocol of 20–25 days. Some studies suggest that women who receive milder approaches in ovarian stimulation could be more prone to face a new treatment attempt compared with women receiving a standard protocol: In fact, the psychological burden of treatment is one of the most frequent causes of dropout, and a significantly lower dropout rate was observed in more patient-friendly "mild" stimulation programs [19, 20].

Low Score of Oocytes or Embryos by Morphology for the Last Conventional Ovary Stimulation Cycle

Several reports [21] demonstrated that the chromosome error rate was higher when more oocytes were retrieved. This study demonstrated that a high oocyte yield resulted in more chromosomally abnormal embryos, particularly in younger women. Study by Katz-Jaffe [22] found similar results, demonstrating that the likelihood of segregation errors seen in early embryo cleavage states is reduced with mild stimulation. A randomized trial concerning the chromosomal constitution of human embryos following mild ovarian stimulation for IVF showed a significantly higher proportion of euploid embryos compared with conventional stimulation, suggesting that through maximal stimulation, the surplus of obtained oocytes and embryos is of lower quality [23]. That means, despite "mild" stimulation obtained significantly fewer oocytes and embryos, both regimens (conventional long protocol and mild stimulation) finally generated the same number of chromosomically normal embryos. This observation suggests that the reduced pharmacological interference with ovarian physiology could generate oocytes of better genetical quality.

Patients Who Could Accept for FET Instead of Fresh Embryo Transfer

One of the main side effects of CC is the effect of the thickness of the endometrium. Endometrial thinner than conventional program may affect embryo implantation rate. Additionally, because gonadotropins may counterbalance the undesired anti-estrogenic effects of the clomiphene on the endometrium [7, 10] which has been held responsible for the relatively low embryo implantation rates observed, this combination might lead to improved pregnancy rates compared with clomiphene alone. In the clomiphene combined gonadotropin cycle, patients with proper thickness of the endometrium (≥ 8 mm) also can have fresh embryo transfer.

Prefer for a Cheaper Treatment Cycle

A mild ovarian stimulation using clomiphene combined with gonadotropin is undoubtedly associated with a lower medication consumption and with a lower cost for purchasing drugs than either the conventional agonist long protocol or the antagonist protocol.

No Pre-ovulation in the Past

HCG day slightly higher LH levels may increase pre-ovulation. Patients who had a pre-ovulation history are unfit for the program of clomiphene combined with gonadotropin.

IVF Cycle Management

1. Preparation before ovary stimulation protocol is essential. The baseline evaluation should be taken place in the first three days of menstruation. If baseline levels of estradiol (less than 183.5 pmol/L or 50 pg/ml), and serum FSH less than 20 IU/L had been achieved, trans-vaginal ultrasound on the third day of menstruation. If the antral follicles diameter is among 4–8 mm, the stimulation procedure could be started.
2. Clomiphene citrate 50 mg/day (Medochemie Ltd.) was administrated orally with an extended regimen from cycle day 3 until the day of HCG trigger. At the same time, the right dosage of gonadotropin (HMG or recombinant FSH 75–150 IU per day) was added in injections. After starting 5 days of co-stimulation, on day 8 of the menopause, serum LH/E_2 and trans-vaginal ultrasound should be monitor for the ovarian response (Fig. 15.5).
3. The dosage of gonadotropin could be adjusted according to the results of the serum hormone and follicle size until the triggering day. GnRH antagonists were not routine used because LH suppression was already obtained by the extensive clomiphene citrate regimen except that the serum

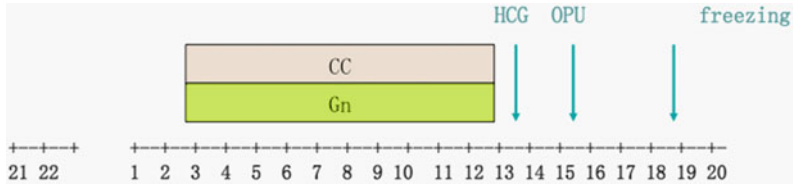

Fig. 15.5 IVF treatment workflow. CC, clomiphene citrate; Gn, gonadotropin

LH is higher than three times of the basal day 3 LH with the small follicle size that could not be trigger. If the follicle size is small and the LH increased, LH suppression may not be attained by using clomiphene citrate alone, and GnRH antagonists should be added and are still effective to prevent the LH surge and the pre-ovulation.

4. When dominant follicle diameter reached 14 mm, indomethacin could be used to prevent the pre-ovulation(0.1 g per day by rectal administration).
5. When at least two leading follicles size reached 18 mm, with appropriate serum E_2 levels, ovulation triggering with human chorionic gonadotropin (HCG) or GnRH agonist was routinely performed (Ovidrel 250ug Hst, EMD Serono, Inc.). On the morning of the trigger day need to monitor LH\P\E_2 data, clomiphene citrate was used until the trigger day.
6. Trans-vaginal ultrasound-guided oocyte aspiration (OPU) was performed approximately 36–37 h after HCG injection.
7. Either IVF or ICSI was performed according to the clinical indication.
8. On the third day of OPU(D3), fresh embryo transfers performed or delayed vitrified–warmed embryo transfer according to the thickness of endometrial, embryo-transfer procedures were performed under abdominal ultrasound guidance using a soft catheter (Sydney, Cook, Australia). No more than two embryos were transferred in order to avoid triplet pregnancies.
9. Frozen embryo transfer (FET) were usually performed during the next-month period following the oocyte retrieval.
10. The luteal phase was supported by administering 20 mg/d Dydrogesterone Tablets (Abbott Healthcare Products B.V.) and Utrogestan 400 mg/d (Progesterone Capsules, Besins Manufacturing Belgium) for 14 days. Pregnancy was assessed by serum hCG assay after 14 days from embryo transfer and then confirmed when a gestational sac was visualized at vaginal US after two to three further weeks.
11. About thawing cycle management. Thawing cycles were performed on a nature cycle or control ovarian stimulation or hormone replacement cycle: Trans-vaginal ultrasound was adjusted to the follicle size or the thickness of endometrium. HCG was used when a dominant follicle reaching 18 mm diameter. A maximal number of two thawed embryos were transferred three days later after the ovulation, or the fifth day of the progesterone used in the hormone replacement cycle;

Our Preliminary Data of Mild Stimulation of Clomiphene Combined with Gonadotropin

Preliminary Data for Poor Responders or Slow Responders

Mild stimulation of clomiphene combined with gonadotropin was used for 63 patients that were poor responders or slow responders in the pre-treatment IVF cycle in 2010–2011 in our center. The average age is 32.5 ± 4.2 years, basal FSH average is 8.6 ± 3.2 IU/L, average antral follicle

Table 15.1 Baseline characteristics of the patients (n = 63)

Characteristics	
Age (year)	32.5 ± 4.2
Basal FSH (IU/L)	8.6 ± 3.2
AFC	5.9 ± 3.2
Gn duration (day)	8.3 ± 1.8
Gn dosage (IU)	831.0 ± 287.8
E_2 on HCG day (pmol/L)	8948.2 ± 5320.2
LH on HCG day (IU/L)	7.2 ± 3.5
P on HCG day (nmol/L)	3.6 ± 2.1
Thickness of endometrium on HCG day (mm)	7.0 ± 1.5

Table 15.2 IVF outcome of the patients (n = 63)

IVF outcome	
Number of oocyte retrieval	5.2 ± 3.2
High score of embryo (%)	163/214 (76.2%)
Cancel rate (%)	15.9% (10/63)
Cumulative clinic pregnancy rate (%)	39.6% (21/53)
Cumulative live birth rate (%)	35.8% (19/53)
Pregnancy loss rate (%)	4.8% (1/21)
Ectopic pregnancy rate (%)	4.8% (1/21)

count is 5.9 ± 3.2, average stimulate duration is 8.3 ± 1.8 days, average total Gn dose is 831.0 ± 287.8 IU, average levels of estrogen are 8948.2 ± 5320.2 pmol/L on the HCG trigger day, progesterone levels are 3.6 ± 2.1 nmol/L, and mean endometrial thickness is 7.0 ± 1.5 mm (Table 15.1). The average number of oocytes retrieved per cycle was 5.2 ± 3.2, the average number per embryos transferred was 1.6 ± 0.5, the cumulative clinical pregnancy rate was 39.6%, cumulative live birth rate was 35.8%, and pregnancy loss rate was 4.8% (Table 15.2).

Preliminary Data for Normal Responders

For the exciting result of clomiphene combined with gonadotropin used for the poor responders or slow responders, we planned the followed treatment for normal response patients. A whole combination of clomiphene and gonadotropins protocol had been applied for 652 IVF/ICSI cycles for normal response in our center in 2012–2013. The baseline characteristics and IVF outcome of the patients of three different stimulation protocols were retrospectively analyzed including conventional agonist long protocol, clomiphene combined gonadotropins (CC + Gn), and antagonist protocol. Although the baseline characteristics were difference (Table 15.3), the average age of CC + Gn group was older than the other two groups. Average basal FSH was higher. But the average duration of ovarian stimulation and average total dose of follicle-stimulating hormone (IU) were decreased. The oocytes per retrieved and average embryos per transfer in CC + Gn group were less (Table 15.4). The implantation rate and term cumulative pregnancy rate were similar in three groups (Table 15.5). The term cumulative live birth rate was little low in CC + Gn group for the less retrieved oocytes and embryo. The moderate or severe OHSS rate (requiring hospitalization) was overall low in CC + Gn group. The premature ovulation had been observed only in 2 cycles. According to retrospective analysis, the outcome of three different treatment programs found that the program can achieve the similar pregnancy outcome as the other two schemes. At the same time, both the dosage of gonadotropin and the OHSS rate was decrease. Above all, this protocol really meets the ends of mild stimulation, and Clomiphene may control the endogenous LH peak.

The New Features of Mild Stimulus Package

1. Mild ovarian stimulation to achieve target number of oocytes 5–8.
2. Clomiphene combined with gonadotropin could lead to a reduction in the amount of gonadotropins required due to the combined synergistic effects.

Table 15.3 Baseline demographics and clinical characteristics of patients

	Agonist long protocol (n = 722)		CC + Gn (n = 671)		Antagonist protocol (n = 912)		P
	Mean	SD	Mean	SD	Mean	SD	
Age (year)	29.00	3.79	31.13	4.32	29.65	3.95	<0.01
Infertility duration (year)	3.96	2.75	4.64	3.16	4.23	2.69	<0.01
BMI (kg/m^2)	21.90	2.68	22.13	2.91	22.02	2.81	0.31
Basal FSH (IU/L)	7.18	1.72	8.00	2.60	7.51	2.27	<0.01
Basal E_2 (pmol/L)	157.71	93.68	143.49	111.67	118.65	85.00	<0.01
Basal LH (IU/L)	5.09	3.46	4.54	2.54	5.16	3.24	<0.01
AFC	14.47	5.28	12.03	5.69	14.46	6.12	<0.01
Duration of ovarian stimulation (days)	9.62	1.58	8.46	1.55	9.07	1.66	<0.01
Total dose of follicle-stimulating hormone (IU)	1664.25	482.78	960.53	370.15	1796.67	554.68	<0.01
Thickness of endometrium (mm)	11.18	2.30	7.57	2.29	10.44	2.12	<0.01
E_2 (pmol/L) on HCG day (pmol/L)	17019.83	8214.37	11265.53	6643.80	14830.78	8267.98	<0.01
LH on HCG Day (IU/L)	3.60	2.14	8.43	5.98	3.66	6.42	<0.01

Table 15.4 Cycle-specific characteristics of IVF cycles

	Agonist long protocol (n = 722)		CC + Gn (n = 671)		Antagonist protocol (n = 912)		P
	Mean	SD	Mean	SD	Mean	SD	
Number of oocytes per retrieval	10.72	5.20	6.36	3.98	11.20	5.69	<0.01
Two pronuclear	6.21	3.91	3.94	2.90	6.90	4.37	<0.01
Cleavage embryo	6.03	3.89	3.81	2.87	6.69	4.32	<0.01
Number of embryos per retrieval	5.15	3.68	3.31	2.62	5.57	3.91	<0.01
High score embryo	4.24	3.39	2.72	2.39	4.63	3.67	<0.01
Number of cryopreserved embryos per fresh embryo transfer cycle	3.64	3.55	2.58	2.52	4.11	3.96	<0.01

Table 15.5 Clinical result for different ovulation programs of IVF treatment

	Agonist long protocol (n = 722)	CC + Gn (n = 671)	Antagonist protocol (n = 912)
Number of embryos per transfer	1.74	1.60	1.75
Embryo implantation rate	36.5% (599/1639)	33.38% (470/1408)	32.71% (721/2170)
Term cumulative pregnancy rate	67.17% (485/722)	59.31% (398/671)	62.39% (569/912)
Term cumulative live-birth rate	64.6% (312/483)	44.32% (195/440)	68.04% (428/629)
Miscarriage rate	11.34% (55/485)	12.31% (49/398)	12.82% (73/569)
Moderate or severe OHSS rate	3.87% (28/722)	2.38% (16/671)	2.6% (24/912)

3. Continued conjunction of clomiphene can prevent premature LH peak and avoid eggs early escape.
4. Clomiphene supports the role of luteal good.
5. The total dosage of gonadotropin is greatly reduced.
6. Clomiphene is cheaper, possible alternatives than antagonists.
7. Endometrial thinner than conventional Gn program may affect embryo implantation rate, due to intimal no fresh embryo transfer.
8. HCG day slightly higher LH levels may increase the risk of egg early escape and yet to be confirmed.

Discussion

It is easy to calculate that the live birth rate/inseminated oocyte is extremely low in human IVF, on the average around 2–4% [24]. Thus, the complex and demanding ovarian stimulation protocols are usually applied in order to compensate for the poor laboratory effectiveness. However, the efficiency of IVF procedure has markedly improved from the 1980s until now: Is it still necessary to work on a high number of oocytes to get a baby? Despite "mild" stimulation obtained significantly fewer oocytes and embryos, both regimens finally generated the same number of chromosomically normal embryos. This observation suggests that the reduced pharmacological interference with ovarian physiology could generate oocytes of better genetical quality.

Clomiphene joint gonadotropin whole stimulus package, give full play to the role of efficient induction of ovulation, gonadotropin dose average reduce about 1000 units compared with conventional stimulation cycle. Clomiphene may control the endogenous LH peak and does not depend on antagonists. Mild stimulation by clomiphene combined with gonadotropin leads to good clinical outcomes, really has a mild stimulus safe, efficient, inexpensive, and comfortable advantage, and can be used as a mainstream program. The "mild" protocol has several advantages in comparison with the classical one: It is more patient-friendly and has less frequent side effects; it is better tolerated, it bears a very low risk of ovarian hyperstimulation syndrome (OHSS), it is cheaper as much less medications are used, and it is quicker as it lasts for only 10–14 days.

Clomiphene additional features because of the central inhibition of estrogen receptor strong endogenous LH are difficult to form, while the secretion of gonadotropin feedback is more durable and easier to produce multiple follicles and follicular sustained growth of the phenomenon, which is characterized by IVF stimulation program needs. In addition to greater risk of embryo freezing, or more surplus embryos and endometrial thickness compliance cycle, usually whole embryo freezing in fresh cycle, after 1–2 cycles and embryo transfer in natural cycles.

The conventional agonist protocol prevents the LH surge and leads to multifollicular recruitment. However, the agonist protocol has many side effects, including formation of ovarian cysts and symptoms of estrogen deprivation such as hot flushes, vaginal dryness, and headaches.

The definition of success could be further refined to incorporate chances for term live birth (or healthy child) per IVF treatment period (which could include several cycles) in relation to cost, patients' discomfort, and risks of complications.

The chance that IVF can produce a healthy baby (or babies) needs to be weighed against the discomfort and risks of complications and costs associated with the treatment. Adoption of the endpoint of IVF treatment would encourage patient-friendly stimulation protocols and single embryo transfer [6].

Conclusion

Mild stimulation by clomiphene combined with gonadotropin leads to good clinical outcomes, really has a mild stimulus safe, efficient, inexpensive, and comfortable advantage, meets the ends of mild stimulation, may represent an important step toward the objective of an easier IVF, and may be used as a mainstream program.

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Mild Stimulation Protocol for Poor Ovarian Responders Undergoing IVF

16

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Introduction

The incidence of poor ovarian responders (PooResp) among infertile women undergoing controlled ovarian stimulation (COS) for in vitro fertilization (IVF) has been estimated at 9–24%, but according to recent reviews, it seems to have slightly increased in the last years [1]. Despite the huge number of trials that have been performed and published in the last two decades with the aim to find more efficient stimulation protocols for the management of PooResp, systematic reviews suggest that we still have insufficient evidence to recommend a specific treatment, the poor ovarian response remaining one of the most challenging tasks in reproductive medicine [2]. Several studies included a too small number of patients, and, above all, an unclear definition of PooResp led to the inclusion in the clinical trials of heterogeneous groups of patients [2]. Although the concept of poor ovarian response was introduced over 30 years ago, we had no clear definition of PooResp until 2011, when the ESHRE Bologna criteria were published [3].

A poor ovarian response to COS could result from the use of a suboptimal COS protocol, and largely more frequently, by the very limited follicular content inside the ovary. Although it is self-evident that "no juice can be obtained by a dry lemon," the effort of finding at least the best protocol among those available goes on.

The Classical Strategy of COS in PooResp

One of the most extensively employed strategies to get a satisfactory follicular response in PooResp has traditionally involved the use of high doses of gonadotropins [4]. However, conflicting data have been reported on this approach: Some authors [5–7], even if not all [8], did not report the enhanced ovarian response and/or better pregnancy rates when the starting dose of gonadotropins was increased up to 450 IU, in both prospective and retrospective studies. More recently, Berkkanoglu and Ozgur [9] confirmed that the increase in FSH starting dose did not result in higher pregnancy rates and also found no differences in IVF outcome when the starting dose of 300, 450, and 600 IU were compared. PooResp have a reduced ovarian reserve and the recruitable follicles are a few; for this reason, even high gonadotropin amounts can just promote the development of the available follicles receptive to stimulation, and cannot, obviously, manufacture follicles de novo [2].

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The Mild Stimulation Strategy for PooResp: Clomiphene Citrate

Mild controlled ovarian stimulation (mCOS), the administration of low doses of exogenous gonadotropins for fewer-than-normal days in a GnRH-antagonist cycle, has been recently proposed as a patient-friendly COS strategy, able to increase the patient's compliance to IVF without compromising IVF effectiveness. Some oral compounds (such as Clomiphene citrate or aromatase inhibitors) have been proposed to be a suitable alternative to conventional IVF COS not only for women with an adequate ovarian reserve, but also for PooResp [10]. The hypothesis at the basis of this strategy is that patients with a few available eggs could benefit from receiving a milder COS not only as far as treatment acceptability is concerned but also producing oocytes with a better competence.

The level of evidence supporting the use of a "mild" stimulation protocol with CC/gonadotropins/GnRH-antagonist in patients with poor ovarian reserve is still poor, as properly designed studies on an appropriate number of patients are still unavailable [11]. Anyway, it has been studied in some trials.

Clomiphene citrate (CC) is known to act as an anti-estrogen on the central nervous system, increasing the pulse frequency of endogenous FSH and LH and giving a moderate gonadotropin stimulus to the ovary [12]. CC has been used for over thirty-five years to induce ovulation in WHO type II anovulatory women, and is still appreciated for the possibility of oral administration and for the low price; the combination with gonadotropins may counterbalance its undesired anti-estrogenic effect on the endometrium and at the same time may reduce the amount of gonadotropins required, thanks to the combined synergic effect on the ovary [11]. Clomiphene citrate, as adjuvant therapy in GnRH-antagonist protocol, is more effective when associated with medications containing LH or hCG rather than FSH alone because when in CC/gonadotropins/GnRH-antagonist cycles the circulating level of LH at the time of hCG drops to less than one-third than it was at the beginning of stimulation, both the pregnancy and the implantation rates are significantly reduced [13].

The first report describing the use of CC/gonadotropins/GnRH-antagonists in PooResp included only eighteen patients [14]; compared to their response in a previous standard GnRH-agonist cycle, light improvements in cycle cancellation rate, oocyte yield and gonadotropin requirement were observed. Unfortunately, neither the number of patients nor the study design allowed to draw definitive conclusions.

Some years later, Takahashi [15] studied 40 poor responders with a story of multiple IVF failures with the "long" protocol: Treating them with CC/FSH/GnRH-antagonist, he obtained an ovarian response comparable to the previous ones, but a significantly higher blastocyst development rate and a very good (41.2%) ongoing pregnancy rate. Again, the study design was not very informative and the number of patients was too little, but it suggested that a mild approach could obtain oocytes of higher competence toward embryo development.

We recently published a large prospective randomized trial comparing in expected PooResp patients the classical long protocol with GnRH-agonist and high daily dose of gonadotropins (300–450 IU) to a "mild" protocol with CC plus low dose gonadotropins (150 IU daily) and GnRH-antagonist [16]. In both groups, hMG or a combination of recombinant FSH plus recombinant LH was used to stimulate the ovary, because some LH activity was proven to be beneficial in PooResp [13, 17]. Patients stimulated with the "mild" protocol had significantly shorter follicular phase, received a significantly lower amount of exogenous gonadotropins (2237 IU vs. 5265 IU), and reached a lower peak estradiol level than patients stimulated with the "long" protocol. We observed that the endometrium at oocyte pickup was significantly thinner in the "mild" group than in the "long" group, both because circulating E2 was lower and because CC is known to exert a peripheral anti-estrogenic action able to affect endometrial proliferation. The "mild" strategy was more frequently associated with a failure in retrieving

oocytes, and the "long" protocol allowed to retrieve significantly more oocytes (4.8 vs. 2.7 per ovum pickup, respectively), finally leading to have more embryos available for transfer. Despite this, however, the clinical pregnancy rate per completed treatment was 23.2 and 19.9% for the "mild" and "long" groups, respectively, with no significant differences between the two stimulation regimens [16]. Our study, that to date is the largest prospective randomized trial comparing a "mild" stimulation protocol with CC to the conventional "long" protocol in PooResp, has the limitation that it was planned and performed before the publication of Bologna criteria and included a population of patients who were shown to have a poor response in a previous COS with a "long" regimen.

Another large, but retrospective, study including 258 PooResp compared the outcome of "mild" versus "conventional" COS protocols. In the mild group, patients were given either 100 mg of CC or 5 mg of letrozole daily for five days, followed by the administration of 150–225 IU of either recombinant FSH or hMG [18]. The control group, instead, received daily more than 300 IU of gonadotropins, in association with GnRH-agonist or antagonist. Once again, the duration of COS, the total gonadotropin dose (901 IU vs. 3165 IU), the serum E2 level on hCG administration day, the endometrial thickness, and the total number of retrieved oocytes (1.5 vs. 1.9) were lower in the mild group, but no significant difference in the clinical pregnancy rate was found (18.4% vs. 22.6% for "mild" and "conventional" protocols, respectively). Moreover, the number of good scored embryos and the cancellation rate were also comparable between the two groups. Again, however, not all the included patients satisfied the Bologna criteria, as poor ovarian response was defined, instead, according to a basal FSH level above 12 IU/mL, a number of retrieved oocytes not exceeding three, and E2 level below 500 pg/mL on the day of hCG administration. In addition, this study has the limitation to be a retrospective design and to include a data analysis which was not stratified on the basis of the therapy used (CC or letrozole) and of the type of gonadotropins administered (FSH only or FSH+LH/hMG).

Another recent study evaluated the effectiveness of CC treatment in PooResp undergoing IVF [19]. Thirteen patients fulfilling the Bologna criteria received first a COS based on hMG alone and subsequently, in another attempt, hMG plus CC. In 20 cycles, 150–300 IU of hMG was given once every second day starting on day 3–5 of the menstrual cycle; whereas in 46 cycles, 150–300 IU of hMG was concurrently administered every other day in association with 100–150 mg/day of CC, started on day 3–5 and continued until the dominant follicle reached 12 mm mean diameter. This study showed that the patients receiving CC needed a significantly lower total dose of hMG (2611 IU vs. 3643 IU, respectively), even though there was no significant difference in peak estradiol concentration. Women treated with hMG alone obtained more oocytes (3.6 vs. 2.5), but the number of good quality embryos was comparable in the two groups. Pregnancy was obtained in 4 out of 13 cases, all treated with CC+hMG. Overall, hMG +CC stimulation was shown to have an acceptable effectiveness in PooResp undergoing IVF; however, a low number of patients were allowed to get a very limited level of evidence.

The Mild Stimulation Strategy for PooResp: Aromatase Inhibitors

Aromatase inhibitors (AI, such as letrozole) have been introduced as alternative ovulation induction agents either alone [20] or in association with gonadotrophins [21]. It was reported that transient inhibition of aromatase activity in early follicular phase (days 5–9) with letrozole resulted in ovarian stimulation similar to CC with no apparent adverse effect on the endometrium [22]. Furthermore, letrozole was observed to cause temporary accumulation of androgens in the ovarian follicles by blocking the conversion of androgens to estrogens, with the effect of increasing the sensitivity of the growing follicles by increasing the FSH receptors [23].

Letrozole is still not approved by FDA for use in ovulation induction and COS [24]. A major concern of using this drug is its possible teratogenicity [25]: Animal data showed embryo and fetal death in rats and rabbits, and congenital malformations (kidney and ureter) and altered sexual function in male offspring [26, 27]. However, data from humans were reassuring as no congenital abnormality was linked to the use of letrozole [28]. This compound has a short half-life (45 h) and there is a sufficient time interval for complete clearance after its administration in the early follicular phase, so the drug should not be present during fertilization and embryo implantation [29].

Studies reported that the addition of letrozole improved ovarian response to FSH in PooResp and reduced gonadotrophins requirement for COS in women with unexplained infertility [30, 31]. Healey [32] demonstrated that the addition of letrozole to gonadotrophins in GnRH-antagonist protocol increased the number of preovulatory follicles without having a negative impact on pregnancy rates. These reports prompted the scientific community to hypothesize that adjunctive use of letrozole in COS protocol could open the future prospects toward a cost-saving stimulation protocol for IVF in women with poor ovarian response.

To date, however, the effectiveness of the use of letrozole for poor responders is still controversial [33]. In most studies, letrozole is administered with high doses of gonadotropins (300–450 IU daily) [24, 34–38] and there are only a few trials testing the efficacy of letrozole in "mild" stimulation protocols. Goswani [39] evaluated in a randomized single-blind controlled trial a "low-cost" IVF protocol for PooResp, consisting of letrozole (2.5 mg daily from day 3 to 7 of the menstrual cycle) plus low dose of recombinant FSH (75 IU daily on day 3 and 8 of the menstrual cycle). Forty-eight women over 35 years of age, who had failed one to three IVF attempts due to poor ovarian response to conventional long GnRH-agonist protocol, were selected for this study. Compared to the classical "long" protocol with high starting dose of gonadotropins (300 IU of r-FSH), the low-cost protocol ensured the same IVF outcome; the two groups, in fact, did not differ significantly with respect to the number of matured follicles, the number of retrieved oocytes, and the number of transferable embryos. Also the pregnancy rates were comparable. The letrozole group, however, received an approximately 20-fold lower total FSH dose and had significantly decreased levels of peak estradiol. This preliminary study had encouraging results, but involved a very small number of patients (13 cases and 25 controls).

In 2011, Lee [40] conducted a randomized trial to compare the sequential use of letrozole and hMG with hMG alone in PooResp undergoing IVF. Women were recruited if they had less than four oocytes retrieved in their previous failed IVF cycles or an antral follicle count lower than five. In the letrozole group (26 patients), patients received letrozole 2.5 mg daily for 5 days (from day 2 to 6 of menstrual cycle) followed by a fixed daily dose of hMG (225 IU). In the control group (27 patients), patients were given a fixed daily dose of 225 IU hMG from day 3 onward. In both groups, the GnRH-antagonist ganirelix (0.25 mg/day) was administered in a flexible way, starting when the leading follicle reached 18 mm in diameter. The letrozole group needed a significantly lower dose of hMG, had a significantly shorter duration of stimulation and lower serum estradiol concentrations when compared with the control group; also the number of retrieved oocytes was significantly lower, while the number of transferred embryos, the implantation rate, the ongoing pregnancy rate, the live birth rate, and the cancellation rate were comparable between the two groups.

These data substantially confirmed the results of the previous trial [39], unless for the number of oocytes retrieved, which was significantly lower in the letrozole group. Actually, the average number of retrieved oocytes in the letrozole group of Lee was similar to that of Goswani's trial (2.0 vs. 1.6, respectively), despite the bigger total gonadopropin dose (1507 IU of hMG vs. 150 IU of recombinant FSH). The difference reported between cases and controls in the study of Lee is linked to the high number of retrieved oocytes in the control group: PooResp treated

with 225 IU/day hMG in association with GnRH-antagonist obtained an average of four oocytes, whereas the PooResp treated by Goswani with the long GnRH-agonist protocol and high doses of gonadotropins (300 IU), only two [39]. Indeed, some studies have shown that GnRH-antagonists protocols could ensure a more natural follicular recruitment than GnRH-agonists with a possibly better outcome in terms of oocyte yield [41, 42].

Another study evaluated the efficacy of letrozole in a "mild" IVF protocol [18]. In this retrospective study, patients who received letrozole in a mild regimen had a lower duration of stimulation, lower total dose of gonadotropins, and lower endometrial thickness than controls. In women treated with the "mild" protocol with letrozole, the average number of retrieved oocytes was 1.5, significantly lower than that in the control group.

A recent randomized controlled trial by Mohsen [43] analyzed the IVF outcome of letrozole/GnRH-antagonist protocol with low hMG dose (150 IU daily from cycle day 7) versus a GnRH-agonist mini-flare-up protocol with 300 IU of hMG. The recruited PooResp were women with previous failed IVF attempts due to poor ovarian response to conventional GnRH-agonist long protocol. The trial showed that the letrozole+GnRH-antagonist protocol, compared to the mini-flare-up GnRH-agonist protocol, did not improve the fertilization rate or the clinical pregnancy rate, but the length of stimulation and the dose of gonadotropins were significantly lower (1764 IU vs. 5392 IU, respectively). The mean number of retrieved oocytes was the same in the two groups: 5.14 in letrozole group versus 5.11 in the mini-flare-up group. Despie the absence of a difference statistically significant between cases and controls, what astonishes is the average number of oocytes recovered in the letrozole group, which is much higher described in the former studies [18, 39, 40]. This difference may be due to the heterogeneity of patient inclusion criteria. Unfortunately, the number of patients was again too little (16 cases and 30 controls) to get an acceptable level of evidence.

Conclusion

Taken together, the results summarized herein suggest that in patients with poor ovarian reserve, "mild" COS with GnRH-antagonist and adjuvant drugs (letrozole, Cce) may give benefits in reducing the total dose and the duration of gonadotrophin administration, ensuring, at the same time, a comparable pregnancy rate. When a mild COS protocol was used in PooResp, even with a low number of retrieved oocytes good quality embryos were produced, and acceptable fertilization rate, clinical pregnancy rate, and live birth rate were observed. Therefore, letrozole/CC +gonadotropins+GnRH-antagonist regimens may represent a valuable and cost-effective alternative for COS of PooResp undergoing IVF.

Some doubts are still not solved due to the heterogeneity between the studies in the definition of PooResp and to the limited number of available observations.

More sufficiently powered randomized studies with large numbers and homogeneous populations, based on a clear definition of poor response, are needed to assess the true value of a "mild" stimulation protocol for PooResp.

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The Application of Mild Stimulated Cycle IVF in Primary Ovarian Insufficiency

17

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Introduction

Primary ovarian insufficiency (POI), commonly referred to as premature ovarian failure (POF), is defined as the cessation of ovarian function with follicle-stimulating hormone (FSH) concentrations exceeding 40 IU/L before 40 years of age, resulting in amenorrhea, infertility, and other systemic consequences (e.g., cardiovascular diseases and osteoporosis) because of estrogen deficiency [1]. POI is first brought to light by Fuller Albright in 1942 [2], and it affects approximately 1% of women at the age of 40, 0.1% of women at the age 30, and 0.01% at the age 20 [3]. About 76% of POI patients developed after normal puberty and establishment of regular menses [4].

Multiple causes contributing to POI include environmental factors, genetic background, autoimmunity, metabolism, and iatrogenic factors. However, the cause of POI remains undetermined in most cases [5]. Familial POI research showed that 4–30% of all subjects with POI had a familial form [6], which implied a genetic predisposition to POI. We found that ESR1 PvuII and XbaI polymorphisms were correlated with POF, while no association was found for FST, adiponectin gene, and FMR1 premutation to POF [7–10]. In addition, we conducted a meta-analysis to investigate the association between gene variants and POF. The results showed that BMP15 538A, FMR1 premutation, and INHA 769A (in Asians alone) may indicate susceptibility to POF [11]. Interestingly, a novel gene HFM1 mutation was identified in Chinese POI patients [12]. A recent study reported that variations in BMP15 gene dosage have a relevant influence on ovarian function and can account for several defects in female fertility. The modulation of BMP15 action may have interesting pharmacological perspectives, and the analysis of BMP15 may become a useful marker in IVF procedures [13].

Women with POI extremely rarely ovulate and achieve pregnancy spontaneously [14], so infertility is an important issue in POI/POF patients. Numerous treatment protocols for follicular development and ovulation induction have been tested in patients with POI, and none have been shown to be effective [15]. There are various therapeutic interventions before IVF included clomiphene citrate, gonadotropins, estrogens, GnRH analogues, oral contraceptives, corticosteroids, dehydroepiandrosterone (DHEA), or a combination of these [16]. Therefore, oocyte donation is chosen to be a unique opportunity in the treatment of infertility with POI, although about 5–10% are able to obtain pregnancy after the POI diagnosis.

Oocyte donation and IVF has been used as an aid for conception for young women with POI for over 20 years. In other words, the IVF with donor eggs is a practical treatment strategy and a hope for women with POI to become a mother. Generally, the cryopreserved embryos have been employed

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for ovum donation in POI with a high pregnancy rate of 30% per transfer [17]. In the reproductive center of our hospital, the First Affiliated Hospital of Nanjing Medical University, analysis of 89 oocyte donation IVF-ET cycles showed that implantation cycle rate was 91.0% (81/89), biochemical pregnancy rate was 40.7% (33/81), and clinical pregnancy rate was 37.0% (30/81).

As all known, the first successful IVF treatment was performed in a natural cycle [18]. Generally, natural cycles in IVF have been used for patients who have shown a poor response in at least two previous attempts with gonadotropin stimulation [19]. Some studies showed that [19, 20] the developed follicles of poor responders in natural cycles may produce fewer oocytes but oocytes of better quality than their hyperstimulated ovaries [21]. Furthermore, it is also less expensive and more time-efficient, avoids most of the risks and complications of ovarian stimulation, spares the endometrium from the adverse effects of ovarian stimulation, and is more psychologically friendly to the IVF patients [21]. Concerning the natural cycle in IVF used for the patients with POI/POF, there has been only one study carried out in Japan, in which they reported four women with POI who achieved pregnancies resulting from intrauterine insemination in combination with cyclic estrogen/progesterone therapy and close monitoring of follicle development [22].

Indeed, POI patients, whether or not they desire pregnancy, should be treated with a combination of estrogen and progesterone hormone replacement therapy (HRT) to minimize the bone loss, decrease the risk of cardiovascular events, and relieve the vasomotor flushes and vaginal dryness [14]. Meanwhile, pregnancy may occur while a woman is undergoing estrogen and progestin therapy, suggesting that this might be a method to improve fertility in POI women. Theoretically, estrogen replacement therapy might improve ovulation rates in women with spontaneous POI by reducing the associated chronically elevated serum LH levels to normal [23]. Interestingly, Naredi et al. [24] reported that the pregnancy rate in women after an oocyte donation cycle was 40% in subjects who received HRT prior to their cycle, while only 25% conceived in the non-HRT group, although the difference did not statistically significant.

We reported two cases of POI who achieved pregnancy by modified natural cycle IVF in reproductive center of the First Affiliated Hospital of Nanjing Medical University as follows.

Case 1

A 29-year-old patient, a nulligravida with 4-year history of primary infertility with POI, was diagnosed in our reproductive center. Her karyotype is 46, XX, without a history of chemotherapy or radiotherapy, but she had received a laparoscopic cystectomy for bilateral endometrial cysts at 25 years. Her basal serum levels of FSH, LH, and E2 were 48.3, 10.6 IU/L, and 125.9 pmol/L, respectively. Only one AFC in the left ovary and one in the right ovary, separately, can be seen by trans-vaginal ultrasonography. She had undergone a total of five natural or mild stimulation IVF cycle treatments in past three years. For her successful cycle, which occurred at 32 years of age, the serum levels of FSH, LH, and E2 were 16.1, 1.5 IU/L, and 235.3 pmol/L, respectively, on day 3 of a natural cycle. On day 9 of the cycle, there was a single follicle with an average diameter of 15.3 mm and serum levels of LH, E2, and P were 24.3 IU/L, 1248.7 pmol/L, and 4.84 ng/L, respectively. Ovulation was triggered with 0.1 mg of Diphereline. After 36 h, one MII oocyte was retrieved and fertilized by ICSI. Eight-cell embryo was transferred on day 3 after fertilization. Pregnancy was confirmed on day 14 after embryo transfer. The patient has achieved ongoing pregnancy, and she delivered her baby at 37 week's gestation.

Importantly, during the POI patients in natural cycle IVF, the serum levels of FSH and E2 are closely monitored each month, which were usually measured at day 2–3 after withdrawal bleeding of HRT. If the serum FSH level is

<20 IU/L, the natural cycle IVF treatment will be performed. The initial serum FSH and E2 levels may help manage POI patients in the IVF cycle, which suggest that the initial FSH and E2 levels may be predictive of subsequent follicle development in the corresponding cycle. This schedule is less burdensome to patients than ovarian stimulation protocols employing exogenous gonadotropins. Tartagni et al. [25] suggested that POI patients in whom the pretreatment FSH level is <15 mIU/mL might ovulate in response to exogenous gonadotropins according to their results.

Besides, the modified natural cycle also seems to be an appropriate strategy for the POI patients according to our reports.

Case 2

The patient, a 30-year-old woman, was diagnosed with POI at 26 years of age with amenorrhea in our outpatient clinic. Her karyotype is 46, XX, without a history of chemotherapy or radiotherapy, and basal FSH level was about 65 IU/L (two times). She has been treated with HRT for one year since the age of 28, and her basal serum FSH level was decreased to below 20 IU/L. She returned to our clinic desiring pregnancy at the age of 30. We monitored closely her FSH level in order to give her IVF treatment. However, the first three cycles of IVF were canceled because the serum FSH level was over 20 IU/L. In the fourth IVF treatment cycle, the serum levels of FSH, LH, and E2 were 18.3, 4.2 IU/L, and 145.9 pmol/L, while there was only one AFC in the left ovary and none AFCs in the right ovary. On day 13 of the cycle, she started to be injected with 75 IU HMG every other day because the diameter of left follicle was 9.5 mm. On day 17, the diameter of dominant follicle was 15 mm, and serum levels of LH, E2, and P were 28.7 IU/L, 933.9 pmol/L, and 1.7 ng/L, respectively. One MII oocyte was retrieved using ultrasound-guided trans-vaginal needle aspiration on day 18 without trigger. The oocyte was fertilized in vitro, and 8-cell embryo was transferred on day 3 after fertilization. Pregnancy was confirmed on day 14 after embryo transfer. The pregnancy was then supplemented with progesterone. The patient has achieved ongoing pregnancy, 24 weeks' gestation.

Conclusion

POI/POF remains a clinically challenging entity because IVF with donor oocytes is currently the only treatment known to be effective. Fortunately, the modified natural cycle IVF may be promising for the POI patients in the future. Importantly, the initial FSH and E2 levels should be monitored and follicle development with trans-vaginal ultrasonography and/or hormonal measurements is recommended for POI patients. Finally, large-scale prospective studies should be performed in order to evaluate these interesting results.

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Mild Stimulation Protocols for Oncofertility Patients

18

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Introduction

Oncofertility is an interdisciplinary field which considers the consequences of gonadotoxicity associated with cancer therapies and tries to facilitate fertility preservation when possible. The effects of cancer on fertility can be severe and vary depending on the age of the patient, the type of cancer and the type of chemotherapy or radiotherapy, or surgical treatment used [3]. The oncology clinic must provide patients with sufficient relevant information regarding fertility preservation to aid them in decision-making concerning possible approaches for fertility preservation before cancer treatment. Fertility is one of the aspects of quality of life that men and women find most important in the years after successful treatment of their cancer [4].

This paper covers the practical aspects of cryopreservation of gametes or embryos for cancer patients prior to the treatment. We will cover the process from point of diagnosis, initial consultation with the fertility team, procedure involved, post-fertility team management and long-term follow-up.

Diagnosis

From the point of diagnosis of cancer, it is important for the oncologist to discuss not only the treatment plan for cancer, but also the options of fertility preservation. Patients with cancer may have reduced fertility as a result of their condition. The oncologist will need to state the potential impact of treatment on fertility, the time frame the patient has prior to the start of the treatment, and the safety of fertility preservation based on the type of cancer and its treatment. This ensures that patients are given sufficient information at an early stage, along with the necessary referral to a specialist in oncofertility. Oncologists are understandably preoccupied with delivery of treatment for a potentially life-threatening condition and may not have future fertility problems on their list of important topics to discuss. However, given the importance that many patients ascribe to fertility, this should be addressed by the oncologist. Fertility clinics can assist by providing up-to-date information and contact details.

Initial Consultation

This will be the first consult with the fertility team. This consult gives an opportunity for the fertility team to assess the risk of damage to gonadal function posed by the proposed therapy, the patient's desire for fertility preservation and their social situation, the patient's overall

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medical condition and the time available prior to the start of cancer therapy. Not all patients will be concerned about loss of fertility. Many may have completed their families and others may be due to receive treatment with low gonadotoxicity, rendering fertility preservation unnecessary. Some may already be too unwell to tolerate the procedures involved or have to receive chemotherapy with such urgency as to make fertility preservation impossible [5].

Strategies for fertility preservation will be discussed in the context of their specific circumstances. These include oocyte cryopreservation, embryo cryopreservation or ovarian tissue freezing for women and sperm cryopreservation or testicular tissue cryopreservation for men. Due to the aggressive nature of some cancers, ovarian stimulation may be not always be feasible, as it requires at least 10–12 days to complete, thus possibly delaying the start of cancer treatment. Factors that affect ovarian reserve, particularly female age, are the most important variables to consider for women. Age has less impact on sperm production, but the cancer may already have produced ill health in the male patient and sperm quality may be low.

Written informed consent for any procedure should be obtained before fertility preservation strategies are implemented. Counselling may be necessary before treatment, particularly as the consent process can confront the patient with decisions about what to do with frozen gametes or embryos after their death. For younger patients, consent should be obtained from the parents or legal guardian. For women, a hormone profile including follicular-stimulating hormone (FSH), anti-Mullerian hormone (AMH), estradiol (E2), luteinising hormone (LH), progesterone (P4), and an ultrasound of the pelvis is useful to determine the status of ovarian reserve. FSH, LH and E2 should be measured in the early follicular phase of the cycle, and there may not be time for this to be done. Enough information can be obtained from the pelvic ultrasound and measurement of random AMH to plan ovarian stimulation. For the male, a semen analysis can usually be performed quickly, with freezing of the remainder of that sample plus others if necessary.

An infectious disease screen should be obtained for both male and female patients. Virology tests including hepatitis B, hepatitis C and HIV, along with chlamydia, syphilis and gonorrhoea are taken. Appropriate psychological counselling will also be arranged. The fertility team will decide on the plan for ovarian stimulation. Complications such as ovarian hyperstimulation (OHSS) will be discussed and minimised as OHSS would potentially delay the start of the cancer treatment. Most patients will commence chemotherapy shortly after oocyte retrieval; therefore, the fertility team will need to balance between the necessity to cryopreserve a good number of oocytes and to avoid OHSS. Hence, the stimulation protocol to be adopted should be a compromise between a truly 'mild' stimulation, with suboptimal yield of oocytes, and an overly high-dose protocol with risk of OHSS which can compromise timely commencement of chemo- or radiotherapy.

Patient Work-Up for Oocyte and Embryo Cryopreservation

The patient will need to undergo an ovarian stimulation cycle before retrieval of oocytes. The antagonist protocol is almost invariably preferable, given the short time frame and need for avoidance of OHSS [6]. The usual duration of stimulation is less than two weeks. Ovarian stimulation involves the administration of daily subcutaneous FSH injections, with a series of ultrasound scans and blood tests to monitor the follicular growth. Dose of FSH should be individualised based on the basal AMH and patient's age [7]. The aim of stimulation is to induce the growth of a reasonable number of follicles to reach the adequate size for oocyte maturity. In the antagonist protocol, oocyte retrieval is usually scheduled when three or more follicles reach a diameter of 17 mm on ultrasound. The timing of oocyte collection can be delayed by up to two days if necessary, although for oncology patients, there may not be time for such delay [8]. If the antagonist protocol is used, then a GnRH agonist trigger can be given. This induces an endogenous

LH surge, which then produces final oocyte maturation. Oocyte collection is performed 34 h after administration of the agonist trigger. Severe OHSS is almost unheard of after use of the agonist trigger [9] and is hence the protocol of choice for oncofertility patients. It is now also clear that stimulation can be started at any time of the menstrual cycle provided that fresh embryo transfer is not planned (as is the case for oncology patients) [10]. A luteal phase start should yield a similar number of oocytes but will not induce growth of a healthy secretory endometrium, ruling out a fresh embryo transfer.

For most patients undergoing IVF, the process of fine-needle ultrasound-guided transvaginal oocyte collection is straightforward. However, care must be taken if the cancer patient is thrombocytopaenic. Haemorrhage within the pelvis from the site of puncture of the vaginal wall or ovary can be difficult to control. A pelvic haematoma can develop. This can later form a nidus for infection if the patient becomes neutropaenic during chemotherapy, leading to possible pelvic abscess formation and risk of death. Intra- and post-procedure antibiotics should be given, an extremely narrow (20–21) gauge needle should be used to minimise trauma, and the patient may need to be observed in hospital after egg collection to follow haemoglobin and white cell count for safety [11].

Following oocyte collection, it is possible either to cryopreserve oocytes (for women without a long-term partner) or, for those in a stable relationship, to fertilise the oocytes with the partners' sperm and cryopreserve embryos. The maximum duration of storage is not known, healthy pregnancies having been reported over 14 years after cryopreservation of embryos [12]. Different jurisdictions may place different legal limits on the period of time for which embryos can be stored, but these are limits determined for ethical and legal rather than biological reasons. However, it is most important to empathise during pre- and post-treatment counselling that there cannot be a guarantee of pregnancy from frozen oocytes or embryos. The likelihood of pregnancy is largely determined by the woman's age at the time of stimulation. Cryopreservation of more than 12 oocytes or 6 embryos for women under 35 years of age gives a >50% chance of a live birth, whereas the requirement is far larger for women over 40 and it is questionable whether oocyte freezing is worthwhile for this group [13].

Ovarian stimulation for cancer patients can present specific challenges. Most importantly, the patient may be unwell during stimulation. This can lead to a poor oocyte yield, which must be covered in the pre-treatment counselling. In addition, certain cancers, particularly ER-positive breast cancers, are oestrogen sensitive, and strategies to reduce exposure to high circulating oestrogen concentrations, such as the use of an aromatase inhibitor during stimulation [14], may have to be considered. Other approaches for patients with ER-positive breast cancers include unstimulated collection of a single oocyte (although this carries a low chance of later pregnancy), ultramild IVF with simulation using tamoxifen or letrozole [15], or, increasingly, in vitro maturation (IVM) [16]. IVM with no or minimal ovarian stimulation now offers acceptable pregnancy rates (although still not equivalent to those seen after IVF) [17] with little or no elevation in serum oestradiol before the collection of the immature oocytes.

Ethical issues must also be considered, particularly in cases of embryo cryopreservation. Whereas the oocyte contains only the DNA of the woman, the embryo is created from both partners and, in many countries, both are seen as having equal rights in deciding how the embryo(s) should be used. For oocyte cryopreservation, once the cancer treatment is over, the oocyte will be thawed and fertilised with sperm from the male partner, and the resulting embryo is transferred into the uterine cavity. However, in contrast, when embryos are cryopreserved, the woman may be in a new relationship by the time she wishes to start a family, and the ex-partner will be unlikely to consent to her conceiving with their joint embryos but with a new partner. Pre-treatment consent should cover this possibility and also cover the question of what should be

done with the embryos should the woman die. In theory, the man could use the embryos to have a child with a new partner, but only if written consent to allow him to make this decision unilaterally was given by the woman.

Ovarian Tissue Cryopreservation

Cryopreservation of ovarian tissue requires a laparoscopic surgical procedure to remove the ovarian tissue. The advantage of this procedure as compared with embryo cryopreservation is that it does not require hormonal stimulation, thus allowing the patient to move on more quickly to start her cancer treatment. This approach is also suitable for both pre- and post-pubertal patients. Some time after the completion of cancer therapy, the ovarian tissue will be implanted within the pelvis or subcutaneously in the anterior abdominal wall, aiming for the resumption of endocrine function and ovulation. Ovulation induction agents can be used post-transfer with a view to oocyte collection and IVF [18]. There are several drawbacks to this approach. Most importantly, the tissue must undergo a thorough pathological assessment to exclude the presence of cancer and avoid risk of regrafting the cancer to the patient [19]. Also, ovulation, pregnancy and live birth rates remain low, with less than 30 births reported in the world literature after cryopreservation of ovarian tissue for cancer patients. However, in some cases, there is no time for ovarian stimulation and oocyte collection, and the patient may be too young to undergo these procedures or may be undergoing laparoscopy for other reasons, offering the opportunity for collection of ovarian tissue.

Post-procedure Management

After the completion of the oocyte or embryo retrieval, women can begin their cancer treatment two or three days later, providing that there are no signs or symptoms of OHSS or pelvic infection. The patient should be contacted to inform them of the outcome of the cryopreservation treatment. Written records should be provided with information concerning the number of gametes or embryos stored, any storage limitations and long-term follow-up plan. Cost to patients will vary from country to country, but all patients must be made aware of any financial liabilities that they may incur, both immediately as payment for the cycle management and cryopreservation and later as annual storage charges and costs of ART procedures such as ICSI and frozen embryo transfer.

Long-Term Follow-up

Many centres have now established late effects clinics which provide a multidisciplinary team of specialists to provide long-term care to cancer survivors [20]. One aspect of care is to detect recurrence of cancer or secondary cancers that may develop as a consequence of chemotherapy or radiotherapy. Late effects clinics also manage other sequelae of treatment, including cardiac, respiratory, renal and cerebral damage, as well as reproductive health. A reproductive medicine specialist will give advice on the consequences of treatment-induced gonadal failure in both sexes, as well as help with fertility treatment using cryopreserved gametes or embryos or donor sperm or oocytes.

Conclusion

As fertility preservation technology improves, a detailed understanding of the issues pertaining to fertility preservation by oncologists as well as reproductive medicine specialists is important in ensuring that patients receive up-to-date and adequate information, both verbal and written. Fertility preservation options should be addressed by the oncologists as part of standard care at an early stage of diagnosis. The tasks involved include identifying patients who are interested in future childbearing, ensuring that they are informed of their options and risks as well as benefits, providing them with appropriate resources and

assisting with making referrals. Not all patients will choose to go ahead with fertility preservation, but those who do must have a clear appraisal of what is involved.

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Minimally Invasive IVF as an Alternative Treatment of Option for Infertility Couples: (INVO) Procedure

19

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Introduction

According to the World Health Organization in 2012, more than 80 million couples around the world are suffering from fertility problems [1]. Couple infertility has become a social problem that results in isolation, domestic violence, low self-esteem, depression, polygamy, and divorce. Also, infertility is considered as a real disease recognizing its effects on people's health and well-being highlighting that 1 from 6 couples at the reproductive age suffers from an infertility problem. In developing countries, more than 90% of infertile couples do not have access to assisted reproductive treatment (ART) due to high costs of treatments, difficult access, lack of knowledge of different options in ART, and very few in vitro fertilization (IVF) centers following social public activities [1]. Therefore, a significant aspect of new ART alternatives must be supported by psychological, economical, and physical benefits brought to the infertile couple. One of the innovative options that researchers have found is a device for incubating embryos inside of the patient's vagina as a safe and effective incubator culture for embryos. This device enable a perfect environment for human embryo development similar to CO_2 incubators used in conventional IVF treatments [2].

The INVO procedure is a simplified alternative to traditional IVF that uses the INVOcell™ device placed into the upper vaginal cavity for incubation [3]. In the procedure, INVOcell™ device is a closed system able to keep temperature and pH stability during 72 h of uninterrupted embryo culture, providing a stable, pure, inexpensive, and easy-access source of the required oxygen/CO_2 concentration. This technique enables us to return to more physiological, natural, and seminatural environment where game culture and embryo development occur providing equivalent embryo implantation results, pregnancy rates, live birth, and single live birth rates to conventional IVF protocols [3]. INVO procedure and natural cycle or mild ovarian stimulation permits less egg collected, less risk of ovarian hyperstimulation syndrome, lower cost in medications, conscious sedation rather than general anesthesia, less discomfort for the patient, lower emotional stress, quicker procedure, and potential ethical advantages [3].

Early Steps on INVOcell Development

Intravaginal culture (IVC), also called INVO (intravaginal culture of oocyte), is a procedure discovered initially by Ranoux et al. in 1988.

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The IVC prototype was a tube for human tissue cryopreservation covered by cryoflex envelope to prevent vagina toxicity (length 4.2 cm, diameter 1.2 cm, and volume about 3 ml) [4, 5]. At the beginning, there were problems derived from the use of this early IVC prototype device:

- The IVC prototype was difficult to handle, and it could be closed and opened once;
- Accidental opening of the IVC prototype allowed leaks of culture media and high risk of lost of embryos;
- High risk of culture media contamination; and
- Difficulties in identifying embryos due to the large volume of culture media (3 ml).

Few years ago, INVOcell^{TM} device was specially designed to overcome these difficulties and improve the results obtained with the early prototype device. This new device has been ISO-10993 tested (and mouse embryo tested) to assess toxicity and biocompatibility and has received the European Union mark declaration of conformity which is equivalent to approval by the Food Drug Administration (FDA) in the USA [2].

INVO procedure has been used worldwide, and results from cycles performed by infertility centers around the world, in countries such as France, Germany, The Netherlands, England, USA, and Japan, have been published [4–14]. More recently, our center has pioneered the use of INVOcell^{TM} device within the Latin American and African regions and has been leader in its recent introduction in countries such as Mexico, Guatemala, EL Salvador, Dominican Republic, Panama Venezuela, Peru, Bolivia, Brazil, and Nigeria.

INVO Principle

The INVO procedure consists of utilizing the natural vaginal cavity environment which provides CO_2, O_2, and temperature for the oocyte fertilization and early embryo development [4]. The INVOcell^{TM} device is a closed system able to keep temperature and pH stability during 72 h or 120 h of uninterrupted embryo culture, providing a stable, pure, inexpensive, an easy-access source of the required O_2/CO_2 concentration. The INVOcell^{TM} device is permeable to CO_2 gas and allows the equilibrium between the pCO_2 of the vaginal/rectal venous plexus (~5–6%) and the pCO_2 of the culture medium (0.04–0.05%). Also, the INVOcell^{TM} device is permeable to O_2 gas and allows the equilibrium between the pO_2 of the culture medium (20%—pO_2 of air) and the pO_2 of the vaginal/rectal venous plexus (~5%) (Fig. 19.1). The INVO procedure offers an in vivo fertilization environment with the effectiveness of an O_2 concentration that closely resembles the uterine cavity atmosphere of less than 5% of oxygen. This concentration of oxygen ensures the energetic metabolism required for a successful gamete viability, activation, fertilization, and embryo development, which takes place under near-anaerobic conditions [15]. On the other hand, CO_2 is a key atmospheric component during embryo development; in the culture media, CO_2 produces carbonic acid which is equilibrated with sodium bicarbonate. As a consequence of the prior chemical reaction, the optimal pH is originated necessary for embryo development [15]. The system gives rise to a pH of the culture medium between 7.2 and 7.4 during the entire period of vaginal incubation. The system also avoids the variations in temperature maintaining an environmental stability for the gametes that achieve comparable results to those obtained with large gas-filled incubators [15, 16].

Infertile couples during conventional assisted reproduction treatments undergo an intense process that involves psychological and economical implications, in addition to the medical procedures that represent risks and secondary effects [18, 19]. A significant aspect of the INVO procedure lies in the psychological benefit that is created among the patients who feel closely involved in the process of fertilization and embryo development, which generates a high level of acceptance of the procedure and giving a more natural approach of the assisted conception.

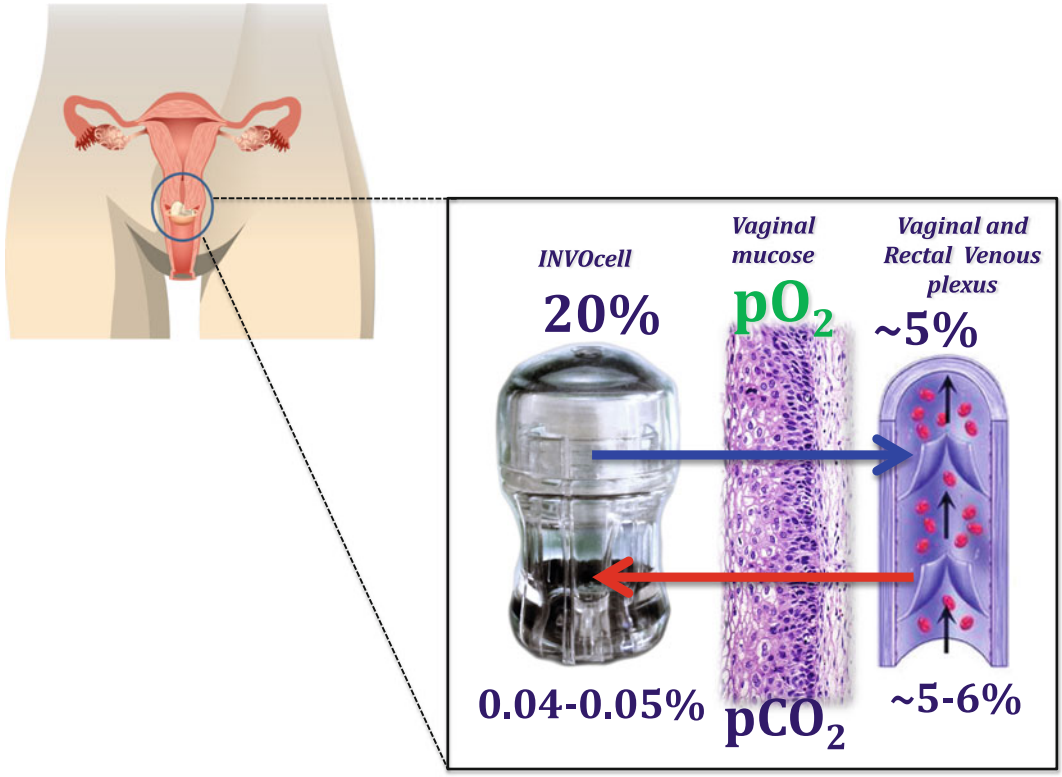

Fig. 19.1 INVO principle. INVOcell device is permeable to CO_2 gas and allows the equilibrium between the pCO_2 of the vaginal/rectal venous plexus (~5–6%) and the pCO_2 of the culture medium (0.04–0.05%). Also, the INVOcell device is permeable to O_2 gas and allows the equilibrium between the pO_2 of the culture medium (20%—pO_2 of air) and the pO_2 of the vaginal/rectal venous plexus (~5%)

INVOcell™ Device Components

The INVOcell™ device is a 2-inch plastic capsule and comprises an inner chamber, an outer rigid shell, and a retention system. The parts of the INVOcell™ are best shown in (Fig. 19.2). The inner chamber and the outer rigid shell are made from biocompatible plastic material (polypropylene) which is permeable to CO_2 gas and O_2 gas. The polypropylene material enable CO_2 gas and O_2 gas permeate the inner chamber wall from the vagina into contained culture medium. The INVOcell™ device is ISO-10993 tested to assess toxicity, biocompatibility, comfort, and retention within the vaginal cavity [2]. Description of each INVOcell™ part is as follows:

Inner Chamber

- Contains the culture medium and the gametes;
- The volume of the chamber is 1.1 ml;
- At the bottom of the main chamber, a microchamber collects the embryos;
- At the top of the chamber, a rotating valve allows several openings and closings without the introduction of air or contamination of the culture medium; and

Fig. 19.2 Component parts of the INVOcell device. **a** Inner chamber. **b** Outer rigid shell. **c** Assembled INVOcell device with its retention system

- The rotating valve has a small orifice at a bottom of small well which prevents variations of pH of the culture medium and loss of gametes due to possible overflow while filling.

Outer Rigid Shell

- Protects the inner chamber from vaginal contaminations;
- Keeps the inner chamber in a sterile environment;
- It has a smooth external surface to prevent any lesions or irritations of the vaginal cavity or cervix; and
- It has a locking position to prevent any unexpected device opening during vaginal incubation.

Retention System

- The diaphragm has a perforated membrane that avoids accumulation of vaginal secretions and preventing vaginal infections during intravaginal culture incubation and
- Avoid the INVOcell^{TM} device expulsion from the vaginal cavity.

INVO Procedures

INVO procedure has contributed to a returned interest in natural and more physiological IVF protocols that produce less embryos and are safer for the female. Using INVOcell^{TM} allows maturation of gametes, fertilization, and embryo development into the best natural incubator, the vaginal cavity. This alternative option for

conventional IVF treatment is considered as a simplified replacement of the IVF complex laboratories. This has reduced the necessity of sophisticated laboratory instrumentation simplifying the process of IVF and embryo development. Natural cycle and mild stimulation protocols with the INVO procedure also have contributed to the simplicity, low complication rates, and low cost of the INVO cycle.

Modified Natural Cycle

The monitoring of natural cycle is simple and inexpensive. Our modified natural protocols begin at the first day of the menstrual cycle evaluating the ovarian reserve by a pelvic ultrasound examination. On day 7th of menstrual cycle, a pelvic ultrasound is made to evaluate follicular size and also to identify a possible dominant follicle. At that moment, we start clomiphene citrate (CC) at doses of 25 mg per day (good ovarian reserve) or 50 mg per day (low ovarian reserve) at 9 am for 5 days to reinforce the follicular recruitment. At 13th day of the menstrual cycle, we start indomethacin (50 mg every 8 h per day, as used in mild protocols and discussed later) if the leading follicle reached 15 mm of diameter and is used to prevent premature ovulation. Triggering of ovulation is performed by GnRH-a (injection of 1 mg of leuprolide acetate) when the size of the follicle reaches 17 mm and the blood estradiol levels are around 250 pg/ml. Oocyte retrieval is performed 36 h after ovulation trigger and continues with the INVO laboratory protocol.

Mild Ovarian Stimulation and INVO: Clinical Experience

Seven years ago, the conventional protocols using high doses of gonadotropins have been discontinued in our clinic. At present, we have designed a new era in assisted reproductive (AR) techniques. Our main goal is to offer more physiological, natural, and safe treatments. This new alternative in controlled ovarian stimulation (COS) has been achieved by using low and mild doses of hormonal medications that support affordable treatments with the purpose of reaching a significant number of infertile couples in our population in terms of cost–benefit. Mild IVF and INVO procedure offer an advantage simplifying the laboratory equipment and manipulations needed, which might decrease the costs and allow a widespread application for patients who cannot afford traditional IVF [20] as it would be the case in developing countries, where the access to cost-effective infertility treatment is limited. We have focused on the following important factors for protocol preparation:

- Increased rates of infertility cases in our population.
- Physical, psychological, and emotional effects derived from conventional COS.
- Multiple risks related to multiple pregnancies.
- Focusing on single embryo transfer.
- Availability of clinically used medications such as non-selective inhibitors of cyclooxygenase type 2 (COX-2) as an economical method to achieve blocking of ovulation.
- Technological advances on different fields of clinical science: endocrinology, ultrasonography, and embryology.
- The cost of conventional COS treatments.
- The current and progressive increase in demand for assisted reproductive treatments.
- The commitment to provide affordable fertility treatments to the public health service in our population.
- The convenience of these fertility treatments to the middle-class population.

Different purposed methods of mild COS have been designed based on the global scientific evidence and joined to adjuvant therapies as hyperbaric oxygen sessions and preconceptional preparation cycle that enhances the effectiveness of AR treatments.

Hyperbaric Oxygen Therapy (HBOT)

HBOT consists of the medical use of oxygen at high doses (oxygen saturation close to 100%), during short periods of time (generally one hour), and at level higher than atmospheric pressure (oxygen pressure comparable to the sea level). The patient breathes pure oxygen in a pressured chamber that induces positive effects to the uterus such as:

- Stimulation of endometrial and follicular angiogenesis.
- Subendometrial, endometrial, and follicular hyperoxia.
- Stimulation of the endometrial pinopode formations.
- Increases sensibility of estrogenic receptors and hormonal mediators that modulate uterine immune response.
- Improves endometrial receptivity.
- Improves embryo implantation rates (Table 19.1).

Table 19.1 Results from treated and non-treated patients under hyperbaric oxygen (HBO) sessions during 2009–2010 at the fertility and sterility Colombian centre (CECOLFES)

	HBO patients (n = 305)	Non-HBO patients (n = 80)
MII	92%	74.3%
Fertilization	80%	90%
48 h	76%	60%
72 h	72%	54%
Clinical pregnancies	129 (42.3%)	22 (28%)
Biochemical pregnancies	5	2
Miscarriages	10	4
Avg. age	26–48 años	23–50 años
Non-tolerance	79 (25%)	
Claustrophobia	1	
Ear pain	78	

Preconceptional Preparation

As a part of COS treatments, we have suggested strategies of previous preparation using birth control pills. Those pills should be started at the first day of the menstrual cycle throughout 21 days. Once the patient takes the 21st pill of preparation, we start the administration of estradiol valerate 2 mg (tablets) for three continuous days. The bleeding will occur on a scheduled basis on the 26th and 29th days of the menstrual cycle. The main goal is to harmonize the FSH and LH levels, the follicular development, and the oocyte–endometrial quality. Also, preconceptional preparation allows a regular endometrial desquamation reducing the possibilities of ovarian cyst formation that impedes the beginning of COS (Fig. 19.3).

Mild Ovarian Stimulation Protocols Used on INVO Procedure

We have established different protocols based on the concentration of medications used for ovarian stimulation: clomiphene citrate (CC) and human menopausal gonadotropin (hMG) and the total days of treatment (Fig. 19.4).

- Protocol I
  hMG, 75 IU, for 5 continuous days, starting first dose at the 3rd day of the menstrual cycle
- Protocol II
  hMG, 150 IU, for 6 continuous days, starting first dose at the 3rd day of the menstrual cycle
- Protocol III
  Combo I: CC 50 mg + hMG, 75 IU, for 6 continuous days
  Combo II: CC 100 mg + hMG, 75 IU, for 6 continuous days
  Combo III: CC 50 mg + hMG, 150 IU, for 6 continuous days
  Combo IV: CC 100 mg + hMG, 150 IU, for 6 continuous days

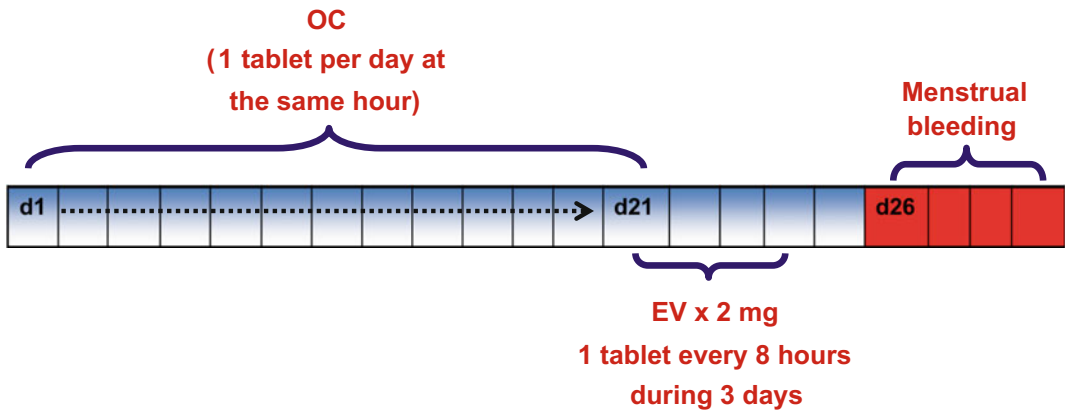

Fig. 19.3 Preparation preconceptional protocol fertility and sterility Colombian Center (CECOLFES) (*OC* Oral contraceptives, *EV* Estradiol valerate)

- Protocol IV
  CC 50 or 100 mg, +75 or 150 IU, at the 6th, 8th, and 10th days of the menstrual cycle
- Protocol V
  CC 50 or 100 mg, for 5 continuous days, starting first dose at the 3rd day or the 5th day of the menstrual cycle.

Blocking of Follicular Rupture

On IVF mild protocols, we have established the use of nonsteroidal anti-inflammatory (NSAID) medicines derived from the indol-3-acetic acid. These medicines, such as indomethacin or acemetacin, are non-selective inhibitors of the production of potent inflammatory mediators derived from COX-2 enzimatic action. COX-2 molecule is essential for the biosynthesis of prostaglandins E2 (PGE2) and F2 (PGF2). Prostaglandins are molecules derived from fatty acids stored in the cell membrane, and its biosynthesis can be divided into three phases: 1) releasing of arachidonic acid from the membrane phospholipids by the phospholipase enzyme action, 2) conversion of the arachidonic acid into an unstable intermediate prostaglandin called PGH2, by the action of COX-2, and 3) conversion of the PGH2 molecule in different specific active prostanoids for each cell [21]. Acemetacin and indomethacin have inhibitory action over COX-2 molecule. Prostaglandins are critical mediators of many biological procedures involved in female fertility such as ovulation, luteolysis, embryo implantation, and delivery [21]. The close relationship between LH surge and PGE2 and PGF2 concentrations in the ovarian follicles, just previous to the ovulation, suggests that the granulose cells might produce those two molecules. Acemetacin and indomethacin have a direct effect on the ovary inhibiting the preovulatory concentration of PGE2 and PGF2 into the follicle giving raise to a lag of oocyte releasing. Also, all molecular events involved in the follicular rupture including increased vascular permeability, collagenolysis, and local inflammatory events are inhibited.

We recommend that follicular rupture blocking should be started once the follicular diameter reaches 14–15 mm measured by pelvic ultrasound (9th–10th day of COS). NSAID doses recommended are as follows: 50 mg oral tablets of indomethacin three times a day or 60 mg oral tablets of acemetacin three times a day until the previous night of oocyte retrieval. On cases of allergies to NSAIDs, we suggest the use of antagonists of gonadotropin-release hormone

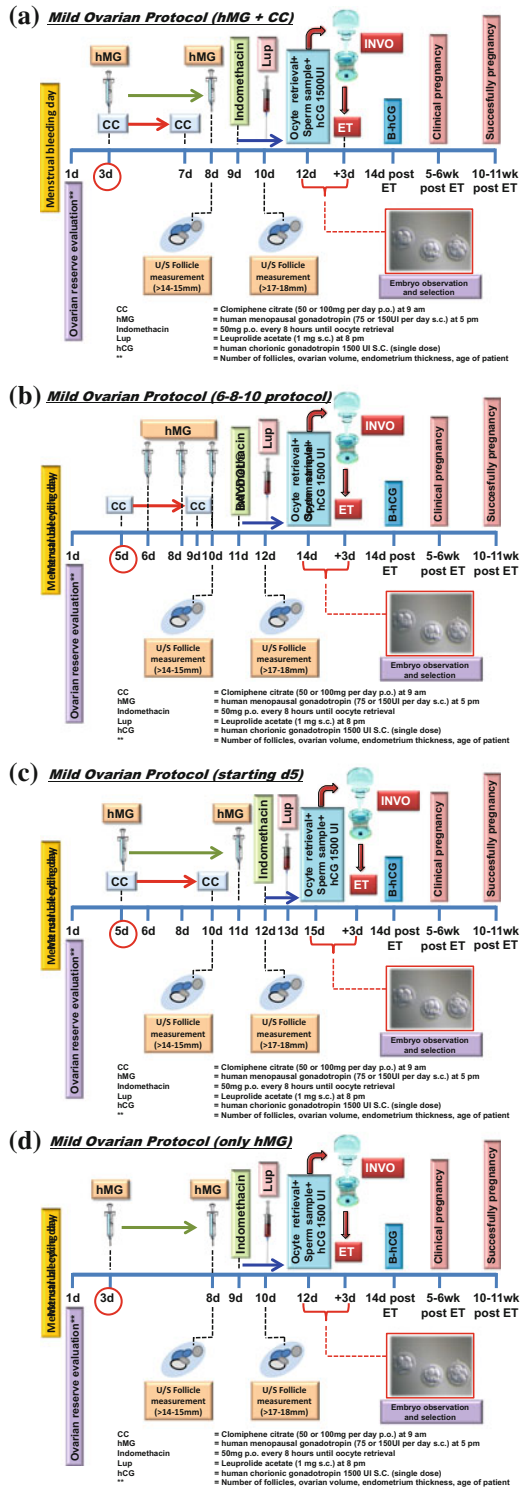

Fig. 19.4 **a–d** Mild ovarian stimulation protocols used on INVO procedure. Further information read the text

(ant-GnRH) single dose of 0.5 mg once follicles reach 14–15 mm of diameter measured by pelvic ultrasound, or multiple daily doses of 0.25 mg until the day of agonist of gonadotropin-release hormone (GnRH-a) injection.

Triggering of Ovulation

Our IVF mild protocols suggest the use of GnRH-a such as leuprolide acetate (Lupron®) at single 1 mg subcutaneous dose when the size of the follicle reaches 18 mm [22]. The use of Lupron® is based on the property of GnRH-a to produce a natural surge of endogenous FSH and LH in a short period of time (flare up effect) for triggering ovulation [23, 24]. The surge of gonadotropins originated by the GnRH-a is significantly shorter (usually 20 h) and induces oocyte maturation in a more physiological way. After 36 h of a single dose of Lupron®, the oocyte retrieval is performed and continues with the INVO laboratory protocol.

Corpus Luteum Rescue

Our IVF protocols suggest the use of a single 1500 IU subcutaneous dose of hCG immediately after the oocyte retrieval to rescue the corpus luteum and improve our embryo implantation rates [24].

Oocyte Retrieval

Transvaginal follicular aspiration using a transvaginal ultrasound probe is performed 36 h after Lupron® injection to get the best oocyte maturity from the grown follicles. The use of conscious sedation makes the retrieval procedure well tolerated by the patient without the need of anesthesia [4, 11]. The use of a 17-gauge needle connected to a closed system of a pump with the control of vacuum pressure (120 mmHg) is recommended for follicular aspiration; if not available, a follicular aspiration using a 10-ml syringe can be done [11].

Luteal Phase Support

After the oocyte retrieval, luteal phase is supported by progesterone injections at doses of 100 mg a day (5 doses), and thereafter, progesterone vaginal tablets (400 mg a day) are continued until the 12th week of pregnancy when the placenta takes progesterone secretion. Estradiol support (6 mg of estradiol valerate a day) is also used in association with progesterone.

Vitrification

Supernumerary oocytes confirmed to be mature by the presence of first polar body after denudation, as well as those good quality embryos that were not transferred, were vitrified for use in subsequent cycles. Vitrification was performed according to the protocol previously published by our group [25].

INVO Laboratory Protocol

INVO has been developed as a physiological IVF technique that comprises maturation of gametes, fertilization, and embryo development in a sealed polypropylene container filled with a suitable culture medium and placed in the vaginal cavity which serves as an incubator [26]. The vagina provides the proper incubation temperature and the correct CO_2 supplementation required for assisted fertilization and embryo development [26]. Steps for INVO procedure are mentioned in the Fig. 19.5.

During three days or five days of vaginal incubation, the couple should not have sexual intercourses. No activity restriction is required for the patient except bath, swimming, or vaginal douche due to the potential changes in vaginal temperature that could affect the incubation.

After three days or five days of vaginal incubation, the retention system and the INVOcell device are removed manually by grasping the ring of the retention system and pulling them out. The device is rinsed with prewarmed saline solution to clean off the vaginal secretions. Then, the device is opened and discarded. Using a Pasteur pipette, the culture media is slowly aspirated from the inner chamber of the INVO and transferred into a culture dish under microscopic observation. Once all embryos are recovered from the inner chamber, they are evaluated to select the best ones for uterine transfer. The selected embryos are then rinsed in fresh medium and loaded as classically into the embryo transfer catheter.

The embryo transfer is performed using ultrasound guidance and an abdominal transducer to visualize the correct position of the catheter in the endometrial cavity. Any bleeding should be carefully avoided during the embryo transfer as it negatively impacts the prognosis of the INVO procedure.

Clinical Results

INVO IVF

In a recent study published by our group, we have assessed the outcome of the INVO procedure, using the specially designed INVOcell^{TM} device, in terms of pregnancy, live birth, and single live birth rates; our results showed comparable successful rates with traditional IVF, highlighting its successfulness as an alternative option treatment in assisted reproduction [3]. In this study, one hundred and twenty (120) infertile couples with over 2 years of infertility were admitted. One hundred and twenty five (125) INVO cycles were performed. Population was ranked by age among four different groups to report clinical pregnancy, live birth, and single live birth rates in order to compare the outcome of the INVO procedure. Patients were distributed in groups as follows: ≤ 29, between 30 and 34, between 35 and 39, and ≥ 40 years old. The results of this study are summarized in the Table 19.2.

Taken together, these results suggest that the INVO procedure could be an alternative treatment for infertile patients ensuring success rates comparable to those in the existing IVF techniques [3]. Cycle secondary outcomes, including mean numbers of retrieved oocytes and oocytes

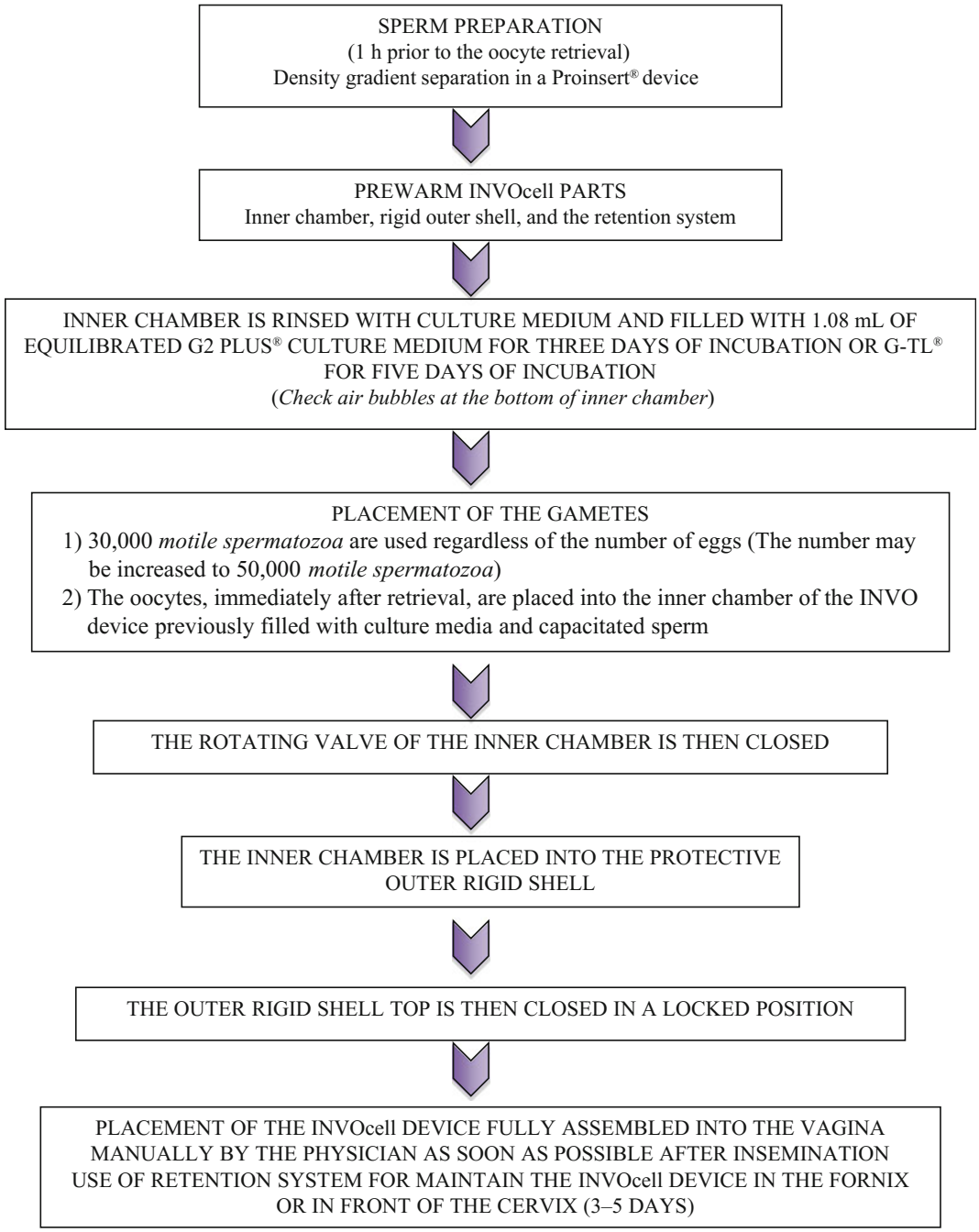

Fig. 19.5 INVO laboratory steps

placed per INVO device, in addition to embryo cleavage and transfer rates after INVO culture, were comparable between the first three established groups of age. However, the last group of age (≥ 40 years old) showed notably lower mean values within these measures.

Table 19.2 Results from IVF–INVOcell™ study

Groups[a]	Cycles (n)	Transfers[b] (n)	Retrieval[c]	INVOcell[d]	Cleavage[e]	ET[f]	Pregnancy[g]
<29	17	16 (94.1)	7.53	4.59	2.7(58.7)	2.3	10 (58.8)
30–34	54	51 (94.4)	6.52	3.8	2.8(73.7)	2.3	22 (40.7)
35–39	48	43 (89.6)	6.23	4.27	2.5 (58.5)	2.0	17 (35.4)
>40	6	4 (66.7)	5.5	5	1.5 (30)	1.5	1 (16.6)
Total	125	114 (91.2)	6.5	4.2	2.6 (63)	2.1	50 (40)

INVO, Intravaginal culture of oocyte; *ET,* Embryo transfer
Values in parentheses are percentages
[a]Ranges of ages
[b]Cycles that ended up in transfer
[c]Mean number of retrieved oocytes per punction
[d]Mean number of oocytes placed for fertilization per INVOcell device (cycle)
[e]Mean number of retrieved viable proper developed embryos per INVOcell device (cycle)
[f]Mean number of transferred embryos per cycle
[g]Number of clinical pregnancies per cycle

Table 19.3 Results from ICSI–INVOcell™ study

Groups [a](n)	Cycles	Transfers [b] (n)	Retrieval [c]	INVOcell [d]	Cleavage [e]	ET[f]	Pregnancy [g]
<29	33	32 (96.9)	6.93	4.63	3.1 (66.9)	2.1	10 (54.5)
30–34	53	50 (94.3)	7.82	4.52	3.2 (70.7)	2.2	22 (41.5)
35–39	71	66 (92.9)	6.42	5.01	2.6 (51.8)	1.8	23 (32.3)
>40	17	13 (86.6)	5.12	4.93	1.7 (34.4)	1.5	2 (13.3)
Total	172	114 (93.6)	6.51	4.7	2.5 (53.1)	2.0	65 (37.9)

INVO, Intravaginal culture of oocytes; *ET,* Embryo transfer
Values in parentheses are percentages
[a]Ranges of ages
[b]Cycles that ended up in transfer
[c]Mean number of retrieved oocytes per punction
[d]Mean number of oocytes placed for fertilization per INVOcell device (cycle)
[e]Mean number of retrieved viable proper developed embryos per INVOcell device (cycle)
[f]Mean number of transferred embryos per cycle
[g]Number of clinical pregnancies per cycle

INVO ICSI (Pioneer Idea)

We have investigated different applications for INVO procedure in patients displaying male factor. A recent study from CECOLFES has evaluated a total of 172 cycles undergone to INVO–ICSI protocol due to low quality of sperm cells [27]. In that study, the ICSI procedure was performed on each retrieved oocyte and all microinjected oocytes were placed into the INVOcell™ device. Later, INVOcell™ device was inserted in the vaginal cavity during 72 h for incubation. Our results show on average that 6.5 oocytes per cycle were retrieved, a mean of 4.7 microinjected oocytes was placed into INVOcell ™ device, and a mean number of transferred embryos per cycle was 2.0. The cleavage rate obtained after the INVO culture was 53.1%. A total number of 65 clinical pregnancies (59 single pregnancies and 6 multiple pregnancies) were achieved corresponding to 37.9% of pregnancy rate per cycle and to 40.3% of pregnancy rate per transfer [27] (Table 19.3).

As a pioneer idea for alternatives in IVF treatments, this study have shown for the first time that INVO–ICSI protocol can be an effective and viable alternative treatment option in assisted reproduction to achieve pregnancy after

ICSI procedure for couples showing male factors of infertility with comparable results to those reported using classical IVF–ICSI procedures.

Conclusion

The INVO procedure is an in vivo conception that occurs in an innovative device placed into the upper vaginal cavity with high level of acceptance among the patients. The results in terms of embryo implantation and pregnancy rates are totally equivalent to those reported from conventional IVF protocols. The advantages of INVOcell™ are enumerated as follows:

- The embryologist training can be done quickly obtaining excellent results. Not strong ART is required.
- Manipulation of gametes and embryos is reduced and simplified on INVO procedure.
- INVOcell™ enables to obtain very good quality of embryos.
- INVO procedure could be used not only for classical IVF but also INVO has been used successfully to vaginally incubate embryos inseminated through ICSI where male factor takes place.
- INVO units require little equipment maintenance, and the use of large, fixed, and complex laboratory equipment is avoided on this technology.
- INVO procedure may also be used in satellite units to extend the geographic influence of the reproductive center.
- The lower cost of treatment places INVO within the reach of a larger infertile population.
- In developing countries, the frequent electric power breakdowns do not have any negative impact on INVO procedure.
- INVOcell™ has a very good acceptance because the patient involvement during the initial stage of embryo development, with very positive and significant psychological impact.
- INVO procedure is a novel solution in assisted reproductive technologies, expanding geographic and affordable access to the global reproductive healthcare community.
- INVO procedure provides a far more personalized and reproducible approach to conception.

The above-mentioned INVO procedure is an ideal option for patients that seek help for fertility at a low cost and a comparable success with traditional IVF treatments.

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Accessible Infertility Care: From Dream to Reality

20

Willem Ombelet, MD, PhD

Introduction

Infertility is a global reproductive health problem. According to Boivin et al., the 12-month infertility prevalence rate ranges from 6.9 to 9.3%. The large majority of childless couples are residents of developing countries (DCs) [1]. Recent data on the worldwide prevalence of involuntary childlessness indicate that 52.6–72.4 million couples could benefit from some form of medical intervention to achieve a pregnancy [2]. Substantial geographical differences are noted, and these differences can be explained by different environmental, cultural and socio-economic influences [1]. In sub-Saharan Africa, infection is the cause of infertility in over 85% of cases among women, compared to 33% worldwide [3, 4]. Approximately 70% of pelvic infections are caused by STDs, while the other 30% are attributable to pregnancy-related sepsis [5]. Similarly, many cases of male factor infertility are caused by previous infections of the male genitourinary tract [6]. The high prevalence of genital infections in developing countries is commonly compounded by a complete lack of diagnosis together with incomplete, inappropriate or no intervention at all. Severe male infertility due to STDs and female infertility due to tubal block can only be treated by "expensive" assisted reproductive technologies (ARTs) which are not available at all or only within reach of those (the happy few) who can afford it, mostly in a private setting. Both conditions are best treated by assisted reproductive technologies (ARTs), but most infertile couples in developing countries cannot afford ART because the techniques are too expensive and mostly limited to a few private centres [7, 8].

The infertility experience has significant negative effects on the individual woman and man as well as the couple and the broader family [9]. Consequences of infertility are numerous: stress, depression, low-self esteem, guilt, marital problems, and sexual problems. So far, infertile people in the Western countries and the resource-poor countries have something in common. However, the differences are emerging mainly for two reasons: (a) sociocultural values surrounding procreation and infertility and (b) availability of infertility treatments.

Consequences of involuntary childlessness are usually more dramatic in DC when compared to Western societies, particularly for women. Often, the woman is blamed for the infertility, even when a male factor is involved. Negative psychosocial consequences are severe: childless women are frequently stigmatized, isolated, ostracized, disinherited and neglected by the entire family and even by the local community [10–18]. This may lead to physical and psychological violence, polygamy and even to suicide. Infertile women—and men—are marginalized, disadvantaged and disempowered. As many families—and elderly people in particular—in DC completely depend on children for economic

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survival, childlessness in DC becomes an important social and public health issue and not only an individual medical problem [19–22]. In the last two decades, the manifold consequences of infertility, most significant within DC, at personal, conjugal, family and community levels, and financially, have been well documented [11, 17, 23]. These studies have also shown that the way people experience, explain and deal with infertility is strongly related with their sociocultural and economic life circumstances as well with the availability and non-availability of healthcare options.

The most important reasons for infertility in DC are as follows: (1) the high incidence of sexually transmitted diseases (STDs)—which affects both men and women—and (2) pregnancy-related infections, due to unsafe abortions and home deliveries in unhygienic circumstances, mainly in rural areas.

The high prevalence of genital infections in developing countries is commonly compounded by a complete lack of diagnosis together with incomplete, inappropriate or no intervention at all. Yet, severe male infertility due to STDs and female infertility due to tubal block can only be treated by "expensive" assisted reproductive technologies (ARTs), which are not available at all or only within reach of those (the happy few) who can afford it, mostly only in private settings [19].

Reduced fecundity in HIV-infected individuals has been described before [24, 25]. Marital instability and polygamy, as a reaction to infertility and childlessness within the conjugal relationship, may in turn increase the spread of HIV-1 infection (and STDs) [20, 26]. Studies have shown that HIV was up to 3 times more prevalent in childless couples compared with fertile couples in the same population [27]. Moreover, expanded access to antiretroviral therapy (ARVs) implies that HIV+ people have increased hopes to live longer and healthier lives, which also has been associated with an increased chance to bear children. HIV+ males could have a child that is HIV− with the assistance of sperm washing procedures commonly used in assisted reproductive procedures such as intrauterine insemination (IUI) and in vitro fertilization (IVF).

Despite the high infertility prevalence and the severe economical consequences of childlessness in DC, infertility care remains a low priority area for local healthcare providers and community leaders. It has been marginalized and neglected by healthcare authorities despite its high prevalence and unmet need [22, 28]. The biggest obstacle in implementing health policies which consider infertility as a problem is the widespread belief that infertility is not a pressing problem in poor developing countries where fatal and contagious diseases remain uncontrolled and because infertility as such is not directly life-threatening neglecting totally the devastating social, psychological, economical and personal burden of being childless in most poor societies.

According to MDG5 (Millennium Developments Goal 5), universal access to reproductive care, including both contraceptive and infertility care, should be adopted by the year 2015. Until today, nothing has been done to help childless couples in developing countries, and according to a recent questionnaire, none of the international organizations, NGOs and foundations is planning to do so in the forthcoming years [22].

Till now, infertility care in most DC has been fragmented between public and private spheres. Inadequate or complete lack of rules and regulations concerning treatment conditions and commercial interests may lead to unethical practices [29]. Overall, very little is known about actual practices and results within clinics providing infertility services in DC.

The Walking Egg Non-profit Organization

The Walking Egg NPO has opted for a multidisciplinary and global approach towards the problem of infertility [21]. The main goal of The Walking Egg Project is to raise global awareness surrounding childlessness and to make infertility care in all its aspects universally available and accessible. Therefore, we need to change and optimize the whole set-up of infertility care in

terms of availability, affordability and effectiveness [30].

To realize this objective, a number of actions are planned including the following: (1) to raise awareness surrounding the problem of childlessness within (a) the donor community, politicians, funding agencies and research organizations through lobbying and publishing, (b) the general population through information, education and counselling on infertility and its consequences; (2) to study the ethical, sociocultural and economical aspects surrounding the issue of childlessness and infertility care in resource-poor countries; (3) to develop new methods to make infertility diagnosis and infertility treatment including ART accessible for a much larger part of the population, by (a) simplifying the diagnostic procedures, (b) simplifying the IVF laboratory procedures and (c) modifying the ovarian stimulation protocols for IVF; and last but not least (4) to work together with other organizations and societies working in the field of reproductive health to reach the goal of "universal access to infertility care".

Research and Innovation

Non-medical

There is an urgent need for more research on sociocultural, ethical, religious and juridical aspects of infertility in poor-resource countries. Reliable data on economical consequences of childlessness in DC are lacking. Ethical considerations and debates on this subject are scarce [31, 32]. Legal aspects and rights dealing with the severe consequences of childlessness for women in DC are almost never mentioned in the literature, and what to say about the legal right to have access to infertility care, agreed upon and mentioned in so many political international statements and commitments [33].

In The Walking Egg Project, we aim to initiate and expand an international network of social science research (in broad sense) in these fields. The first expert meeting on the "Socio-cultural implications of childlessness in developing countries" was organized by The Walking Egg npo in 2009, in cooperation with ESHRE (European Society of Human Reproduction and Embryology) and WHO (World Health Organization). A Monograph with articles of most experts was published in "Facts, Views & Vision in ObGyn" (www.fvvo.eu). This Monograph was distributed to 9000 participants of the annual ESHRE meeting in Rome. A second expert meeting on "Barriers, Access and Ethics of biomedical care in resource-poor countries" was held in 2011 and followed by the publication of another Monograph distributed at the Annual ESHRE meeting in Istanbul in 2012 (www.fvvo.eu). Participants at the 2011 Expert Meeting highlighted the importance of studies addressing barriers to infertility care and studies to prepare, assess and follow up the supply and use of accessible and affordable infertility care in different low-resource contexts. They concluded that to be successful, the project has to be global with a strong sociocultural, ethical and economical component. It will need the support of a reliable network of social scientists supporting the project by discussing the various sociocultural, psychological and ethical aspects of biomedical infertility care in different DC. This network will be crucial in the introduction and follow-up of accessible infertility care services in resource-poor countries.

Medical

From a pure medical point of view, our **first objective** is the establishment of low-cost "one-stop clinics" for the diagnosis of infertility. Simplification of the ART procedures without loss of quality is our **second objective**. Our **final goal** is the implementation of "accessible" infertility services, if possible integrated within healthcare facilities, providing good quality family planning services, reproductive health education and high-standard mother care.

The One-Stop Diagnostic Phase

Standardized investigation of the couple at minimal costs is possible and undoubtedly will enhance the likelihood that infertile couples, both men and woman, will come to the infertility centres. How to organize a one-stop diagnostic clinic has been described before [34]. A questionnaire will be provided to both partners. This questionnaire can be adapted to the local situation in the specific locations and countries. Screening for infections and STDs can be done by using low-cost affordable screening tests which became available recently [35].

Since tubal obstruction associated with previous pelvic infections is the most important reason for infertility in some developing regions, hysterosalpingography and/or hystero-salpingo-contrast-sonography are affordable techniques to detect this problem, easy to perform and without major costs. Combining these techniques with an accurate medical history will identify the majority of women's infertility causes, such as ovulatory disorders, uterine malformations and tubal infertility. A standard gynaecological and fertility ultrasound scanning of the uterus and the ovaries can easily be done.

Male factor infertility can be evaluated by a simple semen analysis [36]. Semen analyses can also be performed by well-trained paramedicals, another important advantage for developing countries. It is also important to calculate the IMC (inseminating motile count). The IMC is the total number of motile spermatozoa after sperm wash procedure. The IMC is very crucial in selecting patients for either IUI (intrauterine inseminations), IVF (in vitro fertilization) or ICSI (intracytoplasmic sperm injection) [37].

Office mini-hysteroscopy to investigate intrauterine abnormalities has been simplified in its instrumentation and technique, so that it can become a non-expensive diagnostic technique accessible for every gynaecologist, provided there has been appropriate training [30, 38]. All the procedures of the one-day diagnostic clinic can be performed by a small team of healthcare providers within a short period of time in an inexpensive setting [30]. A flowchart for the TWE (The Walking Egg) diagnostic clinic is shown in Fig. 20.1.

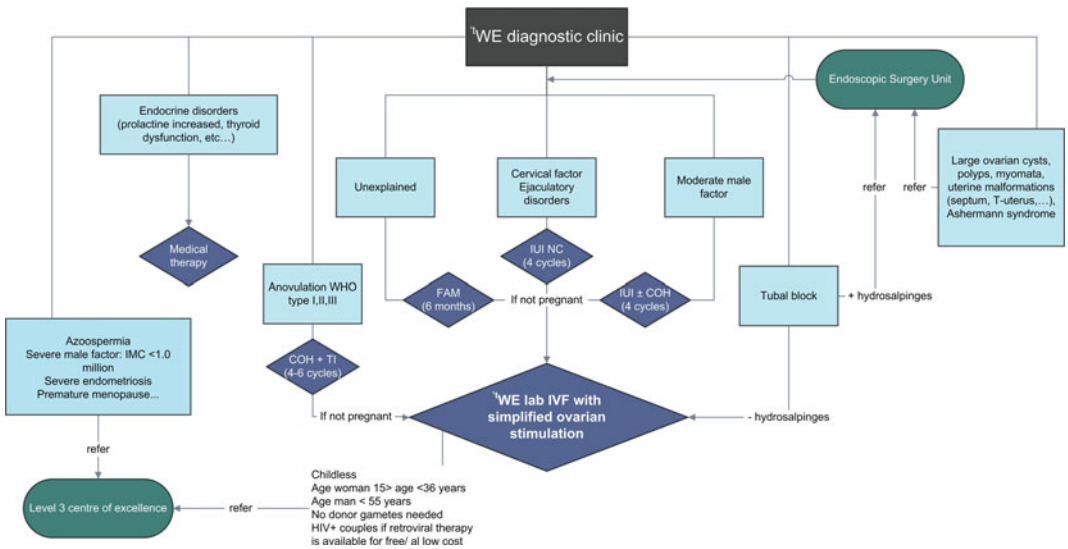

Fig. 20.1 Proposed flowchart for the TWE diagnostic clinic (*TWE* The Walking Egg, *FAM* Fertility awareness methods, *IUI* Intrauterine insemination, *NC* Natural cycle, *COH* Controlled ovarian hyperstimulation, *TI* Timed intercourse, *IMC* Inseminating motile count) [34]

Future studies are planned to assess the reproducibility of "one-stop infertility clinics" in different developing countries.

Simplified Infertility Treatment and Non-IVF Assisted Reproduction

If tubal patency is demonstrated in ovulatory women and if severe male factor subfertility has been excluded, fertility awareness programmes are an inexpensive and efficient first-line approach to infertility management [39, 40]. Fertility awareness counselling to couples about the meaning and detection of cervical mucus secretion can be given by nurses and paramedical staff working in existing reproductive healthcare centres.

For ovulatory dysfunction, representing almost 20% of female infertility, clomiphene citrate (CC) is a very cheap and rewarding option. In case of resistance to CC, a low-dose ovarian stimulation regimen with gonadotrophins aimed at monofollicular growth is advisable, although this medication is more expensive.

In case of unexplained and moderate male factor infertility and provided tubal patency has been documented, intrauterine insemination (IUI) with husband's semen in natural cycles or after mild stimulation is an excellent first-line treatment without major costs and without expensive infrastructure [41, 42]. IUI programmes can be run by well-trained paramedical staff, another advantage for resource-poor countries. Controlled ovarian hyperstimulation (COH), with or without IUI, is associated with the risk of multiple gestations, especially when gonadotrophins are used [43]. Appropriate standardized protocols are available to minimize the risk of multiple pregnancies which is even more important in developing countries because the consequences of multiple pregnancies can be devastating.

Simplified IVF Laboratory Procedures

Another major challenge is to reduce costs of laboratory procedures, namely fertilization and culture of eggs and embryos for IVF. Different options and approaches have been developed or are presently being field-tested with promising results.

Intravaginal fertilization and culturing have been used since many years for low-cost IVF [44]. A tube filled with culture medium containing the oocytes and washed spermatozoa is hermetically closed and placed in the vagina. It is held intravaginally by a diaphragm for incubation for 44–50 h. Over 800 cycles have been published worldwide with a very reasonable clinical pregnancy rate of almost 20% [44].

As part of The Walking Egg Project and based on the previous findings and experience [45, 46], we developed a new simplified method of IVF culturing, called the TWE-lab method. With this new system, specifically designed for low-resource settings, we can avoid the high costs of medical gases, complex incubation equipment and infrastructure typical of IVF laboratories in high-resource settings (see Addendum 1).

For insemination of the eggs, we only use 1000–10,000 motile washed spermatozoa per oocyte, with very promising results, which makes this technique usable for more the 70% of the actual IVF/ICSI population (Genk data, not published). Since development from insemination to transfer is undisturbed and in the same tube until embryo transfer, we can avoid many problems frequently occurring in regular IVF laboratories, such as unwanted temperature changes and air quality problems.

Up to April 2013, twelve healthy babies have been born after using this technique while a prospective study comparing the embryo quality after using TWE-lab versus regular IVF procedures is still ongoing.

Low-Cost Ovarian Stimulation Protocols for IVF

In order to make infertility care more affordable in developing countries, effective, cheap and safe stimulation schemes for intrauterine insemination (IUI) and in vitro fertilization (IVF) need to be established. A review of the literature clearly shows the value and effectiveness of mild ovarian stimulation protocols in ART settings [47]. The success rates of natural cycle IVF can be low per cycle due to high cancellation rates because of premature LH rise and premature ovulation. But the use of indomethacin to block ovulation helps to reduce the cancellations. Cumulative pregnancy and live birth rates after four consecutive cycles could reach 46 and 32%, respectively, making it a cost-effective, safe and patient-friendly option [48]. The use of clomiphene citrate (CC), a very cheap oral drug, has been proven in many studies to be an optimal alternative with acceptable results, minimal side effects and a very low complication rate [47, 49–51].

Monitoring of follicular development in an IVF cycle, as well as the timing of the hCG administration, can be done solely on sonographic criteria with basic inexpensive ultrasound equipment, thereby avoiding the need of expensive endocrine investigations [52].

Nevertheless, although very promising results concerning the different steps of IVF are described, we still have to perform a lot of feasibility studies to examine the value of a one-stop diagnostic phase and to study the value of the simplified TWE-lab system and different low-cost ovarian stimulation protocols in resource-poor settings.

Service Delivery: The Implementation of TWE Pilot Centres in DC

The ultimate aim of The Walking Egg project is the implementation of good quality but low-cost infertility centres in DC, if possible and preferable integrated into existing reproductive healthcare centres. Diagnostic and therapeutic procedures and protocols should be affordable, effective, safe and standardized. Ideally, infertility management should be integrated into sexual and reproductive healthcare programmes.

As developing countries differ in their status of development, three levels of assistance are suggested [53]. A level 1 infertility clinic is a basic infertility clinic capable of offering the following services: basic infertility workout including semen analysis, hormonal assays, follicular scanning, ovulation induction and IUI. In level 2 infertility clinics, IVF can be performed as well.

During many expert meetings, it was decided that level 3 infertility clinics capable of offering ICSI, cryopreservation and operative endoscopy are not part of The Walking Egg Project in the initial phase. Therefore, our first target is the implementation of good quality level 2 centres.

Implementation of level 2 services entails the following activities [53]:

1. ***Equipping the clinics***: Infertility clinics in developing countries should be provided with low-cost and easy serviceable equipment taking into consideration the local problems often encountered (e.g. fluctuating voltage, frequent power cuts, unavailability of servicing facilities, and irregular supply of consumables). This may require negotiations with various manufacturers to supply these tools at affordable prices, particularly if large quantities are ordered.
2. ***Training the staff***: This includes the training of the medical, paramedical as well as the administrative staff. Training courses should tailor to the local conditions and the possible difficulties encountered in developing countries. Table 20.1 gives an overview of the key topics covered in the training courses. Training, quality control, regular audit and systems of accreditation and registration should be implemented in order to maintain appropriate standards of care. Our objective is to organize a one-week course for all members of the team who are involved in the set-up of a pilot centre, part of The Walking

Table 20.1 Key categories of the training courses [34]

• Reproductive healthcare education basic course —Target group: nurses, midwifes
• A general and medical history of and basic clinical examination both partners —Clinician (medical)
• Screening for infections and STDs —Clinician (medical, paramedical)
• How to perform and evaluate a hysterosalpingography and/or hystero-salpingo-contrast-sonography —Clinician (medical, paramedical)
• Standard Operational Procedures for the gynaecological and fertility ultrasound scan —Clinician (medical, paramedical)
• Basic semenology training course according to WHO 2010 manual —Laboratory staff (paramedical)
• Sperm washing procedures —Laboratory staff (paramedical)
• Mini-hysteroscopy —Clinician (medical)
• Documentation and registration —Administrative staff (clerical)

Egg Project. This training will need the support of experts in the field, who are capable to tutor the training courses at the highest level in a very short time, taking into account the experience of the trainees and the quality of facilities that can be expected in the new pilot centres.

3. ***Educating the public***: This necessitates establishing contacts and working relationships with schools, community leaders, traditional healers as well as the media, producing and distributing educational materials (brochures, posters and audio-visual material) etc.
4. ***Running the services***: This should take into consideration the staff salaries, regular purchasing of consumables, cost of equipment maintenance, cost of investigations, cost of medical interventions and the cost of medication. Special servicing contracts should be negotiated with the manufacturers. In addition, simplification of the consumables should be taken into consideration, and laboratory reagents and culture media should have a long shelf life. Special prices for medication should be negotiated with the drug manufacturers, and simple treatment protocols should be put into action in order to reach the best cost-effective therapies.
5. ***Documentation and registration***: We believe that within each pilot centre, online data registration of all ART activities is mandatory. Administrative staff and (para) medicals have to be aware of the importance of correct and trustable data registration. The ultimate goal is to offer all pilot centres a similar registration programme, which should be customer-friendly with a limited but sufficient number of items (increased personnel compliancy) [34]. Continuous monitoring of service activities will be centralized and will provide feedback to clinics for clinical and laboratory policy adjustments, information to couples on clinic performance and information to society. Confidence can then be built and maintained.
6. ***Psychological and sociocultural follow-up***: When implementing low-cost (accessible) infertility services in DC, it is extremely important to study social, psychological, sexual, legal and ethical aspects of infertility and infertility treatment and take study findings into account when setting up gender and cultural sensitive infertility services. Considering psychological and sociocultural follow-up, the most important aims can be summarized as follows:
   - Informing the design of culture- and gender-sensitive treatment and counselling procedures, ethical guidelines and informed consent forms for the selected pilot centres.
   - Describing the psychological well-being of the infertile women and men along the infertility treatment trajectory (before, during, immediately and one year after treatment); their expectations, experiences and suggestions regarding treatment procedures and aspects of quality of care; and the social repercussions and other social

implications infertility treatments may have.
- Enhancing the level of knowledge and understanding with regard to sociocultural, psychical, quality of care and ethical aspects of infertility care in DC.

Selection of Countries/Pilot Centres

Decision-making on infertility treatment in developing countries assumes answers to quite a few questions: How should the infertility problem be defined? How often does infertility occur? What is the income in that specific country and what can be spend on health care? How cheap should IVF be in order to be accessible to a considerable part of the population? With what alternative health interventions should infertility treatment be compared? How cost-effective should IVF be in order to compete with those other interventions?

In this respect, we believe that measurements of the (utility measure-oriented) quality of life over the infertile life course in developing countries are urgently needed.

The selection of countries where the first pilot centres are implemented will be based on the following: (1) available data on the resources, needs and resource gaps for infertility services on a national level; (2) percentage of GDP spent on education and health care; (3) the availability of endoscopic surgery facilities in the neighbourhood; (4) a good quality family planning unit; (5) good quality mother care facilities; and (6) the availability of at least one experienced and dedicated gynaecologist and biologist.

The community/region including the local healthcare authorities should be empowered to support the programme from the beginning. Figure 20.2 gives an overview of The Walking Egg Philosophy and The Walking Egg Charter with the most important recommendations to consider when starting TWE pilot centres in developing countries.

Selection of Patients and IVF Protocol

Decision-making on infertility treatment in developing countries assumes answers to quite a few questions: How should the infertility problem be defined? How often does infertility occur? What is the income in that specific country and what can be spend on health care? How cheap should IVF be in order to be accessible to a considerable part of the population?

In this respect, we believe that measurements of the (utility measure-oriented) quality of life over the infertile life course in developing countries are urgently needed.

The selection of centres where the first pilot centres are implemented will be based on the following: (1) available data on the resources, needs and resource gaps for infertility services on a national level; (2) percentage of GDP spent on education and health care; (3) the availability of endoscopic surgery facilities in the neighbourhood; (4) a good quality family planning unit; (5) good quality mother care facilities; and (6) the availability of at least one experienced and dedicated gynaecologist and biologist.

Potential TWE-centres have to agree on the philosophy and the conditions of The Walking Egg Project as described in Fig. 20.2. The one-stop diagnostic phase with a **standardized investigation of the couple** at minimal costs will enhance the likelihood that infertile couples will come to the infertility centres. A small team of healthcare providers can perform all the procedures of the one-day diagnostic clinic within a short period of time in an inexpensive setting. Future studies are planned to assess the reproducibility of "one-stop infertility clinics" in different developing countries. Minimal (low-cost) ovarian stimulation protocols to avoid multiple

The Walking Egg Charter

The Walking Egg npo is an international non-profit organization based in Genk, Belgium.
The Walking Egg believes that infertility care is a neglected aspect of family planning in resource-poor countries and therefore aims to raise global awareness about childlessness and infertility in developing countries.
The main goal is to make infertility care, including assisted reproductive technologies, available and accessible to a much larger part of the world population.
The Walking Egg has chosen for a multidisciplinary and global approach to realize the implementation of "accessible infertility services", the so-called ***Walking Egg Centres***.
Although the organization is made up mainly of doctors and health sector workers, it is also open to all other professions which might help in achieving its aims.

The Walking Egg Centres agree to honour the following **conditions**:

- Good quality family planning and mother care facilities are available. Ideally infertility management should be integrated into sexual and reproductive health care programmes
- An endoscopic surgery unit is available in the neighbourhood
- At least two dedicated specialists available (gynaecologist, embryologist)
- Training, quality control, regular audit and systems of accreditation and registration will be implemented in order to maintain appropriate standards of care
- To provide affordable infertility care according to the **philosophy** of The Walking Egg considering diagnostics, therapeutical procedures and data registration.

The Walking Egg Philosophy

The Walking Egg actions are guided by medical ethics, the principles of high quality medical care and accountability.

Medical ethics

We carry out our work with respect for the rules of medical ethics, in particular the duty to provide infertility care without causing harm to individuals or groups. We respect patients' autonomy, patient confidentiality and their right to informed consent. We treat our patients with dignity, and with respect for their cultural and religious beliefs. In accordance with these principles, TWE endeavours to provide high-quality medical care to all patients.

High quality medical care

Our decision to offer assistance in any country is based on an independent assessment of people's needs.
If assisted reproductive techniques, including intrauterine insemination and IVF can be offered, the TWE guidelines have to be followed:
Actions to prevent the most common complications of ART are obligatory

- Single embryo transfer (SET) recommended or double embryo transfer (DET) in selected cases to prevent multiple pregnancies
- The use of natural cycle / modified natural cycle protocols or mild ovarian stimulation protocols in IVF to prevent OHSS (ovarian hyperstimulation syndrome)

Using TWE materials for IVF
Only IVF due to the methodology of the simplified IVF system
Preferably childless couples
If HIV-positive: ART only performed if anti-HIV medication is freely available

Accountability

The Walking Egg is committed to regularly evaluating the effects of its activities. We assume the responsibility of accounting a strict follow-up of our Walking Egg Centres and to inform our donors on a regular basis.

Fig. 20.2 Charter and philosophy of The Walking Egg project

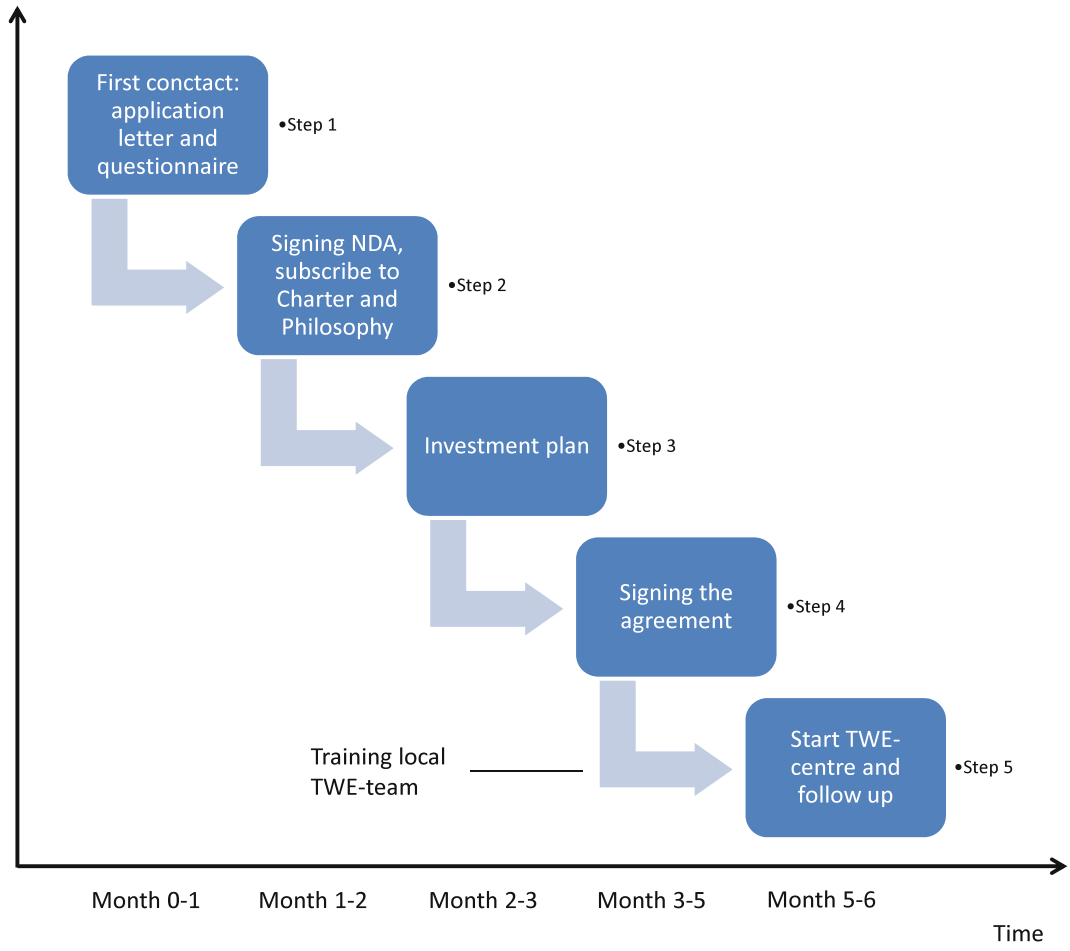

Fig. 20.3 Different steps and time frame to become a Walking Egg centre

pregnancies and severe medical complications such as ovarian hyperstimulation syndrome (OHSS) are mandatory, not only in an IVF setting but also in IUI (intrauterine insemination) programmes. This strategy will drop the cost for assisted reproductive technologies significantly, especially when the simplified TWE IVF procedures are used [30, 45, 55].

Application to Become a TWE-Centre

Every centre participating in the project accepts the rules and philosophy of the concept. This implicates also that a TWE-centre is by preference equipped by the TWE organization. In that way, TWE is also responsible for the validation, implementation and follow-up of the project.

There are 6 steps to be taken over a period of about 6 months, as described in (Fig. 20.3).

Advocacy and Networking

Global access to infertility care can only be implemented and sustained if it is supported by local policy makers and the international community. Many international organizations have already expressed their desire to collaborate including the WHO (World Health Organization), ESHRE (European Society for Human Reproduction and Embryology) and ISMAAR

(International Society for Mild Approaches to Assisted Reproduction). We will also need the media, patient organizations and interested politicians to change the existing moral and sociocultural beliefs that are isolating and ostracizing infertile couples.

Conclusion

The magnitude of childlessness in developing countries has dimensions beyond its prevalence and aetiology. Differences between the developed world and developing world are emerging because of the different availability in infertility care and different sociocultural value surrounding procreation and childlessness. There is a growing belief that individual health needs of impoverished people have a place next to their public health needs. Although reproductive health education and prevention of infertility are number one priorities, the need for accessible diagnostic procedures and new simplified reproductive technologies is very high. The success and sustainability of ART in resource-poor settings will depend to a large extend on our ability to optimize these techniques in terms of availability, affordability and effectiveness. The Walking Egg NPO aims to raise awareness surrounding childlessness in resource-poor countries and to make infertility care in all its aspects, including assisted reproductive technologies, available and accessible for a much larger part of the population. By simplifying the diagnostic and IVF laboratory procedures and by modifying the ovarian stimulation protocols for IVF, assisted reproductive techniques can be offered at affordable prices. The implementation of low-cost infertility centres in resource-poor countries, if possible integrated in existing Reproductive Health Care Centres, will be a crucial step to reach the ultimate goal of "universal access to infertility care".

The selection of pilot-centres will depend on different factors such as budget for education and health care in that specific country, the availability of effective family planning and mother care facilities, a dedicated person who can coordinate the study and shows interest for sociological support, before, during and after treatment. Infertility will likely become one of the more predominant components of future reproductive healthcare practice. Taking advantage of information and communication technologies will increase the effectiveness and accessibility of healthcare services, as well as change the patient's behaviours to seek timely treatment. As evidence-based affordable solutions begin to drive global guidance within both public and private healthcare system solutions, access to care for the infertile couple will become one of the largest emerging fields in global medicine.

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Addendum 1: The TWE-Lab Simplified IVF Procedure Method

The TWE-lab system is based on a simple chemical reaction in a closed environment which removes the need for an expensive IVF laboratory with CO_2 incubators, medical gas supply and air purification systems. CO_2 is necessary to equilibrate the pH of the IVF culture medium to a value between 7.25 and 7.40, optimal for embryo development. Continuous culture at 37 °C is necessary for viability of the embryo and can be achieved by an incubator, heated box or warm water bath. The simplified IVF method uses 2 chemicals, citric acid and sodium bicarbonate, to produce the CO_2 necessary to adjust the pH. The TWE-lab system was designed in collaboration with Jonathan Van Blerkom and is now a standardized procedure to perform an IVF treatment, for which all materials are available in a compact ready-to-use kit. The simplified IVF method uses 2 glass tubes in which the first tube serves as the generator of CO_2 by a chemical reaction between citric acid and sodium bicarbonate to produce the

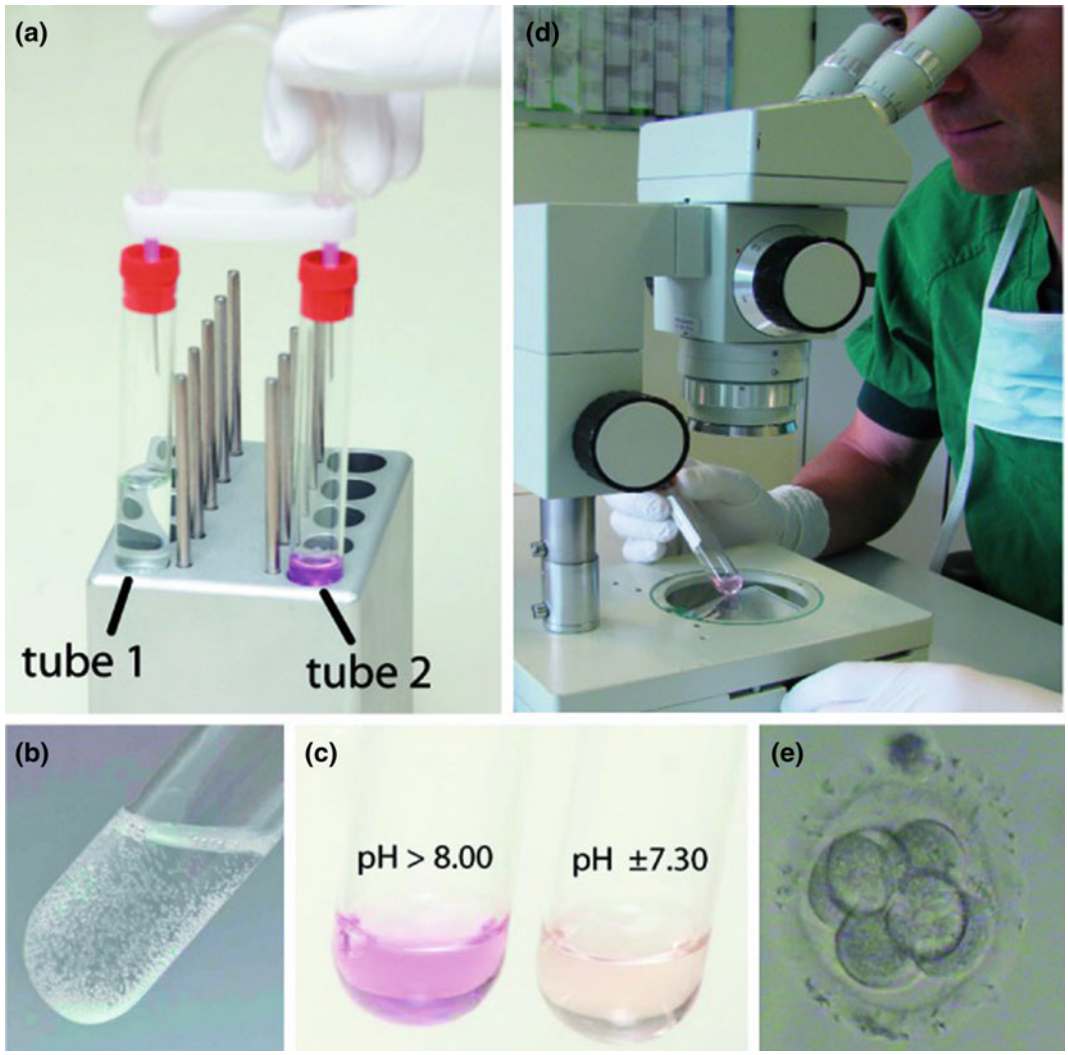

Fig. 20.4 TWE-lab method: **a** equilibration of the culture medium in tube 2 by the CO_2 produced in tube 1, **b** production of CO_2 in the generator tube, **c** phenol red shift from dark pink (pH > 8.00) to salmon pink (pH around 7.30), **d** embryo evaluation through the glass walls of the closed tubes, **e** an embryo visualized in the TWE-lab tube

CO_2 for equilibration of the culture medium in the second tube (Fig. 20.4). A connection between the tubes is established by needles and tubing to transport the produced gas from the generator tube to the tube with culture medium. The connection can easily be removed after the 24-h equilibration of the culture medium has completed. The tubes with culture medium can be used immediately, or they can be stored in a cold (2–8 °C) environment. To keep warm, the tubes are placed in a heated block (alternatively water bath or simple incubator without the need for a gas supply) at 37 °C. After oocytes have been harvested from the follicles, they are inserted individually in the equilibrated tubes using a 1-ml syringe and needle, without opening the tube or disturbing the air environment inside the tube. The TWE-lab system provides a closed

environment to ensure stable culture conditions and protect the gametes and embryos from possible adverse external effects. Between 1000 and 10,000 sperm cells are injected in a similar way to the tubes to inseminate the oocytes. After 24 h, fertilization is assessed by keeping the tubes at a slant and looking through the glass walls at the oocytes to see the 2 pronuclei that are a sign of sperm protrusion. Fertilization and embryo development are assessed through the glass walls of the tube. This simplified method removes the need for expensive air control systems and gives a very stable and safe environment for embryos to develop. The search for the materials needed in the kit has been extensive, and all items have important specifications that ensure good operation of the low-cost IVF method. Changing as little as the tube or the needle can result in a complete loss of function of the TWE-lab system.

Outcomes from the low-cost culture method were compared with those from a conventional IVF culture system in a study conducted at the IVF unit in Genk Belgium and were shown to be identical [45]. As we performed the study in routine IVF cycles, surplus embryos were frozen and these from the TWE-lab system that were thawed and replaced have also led to the birth of healthy children [54]. These studies prove the TWE-lab system to be an alternative low-cost system for a routine IVF procedure. Next step is to implement the TWE-lab IVF system in a centre that is limited in resources. Our goal is to open the first low-cost TWE-lab centres in two African countries in 2015.

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Genetic Aspect of Recurrent Implantation Failure

21

Grace Wing Shan Kong, MBBS, Jin Huang, PhD and Tin Chiu Li, MD, PhD

Introduction

Implantation is a process in which an embryo adheres to the luminal surface of the endometrium followed by migration through the luminal epithelium and invades into the deep layer of the endometrium. Implantation failure refers to the failure of the embryo to reach a stage when there is ultrasonographic evidence of an intrauterine gestation sac [1]. Recurrent implantation failure describes the phenomenon that failure to achieve a pregnancy following 2-6 IVF cycles, in which more than 10 high-grade embryos were transferred to the uterus [2].

The process of implantation involves both a healthy embryo and a receptive endometrium. The reasons for implantation failure may be either (1) the embryo is unable to grow further and implant, or (2) the uterine/maternal environment is hostile for the embryo to grow, or both. The failure of implantation due to embryonic causes is associated with either genetic abnormalities or other factors intrinsic to the embryo that impair its ability to develop in utero, to hatch, and to implant. Maternal factors including uterine anatomic abnormalities, thrombophilia, nonreceptive endometrium, and immunological factors may result in implantation failure. Genetic factor plays an important role in successful implantation. Abnormal genetic material in the embryo or/and endometrium will lead to the failure of implantation. Abnormal expression of the embryo is one of the major reasons for implantation failure and miscarriage. There are also increasing evidence that genetic factors regulating invasion and angiogenesis process in the endometrium are critical in embryo implantation.

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Embryonic Genetic Factors

Age Related Chromosomal Aneuploidies

It is well established that chromosomal abnormality of embryo is a major cause of repeated implantation failure [3, 4]. The majority of aneuploidy is originated from meiosis, and it may be caused by one of the two mechanisms—meiotic nondisjunction or premature separation of sister chromatids. Meiosis is the process of creating haploid gametes from a diploid cell. Once DNA replication is complete, two rounds of chromosomal divisions take place, one each in meiosis I and meiosis II. In meiosis I, homologous chromosomes pair up, line up, and are then split apart. In meiosis II, chromosomes line up and sister chromatids are pulled apart into two sets and results in four haploid cells. The pulling

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apart of either homologous chromosomes or sister chromatids is called disjunction and occurs during anaphase of meiosis I or meiosis II, respectively. Nondisjunction is defined as the failure of homologous chromosomes to segregate symmetrically at cell division. If nondisjunction occurs during anaphase I of meiosis I, this means that at least one pair of homologous chromosomes did not separate. The end result is two cells that have an extra copy of one chromosome and two cells that are missing that chromosome. If nondisjunction occurs during anaphase II of meiosis II, it means that at least one pair of sister chromatids did not separate. In this scenario, two cells will have the normal haploid number of chromosomes. Additionally, one cell will have an extra chromosome and one will be missing a chromosome. Premature separation of the sister chromatids is an alternative mechanism for nondisjunction. In premature separation of chromatids, first, homologues fail to pair during meiosis I. These univalents are prone to predivide, that is separation of the two chromatids, and subsequently these chromatids segregate independently.

This error from meiosis increases with increasing female age [5, 6]. Advanced maternal age is an adverse factor in reproductive outcomes and is more prone to aneuploidies and mosaicism involving sex chromosomes and autosomes 13, 16, 18, 21, and 22. Study from Munne et al. [7] demonstrated that the rates of aneuploidy detected by fluorescence in-situ hybridization (FISH) were 16, 37, and 53% in women aged 20–34, 35–39, and 40–45 years, respectively. Aneuploid embryos have decreased the ability to undergo successful implantation. Thus, aneuploidy accounts for the majority of implantation failures in older women. Besides, aneuploidy is the most frequent abnormality found in normally developing embryos. Many morphologically normal embryos either do not implant or spontaneously abort early in pregnancy because their chromosome number deviates from the normal diploid. Genetic testing of preimplantation embryos from chromosomal aneuploidy allows the selection of chromosomally normal embryos to be transferred into the uterus, which will increase the chance of conceiving, especially in patients with poor prognosis, such as repeated implantation failure and advanced maternal age.

Preimplantation genetic screening (PGS) is a screening test for de novo aneuploidy within embryos produced from parents with normal karyotype to allow the selection of only those chromosomally normal embryos for transfer. It is performed on the polar body or blastomere(s) or trophectoderm cells obtained from an embryo. Theoretically, avoiding the transfer of aneuploidy embryos will reduce the risk of implantation failure and improve the probability of conceiving a viable pregnancy [8].

However, the value of PGS in women with repeated implantation failure is controversial. First, there is no evidence to suggest that the embryos produced by women with repeated implantation failure are more likely to be abnormal. The frequency of aneuploidy (67%) in embryos from women with repeated implantation failure [9] was rather similar to the frequency (64%) in women with good prognosis [10]. Second, the studies of PGS in women with repeated implantation failure have not consistently showed promising results [11, 12].

What are the possible reasons for the limited efficacy of PGS? First, the biopsy procedure per se may damage the growth potential of embryo. Second, the relatively low success rate of FISH-related procedures often results in high rates of embryo without a diagnosis. Third, there is only limited probes available in FISH for genetic analysis with only up to 12-chromosome being screened, instead of the whole genome. Fourth, chromosomal mosaicism, in which different blastomeres have a different chromosomal complement and so the blastomere biopsied for PGS may not be representative of the remainder of the embryo. Mosaism is believed to be the most important contributor for the limited efficacy of PGS.

Effort has been made to improve the efficacy for PGS. Improvement has been achieved in biopsy technique and molecular diagnosis in PGS. Trophectoderm cells biopsy from blastocyst can increase the diagnostic accuracy because it provides more cells than the one or two cells

obtained from cleavage-stage biopsy for genetic analysis which may reduce the risk of amplification failure, allele dropout (ADO), and mosaicism. Kokkali et al. [13] prospectively compared diagnostic accuracy in preimplantation genetic diagnosis cycles after biopsy at the blastocyst versus the cleavage stage. They found that the diagnostic accuracy was significantly higher in the blastocyst group (94%) than in the cleavage-stage group (75%). In addition, removal of trophectoderm cells from blastocyst is less likely to negatively influence the pregnancy rate. New genetic technology of array comparative genomic hybridization (CGH) can help to improve the outcome of PGS by whole genome amplification (WGA). It avoids the drawbacks from FISH method, including hybridization failure and signal overlap, and splitting that can affect the accuracy of the interpretation. Besides, it provides benefit of simultaneous aneuploidy screening of all 24 chromosomes [14]. Moreover, it does not require preclinical validation before each IVF cycle, which is required for FISH. This avoids delay in IVF treatment. All these advances may improve the efficacy of PGS in improving reproductive outcome. However, aneuploidy is not the absolute cause of implantation failure. The cost and benefits of PGS should be considered for individual patients.

Parental Chromosomal Anomalies

Male and female partners are more likely to produce genetically aneuploidy gametes if they are carriers of chromosomal abnormalities. Stern et al. [15] found chromosomal abnormalities in 13 of 514 (2.5%) individuals with implantation failure. Translocations were detected in 7 of 219 couples (1.4%) with implantation failure, a number significantly higher than found among screened neonates.

Chromosomal translocations involve the transfer of genetic material from one chromosome to another and can be reciprocal, involving the breakage of two nonhomologous chromosomes with the exchange of segments, or Robertsonian, involving breakpoints close to the centromere of two acrocentric chromosomes. The importance of translocations relates to the pattern of segregation at meiosis. The patterns of inheritance are complex and depend on the particular chromosomes involved and the size of the rearrangements [16]. Previous study showed that couples with repeated implantation failure have a greater chance of carrying a balanced chromosomal translocation [17]. It is possible that the presence of unbalanced translocation in some gametes may predispose to preimplantation failure of embryo development.

Women with repeated implantation failure and their spouses should undergo karyotyping to rule out balanced translocations. When such an abnormality is detected, the couple should be offered preimplantation genetic counseling and diagnosis.

Sperm DNA Damage

Sperm also contribute to the embryo competence for implantation. Previous studies suggested that poor sperm quality can decrease the success rate of IVF due to abnormalities in chromatin and compactions in the sperm, as well as increased DNA fragmentation [18]. A recent hypothesis put forward by Leduc et al. is that alternations in the steps of chromatin remodeling or the DNA repair mechanism in elongating sperm during spermiogenesis are vulnerable to DNA fragmentation and continue to persist because spermatids lack a repair mechanism [19]. DNA fragmentation is highly correlated with sperm of poor morphology [20]. DNA fragmentation was also found to be associated with reduced natural conception, intrauterine insemination outcome, fertilization rate, embryo quality, implantation rate, and pregnancy rate in IVF cycles [21].

DNA fragmentation can sometimes affect embryo morphology, but it is also possible that the negative effect is only expressed when the paternal genome is activated 3 days post-fertilization. Therefore, the embryo can be morphologically normal when transferred, but stop to develop beyond day 3 in utero. This condition may occur in the couple with repeated

implantation failure despite repeated transfer of morphologically normal embryos. Indeed, male partners of women with repeated implantation failure were reported to have increased incidence of sperm chromosomal abnormalities [22], which may contribute to repeated implantation failure.

The sperm chromatin structure assay (SCSA) was first described in 1980, and it is the pioneering assay to measure sperm chromatin integrity and sperm function via flow cytometry of acridine orange (AO)-stained sperm. SCSA can distinguish the mature haploid and the abnormal diploid mature spermatozoa, cellular fragments and immature germ cells [23]. Results from previous studies showed that SCSA was highly dose-responsive to toxicants, highly repeatable, and provided meaningful biological information on sperm nuclear DNA defects [24]. Routine sperm cell assessment defines normal spermatozoa using low magnification. In fact, some anomalies can only be detected at higher magnification. Intracytoplasmic morphologically selected sperm injection (IMSI), using high magnification of $\times$6000, can exclude sperm with higher probabilities of DNA fragmentation and abnormal chromatin condensation. Sperm sorting for IMSI can improve fertilization, yield embryos with a higher developmental capacity and pregnancy rates in IVF cycles [25].

The clinical value of sperm DNA fragmentation test remains controversial. A meta-analysis reported that DNA integrity was not predictive of pregnancy outcome in assisted reproduction. However, high sperm DNA fragmentation appears to be associated with a reduced pregnancy rate and an increased miscarriage rate [26].

Endometrial Genetic Factors

The endometrium is responsible for implantation and is often considered as the 'soil' for the 'seed' to grow. However, the endometrium is more than the 'soil,' because it undergoes active remodeling and differentiation to provide an optimal environment for embryonic development. There are four stages of endometrium transitions: receptivity, implantation, decidualization, and placentation. Defective receptivity or implantation may cause infertility or miscarriage; suboptimal decidualization can lead to miscarriage or preterm birth, and abnormal placentation may result in preeclampsia [27, 28]. Successful establishment and maintenance of pregnancy involves close cross talks between the endometrium and the embryo, but ethical considerations restrict in-depth studies in human. Our current understanding of the molecular basis of the endometrium and embryo interactions in humans during the process of implantation is derived mostly from animal models and in vitro studies.

Genetic Polymorphisms

So far, 11 gene polymorphisms have been identified as having significant association with repeated implantation failure or recurrent miscarriage (Table 21.1) [29–32]. Although different genetic pathways are involved, most of the genes identified appear to play an important role in endometrium physiology. However, the significance of these associations is often controversial. Genetic polymorphism studies should be followed by well-planned clinical studies, meta-analysis, or functional studies to establish genotype–phenotype correlations [33].

Comparative Studies on Gene Expression Profiles

Implantation is a long continuing process, rather than a discrete event. It may be affected at different stages of the implantation (attachment, migration, and invasion). The precise genetic mechanism involved in implantation among different species may vary, transcriptome profiling around the time of implantation may be used to examine the similarities and differences among different species. However, transcriptome data analyses across species need to be relied on the

Table 21.1 Summary of genetic polymorphisms significantly associated with repeated implantation failure and recurrent miscarriage in meta-analysis studies

Gene	Genotype associated with RIF	Genotype associated with RM	Ethnicity	Other associated phenotypes
Apo E	Nil	E2, E3, E4 [34]	Asian, Caucasian	Higher LDL cholesterol
ACE	Nil	Insertion, deletion [35]	Asian, Caucasian	Thromboembolism, stroke, coronary artery disease
MTHFR	Nil	c.C677T, c.A1298C [36]	Multiple	Hyperhomocysteinemia, thrombophilia
MTHFD	Nil	c.G1958A [36]	Multiple	Hyperhomocysteinemia, thrombophilia
GSTT1	Nil	Null [37]	Multiple	–
HLA-G	Insertion, deletion [38]	Insertion, deletion [39]	Asian, Caucasian	–
VEGF	−1154A/A [40] +405C/C [41]	rs2010963, rs3025039 [42]	Multiple	–
IL-1	IL-1RN*2 [43]	rs16944, rs1143634, VNTR [44]	Multiple	–
P53	p.Pro72Pro [40]	p.Pro72Pro [45]	Caucasian	–
PAI	4G/4G [40]	Nil		
IL-10	Nil	c.G1082A [46]	Multiple	–

VNTR—variable number of tandem repeats

knowledge of orthologous and species-specific genes. So far, many genes for animal models have not been well annotated and under a prediction-based level, and some genes are species-specific which cannot be easily annotated. The main reason is lack of full-length cDNAs sequencing data of the model organisms, which has fundamental implications on RNA-seq data mapping and qualifying. Presently, most of the gene annotation is based on cDNA sequencing data from human, mouse, and rat [27] (Table 21.2).

There were some common pathways in endometrium shared among human and model animals. For example, leukemia-inhibitory factor (LIF), also known as cholinergic differentiation factor, was mapped at chromosome 22q12.2 in human and chromosome 11 in mice [57]. Increased LIF expression can cause recurrent miscarriage [58]. The protein encoded by this gene is a pleiotropic cytokine with roles in multiple stages [59]. Endometrial LIF increases the expression of epithelial growth factors (HB-EGF, Ereg, Ar) and implantation-related genes (Msx-1, Wnt-4) in receptive endometrium; during implantation, LIF produced by endometrium and blastocyst regulates growth and development of the embryo by increasing hCG secretion, enhancing the embryo-endometrium cross talk by increasing pinopodes and adhesion molecules (JAM-2, MUC-1, MUC-4), and synthesis of PGE2, COX-2, and mPGES-1 to stimulate decidualization. LIF can recruit a specific group of leukocytes (Eosinophil, NK cell, T and B lymphocytes) and limit the population and function of macrophages which participate in inflammatory tolerance at the maternal–fetal interface.

However, although some genes share similar pathways among different species, the regulatory mechanism may be different. For example, upregulation of msh homeobox 1 (MSX1), a transcription factor in the WNT signaling pathways, was found in endometrium of mice in the receptive stage, while downregulation was found in WNT signaling pathways of bovine, porcine, and human [27]. Through the recent

Table 21.2 Summary of genes or pathways in murine models account for implantation

Gene title	Gene symbol	Possible etiology
Death effector domain-containing protein	*Dedd*	Defective decidualization [47]
Sphingosine kinase genes	*Sphk*	Abnormal function in sphingolipid metabolic pathway results in defective decidualization with severely compromised uterine blood vessels [48]
Transformation-related protein 53	*Trp53*	Decidual senescence early in pregnancy, preterm birth, and fetal death [49, 50]
Lysophosphatidic acid receptor 3	*Lpar3*	Delayed implantation and embryonic development, and hypertrophic placenta [51]
Wingless-type MMTV integration site family, member 4	*Wnt4*	Defect in embryo implantation and subsequent defects in endometrial stromal cell survival, differentiation, and responsiveness to progesterone signaling [52]
Leukemia inhibitory factor	*Lif*	Blastocysts fail to implant and do not develop [53]
Indian hedgehog	*Ihh*	Embryo implantation [54]
Chicken ovalbumin upstream promoter transcription factor II	*Nr2f2*	Regulated by the progesterone-Ihh-patched signaling axis, defects in decidualization
Heart and neural crest derivatives-expressed 2	*Hand2*	Antiproliferative action of progesterone in uterine epithelium WOI, impaired implantation [55]
Early growth response gene 1	*Egr1*	Follicular development, ovulation, luteinization, and placental angiogenesis [56]

high-throughput transcriptome studies of bovine, porcine and equine endometrium during the estrous cycle and early pregnancy, 42 genes have been identified which share similar up/down expression pattern, while about 2000 genes are differently expressed [60].

Transcriptome Pattern and Endometrium Receptivity

Endometrium cyclic change is regulated by steroidal hormones binding to their respective receptors and interacting with specific co-chaperones or co-regulators. Initially, the endometrium proliferates under the influence of estrogen. After ovulation, the ovary starts to produce progesterone. Progesterone receptors (PR-A and PR-B) and estrogen receptors (ERα and ERβ) are differentially expressed in different cell types in the endometrium. The endometrium becomes receptive to the implantation of embryo 7 days after ovulation, known as the window of implantation (WOI), which usually appears 7–10 days after ovulation. Implantation is unlikely to be successful outside the WOI, or even if it does take place may become defective.

A series of studies have been carried out to delineate the gene expression pattern of the endometrium at the WOI. By comparing the endometrium samples in pre-receptive phase and receptive phase of normal individuals, 134 genes with a threefold differential expression have been identified as signature for endometrium receptivity [61]. To test the reproducibility of this panel of genes, 7 individuals were recruited to sample the endometrium twice at the same day of menstrual cycles with an interval of 2–3 years, and the same array platforms were performed. The reproducibility was 100%; the accuracy in predicting the WOI was significantly higher than that of the conventional method by histologic dating [62]. It was reported that the personalized embryo transfer guided by the result of the array platform improved the pregnancy rate and implantation rate of patients with recurrent implantation failure [63]. Such a novel approach need to be confirmed by prospectively planned clinical trial.

Advances in genomics have triggered a revolution in discovery-based research to understand complex biological systems. With the innovation of technologies, more information about implantation is uncovered, and the enigmatic 'black box' of implantation was soon been opened.

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Part IV
Development of In Vitro Maturation

IVM as Clinical Treatment: Overview 22

Ri-Cheng Chian, MSc, PhD

Introduction

Attempts to mature mammalian oocytes in vitro were started in 1930s. Pincus and Enzmann [1] were using a phosphate-buffered Ringer's solution to culture rabbit immature oocytes. The immature rabbit oocytes were cultured at 38 °C in hollow ground slides sealed with paraffin for in vitro maturation (IVM). In 1937, after obtaining and observing the first two unfertilized human eggs from flushing of excised uterus [2], they tried to culture human immature oocytes with the materials obtained from excision at operation [3]. The human ovaries were collected shortly after excision at operation. The different sizes of follicles were cut, and the collected human immature oocytes were cultured at 37 °C after different treatments briefly. The conclusion was that the removal of the immature oocyte from the follicle is sufficient to initiate a nuclear maturation [3].

The first in vitro-fertilized human oocytes were from in vitro-matured oocytes [4]. Subsequently, nearly 800 human follicular oocytes were isolated from the different sizes of follicles of the ovaries derived from the patients who underwent laparotomy [4]. The obtained immature oocytes were washed in Locker's solution and incubated for 22–27 h in the serum of the same patients, and exposed the oocytes to a washed sperm suspension in Locker's solution for 1 h, and then transferred the oocytes to fresh serum from a postmenopausal patient. They reported the first success of IVF of in vitro-matured human oocytes and obtained the cleaved human embryos in vitro [4, 5].

The similar experiments were repeated by Edwards in 1960s. Ovarian oocytes from mouse, pig, cow, sheep, monkey, and human were cultured in various media: Waymouth's medium, Tissue culture 199 (TC-199) medium, or Hank's saline supplemented with human and/or calf serum, antibiotics, and various other additives. All media were buffered with bicarbonate (pH 7.2) against 5% carbon dioxide in air [6]. As the culture techniques, the 'Falcon' plastic dishes were used. Important observation was made those immature human oocytes from GV stage to M-II stage needs at least 34–36 h of incubation vitro [7]. Afterward, Kennedy and Donahue [8] reported that human immature oocytes can be matured in vitro in a chemically defined medium. In fact, the chemically defined medium was F10 medium supplemented with 4 mg of bovine serum albumin (BSA) per milliliter. At the same time, they indicated that the presence of cumulus cells is essential for oocyte maturation in vitro [9].

With the in vitro-matured human oocytes, many studies on IVF have been done in early days. Edwards et al. [10] used Hank's solution supplemented with 15% fetal calf serum (FCS) for human immature oocyte maturation in vitro. After 38 h in culture, many of the

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oocytes become mature and these in vitro-matured oocytes were used for IVF study. Embryos were produced from in vitro-matured oocytes following IVF, but at that time, it was impossible to use those embryos for transfer in order to produce live birth. However, Edwards et al. [7] clearly indicated that there may be certain clinical uses for human eggs fertilized by this procedure. Although embryos were produced from pre-ovulatory human oocytes that aspirated 30–32 h after injection of human chorionic gonadotropin (hCG) [11], most oocytes obtained were at M-I stage. Therefore, the aspirated oocytes were needed to be incubated for 1–4 h in Ham's F10, Waymouth's medium, or TC-199 medium supplemented with some follicular fluid for final maturation before insemination. It means that the human embryos produced in vitro were from in vitro-matured oocytes rather than in vivo-matured oocytes. Nevertheless, the first live birth of IVF was from in vivo-matured oocytes rather than in vitro-matured oocytes [12].

The first successful IVM births were from immature oocytes collected at Cesarean section for oocyte donation [13], in which the immature oocytes were matured in TC-199 medium supplemented up to 50% follicular fluid. The first live birth of IVM using patient's own immature oocytes was reported by Trounson et al. [14]. The immature oocytes were from the patients with polycystic ovarian syndrome (PCOS) and cultured in TC-199 medium supplemented with 10% FCS.

Oocyte maturation in vitro is profoundly affected by the culture conditions. Although simple medium, such as Krebs-Ringer medium supplemented with pyruvate, lactate, and glucose, can support human oocyte maturation in vitro, the complex culture media, such as TC-199 medium, Hank's F10, and Chang's medium buffered with bicarbonate and supplemented with various sera, gonadotropins (FSH and LH), and steroids (estradiol and/or progesterone), have been most widely used in research or clinical application [15]. Apart from the culture conditions, the source of oocytes, especially the size of follicles, may be more important for oocyte maturation, fertilization, and the subsequent embryonic developments as well as pregnancy and healthy live births.

Based on many studies and clinical trials, it has been demonstrated that priming with follicle-stimulating hormone (FSH) and/or hCG prior to immature oocyte retrieval improves oocyte maturation rates and embryo quality as well as pregnancy rates in infertile women with PCOS [16–19]. To date, the clinical pregnancy and implantation rates have been reached approximately 35–45% and 10–15%, respectively, in infertile women with PCOS [20]. It is estimated that more than 5000 healthy IVM babies were born worldwide so far [21]. Nevertheless, IVM treatment is not popular as conventional (stimulated) cycle for infertile patients who need IVF treatment, because there are some learning curves for IVM treatment, such as immature oocyte retrieval and IVM of immature oocytes in laboratory. In addition, although there are some concerns about epigenetic disorders of IVM treatment, it is not the main issue worried for after understanding the IVM treatment clearly.

Methodology of IVM Treatment

As mentioned above, the clinical application of IVM oocytes has come a long way since the first live birth in the early 1990s. The first pregnancy in a woman with PCOS following IVM of immature oocytes and IVF was reported by Trounson et al. [14]. Another pregnancy was reported in a group of patients with PCOS treated with IVM combined with intracytoplasmic sperm injection (ICSI) and assisted hatching (AH) [22]. Subsequent studies indicated that although immature oocytes recovered from untreated patients with PCOS can be matured, fertilized, and developed in vitro, the implantation rate of these cleaved embryos is disappointingly low [15, 23, 24]. However, data indicate that with an alternative IVM treatment in these patients, using priming with follicle-stimulating hormone (FSH) or hCG before immature oocyte retrieval, the clinical pregnancy and implantation rates can be significantly improved [20]. There is no

unique IVM technology applied in the world. IVM technology can be divided into the following based on the hormone treatment before immature oocyte retrieval: (1) priming with FSH and (2) priming with hCG.

Priming with FSH

As an alternative approach, a truncated course of ovarian stimulation with FSH before immature oocyte retrieval has been applied, indicating that FSH pre-treatment promotes efficient recovery of immature oocytes and maturation in vitro [25]. It has been reported that the immature oocytes from stimulated cycles from normal cycling woman without hCG can be matured and fertilized in vitro as well as obtained pregnancy and live birth [26]. However, another report indicated that FSH priming with a fixed dose (150 IU/day) for 3 days from day 3 of menstrual cycle does not increase the number of oocytes obtained per aspiration and does not improve on oocyte maturation, cleavage rates, or embryo development for women with normal cycling ovaries [27]. Furthermore, Suikkari et al. [28] reported that although using low-dose FSH priming started from luteal phase improves the efficiency of immature oocyte recovery, maturation, and fertilization rates, the average number of immature oocytes collected, the rates of oocyte maturation, and fertilization are not different between women with regular menstrual cycles and women with irregular cycles of PCOS. Nevertheless, it has been reported that priming with rFSH during follicular phase before harvesting of immature oocytes from the patients with PCOS improves the maturational potential of the oocytes and the implantation rate of the cleaved embryos, indicating that significantly higher pregnancy (29%) and implantation (21%) rates were obtained when priming with FSH before immature oocyte retrieval [29]. Mikkelsen et al. [30] also reported that there are no differences in the rates of oocyte maturation, fertilization, cleavage, or implantation between 2 and 3 days that are the time (coasting) interval between FSH priming and aspiration of immature human oocytes for IVM when the normal menstrual cycling women were primed with 150 IU FSH/day for 3 days started at day 3. It seems that optimizing IVM treatment for different groups of patients is important and that after individualized IVM treatment, the pregnancy and implantation rates per embryo transfer can be reached to 23 and 14%, respectively [31].

Interestingly, it has been reported that FSH priming with 75 IU/day for 6 days in combination with hCG priming 36 h before immature oocyte retrieval has no additional benefit for women with PCOS [32]. Although these results are conflicting on the benefits of using FSH priming in women with regular menstrual ovaries or irregular menstrual cycles of PCOS, theoretically, the use of FSH priming at the beginning of follicular or luteal phases may enhance more follicular development and the maturational competence of immature oocytes in vivo.

Priming with HCG

A few GV stage oocytes may be retrieved from the stimulated cycles even 36 h after hCG administration. These immature oocytes are capable of undergoing IVM and then normal fertilization and development. Although the successful pregnancies have been established using those in vitro-matured oocytes [33–36], the pregnancy rate was unacceptably low. It has been noticed that morphological and molecular differences exist between the immature oocytes collected from stimulated cycles and collected from Cesarean section [37]. In addition, it has been found that the time course of germinal vesicle breakdown (GVBD) and oocyte maturation is different between these oocytes, although the final rates of oocyte maturation are not different in the groups [38]. It appears that the oocytes retrieved from follicles in women undergoing ovarian stimulation respond to hCG that may promote the initiation of oocyte maturation in vivo. It has been demonstrated that the time course of oocyte maturation in vitro is

hastened, and the rate of oocyte maturation is increased by priming with 10,000 IU hCG for 36 h before retrieval of immature oocytes from women with PCOS [16, 17]. Therefore, it is possible that pregnancy rate may potentially be improved by priming with hCG prior to immature oocyte retrieval [18]. This hypothesis was confirmed by other reports [32, 39–41].

Lin et al. [32] reported that 36.4% clinical pregnancy rate was obtained from 33 cycles of IVM treatment when priming with hCG before immature oocyte retrieval from women with PCOS, indicating the beneficial effect of hCG priming on IVM treatment. Therefore, it seems that with hCG priming not only promotes some oocytes initiated maturation process to metaphase-I stage from the relatively bigger size of follicles (>10 mm in diameter) but also enhances some GV stage oocytes from the small follicles to acquire maturational and developmental competence in vivo.

Clinical Outcomes

For last 15 years, a significant progress has been made to improve pregnancy and implantation rates from in vitro-matured human oocytes. IVM technology can now be offered as a successful option to infertile women with PCOS. As mentioned above, it has been demonstrated that immature oocytes priming with FSH or hCG prior to immature oocyte retrieval improve oocyte maturation rate and embryo quality as well as pregnancy rates when it is retrieved from women who are infertile with PCOS. The size of follicles may be important for the subsequent embryonic development, but the developmental competence of oocytes derived from the small antral follicles seems not to be adversely affected by the presence of a dominant follicle. It has been collected approximately 1500 IVM babies born from the worldwide (Table 22.1). It seems that there are no differences between IVM babies and naturally conceived babies in terms of birthweight and birth defects [21].

Development of IVM Treatment: Natural Cycle IVF/M

PCOS is the most widespread endocrinological disorder among women of reproductive age as well as the most common cause of anovulatory infertility and has been shown to exist in approximately 10% of the general population [42]. As described above, IVM treatment was most likely applied to infertile women with PCOS as an option of infertility treatment, because this group of patients are very sensitive with exogenous gonadotropins for conventional (stimulated) IVF treatment cycle [43, 44]. Whether or not IVM treatment could be applied to women with normal ovaries, i.e., for women with regular menstrual cycles? Before answering this question, it should be clear that physiology of folliculogenesis in the ovaries. IVM treatment is offered as successful option to infertile women with PCOS, because there are many antral follicles in the ovaries in this group of patients.

In women, although only a single follicle usually grows to the pre-ovulatory stage and releases its oocyte for potential fertilization, there are many small follicles that also developed during the same follicular phase of the menstrual cycle. It seems that approximately 20 antral follicles are selected and developed during each menstrual cycle [45]. As mentioned in Chap. 2, it has been documented that two or three waves of ovarian follicular development in women during menstrual cycle were based on daily transvaginal ultrasonography, challenging that the traditional theory of a single cohort of antral follicles grows only during the follicular phase of the menstrual cycle [46, 47]. Animal model studies also supported these findings that oocyte quality and early embryonic developmental competence of immature oocyte following maturation in vitro are not detrimentally affected by the presence of the dominant follicle in the ovaries [48, 49].

It has been demonstrated in human that atresia does not occur in the non-dominant follicles even after the dominant follicle is selected in the ovary during folliculogenesis, because immature

Table 22.1 Obstetric outcomes and congenital abnormalities in 1421 IVM babies born from 1187 pregnancies [21].

Characteristics from 1187 pregnancies	Singleton pregnancies (n = 960)	Twin gestation pregnancies (n = 221)	Triplet gestation pregnancies (n = 5)	Quadruplet gestation pregnancies (n = 1)
Mean gestational age at delivery (weeks + days)	37 + 4	36 + 5	35 + 2	29 + 0
No. of deliveries at >37 weeks (%)	855 (89)	60 (27)	0 (0)	0 (0)
No. of deliveries at 34–37 weeks (%)	82 (9)	132 (60)	5 (100)	0 (0)
No. of deliveries at <34 weeks (%)	23 (2)	29 (13)	0 (0)	1 (100)
Total of 1421 newborns	Singleton newborns (n = 960)	Twin newborns (n = 442)	Triplet newborns (n = 15)	Quadruplet newborns (n = 4)
Birthweight (mean ± SD) (g)	2965 ± 532	2434 ± 365	1968 ± 472	1330 ± 84
No. of LBW (%)	35 (4)	59 (13)	12 (80)	0 (0)
No. of VLBW (%)	5 (1)	12 (3)	2 (13)	4 (100)
Median Apgar score at 1 min (interquartile range)	9 (7–9)	8 (7–9)	8 (8–9)	–
No. of Apgar score at 1 min less than 7 (%)	133 (14)	31 (14)	0 (0)	–
Median Apgar score at 5 min (interquartile range)	10 (9–10)	10 (9–10)	8 (8–9)	–
No. of Apgar score at 5 min less than 7 (%)	25 (3)	5 (2)	0 (0)	–
Incidence of congenital anomalies (%)	15 (2)	3 (1)	0 (0)	0 (0)

LBW Low birthweight, 1500–2500 g
VLBW Very low birthweight, <1500 g
SD Standard deviation

oocytes retrieved from non-dominant follicles have been successfully matured in vitro, fertilized, and have resulted in several pregnancies and healthy live births [50]. Therefore, one very attractive possibility for the enhancement of the success of natural cycle of IVF treatment is its combination with immature oocyte retrieval and IVM, namely natural cycle IVF/M. If the mature oocyte from the dominant follicle together with immature oocytes from the small follicles was collected as well, the chances of a pregnancy are greatly increased when we manage to mature these immature oocytes and produce several viable embryos. A study indicates that the clinical pregnancy rate can be reached to approximately 45% with per embryo transfer following natural cycle IVF/M in a selected group of patients [51, 52].

The literature reports for pregnancy rate per embryo transfer in natural cycle IVF varied as described in previous chapters. A number of problems arise in natural cycle IVF treatment alone, including an increased risk of empty retrieval during oocyte collection leading to cancellation of treatment cycle [53]. This disadvantage seems not in the case of the natural cycle

IVF/M. It has been shown that more than a half of infertile women who came to infertility clinic for IVF treatment can be treated with natural cycle IVF/M or IVM alone when these treatments have been chosen primarily, and that natural cycle IVF/M is an efficient treatment, especially for women under age of 35 years [52, 53]. Therefore, it has been clearly demonstrated that natural cycle IVF/M is more efficient treatment than natural cycle IVF alone. This may be the future direction for infertility treatment with IVM technology.

Conclusion

Priming with FSH or hCG prior to immature oocyte retrieval improves oocyte maturation and pregnancy rates when the immature oocytes retrieved from women with PCOS. The size of follicles may be an important feature for the subsequent embryonic development, but the developmental competence of oocytes derived from the small antral follicles seems not to be adversely affected by the presence of a dominant follicle. Approximately, 5000 healthy infants have been born following immature oocyte retrieval and IVM. In general, the clinical pregnancy and implantation rates per ET have reached 35–45% and 10–15%, respectively, in women with PCOS. Therefore, as an option, IVM treatment can be offered to women infertility with PCOS. The combination of natural cycle IVF with immature oocyte retrieval followed by IVM is an attractive treatment for women with all types of infertility without recourse to ovarian stimulation with acceptable pregnancy rate.

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Avoidance of Severe Ovarian Hyperstimulation with IVM Treatment

23

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Introduction

Many consider ovarian hyperstimulation syndrome (OHSS) to be the most significant side effect of controlled ovarian hyperstimulation (COH) and of IVF procedures. Severe OHSS occurs frequently enough that anyone considered a practitioner of IVF has managed difficult patients with this condition. Because deaths due to severe OHSS have been reported, management of patients with severe forms of OHSS is a stressful undertaking. Patients may require management in a hospital's intensive care unit since they may experience the same physiological problems as those of the critically ill. However, because of a very different underlying etiology, the necessary management is different from the more routine patients in the intensive care setting. The IVF practitioner may have to personally undertake responsibilities of a critical care physician. Since the stakes of experiencing severe OHSS are so costly, the best management of severe OHSS is to avoid having it occur [1, 2].

Our primary objective in this chapter is to discuss an uncommon approach to avoiding severe OHSS, namely the use of in vitro maturation (IVM) techniques. Awareness of the utility of IVM can change a physician's options in providing IVF care because it offers a much gentler approach to achieving pregnancy for patients at high risk for OHSS. However, it can also expand the tools that the IVF practitioner can provide to his/her patients threatened with the potential development of severe OHSS even in programs that do not offer IVM.

Ovarian Hyperstimulation Syndrome (OHSS)

OHSS presents with a continuum of symptoms. Severe OHSS can become life-threatening. Moderate OHSS needs close follow-up since OHSS is a changing condition and it may become severe OHSS. Mild OHSS is simply uncomfortable for the patient. Over the years, various practitioners have published criteria defining OHSS [3–5]. The most recent widely referenced definition of OHSS is due to Golan [5] and the key determinant of moderate OHSS in his classification is the presence of ascitic fluid on transvaginal ultrasound examination. Golan states that mild OHSS is a common consequence of COH and therefore clinically unimportant. This is consistent with current medical practice. It is sufficiently common for a patient to report that her "ovaries hurt when she moves," that both physicians and patients accept this as a normal side effect of COH rather than a complication of COH. However, earlier classifications viewed these symptoms as constituents of mild or moderate OHSS [3, 4]. The lack of importance of these symptoms of discomfort to physicians may

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be more related to their being unavoidable during COH than to their not imposing a burden on patients to bear as a cost of IVF therapy.

Some researchers note that the incidence of OHSS is likely to be underreported in retrospective reports and in IVF registries [6]. Perhaps this reporting deficit is likely to increase as physician experience with IVF and OHSS becomes more routine. Even severe OHSS can now often be safely managed in an outpatient setting [7]. Managing OHSS has evolved into a part of the IVF management process rather than as a complication of IVF. OHSS is likely to be viewed as a complication of IVF only when the physician feels that the patient requires hospitalization because of it. The US SART IVF registry provides no guidance on when to define OHSS as a complication [8].

There are two different common variants of OHSS seen after COH for IVF. The most frequent is an early-onset form, which generally occurs about three to five days after administration of hCG. The other is a late-onset form of OHSS that presents about 12–15 days after administration of hCG [9, 10]. The symptoms of these forms of OHSS are the same, but the late-onset form usually requires that a multiple gestation has occurred. A less frequently seen form of severe OHSS is often referred to as "familial OHSS." Its occurrence has to do with abnormal activation of the FSH receptor due to mutations coding for the FSH receptor [11]. Perhaps some of the difficulties in predicting which patients will develop severe OHSS is related to natural variants on FSH receptors.

The occurrence of OHSS requires the production of vascular endothelial growth factor (VEGF). VEGF is an angiogenic factor that induces the vascular permeability that is responsible for much of the symptomatology of OHSS [12–15]. VEGF is produced by granulosa cells and is found in a high concentration in the follicular fluid of periovulatory follicles. VEGF production markedly increases with a midcycle LH surge or hCG administration [15–17]. As an angiogenic factor, VEGF is required for the extensive vascular network that forms around the preovulatory follicle and the neovascularization that occurs with the formation of the corpus luteum. VEGF greatly enhances vascular permeability, a cause of many of the problems of OHSS. The enhanced vasculature of the preovulatory follicle is thought to be responsible for the normally increased responsiveness of the dominant follicle to FSH so that FSH can decrease below what is required for the growth of non-dominant follicles while allowing the dominant follicle to continue to grow [18]. The amount of VEGF produced in the in vitro culture also varies with individual granulosa cells (presumably related to individual differences of patients) [13]. After a week in culture, granulosa cells exhibit a sudden increased responsiveness to hCG with the production of high levels of VEGF. This temporally corresponds to the timing of hCG production by the embryo and may also explain why there is a late-onset form of OHSS [13].

Since granulosa cells produce VEGF, their presence is a necessary condition for the development of OHSS. The number of granulosa cells contained in a follicle is directly related to its diameter. Small antral follicles, 4 mm in diameter, contain approximately 1 million granulosa cells. Large antral follicles, 12 mm in diameter, contain approximately 10 million granulosa cells. Preovulatory follicles, 20 mm in diameter, contain about 50 million granulosa cells [19].

The ability of granulosa cells to respond to LH or hCG requires the activity of LH receptors. Significant expression of LH on granulosa cells does not take place until preovulatory follicle maturation, which occurs when the follicle diameter is approximately 16 mm. Until adequate LH receptors have been synthesized by the granulosa cells, the follicle is not able to fully respond to the midcycle gonadotropin surge [18].

In summary, VEGF production is a requirement for OHSS to occur. VEGF is produced by granulosa cells, which are present in large numbers only in larger follicles. VEGF production is required at times of high demand for neovascularization, namely prior to ovulation, at the formation of a corpus luteum, and to rescue the corpus luteum when a pregnancy occurs. In all of these situations, LH (or hCG) appears to be the

primary trigger for VEGF production. Significant LH receptors are present in the follicle only in the late stages of its development.

In Vitro Maturation (IVM) of Immature Oocytes

IVM is an advanced reproductive technology that involves harvesting oocytes from antral follicles. Practitioners differ about the size of the largest antral follicle at which the decision to retrieve oocytes is made, with some practitioners making the decision when all antral follicles have diameters under 10 mm [20], 12 mm [21], or 14 mm [22]. The choice depends on the importance the practitioner places on avoiding either the selection or the impact of a dominant follicle on the IVM procedure. Estrogen may also be used to suppress the development of a follicle likely to become dominant [23]. Even at 14 mm, follicles only have developed a small portion of their future granulosa cell population and thus have at most a small component of their future secretory capacity [19]. Follicles with diameters under 14 mm have not developed the full capacity to respond to an LH surge. In particular, the follicles present during IVM retrieval have a limited capacity to respond to LH or hCG compared to larger follicles.

Practitioners also differ in their use of adjunctive gonadotropins (referred to as "priming") with some using nothing [24], some using small amounts of FSH [23], some using hCG [21], and some using both FSH and hCG [26]. The most common use of FSH is 150 IU for 3 days in the beginning of the cycle [22, 25]. There are also other ways that FSH can be used [27], but the total dose is almost always less than 500 IU during the cycle. FSH use in IVM is an attempt to improve oocyte competence rather than over-stimulate the ovary to maximize the number of mature oocyte produced as in conventional IVF. Human chorionic gonadotropin is not used to trigger the mechanisms of ovulation as it does in IVF, but to primarily enhance the competence of the oocytes to become mature in culture [28]. Human chorionic gonadotropin is usually given 36–38 h prior to oocyte retrieval, and as noted above, at the time when all follicles have diameter less than 14 mm. The combined use of low-dose FSH together with hCG is also used to try to optimize early oocyte competence [26].

The best approach to IVM has not been established since advocates of these differing approaches have all been successful with their programs. All approaches to IVM involve aspiration of follicles with a much simpler architectures and very different secretory capacities than during conventional IVF. Even when FSH and/or hCG is used during an IVM cycle, the timing of oocyte aspiration occurs in a different environment than during conventional IVF. On a theoretical basis, given the role that VEGF plays in the development of OHSS, early-onset OHSS (mild, moderate, or severe) should not occur during an IVM cycle. Late-onset OHSS should also not occur since there is no corpus luteum to stimulate with hCG produced by the pregnancy. For patients with familial OHSS, IVM (without FSH priming) is the only safe management approach. The medical literature supports the theory that use of IVM prevents OHSS. As Guzman et al. stated "…IVM is currently the only ART with no reported cases of OHSS." [29].

The use of IVM on patient populations at high risk for OHSS has been a driving force in the development of IVM [21, 24]. Two studies have compared patients treated by conventional IVF to patients treated by IVM in contemporaneous cycles in the same program [30, 31]. Information about the occurrence of OHSS in these studies is summarized in Table 23.1. Moderate or severe OHSS was not seen in patients treated by IVM. The incidence of severe OHSS in the 204 IVF patients was 9.8%, but the burden of dealing with the risk of severe OHSS was clearly higher. Although the occurrence of mild and moderate OHSS after IVF was not reported, the incidence after IVM was also 0%. All the IVM cases listed in Table 23.1 used 10,000 U hCG as priming medications prior to oocyte harvesting. Since OHSS did not occur, it is likely that the granulosa cell mass and LH expression were inadequate to respond sufficiently to hCG to produce symptoms in these patients.

Table 23.1 Studies comparing OHSS[a] in IVM and conventional IVF

Author	Type of study	Patient diagnoses	Number of cases	CPR/transfer (%)	Cases cancelled for OHSS risk	No transfer (cryo) for OHSS risk	Coasting for OHSS risk	Hospitalized for OHSS risk	Moderate or severe OHSS[b] (% of cases)	Total OHSS burden[c] (% of cases)
Gremeau et al. [30]	Retrospective case control	PCO and PCOS matched for age, diagnosis, and ovulatory status	IVF–97 IVM–97	49/90 (54.4) 19/93 (20.4)	Not stated 0	4 0	9 0	8 0	8 (8.2) 0	14 (14.4) 0
Child et al. [31]	Retrospective case control	PCO pattern matched for age, diagnosis, and month of procedure	IVF–107 IVM–107	36/105 (34.3) 23/105 (21.9)	1 0	0 0	Not stated 0	1 0	12 (11.2) 0	13 (12.1) 0

[a]Golan et al. [5]
[b]As stated in reference
[c]Based on content of reference

Based on the available data, IVM eliminates severe OHSS in patients who are candidates for IVM. If one views the primary benefit of IVM as its ability to reduce the incidence of OHSS, then it is worthwhile to try to understand what burden OHSS presents for IVF. At present, IVM is primarily used for patients with ovulatory PCO and anovulatory PCO (PCOS) [21–24, 29–31]. In 2006, Heijnen et al. published a meta-analysis of case-control studies of IVF cycles in patients with and without PCOS [6]. They found nine studies meeting their criteria, but these uncommonly provided information on cycle cancellations for hyper-response or on the incidence of OHSS. Overall cycle cancellation (for any reason) was four times as frequent for PCOS patients than for non-PCO patients. Three studies provided information on OHSS. In one study, two patients (11%) were hospitalized for moderate to severe OHSS. The second study reported a 16.9% incidence of mild to moderate OHSS and a 3.9% incidence of severe OHSS. The third study reported two cases of OHSS in PCOS patients and one case in a non-PCO patient. At best, we conclude that OHSS is not uncommon in IVF patients with PCOS. Again, our understanding of the factors influencing the development of OHSS and the differences between IVM and IVF suggests that OHSS should not occur with IVM in patients with PCOS. IVM also prevents the discomfort that patients feel due to mild OHSS after IVF. The number of such patients involved is even harder to quantify, but avoiding pain and discomfort in patients is a routine part of good medical care.

Diverse management approaches or treatments for patients either known to be at high risk for OHSS before the start of an IVF cycle or who become high risk for OHSS in the course of an IVF cycle have been advocated. Some of the patients who become high risk for severe OHSS during a cycle are managed by cycle cancellation prior to oocyte aspiration and they incur considerable wasted expense in terms of medications, monitoring costs, and time missed from work. In 2012, more than 2400 women in the USA under age 35 had cycles cancelled prior to oocyte aspiration [8]. At least some of these cancellations were due to OHSS risk. In an ESHRE questionnaire survey, up to 20% of IVF physicians would manage patients having very high estradiol levels by cycle cancellation [1]. In a Web-based Internet study, 18% of physicians from 262 centers and 68 nations would also manage a patient with a very high risk of severe OHSS by cycle cancellation [32].

There are several treatments to mitigate or prevent the development of severe OHSS that are widely used. They are not as effective as IVM because they are less effective in modifying the impact of VEGF on the patient (Table 23.2). Most of these interventions impose extra burdens on patients that they would not experience with IVM (Table 23.3).

The most effective of these (after IVM) is using a GnRH agonist to trigger oocyte maturation. GnRH agonists produce an LH surge that is shorter in duration than a physiological surge. This LH spike is adequate to promote oocyte maturation, but not adequate to establish a corpus luteum. Early luteolysis eliminates the period of highest production of VEGF and also prevents late-onset OHSS since there is no corpus luteum to be rescued. Some of this benefit may be lost with the concomitant use of low doses of hCG, which may be used with a GnRH agonist trigger to improve the pregnancy rate. This is because the half-life of hCG is much longer than that of LH, and hCG activates the VEGF system more effectively than does LH [33, 40]. Table 24.4 is a sample listing of reports in the literature of OHSS occurring in cycles utilizing a GnRH agonist to trigger oocyte maturity. One may hypothesize that these episodes of OHSS are due to severe OHSS occurring prior to the use of GnRH agonist, an atypical response by the patient to GnRH agonist, a confounding effect of low-dose hCG use, or a genetic mutation of the FSH or VEGF receptor. Similar to IVM for which a corpus luteum is not present, when a GnRH agonist is used, the luteal phase and early pregnancy need to be managed with supplemental estrogen and progesterone (Table 23.4).

Table 23.2 Treatments to avoid or mitigate OHSS and their probable impact via VEGF

Treatment	Impact	Comments
Freeze-all approach	Avoids impact of hCG from pregnancy, thus avoiding late increased VEGF production by rescued corpus luteum	Avoids late-onset OHSS. Likely speeds resolution of early-onset OHSS
Coasting	Downregulates VEGF gene expression and decreases potential of granulosa cells to produce VEGF by causing apoptosis [9]	No impact on late-onset OHSS
Albumen infusion	Unclear	Mitigates impact of increased vascular permeability
GnRH agonist	Attenuated LH surge caused by GnRH agonist leads to impaired development of corpus luteum with early and complete lysis of corpus luteum [33]	Effective for both early- and late-onset OHSS. Mild and moderate OHSS commonly occurs. Cases of severe OHSS tied to suboptimal GnRH agonist induced LH surges and to low-dose hCG [33, 34]
Dopamine agonist	Inhibits phosphorylation of VEGF receptor-2 and induces VEGF receptor internalization and lower activation [14, 35]	Long treatment course needed to impact both early- and late-onset OHSS
Pericentesis	May decrease VEGF availability by the removal of fluid containing VEGF	Highest production of VEGF occurs 48 hours after hCG [35]
IVM	Oocytes are removed before adequate granulosa cells are available to produce very high levels of VEGF. HCG is given before the follicular unit is fully capable of responding. No corpus luteum is formed	Effective for both early- and late-onset OHSS

Table 23.3 Advantages and disadvantages of treatments to avoid or mitigate OHSS

Treatment	Advantages	Disadvantages	Comments
Cycle cancellation	Avoids risks and limits discomfort of OHSS. Enables physicians to achieve a better outcome next cycle	Loss of resources used for current cycle. Disruption of patient's plans	Patient may not be able to undertake another cycle for economic or psychological reasons
Freeze-all approach	Avoids worsening a high-risk situation by production of hCG if pregnancy were to occur	Delays time to pregnancy for patient. Incurs additional costs associated with cryopreservation and delayed embryo replacement	Insurance may not pay for additional associated costs
Coasting	Decision to coast can be made late in the ovulation induction	Lengthens cycle and adds monitoring costs. Cycle may need to be cancelled if estradiol drops too quickly or not quickly enough	Significant OHSS may still occur [36]
Albumen infusion	Treats some symptoms of OHSS	Not sufficiently adequate alone	Meta-analysis suggests that this saves one case of severe OHSS for every 18 high-risk women [37]
GnRH agonist	Easy to use as long as all high-risk patients utilize antagonist ovulation inductions	Requires careful management of luteal phase and early pregnancy. Ascertaining adequacy of induced LH surge may be useful. Mild and moderate OHSS can still occur	Lower ongoing pregnancy rate than with hCG in meta-analysis [38]. Dual trigger may make less effective in preventing OHSS [34]

(continued)

Table 23.3 (continued)

Treatment	Advantages	Disadvantages	Comments
Dopamine agonist	Easy to use	Requires management of luteal phase. Some concerns about ergot-derived dopamine agonists leading to cardiac damage. Concerns about drug tolerability of non-ergot-derived dopamine agonists [14]. All forms of OHSS can still occur [39]	Need long duration of medications to reduce both early and late OHSS
Pericentesis	Enables management of severe OHSS as outpatient [7]. Improves hospital management of OHSS	Can be unpleasant for the patient without anesthesia	Patients experiencing a cycle where this was required are unlikely to want to repeat it
IVM	Prevents early- and late-onset OHSS. Symptoms related to high estradiol levels avoided. Simpler, gentler, cheaper, shorter duration cycles than conventional IVF	Lower pregnancy rates per cycle than conventional IVF in most published studies	Requires management of early pregnancy until placental function adequate

Table 23.4 OHSS after use of GnRH agonists to trigger oocyte maturation

Author	Cases	Oocyte maturation	Comments
Van der Meer et al. [41]	Three cases of moderate OHSS in 27 high-risk patient cycles	2.4 mg buserelin acetate	
Gerris et al. [42]	One case of moderate OHSS out of 9 cases	0.5 mg GnRH	
Lee et al. [43]	Clinically significant OHSS in 6 out of 50 cases. Three cases were hospitalized	0.2 mg triptorelin with 500 U HCG given on day of retrieval	
Griesinger et al. [44]	One case of severe early-onset OHSS out of 51 patients	0.2 mg triptorelin	
Griffin et al. [45]	One case of mild OHSS out of 34 with dual trigger. None with GnRHa only (n = 68)	1 mg luprolide acetate. Dual trigger used 1000 U hCG at same time	Clinical pregnancy rate 58.8% with dual trigger and 30.9% without
Shapiro et al. [34]	One case of clinically significant late-onset OHSS out of 182 using dual trigger. No significant OHSS in patients with GnRH agonist only (n = 115)	4 mg luprolide acetate. Dual trigger used 33 U hCG/kg at same time	Highest pregnancy rate with dual trigger. Decrease in pregnancy loss rate with dual trigger or enhanced luteal support
Radesic and Tremellen [46]	One case of severe OHSS requiring hospitalization out of 71 cases	2 mg luprolide acetate with 1500 U hCG at retrieval	
Humaidan et al. [47]	Two cases of moderate late-onset OHSS in high-risk dual trigger group. Two cases of severe late-onset OHSS occurred in low-risk group with extra hCG dose	0.5 mg buserelin with 1500 U hCG at same time and second low risk for OHSS group getting second dose of 1500 U hCG on day of retrieval	

(continued)

Table 23.4 (continued)

Author	Cases	Oocyte maturation	Comments
Seyhan et al. [48]	Six out of 23 women developed severe early OHSS after dual trigger protocol. Five of these women required hospitalization. In three cases, embryo transfer was withheld	1 mg buserelin acetate or 0.2 mg triptorelin with 1500 hCG given at the same time	
Iliodromiti et al. [49]	Two cases of severe OHSS in 275 cycles	Various GnRHa triggers with 1500 U hCG given at aspiration	
Fatemi et al. [50]	Two cases of severe early OHSS after single trigger, freeze all, and cabergoline	0.3 mg triptorelin or 0.2 mg decapeptyl	Cabergoline was started at retrieval for one and six days later for other
Humaidan [51]	One case of late moderate OHSS out of 12 patients	0.5 mg buserelin with 1500 U hCG 35 h later	
Gurbuz et al. [52]	Three cases of severe OHSS with freeze-all strategy		

Rescue IVM and IVF with Early Aspiration Rescue

Experience with IVM or IVM techniques can also be useful to prevent severe OHSS in patients who become high-risk patients in the course of their COH. Physicians may elect a COH approach for some patients using a GnRH agonist who unexpectedly respond by developing early severe OHSS. For example, on day six of gonadotropins, a patient might have an estradiol level of 5000 pg/ml with ovaries containing multiple follicles all less than 12 mm in diameter. The standard approach to this situation could be to cancel the cycle and try again in the future with a different approach to COH. Based on accumulated experience from IVM, a better approach would be to treat the patient with 10,000 U of hCG either on this day or the next and harvest oocytes 36 h after administering hCG. We would expect that 20–30% of the oocytes recovered would be mature and capable of being fertilized. This should provide the patient with one or two good quality embryos to transfer and a chance at pregnancy instead of cycle cancellation. This approach has been referred to as IVF with Early Aspiration Rescue (IVF with EAR) [53]. If the IVF program routinely provides IVM, then the patient may be provided an even better chance at achieving pregnancy by culturing the large number of initially immature oocytes and achieving maturity in approximately 60% of them. This has been termed rescue IVM [53].

As noted above, the number of granulosa cells present in follicles under 14 mm is small compared to the number present in a normal IVF cycle [19]. Also, follicular structures under 14 mm are not able to fully respond to hCG and a corpus luteum is not formed. Thus, severe OHSS will not occur [53, 54]. Rose reported a clinical pregnancy rate for IVF with EAR of 60% with five cases [53]. Lim et al. reported a clinical pregnancy rate of 46.1% with 17 cases (with use of hCG) [54]. Fatum et al. reported on five cases, all of who achieved clinical pregnancies after rescue IVM using hCG [55]. Brigante et al. reported a 37.5% clinical pregnancy rate in eight cases in which IVM without hCG was used [56]. Severe OHSS did not occur in any of these 36 cases.

The key to IVF with EAR or rescue IVM being successful has to do with the number of follicles that a patient has in the 8–14 mm range. The closer the follicles are to 14 mm in size, the higher the probability that a follicle will contain a mature oocyte. Scott et al. found MI or MII

oocytes in 9% of follicles under 11 mm in diameter and in 30% of follicles between 12 and 14 mm collected during conventional IVF [56]. The IVM literature suggests that when oocytes have been exposed to exogenous gonadotropins and hCG (priming with both), then 20% of the oocytes will be mature [26, 57]. The constraint of this approach is that the decision to convert the IVF cycle to IVF with EAR or IVM rescue must be made early enough to avoid the development of severe OHSS (having a maximal follicle less than 14 mm).

Another positive feature of this approach is that routine IVF tools can be used. A special aspiration needle is not required. A different aspiration pressure is not required. All IVF practitioners are used to aspirating oocytes from follicles in the 10–14 mm range (and occasionally slightly smaller). Physicians may continue to use their routine approaches and equipment. Routine laboratory techniques for oocyte identification can also be used. Given the high response to gonadotropins, mature oocytes will have moderate to full expressions of their cumulous. An experienced embryologist should be able to identify oocytes without additional equipment or training.

Conclusion

IVM is the only ART preventive approach for which OHSS has not been reported to occur. Given our current understanding of the central role of the VEGF system in the development of OHSS, OHSS cannot occur in response to an IVM cycle. This is not true of other current popular approaches to prevent or mitigate severe OHSS. Unlike these other approaches, IVM also avoids the patient having to experience mild OHSS. Rescue IVM and IVF with EAR are ART techniques which utilize ideas from IVM. These are additional approaches to prevent severe OHSS that are informed through experience with IVM. They should be available to a wide range of IVF practitioners.

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Immature Oocyte Retrieval for IVM Treatment

24

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Introduction

Oocyte in vitro maturation (IVM) has been evoked by Edwards [1] as an elegant proposition for the majority of in vitro fertilization procedures. The goal of clinical application of IVM is to eliminate or significantly reduce important drawbacks of controlled ovarian stimulation, such as drug costs, burden on patients, and the risk of ovarian hyperstimulation syndrome (OHSS), especially in patients with polycystic ovary syndrome (PCOS). In 2015, the use of IVM is still controversial. Some opponents have no experience with this technique, and others disagree on principle with using a technique that has a higher level of difficulty for oocyte recovery and laboratory procedures than classic IVF. The majority of the team who developed this new technique observed that patients with the lowest ovarian reserve also had the poorest outcomes in embryo implantation and delivery by classic IVF. These patients who have poor ovarian reserve will be the next target for IVM, because their follicles do not develop beyond certain size frequently. The experience of IVM with norm-ovulatory women remains the gold standard for minimizing the burden of classic ovarian stimulation and attenuating the undesirable effects of ART treatment by classic IVF [2]. Good outcome of IVM with PCOS patients is based on important and significant improvements, such as mild follicle-stimulating hormone (FSH) and human chorionic gonadotropin (HCG) priming started by Dr. Chian before aspiration, not only due to improved maturation potential of immature oocyte but also increased the number of puncturable size follicles [3]. The importance of management of endometrial thickness with the adjustment of estradiol supplementation is crucial. IVM is a relatively new option for ART promising significant benefits such as prevention of OHSS, lower cost, and less stress. However, IVM success rates are thought to be less than the conventional ART. We have been using IVM as a routine ART choice mainly for PCO patients for last 11 years. The average clinical pregnancy rate is 28% which should be acceptable for routine clinical use especially without any grade of OHSS. However, IVM is still considered to be a special or experimental treatment among some groups of clinicians with special interests in the world. Another reason why IVM is not applied for routine clinical use is the difficulty of oocyte retrieval. There supposed to be two types of problems: technical difficulty, and what menstrual cycle date or what size of follicles oocyte retrieval should be done. Some studies suggest that successful pregnancy with IVM is correlated with the number oocytes retrieved [4]. The purpose of IVM oocyte retrieval (IVM-OR) is not only the collection of some immature oocytes, but also to aspirate

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small follicles as many as possible to increase the chance of pregnancy as much as possible. In this chapter, details of our method of oocyte retrieval are explained with our protocol of optimal menstrual cycle date for retrievals suggested according to our experience.

Method of Oocyte Retrieval for IVM

IVM protocols used in our clinic are described briefly in Fig. 24.1. Regardless of the choice of priming, HCG is administered before the leading follicle reaches 13 mm, followed by IVM-OR 38 h later. When endometrium is thicker than 8 mm, fresh embryo transfer is performed. Otherwise, all embryos are frozen for subsequent frozen-thawed embryo transfer on either hormone-supplemented or natural ovulation cycle.

Appropriate Timing for Retrieval

Although the main purpose of this chapter is to explain the techniques of oocyte retrieval, it is imperative for deciding the retrieval date to know the proper timing for IVM-OR. We use frozen cycle in the case of thin endometrium <8 mm on the day of HCG administration as shown in Fig. 24.1. We evaluated the most appropriate timing for IVM-ORs in either fresh or frozen cycles as shown in Fig. 24.2. Appropriate cycle day for IVM-ORs are day 15.4 for fresh cycle and day 13.6 for frozen cycle. However, PCO patients have wide range of menstrual cycle days. Therefore, we determined appropriate cycle day for retrieval by the percentage of individual patient menstrual cycle length. From our study, it shows that 33 or 29% of cycle length is appropriate for retrievals in fresh or frozen cycle, respectively.

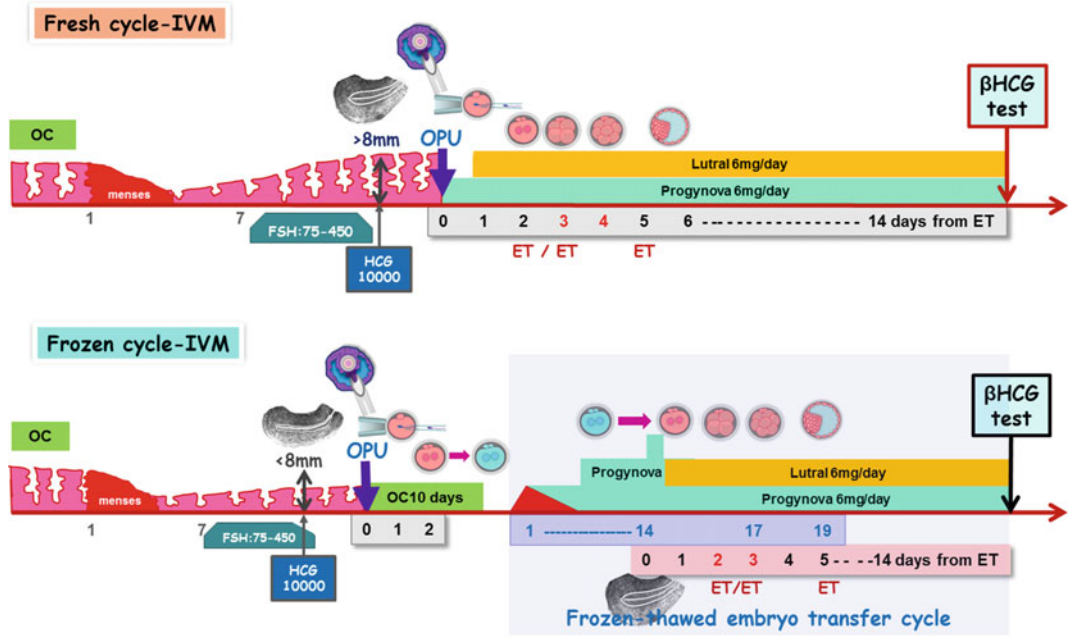

Fig. 24.1 IVM protocols at IVF Osaka Clinic. Fresh or frozen IVM are applied depending on the thickness of endometrium on the day of HCG administration

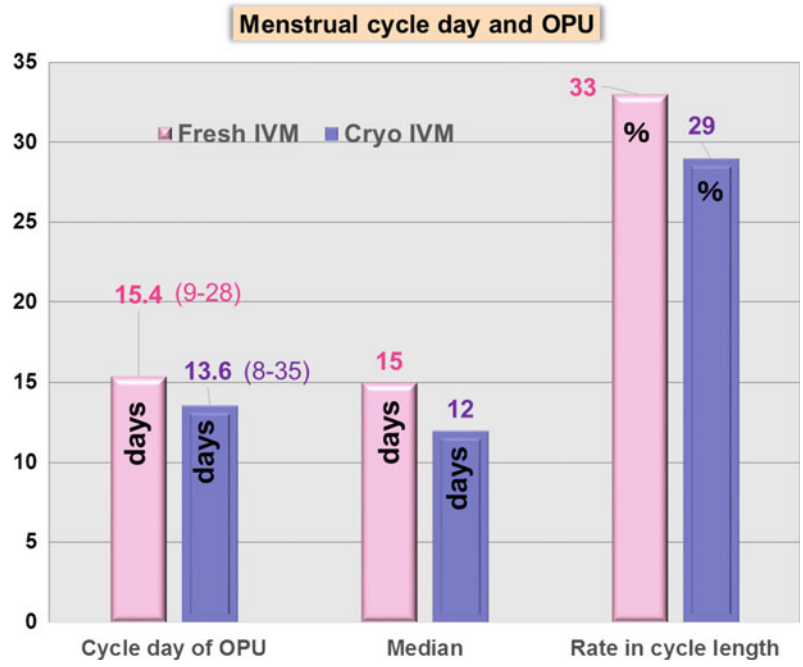

Fig. 24.2 Appropriate timing for IVM oocyte retrievals in either fresh or frozen cycles

Anesthesia for Oocyte Retrieval

Types of anesthesia used for transvaginal follicle aspiration for IVM include iv sedation, local injection, or no-anesthesia at all, but no-anesthesia is not recommended for IVM-OR due to the length of retrieval time. The use of iv sedation for IVM as well as IVF calls for the placement of a secure and accessible venous line. This is necessary not only to administer the medications but also to counteract any side effects using antagonists and to provide fluids in case of needle injury leading to hemorrhage. The use of pulse oximetry is an important part of monitoring patients during iv sedation. The use of low-flow nasal oxygen diminishes or prevents hypoxia during iv sedation. When opioids and benzodiazepines are given concomitantly, the opioid is administered first in a therapeutic dose, followed by the benzodiazepine, which is titrated to the desired effect. This order is based on the ability of opioids to reduce the required dose of a sedative. Propofol (Diprivan) is an intravenous general anesthetic agent that has a rapid onset of action and a rapid recovery after administration. Induction with propofol frequently causes apnea for >60 s and may require ventilatory support. Equipment for this procedure, including an airway, Ambu bag, and supplemental oxygen, should be readily available. Propofol has no effect on pain threshold and therefore requires the concomitant administration of an analgesic. In addition, propofol frequently causes pain, burning, and stinging at the injection site. The use of larger veins, such as those of the antecubital fossa, and the administration of lidocaine before injection of propofol may minimize pain at the injection site. Local anesthesia is an option for IVM, but can cause unnecessary patient discomfort, because more than several puncture should be done for IVM. A local agent can anesthetize only the vaginal mucosa, not the ovary itself. Anyway, it needs a little longer time of anesthesia for IVM compared to IVF.

Procedure of Oocyte Retrieval

Patients were placed in the dorsal lithotomy position, and the vagina was cleansed with sterilized saline or water without antiseptics. The

technique for IVM-OR involves the introduction of a transvaginal ultrasound probe equipped with a needle guide into the vagina. The probe is pushed gently and firmly into the ipsilateral vaginal fornix with the ovary to be aspirated to make an image of aiming ovary as close as possible. Before puncture is initiated, middle-sized vessels in the tissue should be identified by color Doppler to avoid rupturing those vessels on its way to ovary. A needle connected to an aspiration pump is then inserted into the follicles through the vaginal wall and ovarian capsule, while the pump simultaneously applies suction with an aspiration pressure. The number of punctures to the vaginal wall and the ovarian capsule for aspirating should be limited as little as possible to reduce the risk of post-retrieval bleeding, at the same time to retrieve the oocytes as many as possible [5]. The needle tip should be visualized during the entire procedure to avoid injury to adjacent pelvic organs or tissues. IVM-OR is based on the same principle as IVF, but the detail is different. Double-needle system, instead of single needle, is used for IVM-OR to reduce the number of puncture on both vaginal wall and ovarian capsules. To facilitate easier puncturing of small follicles, we developed a new designed needle (IVF OSAKA IVM Needle, Kitazato Medical Co. Ltd., Tokyo, Japan) as shown in Fig. 24.2. Outer needle is 17 gauge with file structure on the tip and used to penetrate the vaginal wall and puncture the ovarian tissue for anchoring there during aspiration (Fig. 24.3). Then, the inner 19-gauge needle is inserted into the outer needle to aspirate small follicles by aspiration pressure of 150 mmHg similar to IVF. However, many other IVM centers use lower pressure compared to IVF. When you finish up aspiration of individual follicle, you should rotate the aspiration needle inside the follicle with continuous negative pressure in order to scrape whole inner

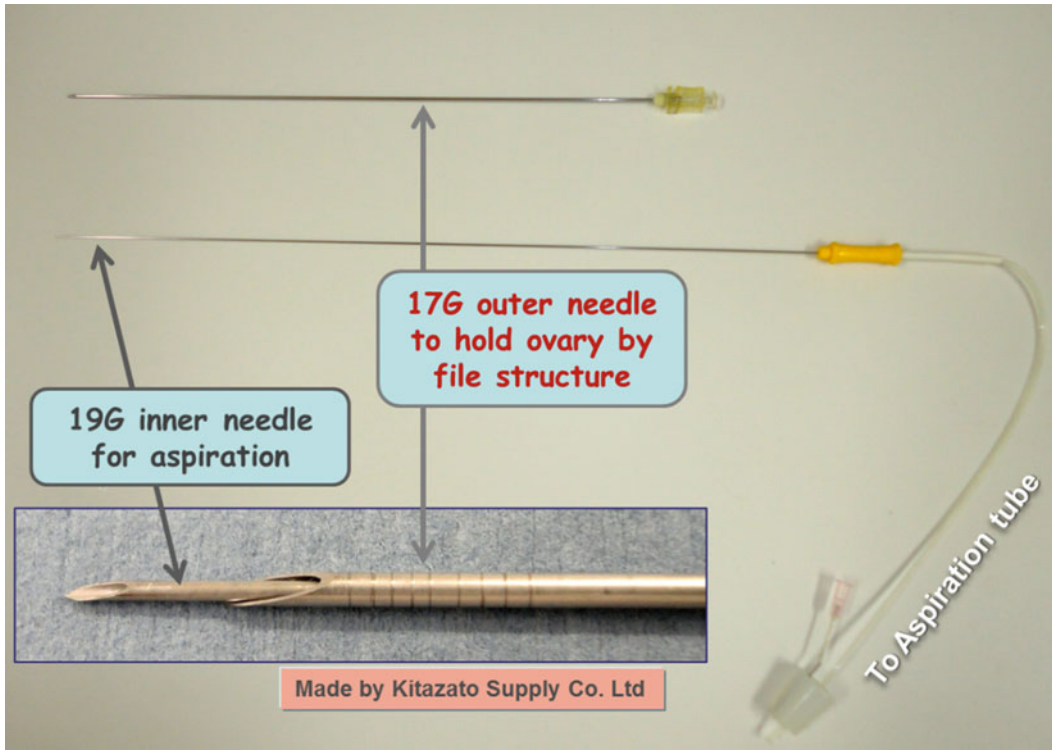

Fig. 24.3 Double-needle system for IVM oocyte retrievals (IVM-OR)

surface of the follicle not to leave immature oocyte. You can puncture the most of small follicles located in all areas of the ovary by changing the direction and depth of outer needle without additional puncture. The outer needle together with inner needle is withdrawn after aspiration of all follicles in an ovary, and the procedure is repeated on the contralateral side. However, in IVM-OR, the majority of follicles to be aspirated measure <10 mm in diameter, and the aspiration needle frequently becomes clogged with blood clots and ovarian tissues during procedure. This occurs not only due to the smaller diameter of the aspiration needle (19 gauge) but also tightly connected granulosa cells, when compared to IVF aspiration with larger gauge needle (17 gauge) and loosely connected mature OCC. Therefore, when you feel insufficient aspiration power, the aspiration needle should be withdrawn from the ovary through the outer needle for flushing its lumen before the next aspiration. Double-needle system does not need multiple punctures of the vaginal wall and ovarian capsule during IVM-OR [6–13]. Moreover, the usage of double-needle system can reduce the undesirable failure IVM-OR with no oocyte retrieved as shown in Table 24.1. All-over failure rate is 1.15%, but none of the PCO patients was failed to retrieve oocyte. This concept has been applied to most IVM centers worldwide [14]. The patients are observed in the clinic for a minimum of 1 h after IVM-OR (Figs. 24.4 and 24.5).

Table 24.1 Clinical outcomes of fresh cycle IVM between PCO and non PCO patients. It is noted that unsuccessful oocyte retrievals were only 4 cases (1.2%) out of 347 attempts, but none for PCO patients

Clinical outcome of fresh cycle IVM–IVF (<40yo) (2003–2012)		
	PCO	Non PCO
OR cycles	243 (70%*)	103 (30%)
Frozen IVM–IVF cycles	90 (40%)	134 (60%*)
No. egg retrieved cycles	0	4 (3.8%)
ET cycles	156 (64%*)	45 (44%)
PR including chemical	57 (37%*)	9 (20%)
Clinical PR	53 (34%*)	9 (20%)
Miscarriage rate	26.4% (14/53)	11.1% (1/9)

*$p < 0.05$

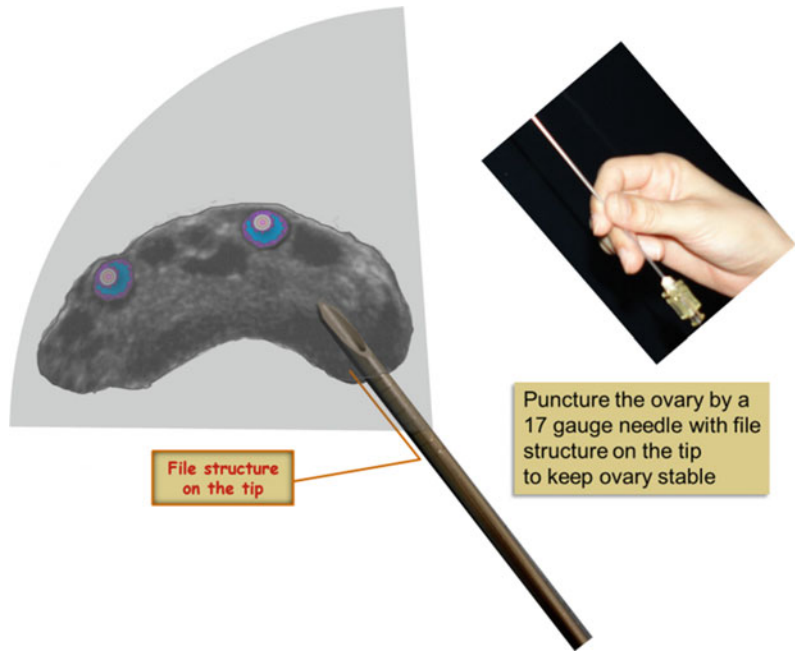

Fig. 24.4 Puncture of ovarian tissue through vaginal wall by outer needle and hold it during aspiration of small follicles by inner needle.

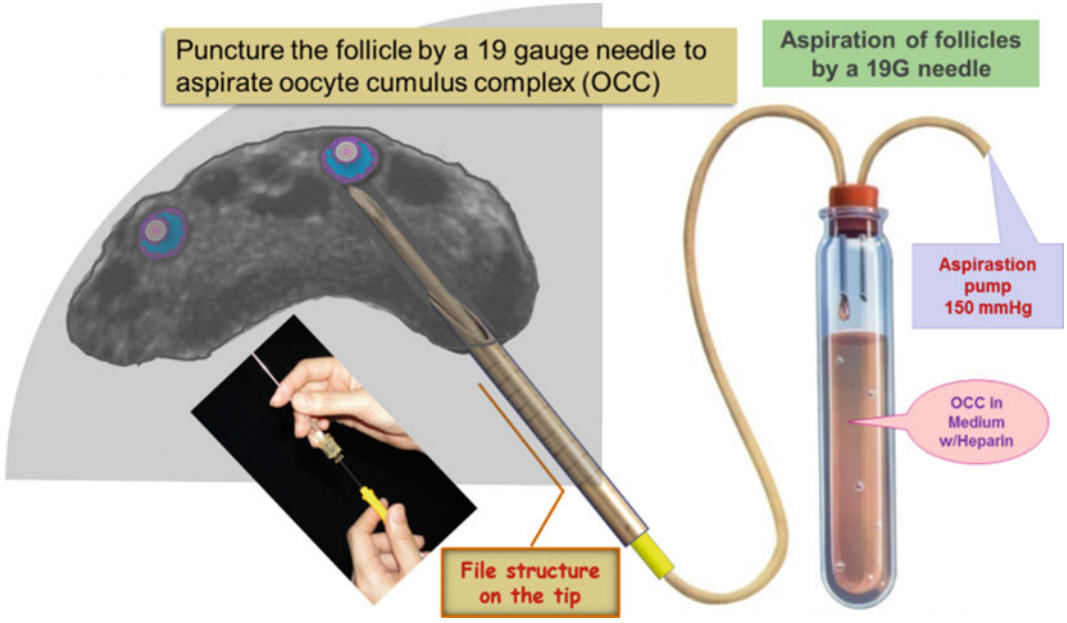

Fig. 24.5 Inner needle is inserted into outer needle in order to puncture small follicles

Complications of IVM-OR

At our clinic, we did not observe a higher complication rate after IVM-OR cycles than in IVF cycles. The patients who underwent IVM-OR did not have higher pain scores than who underwent IVF. It is important to note that the incidence of ovarian bleeding at IVM-OR does not seem to be more than IVF. The tendency toward lower pain scores during IVM-OR than during IVF-OR seems counterintuitive, but several possible mechanisms can be proposed [15]. The smaller diameter of the IVM-OR aspiration needle compared with the IVF-OR needle may make for a more comfortable collection, irrespective of the number of follicular punctures. Enlarged ovaries with multiple large follicles and the larger aspiration needle tip employed in IVF collection may cause more irritation of the ovarian stroma as the follicles collapse. Therefore, IVM-OR does not seem to be associated with an increased risk of post-retrieval complications. (Fig. 24.6).

Summary

1. IVM-OR is a key component of all IVM procedures. If IVM-OR fails, not only disappointment happens on the patients but also physicians who attempt IVM are discouraged to continue IVM practice.
2. The needle system for IVM-OR is critical for the operator. Most suitable needle should be chosen by individual physician. We recommend double-needle system which could reduce unsuccessful retrieval with no oocyte.
3. Ampule anesthesia should be given to the patient either on conscious sedation or intravenous general anesthesia, because IVM-OR requires longer operating time than IVF-OR. Furthermore, patient's discomfort makes IVM-OR more difficult to target small follicles due to the subtle movement of the patient.
4. Application of color Doppler to detect vessels can prevent post-retrieval bleedings.

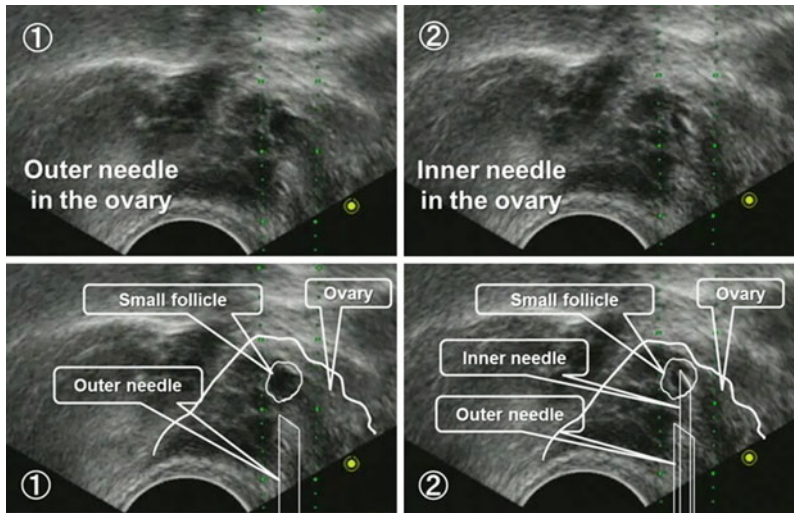

Fig. 24.6 Ultrasonographical images of real oocyte retrievals. Upper-level pictures show outer needle ① and inner needle ② in the ovary. Lower-level pictures, ① and ②, show the same pictures with illustrated captions

5. When you are finishing up of aspirating each follicle, the aspiration needle should be rotated not to leave immature oocytes by scraping inside the follicle.
6. Post-IVM-OR complications are less than IVF in spite of more complicated process with longer procedure.

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Laboratory Aspect of IVM Treatment 25

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Introduction

The source of oocytes, such as the size of follicles, ovarian phase, priming with gonadotropins (FSH and HCG) before IVM, will affect directly on oocyte maturation in vitro. During folliculogenesis, human oocyte grows from 35 to 120 μm in diameter [1]. At the end of oocyte growth, the antrum is formed and the oocyte has acquired the capacity to resume meiosis. Most mRNA and protein are synthesized during the period of oocyte growth. It is a common belief that the ability to complete maturation to M-II and developmental competence is acquired progressively with the increasing follicular size. Although it has been reported that human oocyte has a size-dependent ability to resume meiosis from 90 to 120 μm in diameter [2], non-full-sized oocytes should not be considered when assessing developmental competence, because the non-full-sized oocytes have less products (mRNA and protein) stored in the cytoplasm. Sometimes, small-sized oocytes can be collected from antral follicles, but it is not clear that whether those small-sized oocytes were from non-growing or progressed atretic follicles.

Early studies indicated that the size-dependent ability for meiotic competence depends not only on the size of the follicles and oocytes but also on the stage of the menstrual cycle [3]. It has been confirmed in human that the oocytes derived from different phases of menstrual cycle do not affect adversely oocyte maturation in vitro and subsequent fertilization and embryonic development [4]. It has been demonstrated in animal model studies that the developmental competence of immature oocytes from the small follicles is not immediately affected by the presence of a dominant follicle [5–8].

Priming with FSH alone or both of FSH and HCG before immature oocyte retrieval may promote oocyte maturation in vitro and subsequent embryo development as well as pregnancy outcome [9, 10]. Clearly those results of IVM treatment were related directly to the size of follicles. However, the quality of oocytes from the different sizes of follicles in natural cycle and ovarian stimulated cycle may be different due to the microenvironment of follicles. Most time, immature oocytes obtained from the stimulated cycles are not suitable for IVM, because those oocytes should be matured at oocyte retrieval 36 h after triggering LH surge or HCG injection, but they did not become mature. Therefore, comparing those immature oocytes, most likely from ICSI treatment cycles after denuding cumulus cells from the oocytes, with the immature oocytes from natural cycle will have quite

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different results of IVM treatment in terms of oocyte maturation, fertilization, and embryo development as well as the clinical pregnancy rates.

Interestingly, it has been indicated that anti-mullerian hormone (AMH) in serum may be a predictive marker for the selection of patients with oocyte IVM treatment [11]. Therefore, the source of oocytes is the most critical point for oocyte maturation in vitro in terms of oocyte maturation, fertilization, and embryonic development as well as pregnancy rates, not the oocyte maturation medium.

Immature Oocyte Retrieval

Transvaginal ultrasound-guided follicular aspiration has now become the preferred procedure of choice for immature oocyte retrieval in IVM treatment cycles. The same principles applied to IVF oocyte retrieval are also valid for IVM treatment. A smaller gauge needle (18–21 G) is preferable. This causes less pain and less damage to the smaller follicles, thereby allowing greater numbers of immature oocytes to be collected.

Because the intra-follicular pressure is already higher in small follicles, the aspiration vacuum pressure is reduced to 85 mm Hg, which is approximately half the conventional IVF aspiration pressure. A higher aspiration pressure provokes an increase in the number of denuded oocytes. Good ultrasonographic visualization is the key point for successful immature oocyte retrieval. The follicular sizes vary and certain follicles may be difficult to aspirate or, even if they are aspirated, no oocytes may be recovered, especially from the very small-sized follicles (<4 mm in diameter). Aspirates are collected in 10-mL tubes containing approximately 2 mL of heparinized and warmed flushing medium (usually containing 2 units/mL of heparin). It is possible to use 0.9% saline containing 2 units/mL heparin.

There are two ways to look for and collect immature oocytes from follicular aspirates. (1) Dish search: the follicular aspirates are poured directly into a petri dish and examined for immature oocytes under a stereomicroscope; (2) Cell strainer: the follicular aspirates are filled through a cell strainer (70 μm nylon). After filtering, the collected aspirates can be rinsed with pre-warmed flushing medium and transferred to a petri dish to search for immature oocytes under a stereomicroscope. All handling procedures should be conducted on warming stages or plates at 37 °C.

Typical immature COC with compacting cumulus cells (Fig. 25.1) surrounding oocyte with quite is easy to identify with mature COC, because mature COC is with quite expanded cumulus cells surrounding the oocyte. In order to determine whether the oocyte is mature or not from mature form of COCs, a special observation technique called 'sliding' may be employed. Briefly, the COC is allowed to slide slowly from one side to the other on the bottom of the petri dish, while being observed under the stereomicroscope. During COC sliding, it is possible to observe clearly whether or not the oocyte cytoplasm contains a germinal vesicle (GV) or whether the oocyte has extruded the first polar body (1PB) into perivitelline space (PVS). If neither GV is seen in the oocyte cytoplasm nor 1PB is found in PVS, the oocyte is defined as germinal vesicle breakdown (GVBD) or metaphase-I stage (M-I). If any mature oocytes are found, they should be inseminated by either IVF or ICSI within three hours after collection.

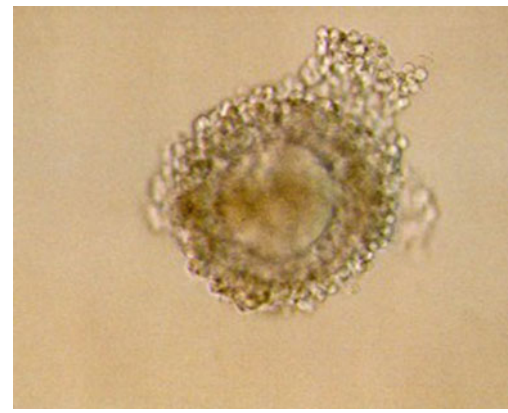

Fig. 25.1 Immature oocyte with compacting cumulus cells surrounding the oocyte

In Vitro Maturation of Immature Oocytes

The source of immature is the most critical point for oocyte maturation, but the culture conditions profoundly affect oocyte maturation in vitro. Although numerous data have been accumulated from the studies, the current rationale for choosing a specific medium for IVM of human immature oocytes appears to stem largely from adapting methods developed from culturing other cell types. Complex culture media, such as TCM-199 medium, Ham's-F10 medium, and Chang's medium buffered with bicarbonate and/or HEPES supplemented with various sera, gonadotropins (FSH and LH), growth factors, and steroids, have been most widely used in research and in the clinical application of oocyte IVM [12, 13]. All existing media for oocyte maturation in vitro are the base of complex culture media supplemented with different substances.

Energy Sources

Different energy substrates can greatly influence oocyte meiotic and cytoplasmic maturation [14, 15]. Lactate, pyruvate, and glucose are the main substrates for energy metabolism in oocytes and embryos. Glutamine can also serve as an energy substrate to improve in vitro nuclear maturation of hamster [16] and rabbit [17] oocytes. Lactate is reversibly converted to pyruvate by lactate dehydrogenase. Pyruvate or oxaloacetate, but not glucose, lactate, or phosphoenolpyruvate, supports the maturation of denuded mouse oocytes through meiosis to M-II [18] Synthesis of pyruvate in cumulus cells from glucose provides evidence that these cells are able to influence the nutritional environment of the maturing oocytes [19].

Oocyte utilization of pyruvate is closely dependent upon cumulus cells that can convert lactate or glucose into pyruvate to be used by oocytes [20]. Pyruvate directly affects nuclear maturation in mouse oocytes [21]. It has been confirmed that mitochondrial oxidative metabolism is much more important than anaerobic glucose metabolism for energy production in the mammalian oocytes [22]. However, sodium pyruvate in non-serum maturation medium supports and promotes nuclear maturation of bovine cumulus-denuded oocytes [23]. It has been reported that pyruvate alone is insufficient for oocyte cytoplasmic maturation [24].

The concentration of pyruvate and lactate in the culture medium for embryo development is 0.25 and 30 mM, respectively [25, 26], in which pyruvate concentration is similar to that found in blood and that lactate is much higher, because high concentrations of lactate were found in rabbit oviductal fluid, and this concentration was increased during the first 3 days of ovulation [27]. Most IVM media adopted the concentration of pyruvate and lactate for embryonic development. A deleterious effect of high lactate concentration on early embryonic development has been reported in mouse [28]. Pyruvate alone or pyruvate with lactate can support preimplantation development of mouse embryos until morula stage, but they cannot support the transition to blastocysts without the addition of glucose [29]. Glucose is necessary for embryonic development from morula to blastocyst [30].

Metabolism of glucose through the Embden–Meyerhof pathway is important during bovine oocyte maturation in vitro [31]. The expression pathway of glycolytic metabolism reflects the presence of different mechanisms involved in gene expression/regulation at the transcriptional and translational levels and their accumulation during human oocyte maturation [32]. In mice, glucose treatment of cumulus–oocyte complexes produced elevated cAMP concentrations, which are associated with a decreased incidence of GVBD in hypoxanthine-supplemented medium [33, 34]. Although it has been indicated that glucose may have an inhibitory effect on cumulus-free human oocyte maturation during culture in vitro [35], other results indicated that oocyte maturation medium with glucose is beneficial to bovine and human oocyte nuclear and cytoplasmic maturation in vitro [13, 15]. It has been reported that the optimal concentration of glucose in IVM medium for bovine oocyte maturation may be at 1 mg/mL [15].

Nitrogen Sources

Although amino acids support hamster [16], rabbit [17], porcine [36], and bovine [14] oocyte maturation in vitro, amino acid requirements for oocyte maturation in culture are not fully understood. Essential and/or non-essential amino acids are commonly added to oocyte IVM media. In many species, it has been believed that the addition of amino acids to the culture medium is beneficial to oocyte maturation [13, 37, 38]. Essential amino acids supplemented to a simple chemically defined medium is absolutely required for bovine oocyte cytoplasmic maturation to support subsequent embryonic development, and non-essential amino acids with essential amino acids have a synergic effect on bovine oocyte cytoplasmic maturation in vitro [39].

Apart from amino acid use for protein synthesis, they play important roles as osmolytes [40], intracellular buffers [41], heavy metal chelators, and energy sources [37] as well as precursors for versatile physiological regulators, such as nitric oxide and polyamines [42]. It has been shown that the culture medium with amino acids affect glucose metabolism in mouse blastocysts cultured in vitro [43]. Therefore, amino acids may not be only as nitrogen sources for protein synthesis but also provide the possibility to modify the metabolic pathway.

In addition, the concentration of amino acids in complex media for somatic cell culture may not be suitable for oocyte maturation in culture. Although there is no direct evidence to prove this hypothesis, it has been shown that reduced concentration of amino acids in culture medium is beneficial to embryonic development in vitro [44–47].

Vitamins

There is a paucity of information about the effects of vitamins in culture medium on the maturational and developmental competencies of immature oocytes. The addition of water-soluble vitamins, particularly inositol, to the embryo culture medium enhances the hatching of rabbit and hamster blastocysts [48, 49]. Vitamins also affect glucose metabolism in mouse [43] and sheep embryos [50]. It has been reported that the presence of vitamins in the oocyte maturation medium is important for subsequent bovine embryonic development [51]. The designed IVM medium containing the essential vitamins showed a better result in terms of nuclear and cytoplasmic maturation of immature human oocytes compared to TC-199 medium [13]. Interestingly, Naruse et al. [52] reported that low concentration of MEM vitamins in IVM medium during porcine oocyte maturation in vitro improves subsequent embryonic development. However, the optimal concentration of vitamins in the IVM medium for human oocyte maturation is needed to be determined.

Antioxidant

Oxygen concentration of female reproductive organs is lower than atmosphere [37]. Embryos cultured in low oxygen (5–10%) improve the development [53]. The beneficial effect of reduced oxygen tension may be due to the decrease in reactive oxygen species (ROS) within the cells. Excess amounts of ROS are known to be the major causes of developmental arrest and cell death [54]. The potential deleterious effects of ROS can be eliminated by antioxidant enzyme activities, free radical scavengers, and iron-binding proteins [55, 56]. Antioxidant-related components, such as glutathione, taurine, selenium, apotransferrin, cysteine, cysteamine, and β-mercaptoethanol, are added to IVM medium [57]. Serum contains many factors, including those antioxygen-related components. Therefore, it seems important for serum-free IVM medium to be supplemented with those antioxygen-related components.

Proteins

In 1960s, it has been known that a fixed protein source is essential for development of 2-cell-stage embryos to develop to blastocyst stage

[25, 26]. Initially, BSA or FCS or fetal bovine serum (FBS) has been the most commonly used protein source in culture media [58, 59]. Serum is considered crucial for oocyte maturation and may also contain factors essential for human oocyte maturation. The important factors in serum for oocyte maturation could be many growth factors. Patient's own serum supplemented to IVM medium for their own oocyte maturation in vitro may be a good option for IVM treatment in order to obtain high oocyte maturation rate. In addition, human follicular fluid (HFF) or human peritoneal fluid (HPF) has been used as a protein source supplemented to the maturation medium [4, 60].

Different sources of protein supplement may have different responses for oocyte maturation in vitro and subsequent development as well as the result of pregnancy. We have proven that 10% serum can be replaced by 10% synthetic serum substitute in the designed IVM medium, resulting in approximately 80% maturation rate and more than 90% fertilization rate when the cumulus-free GV oocytes were retrieved from stimulated IVF and ICSI cycle [13]. The commercially available IVM medium from some companies already contains human serum albumin (HSA); therefore, it seems that the extra protein source does not need to be added to those IVM media.

Serum contains complex components, including growth factors, amino acids, and others. Serum fractions of different molecular weights may have different effects on oocyte maturation in culture. Although it is not clear which serum fraction will affect oocyte maturation in vitro, it has been indicated that some fractions contain embryo developmental inhibitory function [61]. Therefore, it seems not certain which component of serum plays an important role in IVM medium during oocyte maturation in vitro. In addition, it has been reported that fatty acid-free BSA might be the optimal supplement to IVM medium due to the higher transcript level of growth factor coding genes accompanied by lower transcript level of Hsp-70 compared with serum [62, 63]. Indeed, in the designed IVM medium containing gonadotropins (FSH and LH), serum can be replaced by polyvinylpyrrolidone (PVP), resulting in less oocyte DNA fragmentation when the IVM medium is supplemented with 0.3% PVP as serum replacement [64].

Gonadotropins

Oocyte maturation in vitro is gonadotropin-independent. However, most IVM media do supplement with FSH or LH or a combination of FSH and LH. The effect of gonadotropins and their relative importance for oocyte maturation in vitro and subsequent fertilization and early development is still controversial. Oocyte maturation in vitro is unlikely undergoing the elaborate cascade of endocrine and paracrine molecular signals that occurred during oocyte maturation in vivo [65–68]. Although Gilchrist and Thompson [69] indicated that today's IVM system is un-physiological, clinical practice resulted in acceptable pregnancy rate with current IVM system supplemented with gonadotropins [70], while the idea to use FSH and LH is based on the physiological role of FSH and LH in oocyte maturation in vivo. Most likely, FSH and LH act to oocyte maturation-mediated cumulus and granulosa cells because it is believed that there are no FSH and LH receptors on oocytes. Nevertheless, it has been reported that mRNA for FSH and LH receptors are present in mouse and human oocytes, zygotes and preimplantation embryos at different stages [71, 72].

Different concentrations of FSH and LH in the IVM medium have been used. The optimal condition for oocyte maturation should be similar to the physiological concentration of gonadotropins in follicular fluid which contains fully mature oocyte [7, 8, 70, 73, 74]. In addition, the exposure of immature oocytes to different ratios of FSH:LH during maturation in vitro may result in different developmental competencies. Exposure of immature human oocytes to a 1:10 ratio of FSH:LH resulted in significantly higher developmental competence, evidenced by the increased development to the blastocyst stage in vitro compared with FSH alone or no gonadotropins [75]. However, an animal model study

indicated that the ratio of FSH:LH is not important for oocyte maturation and subsequent embryonic development [76].

With mechanism studies of oocyte maturation, especially with mouse model, indicated that LH may not be necessary to supplement to IVM medium [34, 77, 78]. It has been believed that epidermal growth factor (EGF) receptor is a central nexus in propagating to the LH signal from the granulose cells, through the cumulus cells to the oocyte [79, 80]. This signal transduction pathway may not be the only way to control oocyte maturation. The increased production of pyruvate and lactate from the cumulus and granulosa cells occurs in response to LH [81, 82]. Therefore, the beneficial effect of LH on the oocytes during IVM involves many possible factors that affect oocyte nuclear and cytoplasmic maturation. Furthermore, it is possible that IVM medium supplemented with physiological concentration of gonadotropins (FSH and LH) stimulates steroid secretion from the cumulus and granulose cells to act on the oocyte maturation.

Steroids

Estradiol and progesterone are the mediators of normal mammalian ovarian function. The actions of estradiol and progesterone on oocyte maturation might be mediated rapidly through the non-genomic mechanism via cell membrane proteins as described in Xenopus [83]. Estradiol may be important not only in regulating oocyte maturation, but also involved in subsequent embryonic development [84]. Interestingly, there is evidence to support the hypothesis that concentrations of progesterone in follicular fluid are closely associated with human oocyte maturity [85].

To consider the effect of estradiol in IVM medium consensus appears to be a concentration of 1.0 μg/ml, which is the concentration in the follicular fluid of pre-ovulatory follicles shortly after the LH peak in cattle [86]. The presence of estradiol in IVM medium had no effect on the progression of human oocyte maturation but improves the subsequent fertilization and cleavage rates [84]. Evidence has revealed that Ca^{2+} release mechanisms are modified during oocyte maturation [87]. When immature oocytes were cultured in vitro, they acquired the capacity to undergo a single large oscillation of intracellular Ca^{2+} [88]. However, subsequent sustained oscillations were not observed in some immature oocytes, indicating that these oocytes failed to develop a fully competent Ca^{+2} signaling system during maturation in vitro. Steroids may be involved in the modification of oocytes during maturation mediated by non-genomic effects, which is referred as 'oocyte membrane maturation.' Nevertheless, currently less attention was made to 'oocyte membrane maturation.'

There seems to be a negative effect of progesterone on bovine oocyte cytoplasmic maturation when it was added to IVM medium with gonadotropins (FSH and LH) and estradiol (Chian et al. unpublished data). IVM medium supplemented with physiological concentration of FSH and LH stimulates estradiol and progesterone secretion from the cumulus and granulosa cells [73, 89, 90]. Therefore, it may not be necessary to add steroids particularly to IVM medium in the presence of gonadotropins and the cumulus and granulose cells.

Growth Factors

There are several growth factors in follicular fluid which contains fully matured oocytes. EGF and transforming growth factor beta (TGFβ) and members of the TGFβ superfamily (TGFβ, inhibin, and activin) are involved in the pathway of regulation of oocyte maturation. It is clear that cyclic AMP (cAMP) is synthesized in the oocyte by constitutively active G-protein coupled receptors [91]. High levels of intra-oocyte cAMP keep the oocyte meiotically arrested by suppressing maturation promoting factor (MPF) activity by stimulating cAMP-dependent protein kinase A (PKA). The oocyte possesses a potent type-3 phosphodiesterase (PDE3), an enzyme that degrades cAMP [92]. Growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) exist in the oocyte, involving in the

oocyte maturation with a species-specific manner [93] All those signaling mediate to EGF receptor and EGF-like peptide cascade in the cumulus and granulosa cells to control oocyte maturation [94].

EGF alone and associated with gonadotropins induces cumulus expansion and promotes nuclear and cytoplasmic maturation of immature oocytes during culture in vitro [95]. The concentrations of EGF in IVM medium are different from different reports, but most likely use 10.0 ng/ml. It seems that EGF action is mediated by the cumulus and granulosa cells, because denuding oocyte maturation was not affected by the presence of EGF in IVM medium [96, 97]. Therefore, the designed IVM medium can be supplemented with EGF directly when cumulus–oocyte complexes were cultured for maturation in vitro [69, 70, 98].

As mentioned above, serum contains many factors, including the growth factors. Therefore, EGF may not be necessarily added to IVM medium when serum was supplemented to IVM medium for oocyte maturation in vitro [8, 70]. Many commercially available IVM media are ready to use, in which serum was not included in the IVM medium. In this case, it is not clear that whether EGF should be added to IVM medium or not, but it seems better to supplement EGF to the IVM medium when serum was not present in the IVM medium. However, it has to be noted that the active half-life of gonadotropins and growth factors were in IVM medium. Therefore, gonadotropins and growth factors should be supplemented to IVM medium just before use.

Practically, the immature COCs (maximum of 10) can be incubated in an Organ Tissue Culture Dish (Falcon, 60 × 15 mm) containing 1 ml commercially available oocyte maturation medium, in which protein source is already included inside, supplemented with a final concentration of 75 mIU/ml FSH and 75 mIU/ml LH at 37 °C in an incubator with an atmosphere of 90% N_2, 5% CO_2, and 5% O_2 and 100% humidity. Oocyte maturation medium should be prepared for equilibration at least two hours before immature oocyte retrieval (practically, it can be made one day before).

Recently, it has been reported that lysophosphatidic acid (LPA) exerts positive effects on the mouse oocyte IVM process [99]. The addition of LPA to the IVM medium, especially at 30 mM, improved oocyte maturation, fertilization, and subsequent blastocyst development, suggesting that treatment with LPA did not affect the rate of apoptosis of the resultant blastocysts, nor did it had a detrimental effect on spindle normality or the reservation of intact mitochondria in the in vitro-matured mouse oocytes. However, there seem no data available for human immature oocytes.

For human infertility treatment with IVM technology, it seems no breakthrough yet by improving the IVM medium itself. Although there are some 'new' ideas or approaches proposed with animal models for IVM of immature oocytes and resulted in high blastocyst formation and fetal production [100, 101], there is no report indicating the ideas or approaches that can be applied to human infertility treatment. Maybe, the ideas or approaches are acceptable with animal industry to use cell cycle modulating agents or spindle formation inhibitors during oocyte IVM, but the usage of these agents for clinical application with human oocyte maturation in vitro should be carefully discussed for its safety issue before adaption of the methodology.

The immature COCs are cultured in the IVM medium in the incubator and allowed to begin the maturation process for 24–48 h. Twenty-four hours after maturation in culture, all of the COCs are stripped for the identification of oocyte maturity. COCs will be denuded using a finely drawn glass pipette following one minute of exposure to a commercially available hyaluronidase solution for 1 min. The mature oocytes are then subjected to insemination by either IVF or ICSI after stripping. The remaining immature oocytes (GV and M-I) will continue to mature in culture for another 24 h. Forty-eight hours after oocyte retrieval (or oocyte maturation in culture), the remaining stripped oocytes are re-examined and if any have matured (M-II) at this point, they will be inseminated immediately by either IVF or ICSI.

In Vitro Fertilization of Immature Oocytes

After 24 and 48 h of immature oocyte retrieval (or oocyte maturation in culture), the immature COCs are examined and if any have matured (M-II) at this point, they will be inseminated immediately by either IVF or ICSI. Nevertheless, ICSI is recommended for the insemination of in vitro-matured oocytes, because we believe that this method offers a greater chance of successful fertilization than does IVF.

Semen can be collected and prepared for the insemination on the day of oocyte retrieval if a mature oocyte has been retrieved from the dominant follicle. Otherwise, semen collection and preparation should be performed the day after oocyte retrieval. Although it is preferable to prepare sperm freshly before ICSI, it does not appear problematic to use sperm prepared on the day of immature oocyte retrieval or the day after for the in vitro-matured oocytes of 48 h after maturation in culture, if the sperm sample is kept at room temperature in the capped tightly tube with the motile sperm.

Commercially available ICSI medium and PVP solution can be used to prepare the ICSI dish. It is also appropriate to use oocyte washing medium for the preparation of the ICSI droplets because the pH of the oocyte washing medium is quite stable at room temperature and atmosphere. However, it is important to note that the ICSI dish should be prepared at least one hour before ICSI and kept at 37 °C in the incubator or on warming stage or plate for equilibration. After ICSI, the individual oocyte is transferred into a droplet (10 μL) of commercially available fertilization medium or embryo maintenance medium in a petri dish for culture in the incubator.

Embryo Development from In Vitro-Matured Immature Oocytes

Sixteen to 18 h after ICSI or IVF, fertilization of the in vitro-matured oocytes is checked under a microscope for the appearance of two distinct pronuclei (2PN) and two polar bodies (sometimes, the polar bodies are fragmented). The fertilized zygotes are to be cultured in the droplets (10 μL) of commercially available cleavage medium or embryo maintenance medium under mineral (or paraffin) oil for one or two additional days, depending upon the number and quality of embryos achieved.

If blastocyst culture is requested, the cleaved embryos should be transferred to new droplets (10 μL) in a petri dish containing blastocyst culture medium or the same embryo maintenance medium under mineral (paraffin) oil two days after ICSI or IVF. The cleaved embryos can be cultured until blastocyst stage in this blastocyst culture medium or embryo maintenance medium without changing the medium (until day 5 or 6 after ICSI or IVF).

Embryo Transfer

It seems that most embryo transfer (ET) in IVM treatment can be done on day 2 or day 3 after ICSI or IVF, because no extra benefit is derived from culturing the embryos to blastocyst stage if the available number of embryos is small. In general, ET should be performed on day 2 after ICSI if the number of embryos obtained is ≤ 3; ET should be performed on day 3 after ICSI if the number of embryos obtained is ≥ 3. ET with blastocyst should only be considered if a total of more than 3 or 4 good quality 4-cell-stage embryos are achieved on day 2 of embryo assessment after ICSI or IVF.

The scoring of cleavage stage embryos for transfer is very crucial for pregnancy potential. Since the oocytes may not be matured and inseminated at the same time following maturation in culture, the developmental stages of embryos may be variable in the same patient. Therefore, before ET, all embryos for each patient should be pooled and selected for transfer. The final outcome of pregnancy may depend to a great extent on the experience of the embryologist. The cleavage speed of embryos and the morphological marker of each cleaved blastomere are usually used for scoring the

embryo quality of in vitro-matured oocytes. It is recommended that a maximum of 2 embryos be transferred into the uterus, based on the quality of embryos or if blastocysts are obtained the number of embryos for transfer should be only one. It is not true that transferring a greater number of poor quality embryos increases pregnancy and implantation rates.

On the day of ET, endometrial thickness should be measured by transvaginal ultrasound scan. At this point, the endometrial thickness should be at least ≥ 7.0 mm. If the endometrial thickness is <7.0 mm, the embryo should be cryopreserved and transferred in a subsequent cycle.

It seems that selecting a good ET catheter is important for establishing successful pregnancy. The ideal ET catheter should be very soft and its tip should be rounded to prevent any trauma to the endometrial lining. In addition, it should be easy to aspirate embryos through the tip under the microscope without air bubbles. Furthermore, the ET catheter should be smooth enough to prevent the embryos from getting stuck to the inner wall of the catheter. Rinsing the inner wall of catheter with a syringe is very important prior to ET. At this point, the oocyte washing medium (2–3 mL) or other commercially available medium contained in a test tube can be used for rinsing the inner wall of the catheter with a syringe. Embryos with transfer medium loading into the ET catheter should not be more than 2–3 μL (Fig. 25.2).

One of the final key contributory factors to a successful pregnancy is embryo transfer. Careful attention must be paid to both the scientific and clinical aspects of this event. A trial or mock transfer prior to the actual transfer provides very useful information to ensure a curved cervical canal, ascertain the position of the uterus, and any avert foreseeable problems during the actual transfer. It is important that as much mucus as possible is removed from the cervix with a sterile cotton bud before ET. An abdominal ultrasound-guided ET may be recommended in order to confirm that the embryos with the fluid contents of the catheter are in the uterus.

Normally, luteal support for IVM treatment is provided by 200 mg intra-vaginal progesterone three times daily or progesterone injection intramuscularly subcutaneously with instruction starting from the day after immature oocyte retrieval and continuing until the pregnancy test. Normally, luteal support for IVM treatment is provided by 200 mg intra-vaginal progesterone three times daily or progesterone injection intramuscularly subcutaneously with instruction starting from the day after egg collection and continuing until the pregnancy test 14 days after ET. HCG-positive women are scheduled to return for a transvaginal ultrasound scan 2 weeks later to ensure the presence of a fetal sac and heartbeat. It is recommended that women continue taking progesterone until 12 weeks after ET, if a fetal sac and heartbeat are noted by transvaginal ultrasound scan at 4 or 5 weeks after ET. The rest of pregnancy management should be the same as a spontaneous pregnancy. Pregnant women are encouraged to go through amniocentesis but it is not mandatory.

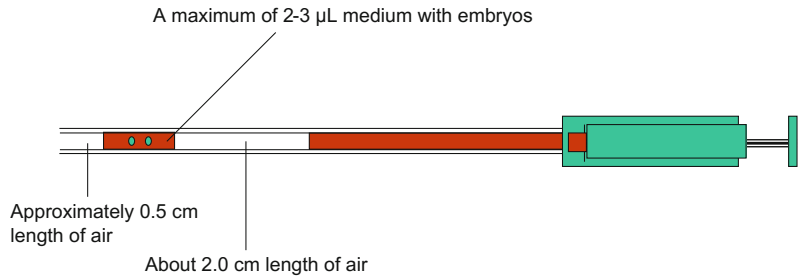

Fig. 25.2 Diagram of embryo loading in ET catheter. Embryo with transfer medium loading into the ET catheter should have maximum 2–3 μL

Conclusion

Recovery of immature oocytes followed by IVM of these oocytes is a potentially useful treatment for women with infertility. Successful pregnancy is directly related to the number of oocytes retrieved and embryos available for transfer. Therefore, the best candidates for IVM treatment seem women under 35 years of age who have more follicles developed in ovaries because younger women have a greater number of follicles that continue to grow through to the pre-ovulatory stage of development during each menstrual cycle. Although immature oocyte retrieval followed by IVM technology might be useful primarily for women who are infertile with PCOS, it is important to mention that IVM treatment may be an attractive alternative for women with all types of infertility. In comparison with conventional (stimulated) IVF treatment, the major advantages of IVM treatment include the following: (1) avoidance of the side effects resulting from gonadotropin stimulation including the risk of OHSS, (2) reduced cost, and (3) simplified treatment.

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Obstetric Outcome of In Vitro Maturation Treatment and Risk of Congenital Malformations

26

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Introduction

The well-being of children conceived with in vitro fertilization and intracytoplasmic sperm injection (collectively referred to as "IVF" in the remainder of the text) has always been a concern. Preterm birth is the leading cause of perinatal morbidity and mortality. Multi-fetal pregnancy associated with IVF is a well-known risk for preterm delivery [1]. However, even studies on IVF singletons consistently report an increased risk of preterm delivery before 37 weeks, as well as risk of very preterm delivery before 32 weeks as compared to naturally conceived singletons [2–7]. This suggests that being conceived by IVF, regardless of the number of fetuses, comprises risk for preterm delivery and associated morbidity. Likewise, even though individual studies examining the association between IVF and major birth defects (MBDs) have reported conflicting results, recent meta-analyses suggest an increased risk of MBDs in children conceived by IVF compared with naturally conceived children [5–8].

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The underlying mechanism(s) for the observed association between preterm delivery, MBDs, and IVF has not been identified. It should be noted that there is evidence that subfertile couples conceiving naturally have an increased risk of preterm delivery and having a child with congenital malformations, suggesting that infertility per se may contribute to the increased risk [9–11].

IVF involves ovarian stimulation (OS) with gonadotropins to allow the collection of several mature oocytes that can be fertilized in vitro, and oocyte and embryo culture with laboratory manipulations. Each of these steps can potentially be responsible for the development of congenital anomalies. More recently, altered endometrial milieu at the time of embryo implantation is also suggested to play a role in adverse obstetric outcomes [12, 13]. Even though conclusive evidence is lacking, supraphysiological sex steroid levels associated with multifollicular growth in conventional OS–IVF cycles is thought to affect endometrial function and increase the risk of preterm delivery and small-for-gestational-age (SGA) babies. A recent retrospective analysis of 65.868 IVF singleton live births reported an increased risk of preterm delivery and SGA baby in women with an excessive ovarian response to stimulation (defined by collection of >20 oocytes) as compared to women with a normal ovarian response (defined by collection of 10–15 oocytes) [14]. Despite its limitations, this finding lends credit to a possible association between OS and adverse

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obstetric outcome. Whether all embryos generated in OS–IVF cycles should be cryopreserved for later transfer in a subsequent unstimulated cycle in order to avoid adverse obstetric outcomes is under debate at the moment [15].

Retrieval of immature oocytes without gonadotropin stimulation followed by in vitro maturation (IVM) has been introduced as an alternative to OS–IVF [16]. IVM does not require OS. Oocytes are collected when the majority is expected to be immature. The immature oocytes collected are matured in vitro before in vitro fertilization. IVM does not only decrease the physical and economic burden of the treatment but also expands the options for women under risk of ovarian hyperstimulation syndrome following OS and for those in need of fertility preservation for whom OS is contraindicated or there is not enough time for OS [17]. However, as in the case of IVF, concerns about the well-being of infants conceived after IVM have been raised. While oocyte retrieval at the immature stage and in vitro maturation procedure can be regarded as additional risk factors over conventional IVF–OS, the absence of multifollicular growth and sex steroid levels being maintained within physiological range in IVM cycles can be regarded as possible advantages for the course of resultant pregnancies.

Risk of Preterm Delivery and Birthweight in In Vitro Maturation Pregnancies

Up to date, obstetric outcome has been reported for few IVM infants in comparison with IVF infants [18–26]. Fetal weight at birth and the incidence of preterm delivery were retrospectively compared with spontaneous conceptions and/or IVF pregnancies in only three studies [21, 22, 24].

Shoo-Chi et al. [21] compared 21 IVM children with 21 spontaneously conceived controls and reported similar birthweight. Buckett et al. compared 31 IVM singleton live births with 133 IVF, 104 ICSI, and 338 spontaneous conception live births. Birthweights of IVM singletons were significantly higher than those of controls. Despite similar proportions of preterm delivery in IVM and spontaneous conception groups, IVF and ICSI singletons were delivered preterm at a significantly higher rate [22]. Fadini et al. retrospectively compared 153 IVM and 148 ICSI singletons. Similar to Buckett et al., they also reported significantly higher birthweight in IVM than in ICSI group; however, the risk of preterm delivery was similar in the two groups [24]. These findings are summarized in Table 26.1.

It should be noted that Shu-Chi et al.'s 21 IVM infants include 4 children from two twin pregnancies [21]. Their inclusion in the calculation of birthweight could have led to the underestimation of birthweights of IVM infants. It is noteworthy that both of the other comparative studies consistently reported higher birthweight in the IVM groups [22, 24]. Moreover, birthweights of IVM singletons in the remaining uncontrolled series were 3252 ± 516 g [18], 3720 g (median) [19], and 3550 ± 441 g [20]. In addition, preterm delivery rates were 2% [19], 5% [20], and 5% [18] in the latter reports. None of these findings suggest either an increased risk of preterm delivery or a decreased birthweight associated with IVM. In our opinion, these findings lend some credit to the suggested association between ovarian stimulation and defective implantation in conventional IVF cycles. It could be argued that most women in the above-mentioned reports have polycystic ovarian syndrome (PCOS) and this could have distorted the birthweight results in favor of IVM infants. However, contrary to the intuitive expectation children born to PCOS mothers are more likely to be SGA rather than large for gestational age, and they are under higher risk of preterm delivery [27, 28]. Therefore, if the above-mentioned results are biased by the presence of a higher proportion of PCOS patients in the IVM groups/series, this would have not caused overestimation, but underestimation of possible advantages of IVM in this regard. If this association can be confirmed in future trials, then IVM or natural cycle IVF/IVM can represent a safer alternative than OS–IVF with regard to obstetric outcomes.

Table 26.1 Comparative studies of obstetric outcome between IVM and IVF or spontaneous pregnancies

	Sun-Chi et al.[a]		Buckett et al.				Fadini et al.	
	IVM	Spontaneous	IVM	IVF	ICSI	Spontaneous	IVM	ICSI
Number of infants	19	21	31	133	104	338	153	148
Birthweight in grams	3074.76 (488.9)*	3133.71 (287.34)	3482[b]	3209	3163	3260[b]	3269[c] (616)	3091[c] (669)
Delivery <37 weeks (%)	1/19 (5%)	0 (0%)	2/31 (6%)	23/133 (17%)	25/104 (24%)	18/338 (5%)	26/153 (17%)	21/148 (14.2)

Only singletons are included in order to avoid confounding by multiple pregnancies. Figures are mean or median as presented in the original reference (standard deviation) unless otherwise mentioned. Figures with the same superscript are statistically significantly different ($p < 0.05$ for bivariate comparisons).* IVM group in the Sun-Chi et al. study includes two twin pregnancies and four infants from these pregnancies are included in the calculation of birthweight. Therefore, it is likely that birthweight of IVM babies is underestimated in this study

Major Birth Defects

We collected information regarding maternal, obstetric, and infant characteristics of successful pregnancies achieved through IVM between years 1999 and 2010 through structured questionnaires sent to 31 assisted reproduction centers located in 22 areas/countries around the world [25]. Specific questions were asked about the presence or absence of any birth defects in the newborns and the description of the defects. The questionnaire drew on data collected at the time of birth, and therefore would include stillbirths but not pregnancy terminations.

Reported birth defects were categorized according to ICD-10, version 2007 [29]. A major birth defect was defined as one that generally causes functional impairment or requires surgical correction [30]. We compared the prevalence of MBDs in IVM infants with two different control groups: (i) infants conceived by IVF and (ii) the general population. First, we conducted a systematic literature review of MEDLINE, EMBASE, and OVID MEDLINE(R) In-Process and Other Non-Indexed Citations databases to identify all studies reporting prevalence of birth defects in IVF infants. Studies with overlapping data were excluded. In case of partially overlapping studies, the study with the larger sample size was included. Studies that did not use a specific definition for MBD, with a sample size of less than 100 IVF children, and studies in which the children were examined after the age of 30 months were also excluded. Annual report of the Centre of the International Clearinghouse for Birth Defects Surveillance and Research (ICBDSR) was used to estimate the prevalence of MBDs in the general population across different countries [31]. ICBDSR is an international organization that affiliated the World Health Organization [32]. ICBDSR conducts worldwide surveillance and research into the occurrence and possible causes of birth defects. ICBDSR operates an international program for regular exchange among its members of information on birth defects in populations covered by the member's surveillance and research programs. The 2009 ICBDSR annual report, used for this study, included data from 38 member programs [31]. Data collected on selected major birth defects, including trisomies 13, 18, and 21, are presented separately for live births, stillbirths, and pregnancy terminations. The number of major birth defects observed in live births and stillbirths were extracted and combined for each member program. Pregnancy terminations were excluded because we did not have available data for pregnancy terminations for IVM pregnancies.

The heterogeneity of available data on the prevalence of birth defects in the selected control groups, e.g., differences in population characteristics and varying definitions of birth defects , prevented converting the data into a single metric. Therefore, we calculated 95% confidence intervals around the reported crude point estimates in the selected studies and registries, as well as around the prevalence observed in IVM

infants. The results were combined in relevant subgroups and sorted in increasing order of point estimates to provide an overview of how the prevalence of MBDs in IVM infants compared with the controls. Of the 1187 IVM infants, a total of 18 were diagnosed with a major birth defect, giving a prevalence of 1.27% (95% CI: 0.81–2.0%).

How Do Major Birth Defects Rates Compare Between IVM and IVF Infants?

Twenty-seven manuscripts were considered eligible for our review. Four of the included studies were prospective [30, 33–35], one had a cross-sectional design [36], and the majority was retrospective. However, population-based registries which prospectively collected data were used to identify IVF children and those with birth defects in eight [37–44] out of 22 retrospective studies. Sample size ranged from 150 to 9175 IVF infants. In total, 54,678 IVF children were included. The prevalence of MBDs observed in IVF infants ranged between 0.37% (95% CI: 0.06–0.021%) [45] and 9.1% (95% CI: 7.9–10.4%) [46]. The observed prevalence of MBDs in IVF infants was higher than the observed prevalence in the IVM cohort in 24 studies, and lower in only four studies [35, 41, 45, 47]. However, the 95% confidence intervals of the observed prevalence of MBD in these four studies and in the IVM cohort were overlapping (Fig. 26.1).

How Do Major Birth Defects Rates Compare Between IVM and Spontaneous Conceptions?

The total number of births, including live and stillbirths, reported by the ICBDSR member programs was 2,637,638. MBDs were reported for 32,572 children, yielding an overall

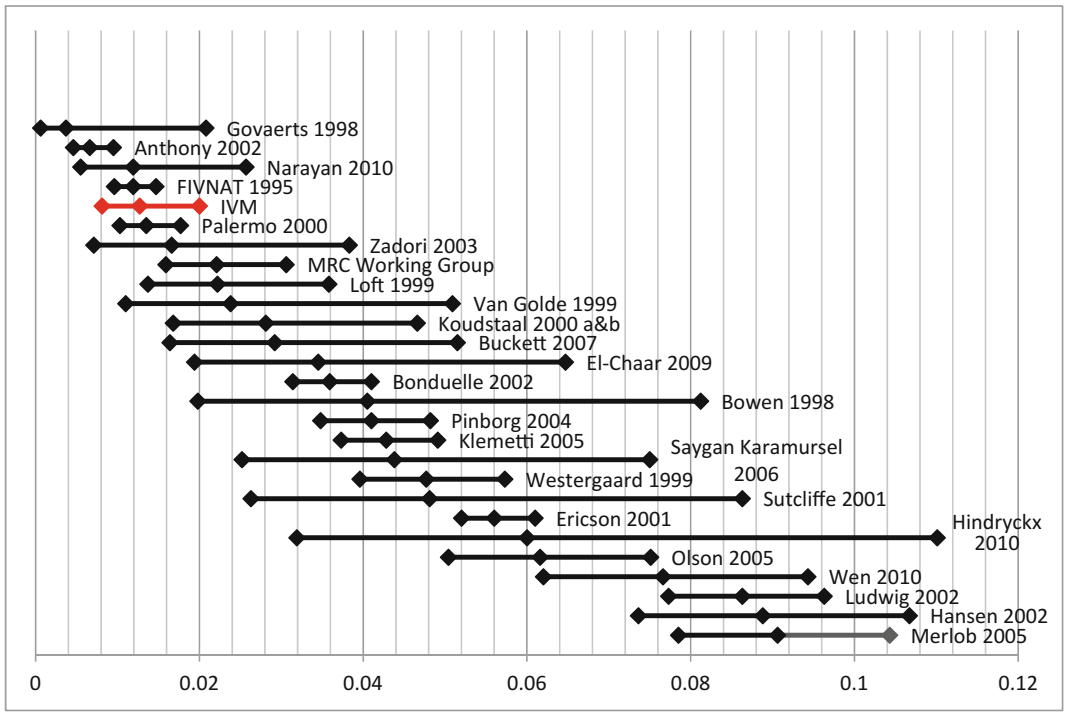

Fig. 26.1 Prevalence of major birth defects in infants conceived with assisted reproductive technologies. Horizontal bars represent the point estimate and 95% confidence intervals of major birth defects observed in individual studies

prevalence of 1.24% (95% CI: 1.22–1.24%). Prevalence of MBD per birth ranged between 0.2% (95% CI: 0.18–0.26%) and 2% (95% CI: 1.9–2.1%) in the individual registries reporting to ICBDSR. Of the registries collecting information until 2 years of age or less, 15 registries reported a lower prevalence of MBDs than that observed in IVM infants, while 16 reported a higher prevalence (Fig. 26.2). The prevalence of MBDs observed in IVM infants was not higher than the overall prevalence from the registries.

The overall MBDs prevalence of 1.27% (95% CI: 0.81–2.0%) per IVM infant born in the largest population of IVM infants to date appears to not exceed the prevalence of MBDs in either IVF children or the general population. In our view, this represents the most reliable estimate of the prevalence of MBDs in IVM infants available so far.

The lack of information on pregnancy terminations, especially on terminations due to congenital anomalies, can have caused underestimation of the

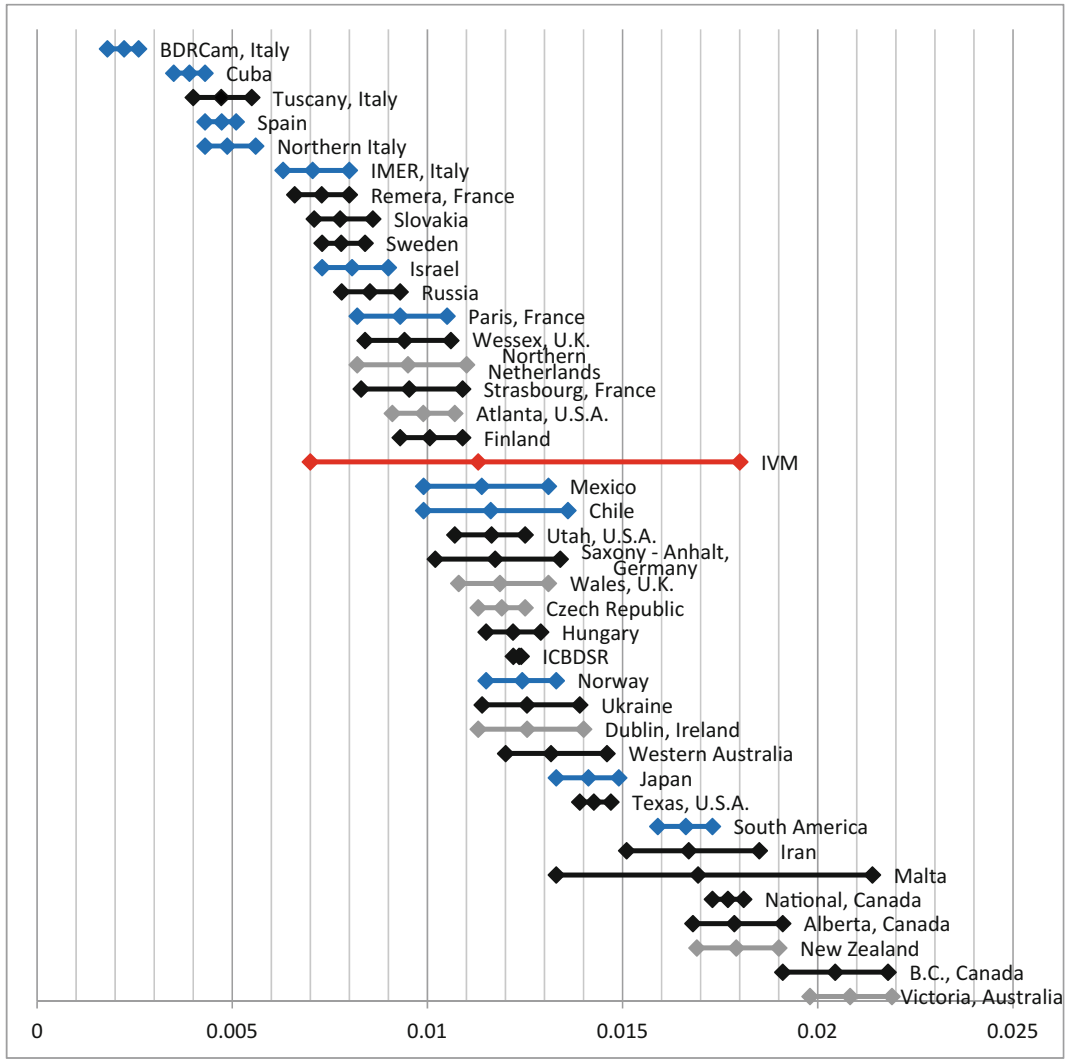

Fig. 26.2 Prevalence of major birth defects in ICBDSR member registries. Horizontal bars represent the prevalence and 95% confidence interval for the prevalence in individual registries

overall prevalence of MBD in IVM pregnancies. However, the figures reported for both control groups also excluded information on pregnancy terminations. While none of the IVF studies included in the first control group presented data on pregnancy terminations, we excluded pregnancy terminations available in the ICBDSR report. Hence, even if the overall prevalence of MBD in IVM pregnancies can be underestimated to some extent, the comparisons with IVF children and population-based registries are unbiased in this regard. The data on IVM infants were mostly collected at birth or shortly after delivery, and this can have led to the underestimation of the true prevalence. However, MBDs are mostly evident at birth, and about two-thirds are diagnosed in the first week of life [38]. In order to prevent a bias in favor of the IVM cohort, we excluded studies in which IVF children were examined for MBDs after the age of 30 months. Similarly, when ICBDSR member registries were categorized according to the time of assessment for MBD, the IVM cohort still remained within the range of the countries where the children were examined no later than the first week of life (Fig. 26.2). Therefore, we believe the comparisons to be unbiased in this regard as well.

The population-based registries contributing to the ICBDSR data did not exclude children conceived after IVF. However, IVF children constitute a small fraction of the total births. Even if their risk of having a malformation at birth is 30–40% higher, as suggested by recent meta-analyses [8, 48], it is unlikely that their presence would distort population-based data to a substantial extent.

Although our cohort represents the largest available IVM population, the numbers are small, especially in order to comment on any specific malformations. For instance, hypospadias was the only MBD observed in more than one IVM infant, resulting in a prevalence of 2.1 per 1000 births. The overall prevalence of hypospadias was 1.7 per 1000 births in the ICBDSR report [31]. Schieve et al. [49] calculated that more than 3000 ART live births were required to demonstrate, as statistically significant (at 5% level), a threefold increase in the prevalence of hypospadias. More than 50,000 ART live births would be required to demonstrate a 1.5-fold increase [49].

Four other studies have reported on the prevalence of birth defects in IVM infants [18, 22–24]. Cha et al. [18] reported on 38 women with PCOS who conceived after IVM and had obstetric follow up at the authors' institution. Following elective reduction in triplets, spontaneous miscarriages (including one fetus with omphalocele and 45X/46XY mosaicism), and one medical abortion for hydrops fetalis (which is not considered a congenital anomaly), there were 20 singleton and 4 twin live births. One out of 28 children born had a cleft palate, resulting in a prevalence of 3.6 per 100 live births. Fifty-five IVM children delivered at the McGill University Health Centre were already included in the data presented above [22]. Foix-L'helias et al. [23] reported on 35 IVM children evaluated at one year of age in the context of a prospective study and reported that prevalence of congenital anomalies was similar to that in IVF children. Fadini et al. [24] did not observe any major congenital anomalies in their group of 196 IVM infants, but observed several minor defects, albeit at a similar rate in ICSI infants.

Considering the available data in its totality, the prevalence of MBD reported in IVM children so far does not seem to substantially exceed the prevalence of MBD in IVF children or in the general population. Despite these reassuring findings, further studies are needed, ideally with matched control groups and longer follow-up.

Growth and Neurologic Development

Shu-Chi et al. [21] also analyzed the chromosomal constitution and mental development of IVM children and compared with those of spontaneously conceived children. All of the IVM children were found to have normal karyotype and mean developmental index score, similar to controls. Soderstrom-Antilla et al. [20] reported similar findings in 46 IVM babies. In the latter study, the neuropsychological development of children was assessed until 24 months and was

found to be within population standards. The physical growth of IVM children also appears to be similar to that of spontaneously conceived children [20, 21]. Currently available data seem reassuring and do not suggest an increased risk of physical or neurological developmental delay in IVM children.

Conclusion

Currently available data do not suggest an increased risk of preterm delivery, SGA, congenital malformations, or developmental delay in IVM children. However, data are limited, and this is certainly an area requiring further well-designed and conducted research. If these findings are corroborated by other studies, IVM can represent a safer alternative than OS–IVF with regard to obstetric outcomes. If future studies also report increased birthweight of IVM babies, this would require particular attention.

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Development of IVM Treatment: Combination of Natural Cycle IVF with IVM

27

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Introduction

Women with polycystic ovary syndrome (PCOS) have abnormal endocrine parameters, anovulation, numerous antral follicles within their ovaries, and frequently infertility. As mentioned in previous chapters, IVM treatment can be an option for infertile women with PCOS, but it seems quite difficult to offer IVM treatment for women with normal ovaries who develop a dominant follicle and ovulate its oocyte during menstrual cycle. In women, follicular development is characterized by the selection of a dominant follicle destined to ovulate from a cohort of growing follicles, and initiation of atresia in those remaining in the cohort. Interestingly, the traditional theory of a single cohort of antral follicles growing only during the follicular phase of the menstrual cycles was challenged that two or three waves of ovarian follicular development in the ovaries during menstrual cycle by daily transvaginal ultrasonography [1, 2]. Therefore, it seems clear that there are several follicles growing in each menstrual cycle as evidenced by baseline ultrasound scan for antral follicle counts (AFC) on day 3 of menstrual cycle, especially if women are healthy and under 35 years of age.

After a dominant follicle has been selected in a spontaneous ovulatory cycle, secondary follicles are generally thought to be atretic with only the dominant follicle possessing the appropriate hormonal milieu necessary to achieve oocyte maturity and ovulation. It is a common belief that the development of a dominant follicle suppresses subordinate follicles and new growth of small follicles occurs only after the dominant follicle has ceased growing [3, 4]. Therefore, IVM treatment may be limited to those women undergoing infertility treatment who have polycystic ovarian syndrome with anovulatory cycles [5, 6].

Fertilization, embryo development, and live births have been achieved following the transfer of embryos produced from oocytes retrieved from secondary follicles in treatment cycles when the development of a dominant follicle in the ovaries [7] was observed. This indicates that oocyte viability is maintained in these secondary follicles in spite of the possibility that the dominant follicle may induce atresia and regression in them. Animal model study indicated that the developmental competence of oocytes from small antral follicles is not adversely affected by the presence of a dominant follicle [8] and phases of folliculogenesis [9]. These results indicate that the maturational and developmental competence of immature oocytes is not affected by the presence of the dominant follicle and the phase of

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folliculogenesis. This notion is not hard to understand from animal industry that oocyte collection is performed several times during one estrus cycle and immature oocytes are aspirated from small follicles from slaughterhouse materials regardless of follicular phases in cattle ovaries. Therefore, this is a very important point to understand how to further develop IVM treatment for infertile women with normal ovaries.

It has been reported that the maturational and developmental competencies of immature oocytes retrieved from women with PCOS are improved by human chorionic gonadotropin (HCG) priming before immature oocyte retrieval [10–12]. As an initial protocol, women with PCOS were excluded when development of a dominant follicle in ovaries occurred by day 8 of the cycle. Women with polycystic ovaries that have taken ultrasound scan but who have a normal menstrual cycle, or those with normal ovaries, usually produce a dominant follicle at the middle of cycle. If following the initial protocol of IVM treatment, most time, IVM treatment will be cancelled by women producing the dominant follicle in the ovaries. Modified IVM treatment has been applied to those women, and natural cycle IVF combined with IVM treatment was proposed accordingly [13].

IVM Treatment for Poor Responders or Over Responders in the Stimulated Cycles

In conventional IVF treatment cycles, some women appear to respond to hormonal stimulation but have a low estrogen level or few or slow growing follicles, in which they have been considered as poor responders. This group of women requires a prolonged stimulation time and higher doses of gonadotropin. Following gonadotropin stimulation, the number of follicles may be normal, but the size of follicles may be smaller than in the usual treatment cycles [14]. In such case, IVM treatment may be an option for those poor responders instead of longer gonadotropin stimulation or treatment cancellation. It has been reported that pregnancies established following the immature oocyte retrieval and IVM with [15] and without [16] HCG administration before oocyte retrieval.

In addition, some women are extremely sensitive to the stimulation with exogenous gonadotropin and are at increased risk of developing OHSS that, sometimes, is a potentially life-threatening complication [17]. Several preventive strategies have been proposed to reduce the incidence and severity of OHSS, and one of them is to cancel the treatment cycle [18–22]. Normally, a woman is considered an over responder when there are more than 20 follicles with a mean diameter >10 mm in both ovaries and extremely high level of estradiol in serum following gonadotropin stimulation for several days. It has been proposed that IVM treatment may be offered as an alternative for these over responders when the leading follicle reached 12–14 mm in diameter, 10,000 IU of HCG was administered, and then oocyte retrieval was performed 36 h later before the treatment cycle cancellation [23].

Development of IVM Treatment

To further develop IVM treatment for women with normal ovaries, the natural cycle IVF combined with IVM was proposed [13]. If the mature oocyte from the dominant follicle together with immature oocytes from the small follicles were collected as well, the chances of a pregnancy are greatly increased when we manage to mature these immature oocytes and produce several viable embryos. Interestingly, mature oocytes can be retrieved from the leading follicles and at the same time immature oocytes also be collected from the small follicles 36 h after HCG injection when the injection was performed at the size of leading follicle reached to 12–14 in diameter, suggesting that some small follicles can respond to an LH surge to trigger oocyte maturation, which might become fully mature in vivo [24]. In fact, in our previous study [10] of women with PCOS undergoing IVM treatment, when immature oocyte retrieval was performed 36 h after HCG administration with

small follicles, it has been reported that approximately 46% of oocytes had initiated the process of oocyte maturation and undergone germinal vesicle breakdown (GVBD).

It may be an important point to mention that when HCG will be administered at the size of leading or dominant follicle reached for this group of women who are undergoing natural cycle IVF combined with IVM in order to prevent ovulation from the dominant follicle due to a spontaneous LH surge when the patients treated with natural cycle IVF combined with IVM. Our experience indicates that 10,000 IU HCG can be administered 36 h before oocyte retrieval when the size of the dominant follicle reached to 10–14 mm in diameter, and most oocytes collected from the dominant follicles were at M-II stage [25].

A selective group of ovulatory women can benefit from natural cycle IVF combined with IVM for treatment with acceptable pregnancy rates [26]. Also it is important to evaluate the efficacy of natural cycle IVF combined with IVM treatment as a clinical treatment for infertile women. We found that natural cycle IVF combined with IVM treatment might be offered to more than 50% of the total infertile women who were seeking infertility treatment, and with more than 40% pregnancy rate [27]. The detailed protocol for natural cycle IVF combined with IVM is shown in Fig. 27.1.

In addition, it has been reported that the clinical outcomes of different infertility causes (tubal factor, male factor, unexplained infertility, combination of tubal and male factors, and other/mixed factors) with natural cycle IVF combined with IVM treatment were evaluated [28]. They found that there were no significant differences in the rates of IVM, IVF, and cleavage as well as in the clinical pregnancy (30.4–46.9%) and live birth rates among the five subgroups, which suggests that natural cycle IVF and IVM treatment are the suitable treatments for infertility of various causes with acceptable pregnancy and live birth rates.

Interestingly, it also has been reported that the blastocysts produced from the immature oocytes derived from small follicles during natural cycle IVF combined with IVM treatment can be safely vitrified and give a healthy live birth, confirming that the presence of the leading follicle during natural cycle IVF combined with IVM treatment does not detrimentally affect the viability and health of the immature oocytes derived from small follicles [29]. Furthermore, it may be interesting to know that still whether or not during natural cycle IVF combined with IVM treatment there are differences in pregnancy and implantation rates between women with and without the presence of mature oocytes obtained at the time of the retrieval. It has been found that although the clinical pregnancy rates are not different regarding the retrieval of mature oocytes or the time of the egg retrieval, the live birth rate is higher ($P < 0.05$) when the mature oocytes are obtained at the time of the egg retrieval [30].

Table 27.1 shows the cycle outcome of Group A (women with mature oocytes) and Group B (women without mature oocytes). In Group A, a total of 739 mature oocytes were obtained at the time of retrieval from 314 cycles of natural IVF combined with IVM treatment with an average of 2.4 ± 1.6 per woman. Of these 739 mature oocytes, 644 (87.1%) were fertilized and 610 (94.7%) cleaved. There were also 2780 immature oocytes of which 1826 (65.7%) matured in vitro and 1457 (79.7%) were fertilized. In Group B, a total of 515 immature oocytes were retrieved from 55 cycles with an average of 9.4 ± 4.9 per patient. Of these 515 immature oocytes, 363 (70.5%) were matured in vitro and 294 (81.0%) were fertilized. There were no differences between the two groups in terms of total fertilization (81.9% vs. 81.0%) and embryo cleavage (92.1% vs. 89.1%) rates. Moreover, the quality of embryos was not different between the groups. Following embryo transfer, the clinical pregnancy rates and implantation rates between the groups were not different (40.1% vs. 34.5% and 16.2% vs. 15.0% in Group A and Group B, respectively). However, the live births per embryo transfer (29.6% vs. 16.4%) and miscarriage per clinical pregnancy (26.2% vs. 52.6%) rates were significantly different between Group A and Group B.

During natural cycle IVF/M, it is impossible to predict the stage of oocyte maturity according

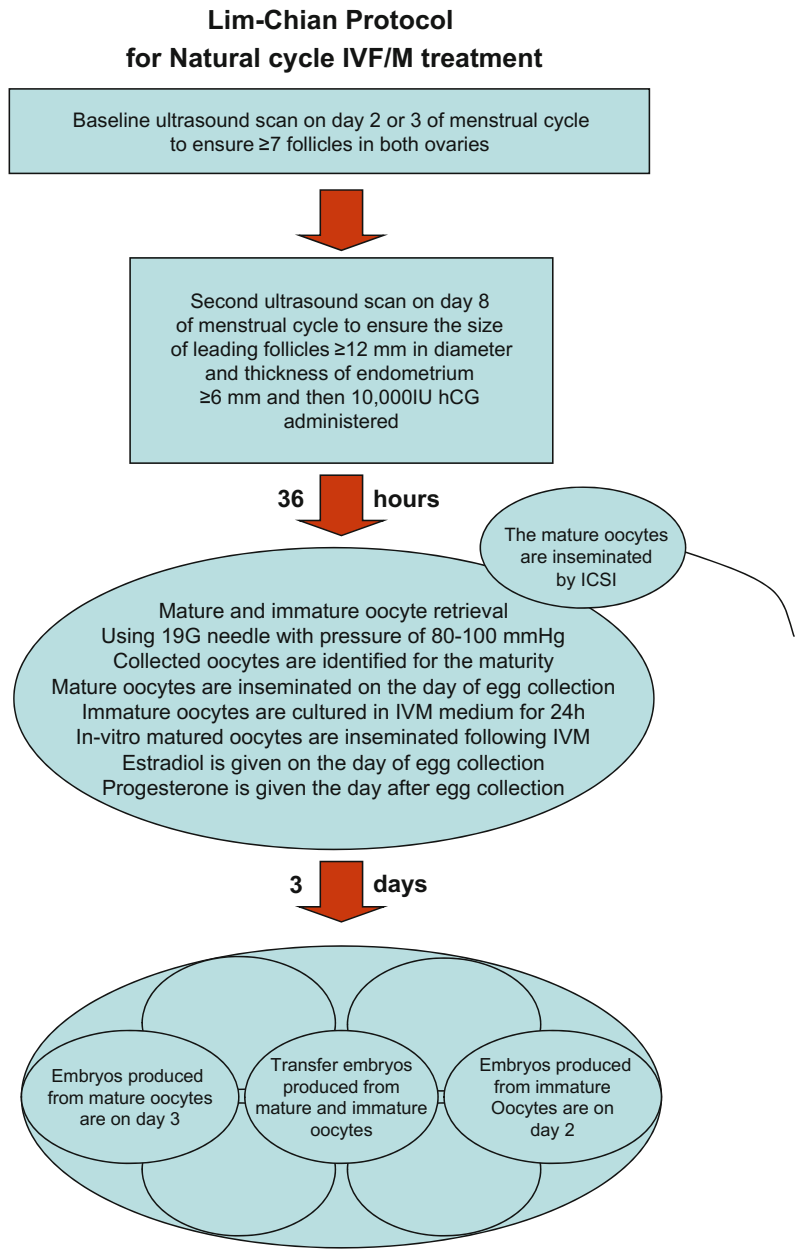

Fig. 27.1 Detailed protocol for natural cycle IVF combined with IVM treatment (Based on Lim et al. [27] with modification)

to the follicular size. In fact, as seen in the patients of Group A, many in vivo mature oocytes were harvested from the small-sized follicles (<12 mm in diameter) than the leading follicles (≥12 mm in diameter). This observation should spur renewed interest in studying the process of folliculogenesis, and the resumption of meiosis during natural cycles and should caution in relying on a particular size of the follicle to predict oocyte maturity.

Lim et al. [27] have shown that the optimal sizes of follicles could be between 12 and 14 mm in diameter for natural cycle IVF combined with IVM treatment, which can prevent premature ovulation [25, 26]. Surprisingly, we found that some mature oocytes can be retrieved from

Table 27.1 Comparison of pregnancy and live birth rates of natural cycle IVF/M treatment in women with or without mature oocytes collected at the time of egg retrieval (Reproduced from Yang et al. [30])

Groups	A	B	*P* value
No. of patients (cycles)	283 (314)	53 (55)	–
Age (mean ± SD)	31.2 ± 3.6	30.4 ± 3.3	NS
No. of mature oocytes retrieved (mean ± SD)	739 (2.4 ± 1.6)	–	–
No. of immature oocytes retrieved (mean ± SD)	2780 (8.9 ± 5.0)	515 (9.4 ± 4.9)	NS
No. of oocytes matured in vitro (%)	1826 (65.7)	363 (70.5)	NS
Total numbers of oocytes matured (mean ± SD)	2565 (8.2 ± 3.5)	363 (6.6 ± 3.5)	NS
No. of oocytes fertilized (%)	2101 (81.9)	294 (81.0)	NS
No. of in vivo-matured oocytes fertilized (mean ± SD)	644 (2.1 ± 1.4)	–	–
No. of in vivo-matured oocytes cleaved (mean ± SD)	610 (1.9 ± 1.4)	–	–
No. of in vitro-matured oocytes fertilized (mean ± SD)	1457 (5.1 ± 1.4)	294 (4.9 ± 2.2)	NS
No. of in vitro-matured oocytes cleaved (mean ± SD)	1324 (4.2 ± 2.6)	262 (4.8 ± 2.7)	NS
No. of zygotes cleaved (%)	1934 (92.1)	262 (89.1)	NS
No. of embryos transferred (mean ± SD)	859 (2.7 ± 0.4)	147 (2.7 ± 0.5)	NS
No. of clinical pregnancies (%)	126 (40.1)	19 (34.5)	NS
No. of embryos implanted (%)	139 (16.2)	22 (15.0)	NS
Live births per cycle (%)*	93 (29.6)	9 (16.4)	0.043
Singleton	74	9	–
Twins	18		–
Triplets	1		–
Miscarriage rate per clinical pregnancies (%)*	33 (26.2)	10 (52.6)	0.019

Group A The patients with mature oocytes retrieved at egg retrieval
Group B The patients without mature oocytes retrieved at egg retrieval
*Significantly different between groups

relatively small follicles (between 8 and 10 mm in diameter). The embryo development from in vivo-matured oocytes had better quality than in vitro-matured oocytes, resulting in higher clinical pregnancy rate in the natural cycle IVF combined with IVM treatment with in vivo-matured oocytes compared to the cycles without in vivo-matured oocytes [25, 26]. However, the data show that there were no significant differences in terms of clinical pregnancy and implantation rates when the natural cycle IVF combined with IVM treatment with or without in vivo-matured oocytes was retrieved at the time of oocyte retrieval (Table 27.1).

Recently, it has been reported that non-dominant small follicles are a promising supplementary source of mature oocytes and that their use increase the live birth rate in natural cycle IVF treatment [31]. Also they demonstrate that the blastocysts from non-dominant follicles are as competent as those from dominant follicles in terms of pregnancy. This report confirms the notion that mature oocytes can be retrieved from non-dominant or leading follicles during natural cycle treatment.

Table 27.2 shows the pregnancy outcomes based on the mature oocytes retrieved from the different-sized follicles at the time of oocyte retrieval. Whether the mature oocytes were retrieved from leading follicles or small follicles, there were no differences in fertilization (81.8, 81.8, and 82.2%, respectively) and embryo cleavage (91.9, 93.9, and 90.5%, respectively) rates among those subgroups. Also the quality of

Table 27.2 Comparison of pregnancy and live birth rates based on the presence of mature oocytes retrieved from the leading or non-leading follicles at the time of egg retrieval (Reproduced from Yang et al. [30])

Subgroups of A	1	2	3	*P* value
No. of patients (cycles)	137 (150)	82 (86)	76 (78)	–
Age (mean ± SD)	31.6 ± 3.8	31.2 ± 3.2	31.0 ± 3.5	NS
No. of mature oocytes retrieved (mean ± SD)	477 (3.2 ± 1.5)	89 (1.0 ± 0.2)	173 (2.2 ± 1.6)	–
No. of immature oocytes retrieved (mean ± SD)	1221 (8.1 ± 4.6)	808(9.4 ± 5.4)	751 (9.6 ± 5.2)	NS
No. oocytes matured in vitro (%)	811 (66.4)	532 (65.8)	483 (64.3)	NS
Total numbers of oocytes matured (mean ± SD)	1288 (8.6 ± 3.5)	621 (7.2 ± 3.4)	656 (8.4 ± 3.5)	NS
No. of oocytes fertilized (%)	1054 (81.8)	508 (81.8)	539 (82.2)	NS
No. of in vivo-matured oocytes fertilized (mean ± SD)	417 (2.8 ± 1.5)	81 (1.0 ± 2.3)	146 (2.0 ± 1.3)	–
No. of in vivo-matured oocytes cleaved (mean ± SD)*	395 (2.6 ± 1.4)	80 (0.9 ± 0.4)	135 (1.7 ± 1.3)	–
No. of in vitro-matured oocytes cleaved (mean ± SD)	574 (3.8 ± 2.6)	397 (4.6 ± 2.7)	353 (4.5 ± 2.5)	NS
No. of zygotes cleaved (%)	969 (91.9)	477 (93.9)	488 (90.5)	NS
No. of embryos transferred (mean ± SD)	410 (2.7 ± 0.4)	232 (2.7 ± 0.5)	217 (2.8 ± 0.4)	NS
No. of clinical pregnancies (%)	66 (44.0)	30 (34.9)	30 (38.5)	NS
No. of embryos implanted (%)	69 (16.8)	34 (14.7)	36 (16.6)	NS
Live births per cycle (%)*	46 (30.7)	23 (26.7)	24 (30.8)	NS
Singleton	41	15	18	–
Twins	5	8	5	–
Triplets	0	0	1	–
Miscarriage rate per clinical pregnancies (%)*	20 (30.3)	7 (23.3)	6 (20.0)	NS

Group 1 Mature oocytes were retrieved from both leading and small follicles
Group 2 Mature oocytes were retrieved from the leading follicles only
Group 3 Mature oocytes were retrieved from the small follicles only
*All embryos that produced from in vivo-matured oocytes were transferred

embryos was not different among the groups assessed by morphology. In addition, there were no statistically significant differences in terms of clinical pregnancy (44.0, 34.9, and 38.5%), implantation (16.8, 14.7, and 16.6%), live birth per embryo transfers (30.7, 26.7, and 30.8%), and miscarriage per clinical pregnancy (30.3, 23.3, and 20.0%) rates among those subgroups.

Interestingly, there are no significant differences in terms of clinical pregnancy rates between Group A (40.1%) and Group B (34.5%) independent of retrieving mature oocytes (Table 27.1). In fact, an average of 1.9 ± 1.4 embryos derived from in vivo-matured oocytes was transferred in Group A compared to Group B in which no embryo was produced from in vivo-matured oocytes transfer. In addition, the implantation rates were not different between these two groups. Surprisingly, there was a higher miscarriage rate in Group B as compared to Group A, indicating that the embryos produced from in vitro-matured oocytes may be associated with a higher miscarriage rate. We cannot reconcile the higher miscarriage rate for in vitro-matured oocytes. Further study may be needed to answer this question.

Conclusion

With the development of IVM treatment for women with PCOS, the combination of natural cycle IVF with immature oocyte retrieval followed by IVM, namely natural cycle IVF/M, was proposed. Natural cycle IVF/M is an attractive treatment for women with all types of infertility without recourse to ovarian stimulation with acceptable pregnancy rate. Natural cycle IVF/M together with IVM-alone treatment can offer more than half of infertile women who are seeking for infertility treatment with an acceptable pregnancy and implantations rates. Although the clinical pregnancy rates are not different in terms of mature oocytes being retrieved or the time of egg retrieval with natural cycle IVF/M treatment, the live birth rate is higher ($P < 0.05$) when the transferred embryos were produced from the in vivo-matured oocytes. Natural cycle IVF/M may be the most suitable treatment for younger women who have regular menstrual cycles.

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