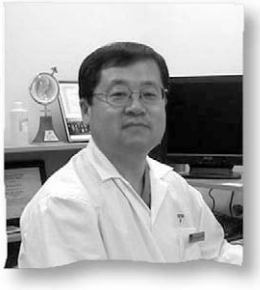
Review

In-vitro maturation of human oocytes



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Abstract

Immature human oocytes can be matured and fertilized *in vitro*. However, subsequent embryonic development is different when the immature oocytes are retrieved in different situations. Exposure to the LH surge *in vivo* may be important for the oocytes to acquire the competence for maturation and subsequent embryonic development. The size of the follicles may also be an important feature for subsequent embryonic development. However, the developmental competence of oocytes derived from small antral follicles does not seem to be adversely affected by the presence of a dominant follicle. Oocyte maturation *in vitro* is profoundly affected by culture conditions. Gonadotrophins are required for oocyte maturation *in vivo*, but any requirement *in vitro* is still unclear. Recent clinical results from in-vitro matured (IVM) human oocytes are promising, although further research remains to be done in order to address the mechanisms of oocyte maturation and to improve culture conditions and also the implantation rate of embryos generated from IVM oocytes.

Keywords: *development, fertilization, in-vitro maturation, oocyte*

Introduction

During the follicular phase of the menstrual cycle, only a single follicle usually grows to the pre-ovulatory stage and releases its oocyte for potential fertilization. Although the first live birth achieved from IVF was from a natural cycle (Steptoe and Edwards, 1978), modern IVF protocols use gonadotrophins to stimulate the ovaries and generate multiple follicles. This is because pregnancy rates are related to the number of good quality embryos available for transfer. Therefore, in conventional IVF treatment, women are usually pretreated for approximately 2 or 3 weeks with gonadotrophin-releasing hormone analogue (GnRHa), then stimulated with human menopausal gonadotrophin (HMG) or purified FSH to induce multiple follicle development. However, some women are extremely sensitive to stimulation with exogenous gonadotrophins and are at increased risk of developing ovarian hyperstimulation syndrome (OHSS). Severe OHSS is associated with occasional severe complications such as thromboembolism, renal impairment, adult respiratory distress (ARDS) and, rarely, death. The long-term side effects of gonadotrophin stimulation are unknown. In addition, the long pretreatment period with GnRHa and FSH can be challenging psychologically and financially.

The recovery of immature oocytes followed by in-vitro maturation (IVM) of these oocytes could be developed as a new method for the treatment of women with infertility. The benefits of IVM include avoiding the side effects of GnRHa and gonadotrophins, avoiding the required ultrasound and serum monitoring (thereby reducing direct and indirect costs), and avoiding the risks of ovarian stimulation, including OHSS. Successful fertilization, embryo development and pregnancies with immature human oocytes matured *in vitro* have been reported (Trounson *et al*., 1994), although most have been limited to women with polycystic ovaries (PCO) or polycystic ovary syndrome (PCOS). Some theoretical concern regarding the safety of IVM has been raised, especially the potential impact in expression of imprinting genes (Fauser *et al*., 2002; Albertini *et al*., 2003). However, based on human oocyte IVM data so far, it is difficult to conclude that IVM increases the risk of imprinting gene damage over that of conventional IVF (i.e. hormone-stimulated IVF cycles). This review will try to describe the mechanisms involved in oocyte maturation and to discuss the recent progress of immature human oocytes matured *in vitro* for clinical applications.

Concepts of oocyte maturation

Human oocytes usually become arrested in prophase I of meiosis during fetal life. At birth, the oocytes remain in the dictyate phase and each ovary has more than 500,000 healthy non-growing or primordial follicles (Baker, 1963). Throughout the reproductive life of the woman, cohorts of oocytes are removed from this non-growing pool and commence growth. The earliest follicular growth phase is determined mainly by an increase in oocyte size and forms a few layers of granulosa cells around the oocyte. Once oocytes build their cytoplasm up, follicular growth becomes concentrated on the granulosa cell proliferation and differentiation. This differentiation drives antral cavity formation in follicles. In the antral phase, which is initiated in response to FSH secreted by the anterior pituitary, fluid accumulates between granulosa cells. A central cavity is formed, with the mural granulosa cells located at the periphery. The oocyte remains surrounded by closely associated granulosa cells, referred to as cumulus cells, forming the compact cumulus–oocyte complex (COC). At this developmental stage, the follicles become gonadotrophin-dependent for further development. Under the influence of FSH, the follicles develop from the early antral stage to pre-ovulatory stage. At late follicular phase (middle of menstrual cycle), the pre-ovulatory surge of luteinizing hormone (LH) induces germinal vesicle breakdown (GVBD) and chromosomes progress from metaphase I to telophase I. The completion of the first meiotic division is characterized by the extrusion of the first polar body and formation of the secondary oocytes, both of which contain a diploid chromosome complement. The second meiotic division is initiated rapidly after completion of the first meiotic division and the oocytes reach the metaphase II stage prior to ovulation. Oocyte maturation is defined as the reinitiation and completion of the first meiotic division from the germinal vesicle stage to metaphase II, with accompanying cytoplasmic maturation necessary for fertilization and early embryonic development. Oocyte maturation is often conceptually divided into nuclear and cytoplasmic maturation. Nuclear maturation is a term that 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 (Cha and Chian, 1998). However, these two processes are not completely separated processes. Nuclear maturation is controlled by cytoplasmic maturation. In addition, increasing evidence indicates that modifications and changes have occurred on the surface of oocyte membrane during maturation. Here reference is made to membrane maturation, in order to distinguish the process from cytoplasmic maturation.

Nuclear maturation

The pre-ovulatory surge of LH *in vivo* initiates GVBD. The mechanisms involved in GVBD are not fully understood. Many potential factors mediate the cumulus cells control of GVBD. High concentrations of cyclic adenosine monophosphate (cAMP) and purine hypoxanthine in the culture medium prevent oocyte GVBD (Törnell and Hillensjö, 1993). The oocyte and cumulus cells are coupled by gap junctions, suggesting that granulosa cells control GVBD via the cumulus cells. The gap junctions permit regulatory molecules, such as steroids, Ca^{2+}, inositol 1,4,5-trisphosphate (IP_3), cAMP and purines, to pass freely between the cytoplasm of the oocyte and cumulus cells.

Protein synthesis is required for GVBD (Schultz *et al*., 1988; Chian *et al*., 1997). After GVBD, not all oocytes can mature and extrude the first polar body to become nuclear maturation. Protein synthesis is needed for the progression of oocytes from the germinal vesicle stage to metaphase II, as well as for the maintenance of the metaphase II arrest (Gerhart *et al*., 1984). Inhibition of protein synthesis in oocytes results in failure to activate maturation promoting factor (MPF) activity (Hashimoto and Kishimoto, 1988). Cytoplasmic proteins, MPF and cytostatic factor (CSF; Shibuya and Masui, 1989), regulate oocyte nuclear maturation.

Molecular characterization of MPF has shown that active MPF is a protein dimer composed of catalytic $p34^{cdc}$ serine/threonine kinase, and regulatory cyclin B subunits (Gautier *et al*., 1988, 1990). The $p34^{cdc2}$ serine/threonine kinase is the product of the *cdc2* gene, first identified in fission yeast. The $p34^{cdc}$-cyclin heterodimer, a protein kinase, has four phosphorylation sites that are regulated by kinase and phosphatase activities. 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 (Sagata *et al*., 1989). Therefore, the metaphase II arrest is due to the transcription of *c-mos* as the oocyte matures.

Mitogen-activated protein kinase (MAPK) was first identified in somatic cells and has now 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 (Sun *et al*., 1999a). A product of *c-mos* stimulates MAPK activity, but does not activate $p34^{cdc2}$ (Nebreda and Hunter, 1993). 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 (Sun *et al*., 1999b). Cyclin was originally identified in sea urchin oocytes as a protein whose concentration was greatly increased upon fertilization and subsequently oscillated during the early cell divisions of the embryo (Evans *et al*., 1983). 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 (Hunter, 1991). Two isoforms of cyclin B have been described in the mouse (Chapman and Wolgemuth, 1992, 1993). The expression patterns of cyclin B1 and B2 differ, with the cyclin B1 isoform predominantly expressed in the oocytes. Cyclin B is also phosphorylated and dephosphorylated during oocyte maturation (Whitaker and Patel, 1990). Cyclin B1 was expressed in oocytes, embryos and granulosa cells from both the human (Heikinheimo *et al*., 1995, 1996), indicating that the expression patterns of *c-mos,* cyclin B1 and β-actin are important for proper oocyte development in human oocytes

and preimplantation embryos. However, the mechanisms involved in GVBD, as well as the cell signalling pathways driving the oocyte into metaphase II in response to pre-ovulatory gonadotrophin surge, are not fully understood.

Cytoplasmic maturation

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 (De Sousa *et al*., 1998; De La Fuente and Eppig, 2001). 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, especially at the end of the oocyte growth phase, when oocytes resume maturation prior to ovulation. RNA synthesis continues at a low level to within 1 h of GVBD and some of the newly synthesized RNA is released into the cytoplasm before GVBD (Wassarman and Letourneau, 1976). Protein synthesis increases obviously before GVBD in human oocytes during maturation culture, suggesting that these newly synthesized proteins may be important for subsequent embryonic development (Chian *et al*., 1997). It has been known that the metabolism of the oocyte is characterized by active transcription and translation during the pre-ovulatory period (Wassarman and Kinloch, 1992). However, transcription ceases at the time of ovulation, so the oocyte and early pre-embryo are dependent upon the pool of mRNA and protein accumulated during the pre-ovulatory period (Telford *et al*., 1990). Some maternal transcripts are even stored after the maternal to embryonic transition of gene expression has been completed (Memili and First, 2000). Cytoplasmic factors of the oocyte are also dependent on maternal genetic background, and may be responsible for maternal effects on de-novo methylation, gene expression and congenital aberrations (Picard *et al*., 2001).

The mammalian oocyte not only provides those proteins that are essential for the initial embryo division, but it is also implicated in regulating expression from the paternal genome after fertilization. It has been reported that MSY2, a member of the Y-box protein family, is expressed in growing oocytes and is one of the most abundant proteins in the oocytes (Yu *et al*., 2001). It is known that insufficient cytoplasmic maturation of the oocyte will fail to promote male pronucleus formation and will thus increase chromosomal abnormalities after fertilization (Thibault *et al*., 1975). Oocytes prepare for fertilization and embryonic development by accumulating essential maternal materials and by undergoing genomic modifications during oocyte growth, and the final preparations are made during oocyte maturation (from the germinal vesicle stage to metaphase II). However, the precise kinetics of the molecular specific accumulation of maternal molecules during oocyte maturation is still unknown.

Until recently, it was believed that mammalian oocytes have no polarity or axes. However, more and more evidence indicates that polarity and axes are established as early as the oocyte in the follicle and that these polarities determine the later positional and morphological polarity (see reviews by Edwards, 2000; Scott, 2000). It is also becoming clear that early molecular events drive and control these physical phenomena, for example, granulosa cell gene products that are transferred to and localize in a polarized manner in the oocytes are STAT3 and leptin (Antczak and Van Blerkom, 1997). STAT3 is a protein that is involved in signal transduction and activation of transcription, and leptin is a cytokine product that is involved in the activation of STAT3. Therefore, it seems that the morphology or polarity of the oocyte plays a major role in the determination of axes of the embryo and the overall global pattern of the embryo. Nevertheless, very little is known about the polarity and the axes of maternal mRNA in mammalian oocytes, especially during oocyte growth and maturation.

Cumulus cells respond to gonadotrophins and are known to secrete various substances. These substances not only control nuclear maturation, but also perform an important role in cytoplasmic maturation. Beneficial effects of cumulus cells on early development have been reported for many species, including human oocytes. Besides cytoplasmic changes involved in the control and direction of meiotic progression, other important cytoplasmic changes occur during oocyte maturation. In pigs and cattle, the formation of a male pronucleus within a fertilized oocyte depends on the presence of cumulus cells during oocyte maturation (Mattioli *et al*., 1988; Chian *et al*., 1994). These results suggest that the ability for male pronucleus formation is acquired during late folliculogenesis. 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 (Chian and Sirard, 1995; Chian *et al*., 1997).

FSH and LH are produced by the pituitary gland, and are essential for normal sexual development and reproductive function. 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 gonadotrophins (FSH and LH) in gonadal development and reproductive function has been established, the mechanism of gonadotrophins on follicle growth and oocyte maturation is not fully understood. Follicular growth and oocyte maturation *in vivo* are FSH dependent. In the ovary FSH binds to FSH receptors located on mural granulosa cells and acts via the cAMP-dependent protein kinase pathway. Therefore, both FSH and LH use the cAMP system as intracellular second messenger. 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 (Richards and Hedin, 1988). Oestradiol is synthesized in granulosa cells from thecal androgens, by two successive enzyme-catalysed reactions (Fortune, 1986; Miller, 1988). FSH-binding in the presence of androgen also stimulates progesterone biosynthesis, of which cholesterol side-chain cleavage is the rate-limiting step (Toaff *et al*., 1983). FSH also induces the gene for tissue-type plasminogen activator (tPA), a protease that leads to digestion of the follicular wall at ovulation (Ny *et al*., 1985; Galway *et al*., 1990). The more extensive studies from fish and amphibian models show that action of gonadotrophin on oocyte maturation is dependent on new mRNA and protein synthesis, but is not mediated by increases in steroid synthesis (Thomas *et al*., 2001).

During follicular growth up to the pre-ovulatory stage, numerous genes are activated and inactivated in the developing oocyte and surrounding mural granulosa and cumulus cells. The induction of LH receptors by FSH is one of the hallmarks of the differentiating mural granulosa cells (Richards and Kersey, 1989), and is mediated by the FSH-induced increase in intracellular cAMP (Erickson *et al*., 1982). Theca cells constitutionally contain LH receptors. LH is capable of stimulating androgen substrate production from theca cells into FSH-stimulated granulosa cells to transform oestrogen (Karnitis *et al*., 1994) and that the thecal layer is the major cellular source of follicular androgen and LH stimulates thecal androgen production (Nahum *et al*., 1995). In addition, LH is thought to stimulate progesterone production of mural granulosa and cumulus cells in pre-ovulatory follicles (McNatty *et al*., 1979; Chian *et al*., 1999c). LH may synergize with FSH to sustain follicle development as well as to prepare it for the mid-cycle LH surge that triggers ovulation. Although it has been reported that LHR can be down-regulated by high concentrations of human chorionic gonadotrophin (HCG) or LH once the LH receptor has been induced by FSH in mural granulosa cells (Schwall and Erickson, 1983), the reaction of LH on LH receptor mRNA in the mural granulosa cells and cumulus cells is poorly understood. Possible participating factors in human oocyte maturation during culture *in vitro* is shown in **Figure 1**.

In addition, in-vitro studies on mouse oocytes have shown that two closely related sterols, subsequently named meiosis-activating sterols (MAS), can overcome the inhibitory effect of hypoxanthine on the resumption of meiosis (Byskov *et al*., 1995). Two sterols (4,4-dimethyl-5α-cholest-8,14,24-triene-3α-ol from follicular fluid and 4,4-dimethyl-5α-cholest-8,24-diene-3β-ol), as well as two closely related synthetic C_{29} sterols, modestly increased the proportion of maturing mouse oocytes cultured with hypoxanthine as an inhibitor. FSH triggers the synthesis by cumulus cells of MAS that may reach the oocyte through the gap junction pathway to bring about meiotic resumption (Leonardsen *et al*., 2000). The proposed mechanism of MAS on oocyte maturation is that MAS produced by the somatic compartment of follicle and acts directly on the oocyte to stimulate maturation (Leonardsen *et al*., 2000). However, recent studies suggested that MAS is not an obligatory step in the stimulation of the resumption of meiosis, indicating MAS production in oocytes rather than its transport from the somatic cells as implied by the proposed role of MAS as a cumulus–oocyte signal molecule (Downs *et al*., 2001; Vaknin *et al*., 2001). Therefore, it seems that a role for MAS in meiotic regulation and the effect of gonadotrophin stimulation on the sterol biosynthetic pathway remain inconclusive.

Membrane maturation

Small antral follicles produce oestrogen, but elevated amounts of this steroid derive mostly from large pre-ovulatory follicles (Fritz and Speroff, 1982). The actions of oestrogen are mediated through binding specifically to nuclear oestrogen receptors, ligand-activated regulatory proteins that act as dimers on specific target genes containing defined DNA sequences called oestrogen response elements (Beato and Klug, 2000; Gruber *et al*., 2002). The human oestrogen receptor was cloned from a cDNA expression library produced from the breast cancer cell line MCF-7 (Walter *et al*., 1985). Oestrogen receptor binding to oestrogen response elements can result in induction or suppression of responsive genes. Oestrogen mRNA and protein concentrations in various target tissues can be influenced by several physiological factors, including oestrogens. Oestrogen receptors been identified within the oocyte (Wu *et al*., 1993; Wu and Wolgemuth, 1995). Therefore, oestrogen may be involved in the events of cytoplasmic maturation of the oocyte. In addition, recently there is increasing evidence for direct non-genomic steroid effects in various cell types. 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. Many of these rapid steroid actions are non-genomic and initiated at the surface of the target cell by binding to membrane receptors (Revelli *et al*., 1996; Watson and Gametchu, 1999). It has been suggested that oestrogen may act at the oocyte surface by producing changes in reactivity of its Ca^{2+} release system during cytoplasmic maturation (Tesarik and Mendoza, 1995). Oocytes need to be primed with oestradiol to develop Ca^{2+} oscillations during maturation. Therefore, the process can be referred as oocyte membrane maturation (**Figure 1**).

Evidence has revealed that Ca^{2+} release mechanisms are modified during oocyte maturation (Mehlmann and Kline, 1994). When immature oocytes were cultured *in vitro,* they acquired the capacity to undergo a single large oscillation of intracellular Ca^{2+} (Herbert *et al*., 1997). However, subsequent sustained oscillations were not observed in some immature oocytes, indicating that these oocytes failed to develop a fully competent Ca^{2+} signalling system during maturation *in vitro*. Sperm penetration triggers the events that signal the oocyte has been activated and has entered the programme of embryonic development. The activated oocyte causes the exocytosis of the cortical granules, which prevents polyspermy and the completion of meiosis to start the first embryonic mitosis. These events of oocyte activation at fertilization are mediated by a sperm-induced increase in the concentration of intracellular free Ca^{2+} (Kline and Kline, 1992; Whitaker and Swann, 1993). Therefore, the development of the ability of the oocyte to release Ca^{2+} in response to the fertilizing spermatozoa is an essential step during oocyte maturation (Carroll *et al*., 1996). LH-supported oestrogens may be involved in membrane maturation of oocytes mediated by non-genomic (oocyte surface) effects.

It seems that oocytes also require a specific intrafollicular progesterone environment for the inductive signals of membrane maturation, because pre-ovulatory follicular fluid contains certain concentrations of progesterone (Seibel *et al*., 1989). 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 follicular maturation is associated with a shift from oestradiol to progesterone production by the granulosa cells, which is reflected in the ratio of progesterone to oestradiol. It appears that the ratio of progesterone to oestradiol in follicular fluid may be an indicator of oocyte maturity (Kreiner *et al*., 1987). Progesterone has been shown to cause an immediate increase in free cytosolic calcium in both capacitated and non-capacitated spermatozoa (Blackmore *et al*., 1990). Therefore,

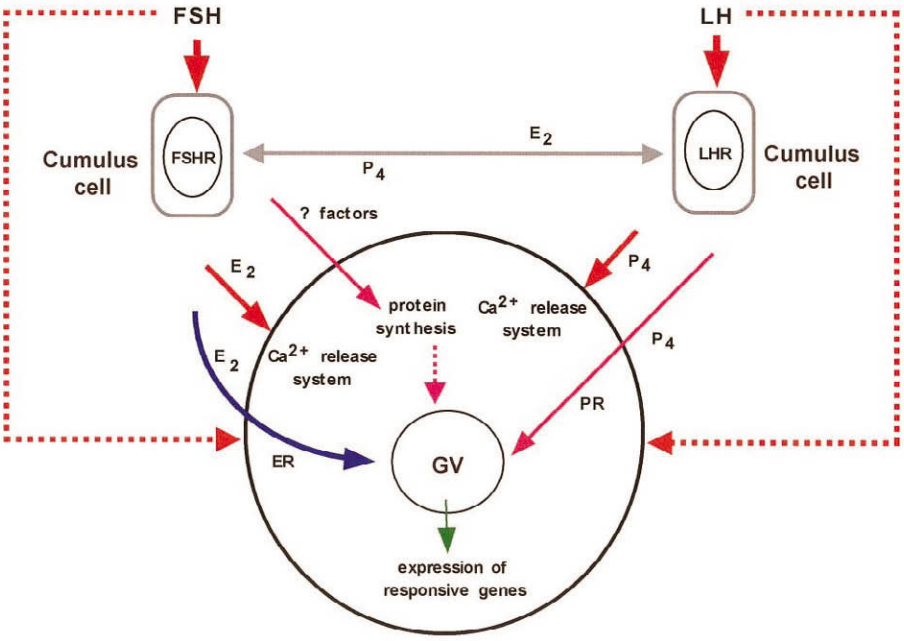

Figure 1. Hypothetical model for the possible participation of factors in human oocyte maturation during culture *in vitro*. FSH = follicle-stimulating hormone; LH = luteinizing hormone; FSHR = FSH receptor; LHR = LH receptor; GV = germinal vesicle; E_2 = oestradiol; P_4 = progesterone; ER = oestradiol receptor; PR = progesterone receptor.

it is possible that progesterone may also be involved in the development of an oocyte membrane Ca^{2+} release system. Besides non-genomic effects of progesterone, the actions of progesterone are mediated through binding specifically to nuclear progesterone receptors. In most species studied to date, progesterone receptors are expressed as structurally related but functionally distinct (Vegeto *et al*., 1993) A and B isoforms, encoded by a single gene but transcribed into separate mRNAs (Kastner *et al*., 1990). Expression of both progesterone receptor A and B isoforms are regulated by oestradiol as well as by their cognate ligand, progesterone (Szabo *et al*., 2000). However, the role of progesterone in the oocyte cytoplasmic and membrane maturation is not fully understood.

In addition, several studies suggest the existence of a sperm receptor on the oocyte plasma membrane (Boldt *et al*., 1988; Kellom *et al*., 1992). Capmany and Bolton (1993) demonstrated a modification of total proteins in unfertilized human oocytes compared with 4- and 8-cell-stage supernumerary or arrested human embryos. It has also been indicated that during human oocyte maturation, the total amount of membrane proteins decreased dramatically from the germinal vesicle to the metaphase II stage, while the relative proportion of the 71 kDa band increased from 9.9 to 13.1 and 27.4% in germinal vesicle, metaphase I-, and metaphase II-stage oocytes respectively (Ji *et al*., 1997). Although the functional role of these changes is unclear, it is possible that these modifications may be related to the oocyte membrane maturation.

In-vitro maturation of immature oocytes

Mammalian oocytes acquire a series of competences during follicular development (oocyte growth and maturation) that play critical roles at fertilization and subsequent early embryonic development. Early studies have shown that nuclear maturation can occur spontaneously following culture *in vitro* of animal and human immature oocytes. However, the developmental competence after fertilization of these oocytes is questionable. Oocyte maturation *in vitro* is profoundly affected by culture conditions. The percentage of oocytes that can develop to the blastocyst stage is generally considered a suitable indication of developmental competence. However, recent data from animal studies suggested that 'blastocyst formation' is a limited predictor of development (Duranthon and Renard, 2001). The successful production of morphologically normal blastocyst stage embryos has not proved reliable in indicating whether a successful pregnancy will be established. There are differences in the relative abundance of a set of developmentally important gene transcripts in bovine morulae and blastocysts between culture systems and protein supplements (Wrenzycki *et al*., 2001; Rizos *et al*., 2002). Therefore, expression of these genes could be a potentially important marker for assessing embryo viability and implantation. However, it is difficult for clinical practice to use these important markers at the present time. Further research is required to develop reliable markers for assessing oocyte and embryo viability.

Follicular size

During folliculogenesis, the human oocyte grows from 35 μm to 120 μm in diameter (Gougeon, 1996). At the end of oocyte growth, the antrum is formed and the oocyte has acquired the capacity to resume meiosis. As discussed, most mRNA and protein are synthesized during the period of oocyte growth. 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 (Eppig *et al*.,

1992). Although it has been reported that the human oocyte has a size-dependent ability to resume meiosis from 90 to 120 μm in diameter (Durinzi *et al*., 1995), 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. Sometimes, small-size oocytes can be collected from antral follicles and matured to metaphase II following proper in-vitro culture. Therefore, it seems that these oocytes are still growing in size during antrum formation in human and some animal species (Fair *et al*., 1995).

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 (Tsuji *et al*., 1985). In the follicular phase of the menstrual cycle, the percentage of oocyte maturation in the large-follicle group (9–15 mm in diameter; 34.5%) was significantly ($P < 0.05$) higher than that of the small-follicle group (3–4 mm; 8.8%). During the follicular phase, normally the largest healthy follicle (5.5–8.2 mm in diameter) appears to be selected (Gougeon and Lefevre, 1983). The selected follicle becomes the dominant follicle and will be destined to ovulate. The remaining cohort of follicles will be terminated by atresia. The mechanism of the selection of the follicle for ovulation is still unclear, but it has been suggested that it may be related to FSH-induced aromatase activity of the granulosa cells (Hillier, 1985). Prolonged exposure to androgens in the remaining cohort of follicles may have an adverse effect on oocyte viability and developmental competence (Anderiesz and Trounson, 1995). In cattle, the oocytes from follicles <3.0 mm not only show significantly lower maturation rates but also have very low blastocyst development rate (Blondin and Sirard, 1995). However, it has been reported that the developmental competence of oocytes from the small antral follicles is not adversely affected by the presence of a dominant follicle (Smith *et al*., 1996). Furthermore, recent results indicate that rates of oocyte maturation, fertilization and early embryonic development are not affected by the phases of folliculogenesis when the immature oocytes were aspirated from the similar sizes (2–8 mm in diameter) of follicle group (Chian *et al*., 2002). Therefore, further investigation is needed to clarify when the remaining cohort of follicles undergoes atresia.

In current gonadotrophin-stimulated IVF protocols, gonadotrophins are used to induce multiple follicular development and oocyte maturation *in vivo*. FSH is necessary for the growth of pre-ovulatory follicles. LH supports follicular growth by providing androgen substrate for the granulosa cell aromatase and also triggers the resumption of oocyte maturation. The use of gonadotrophins has resulted in follicle asynchrony (Bomsel-Helmreich *et al*., 1987). Mature oocytes are retrieved by transvaginal aspiration 36 h post-HCG administration. The size of the leading follicle does not affect the fertilization and cleavage rates of cohort oocytes from gonadotrophin-stimulated cycles (Wittmaack *et al*., 1994). 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 (Dubey *et al*., 1995; Salha *et al*., 1998). Recently, it has been shown that the development of embryos in cohort follicles from stimulated cycles appeared to be independent of the diameter of the leading follicle at the time of HCG injection (Jones *et al*., 1998; Trounson *et al*., 2001). It must be noted, however, that immature oocytes are retrieved frequently after HCG administration even from the size of follicles >10 mm in diameter, and these immature oocytes can be matured and developed *in vitro* (Cha and Chian, 1998; Chian and Tan, 2002).

Media

Although numerous data have been accumulated from animal studies, the current rationale for choosing a specific medium for IVM of immature oocytes appears to stem largely from adapting methods developed from culturing other cell types. Complex culture media, such as tissue culture medium 199 (TCM-199), Ham's-F10, and Chang's medium buffered with bicarbonate or HEPES, supplemented with various sera, gonadotrophins (FSH and LH) and oestradiol, have been most widely used in research or the clinical application of oocyte IVM (Trounson *et al*., 1998). Although major beneficial components seem already present in these media (Bevers *et al*., 1997), more attention is required to determine specific metabolic needs and optimal culture conditions required by maturing oocytes for appropriate gene expression and regulation.

Different energy substrates and nutrients can greatly influence oocyte meiotic and cytoplasmic maturation (Rose-Hellekant *et al*., 1998; Chung *et al*., 2002). Glucose, pyruvate and lactate are the main substrates for energy metabolism in somatic cells and oocytes. Glutamine can also serve as an energy substrate to improve in-vitro nuclear maturation of hamster (Gwatkin and Haidri, 1973) and rabbit (Bae and Foote, 1975) oocytes. Oocyte utilization of pyruvate is closely dependent upon cumulus cells that can convert glucose or lactate into pyruvate to be used by oocytes (Leese and Barton, 1985). Pyruvate or oxaloacetate, but not glucose, lactate or phosphoenolpyruvate, support the maturation of denuded mouse oocytes through meiosis to metaphase II (Biggers *et al*., 1967). It has been confirmed that mitochondrial oxidative metabolism is much more important than anaerobic glucose metabolism for energy production in the mammalian oocytes (Gandolfi *et al*., 1998). Synthesis of pyruvate in the cumulus cells from glucose provides additional evidence that these cells are able to influence the nutritional environment of the maturing oocytes (Leese and Barton, 1984). Recently, it has been shown that sodium pyruvate in non-serum maturation medium supports and promotes nuclear maturation of bovine cumulus-denuded oocytes (Geshi *et al*., 2000). However, it has been reported that pyruvate alone is insufficient for oocyte cytoplasmic maturation (Zheng *et al*., 2001). Nevertheless, it has been indicated that the expression pathway of glycolytic metabolism reflects the presence of different mechanisms involved in gene expression/regulation at the transcriptional and translational level and their accumulation during human oocyte maturation (Mouatassim *et al*., 1999). In addition, it has been indicated that metabolism of glucose through the Embden–Meyerhof pathway is important during bovine oocyte maturation *in vitro* (Krisher and Bavister, 1999).

Spontaneous mouse oocyte maturation *in vitro,* in either the presence or the absence of meiotic inhibitor, is associated with a decrease in oocyte cAMP concentrations (Downs *et al*., 1989). 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 (Downs, 1995). Pyruvate directly affects nuclear maturation in mouse oocytes (Haekwon and Schuetz, 1991). Although it has been indicated that glucose may have an

inhibitory effect on cumulus-free human oocyte maturation during culture *in vitro* (Cekleniak *et al*., 2001), recent results indicate that oocyte maturation medium with glucose is beneficial to bovine and human oocyte nuclear and cytoplasmic maturation *in vitro* (Chian and Tan, 2002; Chung *et al*., 2002).

Essential and/or non-essential amino acids are commonly added to serum-supplemented or serum-free culture media for mammalian embryo development *in vitro*. In many species, it has been known that addition of amino acids to the culture medium is beneficial for embryonic development (Lane and Gardner, 1998). Apart from amino acid use for protein synthesis, they play important roles as osmolytes (Biggers *et al*., 1993), intracellular buffers (Edwards *et al*., 1998), heavy metal chelators and energy sources (Bavister, 1995) as well as precursors for versatile physiological regulators, such as nitric oxide and polyamines (Wu and Morris, 1998). It has also been shown that the culture medium with amino acids affect glucose metabolism in mouse blastocysts *in vitro* (Lane and Gardner, 1998). Although it has been shown that amino acids support rabbit (Bae and Foote, 1975), hamster (Gwatkin and Haidri, 1973), porcine (Ka *et al*., 1997) and bovine (Rose-Hellekant *et al*., 1998) oocyte maturation, amino acid requirements for oocyte maturation in culture are not fully understood. Recent data indicate that 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 oocyte cytoplasmic maturation (Rezaei *et al*., 2003).

It has been reported that the addition of water-soluble vitamins, particularly inositol, to the embryo culture medium enhances the hatching of rabbit and hamster blastocysts (Kane and Bavister, 1988; Fahy and Kane, 1992). Vitamins affect glucose metabolism in mouse (Lane and Gardner, 1998) and sheep embryos (Gardner *et al*., 1994). However, there is a paucity of information about the effects of vitamins in culture medium on the maturational and developmental competence of immature oocytes. The results from this laboratory demonstrate that the presence of vitamins in the oocyte maturation medium is important for subsequent bovine embryonic development (Abdul Jalil *et al*., 2002). Based on animal studies, a new IVM medium has been designed and shown to be beneficial for nuclear and cytoplasmic maturation of immature human oocytes derived from stimulated IVF and intracytoplasmic sperm injection (ICSI) cycles (Chian and Tan, 2002).

Supplements

Serum

Earlier studies cultured immature human oocytes with TCM-199 or Ham's F-10 medium supplemented with 10% fetal calf serum (FCS) or fetal bovine serum (FBS). Normally, successful oocyte maturation media for animals contain a large quantity of FBS (Younis *et al*., 1989). FBS is considered crucial for animal 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. Although a few pregnancies and live births were achieved when using 50% human follicular fluid (HFF) or 50% human peritoneal fluid (HPF) as supplements in the oocyte maturation medium (Cha *et al*., 1991, 1992), rates of oocyte maturation were relatively low. Pregnancy and the first birth of a normal baby occurring in an anovulatory polycystic ovarian syndrome (PCOS) patient following immature oocyte maturation *in vitro* was with TCM-199 plus 10% FBS supplemented with gonadotrophins (Trounson *et al*., 1994). Chian and Tan (2002) have been using a designed IVM medium plus 10% synthetic serum substitute, resulting in approximately 80% maturation rate and >90% fertilization rate when the cumulus-free germinal vesicle oocytes were retrieved from stimulated IVF and ICSI cycles. It has been confirmed that FBS can be replaced by the patient's own serum for immature human oocyte maturation *in vitro* (Chian *et al*., 1999a). Therefore, concerns about potential transmission of infectious agents could be resolved in the clinical application of oocyte maturation *in vitro*.

Gonadotrophins

As mentioned above, gonadotrophins (FSH and LH) play an important role in the development and function of pre-antral, antral and pre-ovulatory follicles *in vivo*. However, it is important to determine whether these gonadotrophins could play the same role in promoting oocyte maturation *in vitro*. Currently most IVM protocols do supplement FSH or LH or a combination in culture medium. However, the effect of gonadotrophins and their relative importance for oocyte maturation and subsequent fertilization and early development is still controversial. While the idea to use FSH and LH is based on the physiological role of FSH and LH in oocyte maturation *in vivo*, it is most likely the mechanisms of oocyte maturation are different between *in vitro* and *in vivo,* because most IVM experiments are performed with oocytes from small and medium-sized follicles. Those follicles differ in many aspects from pre-ovulatory follicles. In in-vitro conditions, LH probably acts to induce GVBD by an indirect action mediated by cumulus cells because it is believed that there are no LH receptors (LHR) on oocytes (Dekel *et al*., 1981). Nevertheless, it has been reported that mRNA for FSHR and LHR are present in mouse oocytes, zygotes and preimplantation embryos, indicating that a potential role for gonadotrophins in the modulation of meiotic resumption and the completion of oocyte maturation (Patsoula *et al*., 2001). Recently the same authors reported that mRNA for FSH and LH receptors are observed in human oocytes and preimplantation embryos at different stages (Patsoula *et al*., 2003). Recent results also indicate that mRNA for FSHR exists on bovine oocytes (Chian *et al*., unpublished data; **Figure 2**). Therefore, the mechanisms of FSH and LH on oocyte maturation *in vitro* need to be verified.

It has been indicated that only FSH, not LH, influences the IVM of bovine oocytes (Bevers *et al*., 1997), suggesting that the contradictory reports supporting LH as a major hormone involved in IVM are most likely caused by FSH contamination of the applied LH preparations (Harper and Brackett, 1993). However, it has been shown that FSH does not have a beneficial effect on mouse oocyte development *in vitro* (Eppig *et al*., 2000). Therefore, FSH and LH, apart from regulating oocyte growth and ovulation, also must directly or indirectly act on oocyte or cumulus cells to promote cytoplasmic maturation. The addition of FSH to oocyte culture medium

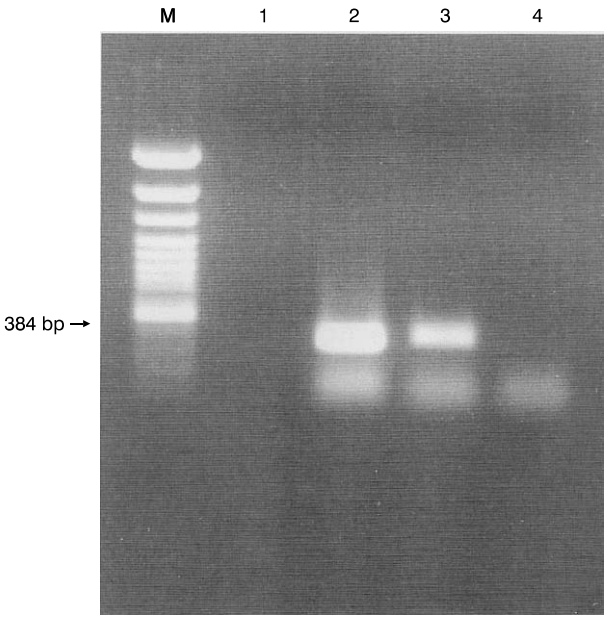

Figure 2. Reverse transcriptase-polymerase chain reaction amplification of bovine GV-stage oocytes for FSH and LH receptors' mRNA. M: DNA marker; lane 1: oocytes without RT; lane 2: β-actin; lane 3: oocytes with RT for FSH receptor (estimated 384 bp); lane 4: oocytes with RT for LH receptor (estimated 552 bp).

does not significantly increase the ability of the oocyte to reach metaphase II (Durinzi *et al*., 1997). However, maturation medium with FSH significantly increases fertilization (Morgan *et al*., 1991). The ability of FSH to increase the developmental capacity of mouse oocytes maturing *in vitro* varies depending on the age and prior gonadotrophin priming *in vivo* (Eppig *et al*., 1992). Addition of FSH into culture medium does not increase the nuclear maturation of rat (Vanderhyden and Armstrong, 1989), monkey (Morgan *et al*., 1991) or human (Durinzi *et al*., 1997) oocytes, and FSH initially has an inhibitory action on mouse oocyte maturation (Downs *et al*., 1988). It has been reported that mouse oocyte GVBD is inhibited by FSH, due to its effect on the rise in concentrations of cAMP in cumulus cells (Schultz *et al*., 1983). FSH concentrations in the follicular fluid of metaphase I and metaphase II oocytes were found to be significantly higher than in follicles containing GV oocytes in women undergoing oocyte retrieval for stimulated IVF cycles (Laufer *et al*., 1984). FSH is important in the development of pre-ovulatory follicles. In addition, data from animal studies indicated that protein synthesis in oocytes is modified by FSH during culture of cumulus–intact oocytes *in vitro* (Chian and Sirard, 1995).

In-vitro maturation of bovine oocytes in the presence of LH resulted in increased embryonic development after IVF (Zuelke and Brackett, 1990). Denuded cumulus cells from oocytes do not respond to LH, thereby implicating the cumulus cells as the mediator of the LH effect (Zuelke and Brackett, 1990). One mechanism by which LH enhances IVM of bovine oocytes is through changing glucose metabolism in cumulus-enclosed oocytes and through modifying the nutritional environment of the oocyte (Zuelke and Brackett, 1992). It has been reported that increased pyruvate and lactate production from rat cumulus granulosa cells occurs in response to LH (Hillensjo, 1976; Billing *et al*., 1983). It has been reported that LH-enhanced IVM of bovine oocytes needs the presence of either 20% FBS or 3 mg/ml bovine serum albumin (BSA) (Zuelke and Brackett, 1990). To achieve efficient FSH and LH dependent steroid production cumulus cells require the presence of FBS (Chian *et al*., 1999d). Therefore, these data suggest that the beneficial effect of LH on the oocytes during IVM involves many possible factors that affect oocyte nuclear and cytoplasmic maturation.

The concentration of FSH and LH in the oocyte maturation medium should be the same as that in the follicular fluid containing fully mature oocytes. Nevertheless, an extremely high concentration of FSH and LH in the oocyte maturation medium has been used by some investigators (Nagy *et al*., 1996; Liu *et al*., 1997). There seems to be no specific reason for using such high concentrations (10 IU/ml) of pregnant mare's serum gonadotrophin (PMSG) and human chorionic gonadotrophin (HCG) in the maturation medium. PMSG has approximately 50:50 ratio FSH and LH bioactivity. Many reports have indicated that the optimal condition for IVM of immature human oocytes should be similar to the physiological concentration of gonadotrophins in follicular fluid that contains fully mature oocytes (Chian *et al*., 1999a,b, 2000). In addition, exposure of immature oocytes to different ratio of FSH:LH during maturation *in vitro* may result different developmental competence. It has been reported that exposure of cattle and human oocytes to a 1:10 ratio of FSH:LH resulted in significantly higher developmental competence evident by increased development to the blastocyst stage *in vitro* compared with FSH alone or no gonadotrophins (Anderiesz *et al*., 2000). However, a recent report from an animal study indicated that the ratio of FSH:LH is not important for oocyte maturation and subsequent embryonic development (Choi *et al*., 2001). Furthermore, previous results indicated that culture medium supplemented with physiological concentration of FSH stimulates oestradiol secretion from the cumulus cells derived from mature and immature human oocytes, suggesting that it may not be necessary to add oestradiol to the oocyte maturation medium when the oocytes are cultured with the cumulus cells (Chian *et al*., 1999d).

Steroids

Oestradiol and progesterone are mediators of normal mammalian ovarian function. Oestradiol may be important not only in regulating oocyte maturation, but also involved in subsequent embryonic development (Tesarik and Mendoza, 1995). Progesterone is required for fertilization and maintenance of luteal function (Hibbert *et al*., 1996). There is evidence to support the hypothesis that concentrations of progesterone in follicular fluid are closely associated with oocyte maturity (Seibel *et al*., 1989). The actions of oestradiol and progesterone on oocyte maturation might be mediated rapidly through the non-genomic mechanism via cell membrane proteins (receptors?) as described in *Xenopus* (Bayaa *et al*., 2000). Morrison *et al*. (2000) indicated that in *Xenopus,* progesterone operates through a membrane-associated tyrosine kinase to activate phospholipase.

Inhibition of steroid synthesis in whole cultured follicles from sheep impairs subsequent fertilization and developmental

capacity following oocyte maturation (Moor *et al.*, 1980). The presence of oestradiol in the culture medium of in-vitro matured (IVM) human oocytes had no effect on the progression of meiosis but improves the fertilization and cleavage rate (Tesarik and Mendoza, 1995). To consider the effect of oestradiol in bovine culture medium consensus appears to be a concentration of 1 μg/ml, which is the concentration in the follicular fluid of pre-ovulatory follicles shortly after the LH peak (Dieleman *et al.*, 1983). There is little information about the effect of progesterone contained in culture medium on oocyte maturation. There seems to be a negative effect of progesterone on bovine oocyte cytoplasmic maturation when it was added to culture medium with gonadotrophins (FSH and LH) and oestradiol (Chian *et al.*, unpublished data).

Growth factors

There are several growth factors and their receptors in follicular fluid. The intra-ovarian regulators include epidermal growth factor (EGF)/transforming growth factor beta (TGFβ) and members of the TGFβ superfamily (TGFβ, inhibin, and activin). In-vitro studies using growth factors have shown that meiotic resumption in cumulus–oocyte complexes can be induced by EGF (Lorenzo *et al.*, 1994), TGFα and TGFβ (Brucker *et al.*, 1991; Coskun and Lin, 1994a), and activin-A (Coskun and Lin, 1994b). EGF alone and associated with gonadotrophins induces cumulus expansion and promotes nuclear and cytoplasmic maturation of immature oocytes during culture *in vitro* (De La Fuente *et al.*, 1999). Nuclear maturation is not affected when denuded oocytes were cultured with EGF, indicating that EGF action is mediated by cumulus cells (Lorenzo *et al.*, 1994; Wang and Niwa, 1995). It seems that stimulating the activity of EGF is independent of the cAMP pathway (Coskun and Lin, 1994a). Recent results from the bovine model indicate that there are no obvious beneficial effects on oocyte nuclear and cytoplasmic maturation when cumulus–oocyte complexes are cultured with presence of EGF and insulin (Chian *et al.*, unpublished data).

Insulin is an anabolic hormone involved with energy storage. Primarily, this action is manifested as an increase in glucose and amino acid transport into cells and the stimulation of conversion of these precursors into storage forms, such as glycogen, protein, and triglycerides. Insulin and its receptor are expressed on many different cell types, where they are likely to regulate glucose homeostasis and gene expression (Wozniak *et al.*, 1993). The insulin receptor is tyrosine kinase linked, but many of its actions require accessory molecules known as insulin receptor substrates (IRS-1, IRS-2, and IRS-3). Mural granulosa cells contain insulin receptors, and insulin can bind to the IGF-I receptor. The IGF–insulin receptor complex is a heterotetramer with two α- and two β-subunits in a structure similar to that of the insulin receptor. Insulin can bind to the α-subunit ligand-binding domain and activate the β-subunit (Voutilainen *et al.*, 1996). Thus, insulin can modulate ovarian cellular functions either through its own receptor or through the IGF–insulin receptor complex (Giudice, 1995). Although insulin and IGF-I seem to stimulate oocyte maturation and morphological development of mouse (Reed *et al.*, 1993) and bovine blastocysts (Ocana-Quero *et al.*, 1998), its action on oocyte maturation is largely unknown.

Fertilization of in-vitro matured oocytes

Cortical granules are small, spherical, membrane-limited organelles found beneath the plasma membrane of mature unfertilized oocytes (Austin, 1956). These granules subsequently disappear during fertilization. The contents of the cortical granules include various enzymes that are released into the perivitelline space and enter the zona pellucida at fertilization, altering the physical and chemical characteristics of the zona pellucida, and causing zona hardening. Zona hardening is also thought to result from changes of zona pellucida structure catalysed by a protease released by precocious cortical granule exocytosis during IVM (Ducibella *et al.*, 1990). Previous studies from animals showed that oocyte maturation in serum-free medium resulted in zona hardening, leading to poor sperm penetration and failure of fertilization (Kito and Bavister, 1996). However, it has been reported that zona hardening does not occur during IVM of rhesus monkey oocytes in protein-free medium (Zheng *et al.*, 2001). It also seems that premature cortical granule exocytosis does not occur during IVM of bovine and porcine oocytes (Kekintepe and Brackett, 1996; Abeydeera *et al.*, 1998). Some factors in serum, such as fetuin, an α-trypsin inhibitor, can inhibit mouse oocyte zona hardening in a dose-dependent manner (Schroeder *et al.*, 1990).

Extremely low fertilization rates are usually obtained after standard insemination of IVM human oocytes, suggesting that ICSI is the best option even when the sperm parameters are not impaired (Nagy *et al.*, 1996). It is likely that these IVM oocytes were retrieved from stimulated cycles. The ability of the oocyte fertilization was different between oocytes retrieved from stimulated and unstimulated ovaries (Cha and Chian, 1998). Recently, it has been demonstrated that ICSI is not essential for the fertilization of IVM oocytes retrieved from unstimulated infertile women with PCOS when sperm parameters are normal (Chian *et al.*, 2000b). However, it is still unclear whether ICSI is essential for IVM human oocytes retrieved from stimulated cycles, and further work is required to verify whether ICSI is superior in these oocytes even when the maturation medium contains serum. Nevertheless, in clinical practice, ICSI is usually performed for IVM human oocytes, because it reduces the risk of unexpected poor fertilization when compared with conventional IVF.

Developmental competence of in-vitro matured oocytes

Oocytes from stimulated cycles

Ovulation induction results in follicular asynchrony (Bomsel-Helmreich *et al.*, 1987). Oocytes retrieved after HCG administration are frequently maturing (metaphase I) and pre-ovulatory (metaphase II) oocytes from the cohort of developing follicles. At the same time, a few germinal vesicle-stage oocytes may be retrieved, despite having been exposed to an ovulatory dose of HCG 36 h before aspiration (**Figure 3**). Normally, 85–90% of the oocytes retrieved from stimulated cycles are in metaphase II stage, while, 10–15% of oocytes derived from stimulated cycles are still at germinal vesicle or metaphase I stage (Cha and Chian, 1998; Kim *et al.*, 2000).

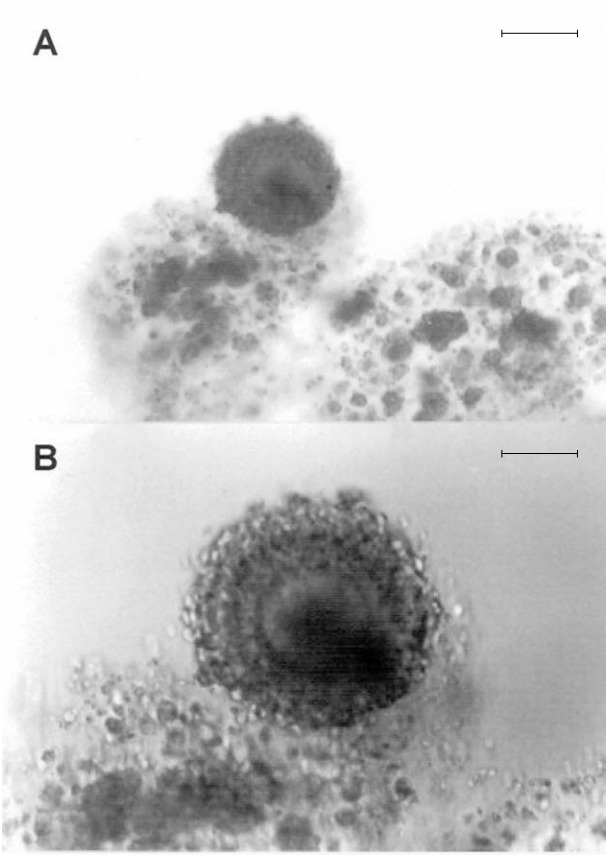

Figure 3. Immature human oocyte retrieved from stimulated ovaries. The expanded granulosa cells are around the oocyte, but with several layers of compact cumulus cells. The same oocyte was observed with different magnification (**A**, scale bar = 80 μm; **B**, scale bar = 40 μm).

These oocytes are capable of undergoing spontaneous nuclear maturation *in vitro,* and then normal fertilization. It has been reported that cumulus-free germinal vesicle-stage oocytes retrieved from stimulated cycles can be matured *in vitro* by co-culturing with Vero cell monolayers (Janssenswillen *et al*., 1995). However, there is a question about these oocytes' developmental competence because the oocytes were freed from cumulus cells at the germinal vesicle stage. Nevertheless, a few pregnancies have been obtained following transfer of embryos from IVM oocytes from stimulated cycles (Edirisinghe *et al*., 1997). It has been reported that the maturation rate of germinal vesicle-stage oocytes from follicles stimulated with gonadotrophin is not affected by the presence or absence of cumulus cells, but the proportions of oocytes with two pronuclei and embryos cleaved to the 16-cell stage following ICSI are significantly lower in the oocytes matured *in vitro* than in the oocytes matured *in vivo* (Kim *et al*., 2000). Recently, it has been reported that cumulus-free oocytes derived from stimulated cycles can be matured in a designed IVM medium and developed to blastocyst stage following fertilization by ICSI (Chian and Tan, 2002).

The time course of oocyte GVBD and maturation are different between the oocytes retrieved from stimulated and unstimulated ovaries (Cha and Chian, 1998). The first GVBD was observed 6 h after culture *in vitro* in the oocytes retrieved from stimulated ovaries, but GVBD occurred 12–15 h after culture *in vitro* in the oocytes retrieved from unstimulated ovaries. However, the final maturation rates are not different between immature GV stage oocytes retrieved from stimulated or unstimulated ovaries. It has been suggested that the structure of GV stage oocytes are different between oocytes retrieved from stimulated and unstimulated cycles (Chian *et al*., 1997). The profile of protein synthesis is also different in these two groups, resulting in different time courses of oocyte GVBD and maturation *in vitro*. The different time courses of GVBD and maturation between the GV oocytes retrieved from stimulated and unstimulated ovaries may be due to the oocytes already having been exposed to the action of LH/HCG in the follicle from the stimulated ovaries.

Cellular, metabolic and cytoplasmic studies of mature human oocytes obtained from stimulated cycles provide explanations for some types of early developmental failures. Developmentally lethal chromosomal abnormalities are a common defect in mature human oocytes that may affect more than 25% of normal-looking mature human oocytes retrieved from stimulated cycles (Delhanty and Handyside, 1995; Wall *et al*., 1996). Recently, Kuliev *et al*. (2003) reported that 52.1% of which were aneuploid based on 6733 normal-looking mature human oocytes obtained in 1297 stimulated IVF cycles from patients in advanced maternal age (mean 38.5 years). Specific cytoplasmic pathologies present in human oocytes at the time of insemination are related both to the ability to fertilize and the ability of the fertilized oocyte to develop progressively (Van Blerkom, 1994). Metabolic differences between morphologically equivalent metaphase II human oocytes retrieved from the same ovary have been observed and related to the developmental ability of the resulting embryo (Van Blerkom *et al*., 1995; Van Blerkom, 1996). Very high frequencies of chromosomal aneuploidy have been observed in human oocytes with cytoplasmic defects, suggesting that these genetic abnormalities may develop during the pre-ovulatory stages in association with or as a result of degenerative cytoplasmic alteration (Van Blerkom and Henry, 1992). Some chromosomal anomalies are first detectable during early meiotic metaphase, although it is possible that the damage to DNA may occur in earlier oogenesis (Ashwood-Smith and Edwards, 1996). Therefore, the microenvironment in the follicle during follicular stimulation is important for oocyte development.

Oocytes from unstimulated cycles

As discussed, the first successful human pregnancy from IVF was achieved with oocytes from unstimulated natural cycles (Steptoe and Edwards, 1978). Subsequently, the first successful series of patients conceived following IVF was with mature oocytes retrieved from unstimulated cycles (Edwards *et al*., 1980). Currently, most IVF programmes use controlled ovarian stimulation during the follicular phase of the menstrual cycle, because it increases the number of oocytes and thus increases the available numbers of embryos for transfer. However, the number of normal embryos produced from each stimulated cycle is still unknown.

It has been shown that immature human oocytes can be collected from ovaries during the follicular and luteal phase of

ovulating cycles (Cha *et al*., 1991, 1992). The first successful IVM birth was from immature oocytes retrieved at Caesarean section from an egg donor (Cha *et al*., 1991). Although the data are insufficient, it has been suggested that age, pathology, day of the menstrual cycle, and cyclic versus non-cyclic ovaries, are factors influencing the number of immature oocytes retrieved (Cha *et al*., 1992). The number of immature human oocytes collected from an ovary decrease as a patient's age increases. However, there is no statistical difference in the rate of oocyte maturation and cleavage among donors in the different age groups (Cha *et al*., 1992). Oocytes from women with regular ovulatory cycles had a better cleavage rate than those who were anovulatory or had irregular menstrual cycles (Barnes *et al*., 1996). Interestingly, it has also been shown that immature oocytes from the luteal phase had a significantly higher maturation rate than those obtained during the follicular phase (Cha *et al*., 1992). Furthermore, it has been reported that co-culture embryos with human ampullary epithelial cells enhance the development of blastocysts, in which immature human oocytes were aspirated from small follicles at Caesarean section followed by IVM/IVF (Hwu *et al*., 1998).

Because of the disadvantages of ovarian stimulation, including side effects of gonadotrophins, many attempts have been made to perform IVF without the use of gonadotrophins to induce multiple follicles – a procedure called 'natural cycle IVF'. Natural cycle IVF treatment usually involves giving an HCG injection when the dominant follicle reaches >19 mm in diameter. Recently it has been shown that natural cycle IVF treatment may have similar or higher implantation rate compared with stimulated cycles. Therefore, the efficiency and clinical implication of natural cycle of IVF treatment should be re-evaluated. Life-table analysis of data suggests that per natural cycle, the rate of pregnancy does not decline during successive cycles and thus a decrease in per natural cycle success rate may be offset when more cycles are performed (Janssens *et al*., 2000; Lukassen *et al*., 2003). After four natural cycle IVF treatments, the cumulative probability of pregnancy was 46% with an associated live birth rate of 32% (Nargund *et al*., 2001). This is comparable with conventional stimulated IVF treatment.

One very attractive possibility for enhancement of the success of natural cycle of IVF treatment is its combination with immature oocyte retrieval and maturation. In humans, during the follicular phase of menstrual cycle, multiple follicles often exist in the ovaries. It seems that atresia does not occur in the non-dominant follicles even after the dominant follicle was selected during folliculogenesis, because immature oocytes retrieved from non-dominant follicles have been successfully matured *in vitro,* fertilized, and resulted in several pregnancies and healthy live births (Paulson *et al*., 1994; Thornton *et al*., 1998; Chian *et al*., unpublished data). Animal model studies also indicate that oocyte quality and early embryonic developmental competence of immature oocyte following maturation *in vitro* are not affected by the presence of the dominant follicle in the ovaries (Smith *et al*., 1996; Chian *et al*., 2002). Therefore, natural cycle IVF treatment combined with IVM may be a useful approach for infertile patients.

Pretreatment with FSH or HMG

Studies in rhesus monkeys have indicated that mild ovarian stimulation in the early follicular phase prior to retrieval markedly improved the proportion of oocytes that are meiotically competent, and that after fertilization would support normal embryonic development (Schramm and Bavister, 1994). Wynn *et al*. (1998) examined potential benefits of FSH priming on human oocyte IVM. The stimulation group received a truncated course of recombinant FSH (rFSH), 300 IU on day 2, with additional doses of 150 IU FSH on days 4 and 6 of the menstrual cycle. The results suggested that there are significantly more follicles and hence immature oocytes recovered by FSH priming. Unfortunately, there are no data concerning fertilization and embryonic development to support the findings. However, it has been suggested that the treatment of women for 1 or 3 days with rFSH in the early follicular phase resulted in no difference in the recovery rate of immature oocytes, oocyte maturation, fertilization or development in culture (Trounson *et al*., 1998, 2001). These results were further confirmed by Mikkelsen *et al*. (1999), who also gave women rFSH for 3 days in the early menstrual cycle and found no improvement in oocyte recovery, maturation or developmental competence. However, recently the same authors have indicated that priming with rFSH before harvesting of immature oocytes from patients with PCOS may improve the maturational potential of the oocytes and the implantation rate of the cleaved embryos (Mikkelsen and Lindenberg, 2001). Furthermore, it has been indicated that although higher pregnancy rate were achieved after 3 days of FSH followed by 3 days of coasting compared with 2 days of coasting, there were no differences in apoptosis of granulosa cells and embryonic developmental competence of oocytes obtained after coasting for 3 days compared with coasting for 2 days (Mikkelsen *et al*., 2003). Theoretically, the provision of additional FSH could promote follicular granulosa cell proliferation, steroid production and through intercellular signaling, additional RNA and protein synthesis. Thus, pretreatment with FSH or HMG should improve the recovery rate of oocytes, oocyte maturation, fertilization, and developmental rates following in-vitro culture. Nevertheless, it must be emphasized that there would be no clinical application of pretreatment of women with FSH or HMG before collection of immature oocytes, unless a very substantial benefit could be demonstrated for maturation and development to term (Trounson *et al*., 2001). Recent data also show that pretreatment with FSH during early days of menstrual cycle and then priming with HCG 36 h prior to immature oocyte retrieval has no additional beneficial effect on human oocyte IVM outcome (Lin *et al*., 2003; personal communication).

Priming with HCG before immature oocyte retrieval

Gomez *et al*. (1993) have reported that the oocyte maturation rate is significantly higher from GV-stage oocytes retrieved from stimulated rather than from unstimulated ovaries. It has been indicated that the time course of oocyte GVBD and maturation are different between the oocytes retrieved from stimulated and unstimulated ovaries (Cha and Chian, 1998), but the percentage of metaphase II oocytes following IVM are not different between two groups. When the immature germinal vesicle-stage oocytes were retrieved from stimulated cycles, most oocytes reached metaphase II at 20–24 h after culture *in vitro*. In contrast, the majority of immature germinal vesicle-stage oocytes retrieved from unstimulated cycle were matured at

36–38 h after culture *in vitro* (Cha and Chian, 1998). It appears that the oocytes retrieved from the small follicles in women undergoing ovarian stimulation respond to the HCG switch to promote the initiation of oocyte maturation. Similarly, it also appears that the oocytes retrieved after HCG administration show a shorter period of maturation *in vitro* (Chian *et al*., 2000a; **Figure 4** and **Figure 5**). It must be noted that there are approximately 10% immature oocytes retrieved from women with or without PCOS that degenerate 24 h after culture *in vitro* (Chian, unpublished data). These immature oocytes may be collected from the 'real' atretic follicles.

It has been shown that the rates of oocyte maturation, fertilization and development, as well as pregnancy rate are improved by HCG priming 36 h before immature oocyte retrieval in patients with PCO or PCOS (Chian *et al*., 2000a; Tan and Child, 2002). These results were further confirmed by Son *et al*. (2002), indicating that HCG priming 36 h before immature oocyte retrieval improves maturational and developmental competence of human oocytes. Recently, it has been indicated that HCG priming combined with laparoscopy for immature oocyte retrieval, IVM and ICSI may open up new therapeutic strategies, even in patients without PCOS and those with regular menstrual cycles, undergoing laparoscopy for other cases of infertility such as tubal factors or endometriosis (Nagele *et al*., 2002). To date, approximately 800 cycles of IVM treatment have been performed using the HCG priming method at the McGill Reproductive Centre (**Table 1**), the Maria Infertility

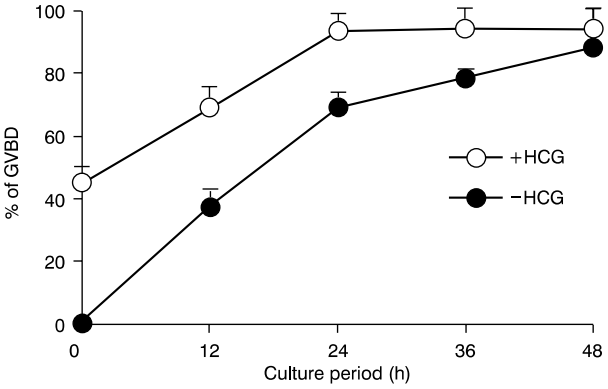

Figure 4. The time course of human oocyte germinal vesicle breakdown (GVBD) during culture *in vitro*. At the time of retrieval in the human chorionic gonadotrophin (HCG)-priming group, 46.2 ± 5.2% of oocytes showed GVBD. At 48 h culture, the percentage of GVBD was similar in oocytes retrieved from the HCG-primed (—○—) and non-HCG-primed (—●—) groups (total 183 oocytes). Reproduced from Chian *et al*. (2000).

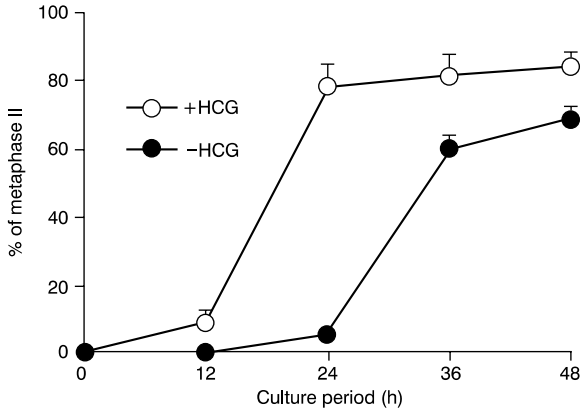

Figure 5. The time course of human oocyte maturation during culture *in vitro*. At 24 h after culture in the human chorionic gonadotrophin (HCG)-priming group, 78.2 ± 7.1% of oocytes were matured. Final percentages of oocyte maturation were significantly different between oocytes retrieved from the HCG-primed (—○—) and non-HCG-primed (—●—) groups (total 183 oocytes). Reproduced from Chian *et al*. (2000).

Table 1. Results of in-vitro maturation and fertilization of oocytes followed by embryo transfer in women with polycystic ovary and polycystic ovary syndrome (from 1998 to 2002 at the McGill Reproductive Centre).

Cycles[a]	254
Age (years)[b]	32.6 ± 3.7
No. of oocytes collected	
Total	3079
Mean[b]	11.9 ± 6.2
No. of oocytes matured (%)	2426 (78.8)
No. of mature oocytes fertilized (%)	1679 (69.2)
No. of fertilized oocytes cleaved (%)	1509 (89.9)
No. of embryos transferred	
Total	865
Mean[b]	3.4 ± 0.9
No. of clinical pregnancies (%)	61 (24.0)
No. of implantations (%)	96 (11.1)

[a]Includes 79 cycles of patients who have had at least three attempts at conventional IVF treatments at the McGill Reproductive Centre or other clinics without pregnancy.
[b]Values are mean ± SD.

Table 2. Results of in-vitro maturation and fertilization of oocytes followed by embryo transfer at Maria Infertility Hospital, Seoul, Korea (from November 2000 to October 2002).

Cycles	419
Age (years)[a]	32.2 ± 4.3
No. of oocytes collected	
Total	6860
Mean[a]	16.4 ± 7.1
No. of oocytes matured (%)	5021 (73.2)
No. of mature oocytes fertilized (%)	3967 (79.0)
No. of embryos transferred	
Total	1816
Mean[a]	4.3 ± 0.9
No. of clinical pregnancies (%)	137 (32.7)
No. of implantations (%)	211 (11.6)

[a]Values are mean ± SD.

Table 3. Results of in-vitro maturation and fertilization of oocytes followed by embryo transfer in women with polycystic ovary syndrome at the Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan.

Cycles	68
Age (years)[a]	30.7 ± 3.5
No. of oocytes collected	
Total	1528
Mean[a]	22.5 ± 10.1
No. of oocytes matured (%)	1134 (74.2)
No. of mature oocytes fertilized (%)	825 (72.8)
No. of fertilized oocytes cleaved (%)	733 (88.8)
No. of embryos transferred	
Total	258
Mean[a]	3.8 ± 0.9
No. of clinical pregnancies (%)	23 (33.8)
No. of implantations (%)	27 (10.5)

[a]Values are mean ± SD.

Table 4. Results of in-vitro maturation and fertilization of oocytes followed by embryo transfer in women with polycystic ovary syndrome at the Hôpital Antoine Béclère, Paris, France (from September 2002 to April 2003).

Cycles	17
Age (years)[a]	32.1 ± 3.6
No. of oocytes collected	
Total	237
Mean[a]	13.2 ± 7.0
No. of oocytes matured (%)	173 (73.0)
No. of mature oocytes fertilized (%)	119 (68.8)
No. of fertilized oocytes cleaved (%)	106 (89.1)
No. of embryos transferred	
Total	42
Mean[a]	2.6 ± 0.7
No. of clinical pregnancies (%)	4 (23.5)
No. of implantations (%)	4 (9.5)

[a]Values are mean ± SD.

Table 5. Results of blastocyst transfer produced from IVM oocytes at the Maria Infertility Hospital, Seoul, Korea.

Cycles	80
Age (years)[a]	31.9 ± 3.8
No. of oocytes collected	
Total	2108
Mean[a]	26.4 ± 9.9
No. of oocytes matured (%)	1624 (77.0)
No. of mature oocytes fertilized (%)	1288 (79.3)
No. of embryos developed to blastocyst (%)	468 (36.3)
No. of blastocysts transferred	
Total	246
Mean[a]	3.1 ± 0.4
No. of clinical pregnancies (%)	43 (53.8)
No. of implantation (%)	67 (27.2)

[a]Values are mean ± SD.

Hospital in South Korea (**Table 2**), the Shin Kong Wu Ho-Su Memorial Hospital in Taiwan (**Table 3**), and the Hôpital Antoine Béclère in France (**Table 4**). Interestingly, high pregnancy and implantation rates were achieved when blastocyst transfer was combined with IVM in South Korean group (**Table 5**). Approximately 200 healthy infants have been born following HCG priming IVM. In general, the clinical pregnancy and implantation rates have reached approximately 25–30% and 10–15% respectively in infertile women with PCO or PCOS.

The mechanism of HCG priming on immature oocytes in the small follicles of ovaries with or without PCOS is poorly understood. The hypothesis is that the small follicles in ovaries may possess some HCG receptors to respond to HCG priming. Although it has been shown that the recovered oocytes were mature 36 h after HCG injection even from follicles as small as 6 mm in diameter (Trounson *et al*., 2001), experience suggests that at least half of oocytes retrieved from the small follicles (between 2 and 10 mm in diameter) were still at the germinal vesicle stage 36 h after HCG injection. This may explain that some oocytes (10–15%) retrieved from stimulated cycles are still at germinal vesicle stage even if the size of follicles was ≥10 mm in diameter (Cha and Chian, 1998). Similar results have been reported by Scott *et al*. (1989), indicating that only 30% of oocytes were mature in follicles between 12 and 14 mm in diameter 36 h after HCG administration to trigger oocyte maturation. The action of HCG in the small follicles to promote oocyte acquisition of maturational and developmental competence needs further study.

Conclusions

Oocyte maturation is a complicated process involving many factors in the follicle between the supporting cells and the oocyte itself. Maturation *in vitro* of human oocytes can be completed morphologically, but it is accomplished with a loss of developmental competence following in-vitro culture. Loss of developmental competence may be corrected by improving culture condition *in vitro*. Encouraging pregnancy rates have been achieved from immature human oocytes following IVM when the immature oocytes were retrieved from infertile women with PCO or PCOS. It is possible that the combination of natural cycle IVF with immature oocyte retrieval followed by IVM could offer women with all types of infertility reasonable rates of pregnancy without recourse to ovarian stimulation.

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