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Maturation *in vitro* of immature human oocytes for clinical use

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Human oocyte maturation is considered as the reinitiation and completion of the first meiotic division from the germinal vesicle stage (prophase I) to metaphase II, and the accompanying cytoplasmic maturation for fertilization and early embryonic development. Immature human oocytes obtained from patients undergoing gynaecological surgery, or ovulation induction or having polycystic ovary syndrome (PCOS) can be matured and fertilized *in vitro*. To date, 80% of immature oocytes matured to metaphase II when cultured in maturation medium supplemented with gonadotrophins and 85% of matured oocytes fertilized and cleaved *in vitro*. Following transfer of these embryos, pregnancies and live births have been achieved. However, the capacity for oocyte maturation was different when the immature oocytes were retrieved from PCOS patients and when the oocytes were cryopreserved at germinal vesicle stage.

Key words: fertilization/germinal vesicle/human/maturation/oocyte

Introduction

Since the first successful human pregnancy from in-vitro fertilization (IVF) was achieved (Steptoe and Edwards, 1978), assisted reproductive technology has become the frontier of both infertility treatment and research. The core of an assisted reproduction programme is oocyte quality because one of the major problems encountered by IVF clinics is to recognize the maturation state of oocytes obtained from stimulated or unstimulated follicles. The cytoplasm of the oocyte is of key interest in oocyte maturation. The best way to improve embryo quality is to improve oocyte quality. It is known that insufficient cytoplasmic maturation of the oocyte will fail to promote male pronuclear formation and will thus increase chromosomal abnormalities after fertilization (Thibault *et al*., 1975). Successful fertilization, development and pregnancy have been achieved with immature human oocytes matured *in vitro* in some infertility centres (Veeck *et al*., 1983; Prins *et al*., 1987; Cha *et al*., 1989, 1992, 1996; Paulson *et al*., 1994; Trounson *et al*., 1994; Barnes *et al*., 1995, 1996; Nagy *et al*., 1996; Edirisinghe *et al*., 1997; Jaroudi *et al*., 1997; Liu *et al*., 1997; Russell *et al*., 1997); however, little is known about what conditions are suitable for in-vitro maturation of human oocytes, enabling IVF and embryo development in culture. Although our understanding is currently incomplete, it is now clear that addition of gonadotrophins [follicle stimulating hormone (FSH), luteinizing hormone (LH)] to the culture medium is beneficial to cytoplasmic maturation *in vitro*. In this paper, we will review research and clinical applications utilizing immature human oocytes.

Regulation of human 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 ~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. Near the completion of growth, oocytes acquire the ability to reinitiate meiosis (Pincus and Enzmann, 1935). Following resumption of meiosis, the

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nuclear membrane dissolves (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 oocyte, both of which contain a haploid chromosome complement. The second meiotic division is initiated rapidly after completion of the first meiotic division and the oocytes reach metaphase II 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. Most knowledge of the regulation of oocyte maturation comes from animal experiments. The reader should keep this in mind although similar concepts may be relevant to human oocytes.

Nuclear maturation

GVBD is initiated by either the pre-ovulatory surge of gonadotrophins (LH) or the atretic degeneration of the follicle *in vivo*. The competence to undergo GVBD was previously thought to be acquired during follicle growth. However, it has been reported that the acquisition of GVBD competence is not dependent on oocyte growth (Canipari *et al*., 1984). Therefore, the acquisition of the competence to undergo GVBD could be a multi-step process. In humans, some germinal vesicle oocytes collected from both stimulated and unstimulated ovaries fail to undergo GVBD during culture *in vitro*. It seems that only the competence to undergo GVBD is only acquired during follicle growth, suggesting that an inherent capability for GVBD is still important.

Many potential factors mediate the cumulus cells' control of GVBD (Figure 1). High levels of cyclic adenosine monophosphate (cAMP) and purine hypoxanthine in the culture medium prevent oocyte GVBD (Heller *et al*., 1981; Downs *et al*., 1985; Eppig *et al*., 1985; Hillensjo *et al*., 1985; 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 (Dekel *et al*., 1981). Addition of luteinizing hormone (LH) to the culture medium induces GVBD. LH probably induces GVBD by an indirect action mediated by cumulus cells because there are no LH receptors on oocytes (Dekel, 1988). The mechanism involves LH inducing the loss of communication between oocyte and cumulus cell complex, thus terminating the flow of these substances into the oocyte (Berridge and Irvine, 1984; Larsen *et al*., 1987; Downs *et al*., 1988; Fagbohun and Downs, 1991). LH-induced GVBD may be also mediated by the IP_3/Ca^{2+} pathway (for review see Eppig, 1993). It has been suggested that the hormone signalling mediate Ca^{2+} transients may be important at the time of GVBD (for review see Whitaker, 1996).

Results from animal experiments indicate that protein synthesis is required for GVBD (Stern *et al*., 1972; Schultz and Wassarman, 1977; Sirard and First, 1988; Sirard *et al*., 1989). It seems that protein synthesis is also needed for GVBD of human oocytes because protein synthetic patterns differ before and after GVBD (Gifford *et al*., 1987; Schultz *et al*., 1988). After GVBD, not all oocytes can mature and extrude the first polar body. The competence to undergo GVBD and to progress from metaphase I to metaphase II is acquired separately during culture *in vitro* (Szybek, 1972; Iwamatsu and Yanagimachi, 1975; Sorensen and Wassarman, 1976). Some human oocytes undergo GVBD when cultured *in vitro*, but become arrested at metaphase I. Thus, the competence to undergo GVBD and competence to progress through metaphase I to metaphase II are separately and sequentially acquired. It is not clear, however, what causes oocytes to complete maturation from metaphase I to metaphase II. It is well known that the cytoplasmic maturation during this period is very important in order to support early development. 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 (Clarke and Masui, 1983; Gerhart *et al*., 1984).

Inhibition of protein synthesis in oocytes results in failure to activate maturation promoting factor (MPF) activity (Masui and Clarke, 1979; Maller, 1985; 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 $p34^{cdc2}$ serine/ threonine kinase, and regulatory cyclin B subunits (Dunphy *et al*., 1988; Gautier *et al*., 1988; 1990; Pines and Hunter, 1989). The $p34^{cdc2}$ serine/threonine kinase is the product of the *cdc2* gene, first identified in fission yeast. The $p34^{cdc2}$-cyclin heterodimer, a protein kinase, has four phosphorylation sites that are regulated by kinase and phosphatase activities. Activation of MPF requires dephosphorylation at Thr 14 and Tyr 15 and phosphorylation of Thr 161 (for review see Piwnica-Worms *et al*., 1993). Kinases and phosphatases capable of modifying $p34^{cdc2}$ have been identified by genetic analysis in yeast

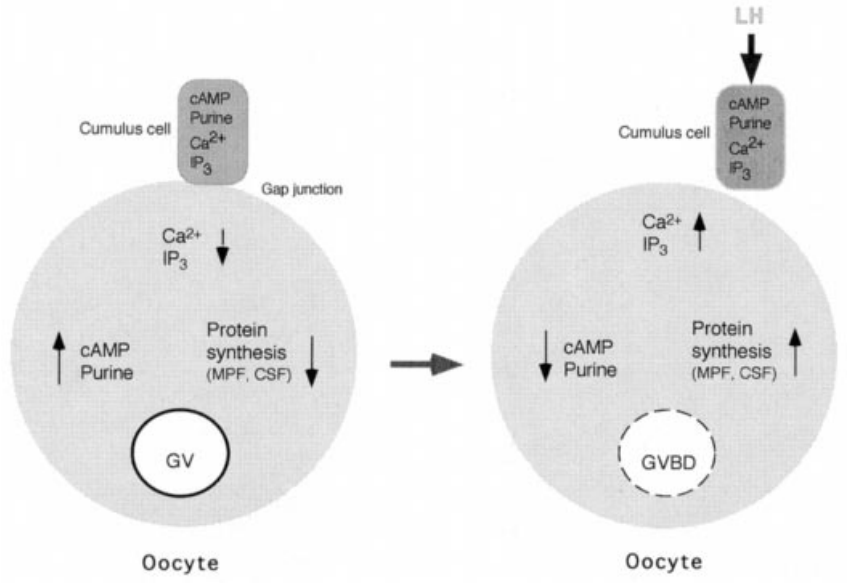

Figure 1. Hypothetical model for the possible participation of factors in germinal vesicle breakdown (GVBD) of human oocytes. LH = luteinizing hormone; GV = germinal vesicle; MPF = maturation promoting factor; CSF = cytostatic factor; IP_3 = inositol 1,4,5-trisphosphate.

(wee1, cdc25) and in frog oocytes (cak: cdc activating kinase) (for review see Whitaker, 1996).

G2/M arrest always finds the oocyte with a high content of inactive $p34^{cdc2}$ complex in its tyrosine-phosphorylated form. It induces GVBD when $p34^{cdc2}$-cyclin is activated (Ookata *et al*., 1992). Before fertilization, human oocytes arrest firmly at metaphase II. At this point, MPF kinase activity is high and cyclin destruction has been inhibited. This is due to CSF. It is known that mos protein, the product of the *c-mos* proto-oncogene, has the same effect as CSF in arresting mitosis at metaphase with high $p34^{cdc2}$ activity. Clearly, meiotic arrest is usually explained in terms of activation and inactivation of cell cycle control proteins, particularly $p34^{cdc2}$. Oocytes need $p34^{cdc2}$ activation to enter meiotic metaphase and $p34^{cdc2}$ inactivation to proceed through anaphase into the next cell cycle. Therefore,the metaphase II arrest is due to the transcription of *c-mos* as the oocyte matures.

The *c-mos* gene plays a critical role in regulation of MPF activity during meiosis. The product of *c-mos* is expressed early in oocyte maturation and disappears immediately after fertilization (Sagata *et al*., 1989; Watanabe *et al*., 1989). *c-mos* encodes a protein-serine/threonine kinase known as Mos. Mos has been proposed to enhance MPF activity via several mechanisms (Figure 2). Direct activation of MPF occurs through phosphorylation of the cyclin-B subunit of MPF (Roy *et al*., 1990), or indirectly via mitogen-activated protein kinase (MAP kinase) (Nebreda and Hunter, 1993). In addition, Mos may inhibit proteolytic degradation of cyclin-B between meiosis I and meiosis II (O'Keefe *et al*., 1991), and thus maintain high MPF activity (Kubiak *et al*., 1993) because Mos is required for cyclin B accumulation and this newly synthesized protein is required to maintain MPF activity during meiosis of oocytes (O'Keefe *et al*., 1989; Minshull *et al*., 1991). Thus, inhibition of protein synthesis will block the effects of Mos activity. The *c-mos* gene is an abundant maternal message in the oocytes. Expression of *c-mos* has recently been reported in human oocytes (Goldman *et al*., 1987; Mutter and Wolgemuth, 1987; Pal *et al*., 1994), suggesting that Mos may be a critical regulator of human oocyte meiosis. Surprisingly, using reverse transcriptase–polymerase chain reaction (RT–PCR), Li *et al*. (1993) detected *c-mos* mRNA in various human cell lines, suggesting a wider role for *c-mos* kinase.

Like Mos, MAP kinase was first identified in somatic cells and has now been revealed as central to the regulation of meiotic arrest in oocytes. MAP kinase is also a serine/threonine kinase but is activated, not inhibited, by tyrosine phosphorylation. It is known that MAP kinase is activated during oocyte maturation, that it regulates meiosis and has CSF activity. Activation of MAP kinase precedes activation of $p34^{cdc2}$. Blocking MAP kinase activity prevents GVBD. Mos protein stimulates MAP kinase activity, but does not activate $p34^{cdc2}$ (Nebreda and Hunter, 1993). The time course of MAP kinase activation in oocytes parallels that of *c-mos* transcription (Sagata *et al*., 1988; Ferrell *et al*., 1991); therefore, it seems that the MAP kinase activity in oocytes is due to Mos. The phosphorylation cascade of Mos and MAP kinase may play an important role

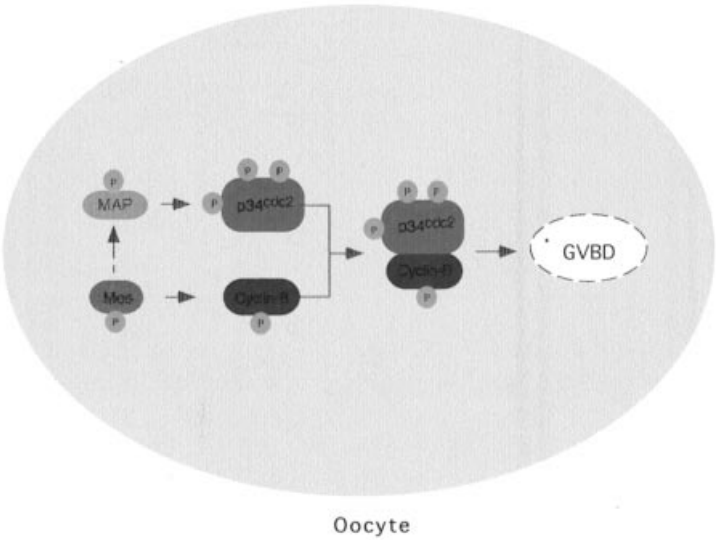

Figure 2. The regulatory pathways surrounding maturation promoting factor (MPF). $p34^{cdc2}$ combines with its activating subunit, cyclin B. The $p34^{cdc2}$-cyclin B heterodimer, a protein kinase, has four phosphorylation sites that are regulated by kinase and phosphatase activities. The phosphorylation cascade of Mos and MAP kinase plays an important role in MPF activity for germinal vesicle breakdown (GVBD).

in meiotic and mitotic cell cycles. Mos activates MAP kinase to arrest oocytes at metaphase II; however, the connection between Mos/MAP kinase and $p34^{cdc2}$ activation at a molecular level is not clear. After fertilization, Mos activity is lost (Lorca *et al*., 1993). Cyclin disappears before Mos (Kubiak *et al*., 1993). The evidence indicates that calcium is the signal to break the metaphase II arrest of oocytes (for review see Swann, 1996).

Cyclin was originally identified in sea urchin oocytes as a protein whose level greatly increased upon fertilization and subsequently oscillated during the early cell divisions of the embryo (Evans *et al*., 1983). The level 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 the timing of their appearance during the cell cycle (Minshull *et al*., 1989; Hunter, 1991; Pines and 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 and monkey (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.

Cytoplasmic maturation

During oocyte growth and maturation, products are synthesized and stored. These products support development after fertilization until the embryonic genome becomes transcriptionally active and embryo-derived messages begin to regulate embryogenesis. Most of the RNA present in mammalian oocytes is synthesized and accumulated during the period of oocyte growth (Bachvarova, 1974; Jahn *et al*., 1976; Bachvarova and DeLeon, 1980; Sternlicht and Schultz, 1981; Piko and Clegg, 1982). 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 (Bloom and Mukherjee, 1972; Rodman and Bachvarova, 1976; Wassarman and Letourneau, 1976). It is known that the metabolism of the oocyte is characterized by active transcription and translation during the pre-ovulatory period (Telford *et al*., 1990; 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).

The quality of the oocyte predominantly depends upon the maturity of the ooplasm. The cytoplasm prepares for fertilization and early embryonic development in parallel with nuclear maturation. If cytoplasmic maturation does not occur properly, the oocyte will fail to fertilize and develop successfully (Thibault *et al*., 1975; Thibault, 1977). Mammalian oocytes are surrounded by a compact cumulus

cell layer during folliculogenesis. 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 rabbit (Robertson and Baker, 1969), mouse (Cross and Brinster, 1970), rat (Vanderhyden and Armstrong, 1989), human (Kennedy and Donahue, 1969; Gregory and Leese, 1996), ovine (Staigmiller and Moor, 1984), and bovine (Sirard *et al*., 1988; Fukui, 1990; Chian *et al*., 1994) oocytes. Besides cytoplasmic changes involved in the control and direction of meiotic progression, other important cytoplasmic changes occur during oocyte maturation (Mattioli 1992). In pigs, the formation of a male pronucleus within a fertilized oocyte depends on the presence of cumulus cells during oocyte maturation (Mattioli *et al*., 1988). The events of male pronucleus formation after sperm penetration are also affected by the presence or absence of cumulus cells during bovine oocyte maturation *in vitro* (Chian *et al*., 1994). These results suggest that the ability for male pronuclear 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).

The cumulus cells are distinct from the mural granulosa cells. The cumulus cells have different levels of progesterone secretion and different responses to gonadotrophins (Dirnfeld *et al*., 1993; Goldman *et al*., 1993). The cumulus cells also differ in the expression of specific mRNA in comparison with the mural granulosa cells (Braw-Tal, 1994). Several regulatory factor transcripts have been demonstrated in human oocytes by RT–PCR, including oestrogen receptor, *c-mos* and *c-raf* mRMA (Wu *et al*., 1993; Wu and Wolgemuth, 1995). Therefore, these regulatory factors may be involved in the events of cytoplasmic maturation of the oocyte. Thyroid hormone, tri-iodothyronine (T_3), synergizes with FSH to increase LH receptors and to increase progesterone secretion by granulosa cells from porcine follicles (Maruo *et al*., 1987, 1992). A recent report indicated that T_3 may also be important for oocyte maturation since four isoforms of thyroid hormone receptor (TR) mRnA (TR-α-1, TRβ-1, TRβ-2 and c-erbAα-2) are expressed in the human oocyte, and the granulosa cells as well as on the cumulus cells (Zhang *et al*., 1997). These results suggest that thyroid hormone may both have direct effects on the oocytes, and indirect effects via the cumulus cells. The expression of TR mRNAs in the oocyte, cumulus cells and granulosa cells raises the question of whether addition of T_3 to the maturation medium would improve maturation *in vitro* of immature human oocytes in a more rapid or normal manner (Zhang *et al*., 1997).

It is also known that different culture conditions for oocyte maturation *in vitro* affect fertilization and embryonic development. Normally, FSH is added to the culture medium for oocyte maturation *in vitro*, but addition of FSH does not increase the nuclear maturation of rat (Vanderhyden and Armstrong, 1989), monkey (Morgan *et al*., 1991) and 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 the rise in the levels of cAMP in cumulus cells (Eppig *et al*., 1983; Schultz *et al*., 1983).

FSH is important in the development of pre-ovulatory follicles. The enhanced FSH responsiveness of pre-ovulatory follicles appears to result from an increase in the content of the stimulatory G-protein of the adenyl cyclase system (for review see Richards and Hedin, 1988). FSH is necessary for the induction of LH receptors in pre-ovulatory follicles; however, the mechanism of FSH on nuclear and cytoplasmic maturation of oocytes is not clear. It was found that FSH levels in the follicular fluid of metaphase I and metaphase II oocytes were significantly higher than in follicles containing germinal vesicle oocytes in women undergoing oocyte retrieval for stimulated IVF cycles (Laufer *et al*., 1984). The addition of FSH to oocyte culture medium 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 (Schroeder *et al*., 1988) and early embryo development (Jinno *et al*., 1989; 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).

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). 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 (for review see Carroll *et al*., 1996). Recent evidence has revealed that Ca^{2+} release mechanisms are modified during oocyte maturation (Chiba *et al*., 1990; Fujiwara *et al*., 1993; Mehlmann and Kline, 1994). When immature human oocytes were cultured *in vitro*, they acquired

the capacity to undergo a single large oscillation of intracellular Ca^{2+}; however, subsequent sustained oscillations were not observed in some immature oocytes, indicating that these oocytes failed to develop fully competent Ca^{2+} signalling mechanisms during maturation *in vitro* (Herbert *et al*., 1997). It seems that the maximal sensitivity of Ca^{2+} release is reached in the final stages of oocyte maturation, just before the optimal time for fertilization.

In oocytes, cytosolic free Ca^{2+} is increased by the release of Ca^{2+} from intracellular Ca^{2+} stores in the endoplasmic reticulum. The release of Ca^{2+} from the stores is controlled by two types of receptors/Ca^{2+} release channels, one sensitive to IP_3 and the other to ryanodine (Lai *et al*., 1988; Furuichi *et al*., 1989). These two Ca^{2+} release channels are sensitive to cytosolic Ca^{2+} such that low concentrations stimulate opening while high concentrations inhibit the receptors (Bezprovzanny *et al*., 1991; Iino and Endo, 1992). The mechanism of the Ca^{2+} oscillations that occur during fertilization of mammalian oocytes is still unclear. There is considerable evidence to indicate that IP_3 plays a primary role in generating Ca^{2+} signals in mammalian oocytes (Miyazaki *et al*., 1992; Tesarik and Sousa, 1996; Berridge, 1996; Swann and Lawrence, 1996). It has been suggested that human oocyte activation at fertilization is mediated by the IP_3 Ca^{2+} release system because the Ca^{2+} oscillations were inhibited by caffeine (Herbert *et al*., 1997). The development of Ca^{2+} release mechanism during oocyte maturation so that the fertilizing sperm initiates a Ca^{2+} signal sufficient to stimulate oocyte activation is still a mystery. Changes may be brought about by modifications in the sensitivity of Ca^{2+} release through the IP_3 receptors. Ca^{2+} release channels are also regulated by phosphorylation (Nakade *et al*., 1994) and changes in redox state (Salama *et al*., 1992) as well as by interactions with cytoplasmic proteins such as ankyrin (Bourgguignon *et al*., 1993) and FK506-binding protein (Brillantes *et al*., 1994; Cameron *et al*., 1995). Therefore, it is an important that the oocyte develops the ability to respond to spermatozoa with Ca^{2+} transients during cytoplasmic maturation.

Oocyte polarity and spatial patterning during maturation should also receive attention (for review see Gardner, 1996; Edwards and Beard, 1997). Microvilli are distributed evenly during human oocyte maturation (Pickering *et al*., 1988) and a microvillus-free region was observed as a consequence of ageing in culture in human oocytes (Santello *et al*., 1992). Several proteins display a polarized cortical distribution at different maturation stages of human oocytes (Ji *et al*., 1996). The maternally encoded mRNA to establish a critical polarity and spatiality seems to be involved in oocyte growth and maturation. However, very little is known about the polarity and the spatiality of maternal mRNA in mammalian oocytes, especially during oocyte maturation. Moreover, leptin, a 16 kDa protein product of the obese gene, and its receptor isoforms are in human pre-ovulatory follicles and in mature oocytes, suggesting that leptin may play some role in transcriptional regulation of the oocyte and early embryo development (Cioffi *et al*., 1997).

It is only after activation of the embryonic genome that the embryo begins to be controlled by its own unique genome. The information required for the embryonic cells to reorganize their surface according to their special orientation within the embryo is probably part of the maternal genetic message inherited from the oocyte in the form of pre-synthesized RNA or proteins and utilized to guide developmental processes in the early embryo. In humans, this is thought to occur between the 4- and 8-cell stages (Tesarik *et al*., 1986; Braude *et al*., 1988). Early cleavage arrest has been demonstrated in most animals (Flach *et al*., 1982; Davis, 1985; Crosby *et al*., 1988; Frei *et al*., 1989; Sakkas *et al*., 1989). In human pre-embryos, the *c-mos* transcripts disappear rapidly and are not detectable beyond the 5-cell stage and expression of cyclin-B1 is somewhat paralleled and increased after the 6-cell stage (Heikinheimo *et al*., 1995). This suggests that active transcription of these genes in human pre-embryos is from the 4-cell stage onwards. However, Artley *et al*. (1992) have examined the relationship between gene expression and cleavage arrest by investigating the patterns of protein synthesis in cleavage-arrested human pre-embryos cultured *in vitro*, indicating that cleavage arrest is not always accompanied by failure of activation of the genome. The underlying mechanisms responsible for cleavage arrest are unclear. Possible causes include inadequate culture conditions (Gandolfi and Moor, 1987; Menezo *et al*., 1990), inherent or induced abnormality (Macas *et al*., 1990; Pickering *et al*., 1990) and failure of embryonic gene expression (Braude *et al*., 1990). The optimal culture condition *in vitro* seems to promote the expression of the embryonic genome. It has been reported that transcription activity of mouse blastocysts can be enhanced by alterations in culture conditions (Ho *et al*., 1994). Thus, it is important for effective gene transcription of the human pre-embryo that the culture conditions of assisted reproductive techniques are correct.

Utilization of immature human oocytes

Attempts to study human fertilization in culture were first reported by Rock and Menkin (1944). It was confirmed by Edwards (1965a,b) that human follicular oocytes are able to mature *in vitro* when they are isolated from follicles and placed in appropriate culture medium. Human oocytes surrounded by cumulus cells have a higher maturation rate

than those without these cells (Kennedy and Donahue, 1969). The first successful fertilization *in vitro* of human oocytes matured *in vitro* was reported by Edwards *et al.* (1969). Subsequently, the first successful human pregnancy from IVF of the oocytes retrieved from natural ovulatory cycles was achieved by Steptoe and Edwards (1978). Thereafter, the successful fertilization, development and pregnancy with immature human oocytes matured *in vitro* has been reported continuously (Veeck *et al*., 1983; Prins *et al*., 1987; Cha *et al*., 1991, 1992, 1996; Paulson *et al*., 1994; Trounson *et al*., 1994; Barnes *et al*., 1995, 1996; Nagy *et al*., 1996; Edirisinghe *et al*., 1997; Jaroudi *et al*., 1997; Liu *et al*., 1997; Russell *et al*., 1997). However, very little is known about the maturation and developmental capacity of immature human oocytes for clinical application.

Oocytes from stimulated ovaries

For clinical application, most oocytes are obtained from ovarian follicles of women who have been treated with hormones to induce multiple follicular growth. The use of superovulation hormones has resulted in follicular asynchrony (Laufer *et al*., 1984; Bomsel-Helmreich *et al*., 1987). When oocytes were retrieved from stimulated ovaries before the LH surge, most of the oocytes were at the germinal vesicle stage (Bomsel-Helmreich *et al*., 1987); however, oocytes retrieved after human chorionic gonadotrophin (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 oocytes may be retrieved, despite having been exposed to an ovulatory dose of HCG 36 h before aspiration. These oocytes are capable of undergoing spontaneous nuclear maturation *in vitro*, and then normal fertilization and development. Studies on oocytes from animals have shown that the endocrine environment plays an important role in ensuring normal cytoplasmic maturation with respect to subsequent fertilization (Moor and Trounson, 1977). The inclusion of human menopausal gonadotrophin (HMG) in the culture medium for immature human oocytes results in improved maturation and fertilization rates (Prins *et al*.,1987; Table I). It has been reported that pregnancies have been obtained following the transfer of immature oocytes from stimulated cycles (Veeck *et al*., 1983; Prins *et al*., 1987; Nagy *et al*., 1996; Liu *et al*., 1997; Jaroudi *et al*., 1997; Edirisinghe *et al*., 1997).

In addition to the nuclear events, oocyte cytoplasmic maturation is necessary for normal fertilization and subsequent embryonic development. Human IVF programmes have indicated that delaying insemination improves fertilization rates, presumably by allowing the completion of cytoplasmic maturation for those oocytes that have not completely matured at the time of retrieval (Trounson *et al*., 1982). Increasing FSH levels within the follicle is coincident with the generation of a positive signal necessary to complete oocyte maturation in humans *in vivo*. This suggests that this signal may be linked to the dynamics of growth factors within the follicle itself (Gómez *et al*., 1993a,b). Recently, it has been reported that LH is required during the peri-ovulatory period for normal embryo development *in vivo* (Weston *et al*., 1996). Fertilization and cleavage rates were improved by the addition of 150 mIU/ml HMG into the medium if oocytes derived from different size of follicles were cultured *in vitro* shortly before insemination (Zhang *et al*., 1993; Table II). This indicates that the presence of gonadotrophin during the short period of culture before insemination may also have provided the oocytes with a proper hormonal environment for both nuclear and cytoplasmic maturation.

Table I. Meiotic maturation and fertilization rates of immature human oocytes (germinal vesicle) derived from stimulated cycles[a]

	Group I (no HMG)	Group II (+ HMG)
Total no. of oocytes	31	34
Oocytes matured (1st polar body) (%)	11 (35.6)	25 (73.5)[b]
Oocytes fertilized/inseminated oocytes(%)	4/11 (36.4)	16/25 (64.0)[b]
Oocytes fertilized/total oocytes (%)	4/31 (12.9)	16/34 (47.1)[b]

[a]Oocytes were cultured with Ham's F-10 medium supplemented with 7.5% maternal serum in the absence (group I) and pres ence (group II) of 75 mIU human menopausal gonadotrophin (HMG). Data from Prins *et al.* (1987).
[b]Significantly different from group I.

Different ovulation induction protocols may result in variations in oocyte quality, fertilization and cleavage rates (Boué and Boué, 1973; De Sutter *et al*., 1991a,b). Fertilization rates are different when using clomiphene citrate (CC)/HCG and gonadotrophin analogue (GnRHa) protocols (De Sutter *et al*., 1992). GnRHa is now used routinely to prevent spontaneous gonadotrophin surges during ovarian stimulation for IVF programmes. The short and long GnRHa protocols are usually used and both regimens prevent premature LH surges. Although there is no significant difference with respect to clinical pregnancy rates per cycle between the long and short protocols (Frydman *et al*., 1988; Garcia *et al*., 1990; Maroulis *et al*., 1991; Acharya *et al*., 1992; Hughes *et al*., 1992), the oocyte maturity, fertilization and cleavage rates are different (Greenblatt *et al*., 1995). Furthermore, several studies have suggested that high concentrations of LH during the follicular phase of stimulation can have a negative effect on oocyte quality, pregnancy rate and the incidence of miscarriage (Stanger and

Yovich, 1985; Howles *et al*., 1986; Homburg *et al*., 1988; Regan *et al*., 1990). HMG, which is a 1:1 mixture of FSH and LH, and some centres have substituted HMG partially or completely by pure FSH (Venturoli *et al*., 1986). The highly purified FSH short-term stimulation protocol synchronizes oocyte maturation better than a comparable stimulation with HMG (Imthurn *et al*., 1996). Moreover, increased fertilization rates have been reported with the addition of granulosa cells to culture medium used to mature oocytes with compact cumulus cells for 5–8 h after recovery in CC/HCG-stimulated protocols (Dandekar *et al*., 1991). In addition, it must be noted that 18% of in-vitro maturing oocytes had gross meiotic aberrations (Racowsky and Kaufman, 1992). It is possible that these abnormalities also occur during maturation *in vivo* and after gonadotrophin stimulation of follicular growth for mature oocyte recovery. It is reported that aneuploidy occurs at high rates in mature oocytes (20–31%) recovered in stimulated cycles (Wramsby and Lìedholm, 1984; Martin *et al*., 1986; Bongso *et al*., 1988; Djalali *et al*., 1988; Papadopoulos *et al*., 1988; Plachot *et al*., 1988; Ma *et al*., 1989; Pellestor, 1991; Gras *et al*., 1992; Racowsky *et al*., 1997).

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 ≥25% of normal-looking mature human oocytes retrieved from stimulated cycles (Plachot *et al*., 1988; Tarin *et al*., 1991; Delhanty and Handyside, 1995; Wall *et al*., 1996). Specific cytoplasmic pathologies present in the oocyte at the time of insemination correlate with both the ability to fertilize and the ability of the fertilized oocyte to develop progressively (Van Blerkom, 1994). Metabolic differences between morphologically equivalent metaphase II oocytes retrieved from the same ovary have been observed and related to the developmental ability of the resulting embryo (Van Blerkom *et al*., 1995; Barnett and Bavister, 1996; Van Blerkom, 1996). Very high frequencies of chromosomal aneuploidy have been observed in 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 alterations (Van Blerkom and Henry, 1992). Some chromosomal anomalies are first detectable during early meiotic metaphase, therefore it is also possible that the damage of DNA may occur in earlier oogenesis (for review see Ashwood-Smith and Edwards, 1996). Increasing maternal age in naturally cycling women (Battagalia *et al*., 1996a) and the unsuitable micro-environment for cytoplasmic maturation *in vivo* (Delhanty and Handyside, 1995), such as a lowering of intracellular pH (Van Blerkom, 1996) may be associated with high frequencies of spindle defects and chromosomal segregation disorders. Cytoplasmic maturation is also important to the development of microtubule organizing centres (MTOC) in the oocyte (Van Blerkom, 1991; Wickramasinghe *et al*., 1991). A recent report indicated that the human oocyte develops MTOC as meiotic maturation proceeds beyond the prophase I arrest (Battaglia *et al*., 1996b).

Anaesthesia might also affect oocyte maturation, fertilization and early cleavage *in vitro*. Normally, the procedure of ultrasound-guided transvaginal oocyte retrieval is carried out under either general, epidural or local anaesthesia. Propofol (diprivan) is one of the commonest medications used for anaesthesia in this situation. Propofol has been reported to accumulate in the follicular fluid (Palot *et al*., 1988). Its concentrations in follicular fluid seem to increase steadily throughout the oocyte retrieval procedure, suggesting that Propofol may have deleterious effects on reproductive outcome following IVF (Coetsier *et al*., 1992). Using a mouse IVF model, it has been suggested that Propofol is detrimental to oocyte maturation, fertilization and subsequent cleavage *in vitro* (Depypere *et al*., 1991; Janssenswillen *et al*., 1996; Alsalili *et al*., 1997). However, it is unclear how Propofol affects oocyte maturation during culture *in vitro*. It is possible that the oocyte chromatin may be damaged by such anaesthetic agents since it is known that mammalian oocytes are unable to recognize damaged chromatin but will continue meiosis (Chian *et al*., 1992; Pyrzynska *et al*., 1996; Fulka, *et al*., 1997).

Table II. Effect of human menopausal gonadotrophin (HMG) in maturation medium (MT6 medium +10% maternal serum) on in-vitro fertilization and cleavage of human oocytes derived from stimulated cycles[a]

Follicle size (ml)[b]	No. of oocytes	Cumulus expanded	HMG (mIU)	Fertilization (%)[c]	Cleavage (%)[c]
32	44	3	0	10 (23)	4 (40)
32	51	5	150	25 (49)	19 (76)
>2	178	178	0	116 (65)	113 (97)

[a]Data from Zhang *et al.* (1993).
[b]Volume of follicle fluid aspirated.
[c]Significantly different from each other ($P < 0.05$).

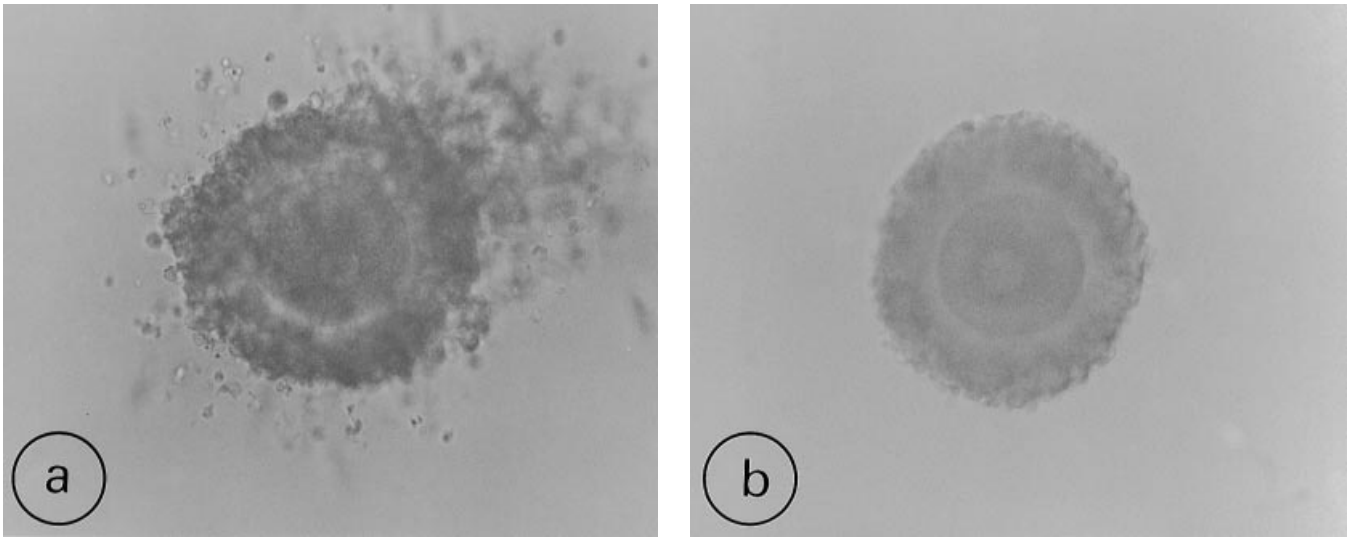

Figure 3. Human germinal vesicle oocytes retrieved from stimulated (**a**) and unstimulated ovaries (**b**). Both oocytes display compact cumulus cell layers. The intact germinal vesicle of the oocytes could be observed clearly under a microscope (original magnification ×400).

Normally, 85–90% of the oocytes retrieved from stimulated cycles in IVF programmes are in metaphase II; however, 10–15% of oocytes derived from stimulated cycles are still immature (K.-Y.Cha *et al*., unpublished data). Therefore, it is also important to improve the quality of these oocytes for clinical purposes. It has been reported that cumulus-free germinal vesicle oocytes retrieved from stimulated cycles can be matured *in vitro* by co-culturing with Vero cell monolayers (Janssenswillen *et al*., 1995). The time course of oocyte GVBD and maturation were different between the oocytes retrieved from stimulated and unstimulated ovaries (Figure 3) and that the ability of the oocyte fertilization was also different between these two groups (R.-C.Chian *et al*., unpublished data). 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 stimulated ovaries (Figure 4). However, the final percentages of GVBD were not different between the oocytes derived from stimulated (83.3%) and unstimulated (87.5%) cycles. GVBD occurred significantly earlier in the oocytes retrieved from stimulated ovaries than unstimulated ovaries. As discussed above, LH probably induces GVBD by an indirect action mediated by cumulus cells because there are no LH receptors on the oocytes (Dekel, 1988). Many factors may mediate the cumulus cells' control of GVBD; the mechanism involves LH inducing the loss of communication between oocyte and the cumulus cell complex, thus terminating the flow of some substances into the oocytes (Berridge and Irvine, 1984; Larsen *et al*., 1987; Downs *et al*., 1988; Fagbohun and Downs, 1991; Downs, 1995). From this

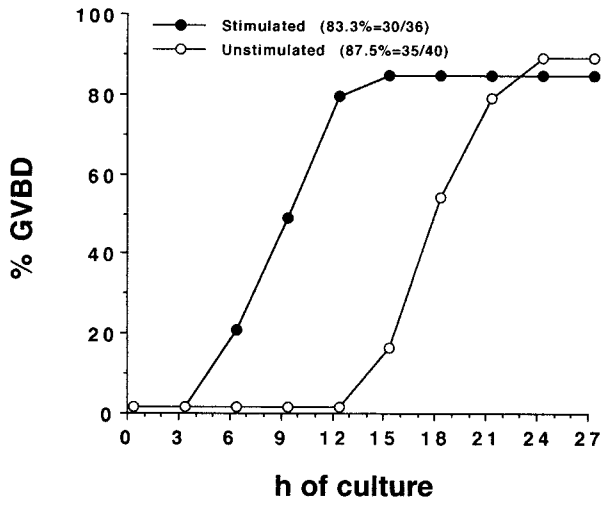

Figure 4. The time course of human oocyte germinal vesicle breakdown (GVBD) during culture *in vitro*. The final percentages of GVBD were not different between the oocytes retrieved from stimulated (–•–) and unstimulated (–○–) ovaries.

we may explain why GVBD occurs earlier in the oocytes derived from stimulated ovaries than from unstimulated ovaries, because the oocytes retrieved from stimulated ovaries have already been exposed to the action of LH/HCG stimulation in the follicles.

Extremely low fertilization rates are usually obtained after standard insemination of matured germinal vesicle oocytes from ovulation induction, suggesting that intracytoplasmic sperm injection (ICSI) is the best option, even where the sperm parameters are not impaired (Nagy *et al*., 1996). It is not clear at the present time why the fertilization rates are so

low. It may be due to alterations of the zona pellucida (zona hardening) as a result of the oocyte maturation *in vitro* in these oocytes.

Oocytes from unstimulated ovaries

There are not many studies on immature human oocytes matured *in vitro* because of the limitation of materials. For clinical application and research, immature follicular oocytes can be obtained from consenting patients undergoing tuboplasty, Caesarean section, and oophorectomy. The method of collection of follicular immature oocytes from surgically removed ovaries were described previously by Cha *et al*. (1991). In the case of donors who underwent tuboplasty or Caesarean section, immature follicular oocytes were aspirated by puncturing visible follicles with a 21-gauge needle attached to a syringe filled with culture medium. The results indicate that the age, pathology, day of the menstrual cycle, and cyclic versus non-cyclic ovaries of donors, are factors influencing the number of immature oocytes (Cha, 1992). Immature oocytes from the luteal phase had a significantly higher maturation rate than those obtained during the follicular phase. The number of immature human oocytes collected from an ovary decreased as the patient's age increased; however, there was no statistical difference in the rate of oocyte maturation and cleavage among donors in the different age groups (Cha *et al*., 1992).

Table III. Details of seven pregnancies using immature follicular oocytes (Cha, 1995)

Number	Maturation media[a]	Age	No. of embryos transferred	Results
1	Ham's F-10 +50% HFF	32	5	Triplets delivered
2	Ham's F-10 +50% HFF	43	1	Singleton delivered
3	Ham's F-10 +50% HPF	51	3	Clinical abortion
4	Ham's F-10 +50% HPF	33	5	Clinical abortion
5	TCM-199 +50% HPF	32	10	Clinical abortion
6	TCM-199 +50% HPF	31	5	Twins delivered
7	TCM-199 +50% HPF	29	3	Singleton delivered

[a]HFF: human follicular fluid; HPF: human peritoneal fluid.

It seems that there is a relationship between follicular size in the human ovary and oocytes capable of resuming meiosis *in vitro* (Tsuji *et al*., 1985). In the follicular phase, the percentage of oocyte maturation in the large-follicle group (9–15 mm diameter; 34.5%) was significantly ($P < 0.05$) higher than that in the small-follicle group (3–4 mm; 8.8%). It has been reported that the unstimulated human oocyte appears to have a size-dependent ability to resume meiosis and complete maturation, indicating a significant difference in oocytes measuring 86–105 μm (22.2%) versus those measuring 106–125 μm (60.0%) (Durinzi *et al*., 1995).

Table IV. Maturation and fertilization rates of human oocytes derived from unstimulated cycles[a]

	Maturation (%)[b] (*n* = 199)	Fertilization (%)[c] (*n* = 27)
No. of oocytes	158 (79.4)	25 (92.6)

[a]Oocytes were cultured in TCM-199 + 20% fetal bovine serum supplemented with gonadotrophins (10 IU/ml pregnant mare's serum gonadotrophin and 10 IU/ml human chorionic gonadotrophin). Because of ethical problems, some egg donors did not want their eggs to be mixed with donor spermatozoa. Thus only a small proportion of mature oocytes were used in the fertilization attempt, the remainder going to cryopreservation experiments.
[b]Extrusion of first polar body.
[c]Formation of both pronuclei.

An earlier study indicated that 42% (52/124) of immature oocytes displayed GVBD and 31% (39/124) progressed to metaphase II when the oocytes were cultured with TCM-199 or Ham's F-10 supplemented with 10% fetal calf serum for 42 h (Shea *et al*., 1975). The maturation and fertilization rates of immature human oocytes were 63% and 60%, respectively, when using human follicular fluid or peritoneal fluid as supplements in the maturation medium (Cha *et al*., 1989). The first live birth from immature human oocytes obtained from unstimulated ovaries was in our ovum donation programme (Cha *et al*., 1991). Five embryos were transferred to a woman with premature ovarian failure and she delivered healthy triplet girls. Shortly thereafter, from 21 transfer cycles, viable embryos were derived from follicular immature oocytes matured *in vitro* in Ham's F-10 medium supplemented with 50% follicular fluid or 50% peritoneal fluid, resulting in another two live births (Cha *et al*., 1992). In our centre, the human ovum donation programme has subsequently obtained six more pregnancies by using follicular immature oocytes derived from donors who underwent tuboplasty or Caesarean section (Cha, 1995; Table III). Recently, we have been using TCM-199 + 20% fetal bovine serum (FBS) and supplemented with gonadotrophins [10 IU/ml pregnant mare's serum gonadotrophin (PMSG) and 10 IU/ml HCG], resulting in ~80% maturation rate and >90% fertilization rate when the

germinal vesicle oocytes were retrieved from unstimulated ovaries (Table IV).

The time courses of GVBD and maturation of the oocytes were different between the immature oocytes retrieved from stimulated and unstimulated ovaries (Figures 4 and 5). Most oocytes matured to metaphase II 45 h after culture *in vitro* when the immature germinal vesicle oocytes were retrieved from the patients undergoing Caesarean section (Figure 5). However, when the immature germinal vesicle oocytes were recovered from stimulated cycles, most oocytes reached metaphase II at 30 h after culture *in vitro*. The final percentages of the oocyte maturation were not different between the immature oocytes derived from stimulated (75.0% = 27/36) and unstimulated (77.5% = 31/40) cycles. However, the percentage of metaphase II oocytes was significantly higher for germinal vesicle oocytes derived from stimulated rather than unstimulated ovaries (Gómez *et al*., 1993a). Combination of the results from Toth *et al*. (1994a,b) also indicated that the maturation rates are different between the immature human oocytes retrieved from stimulated and unstimulated cycles. These differences might be due to the use of different maturation medium and different culture supplements during maturation in each research group. Ninety per cent (18/20) of germinal vesicle oocytes retrieved from patients undergoing Caesarean section presented a nucleolus in the oocyte germinal vesicle, but only 20% (2/10) of oocytes retrieved from stimulated cycles contained a nucleolus in the germinal vesicle. The profile of protein synthesis is also different in these two groups (Chian *et al*., 1997). These results suggest that the differences between the germinal vesicle oocytes retrieved from stimulated and unstimulated cycles may subsequently relate to time courses of GVBD and maturation of oocytes cultured *in vitro* in these two groups.

The disadvantages of ovulation induction include the side-effects from the gonadotrophins, which include weight gain, bloating, nausea, vomiting, mood swings, ovarian hyperstimulation with possible hospitalization, and the unknown long-term cancer risks (Bergh and Navot, 1992). Therefore, many attempts have been made to perform IVF without the use of exogenous gonadotrophins (natural cycle) to induce multiple follicles. One very attractive possibility for enhancement of the success of natural cycle IVF is the combination of oocyte maturation techniques with immature oocyte retrieval, and this technique has yielded live births (Paulson *et al*., 1994; Russell *et al*., 1997). Recently it has been reported that an average of 11 oocytes per patient (from unstimulated ovaries) were retrieved transvaginally with ultrasound guidance, which suggests that unstimulated immature oocyte retrieval can be performed successfully in patients

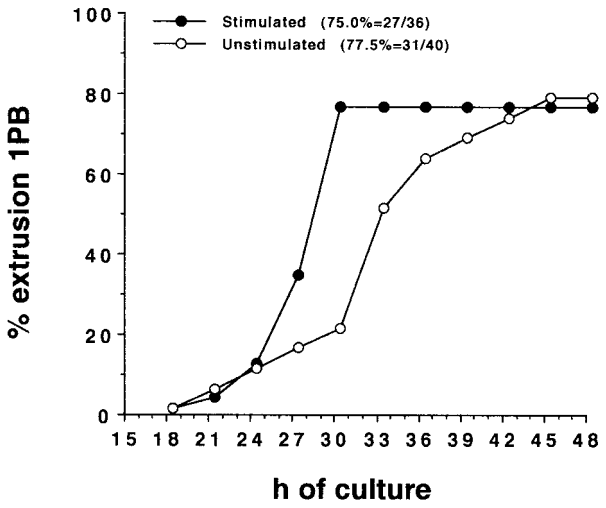

Figure 5. The time course of human oocyte maturation [polar body (PB) exclusion] during culture *in vitro*. There was no significant difference in maturation rates between the oocytes retrieved from stimulated (–•–) and unstimulated (–○–) ovaries.

who are candidates with different diagnoses for assisted reproductive treatment (Russell *et al*., 1997). This indicates that the immature oocyte collection and in-vitro maturation procedures could possibly replace the standard stimulated IVF cycle in the near future.

Oocytes from polycystic ovarian syndrome patients

Patients with polycystic ovarian syndrome (PCOS) are characterized by abnormal endocrine parameters, anovulation, numerous antral follicles within their ovaries and frequently infertility (Goldzieher and Green, 1962). PCOS patients may be extremely sensitive to exogenous gonadotrophin and may be at risk of ovarian hyperstimulation syndrome when treated with gonadotrophins for assisted reproduction. Immature oocyte recovery could be developed as a new method for the treatment of patients with infertility due to PCOS because the oocytes of these patients retain their maturational and developmental competence (Trounson *et al*., 1994). They reported that 65% of oocytes cultured in medium with gonadotrophins, oestrogen and fetal calf serum matured to metaphase II by 43–47 h, and 81% were matured at 48–54 h of culture. Of the inseminated oocytes, 34% fertilized and 56% of the cultured pronuclear oocytes cleaved to eight cells or more. A pregnancy and the first birth of a normal baby occurred in one of the anovulatory PCOS patients receiving an abbreviated steroid replacement protocol after embryo transfer. A second birth was achieved by in-vitro maturation of primary oocytes derived from a PCOS patient, combined with ICSI and assisted hatching (Barnes *et al*., 1995), indicating that the developmental capacity of

primary oocytes is higher in regular cycling women than in irregular cycling and anovulatory women with PCOS (Barnes *et al*., 1996).

In our centre, the PCOS patients are diagnosed when the following conditions are present: physical characteristics such as obesity and hirsutism, menstrual disturbances (oligomenorrhoea, amenorrhoea), LH/FSH ratio >3.0 and the characteristic polycystic ovarian appearance at ultrasonographic assessment. For maturation *in vitro* of immature oocytes retrieved from these PCOS patients, TCM-199 + 20% FBS and supplemented with 10 IU/ml PMSG and 10 IU/ml HCG were used. It has been found that the maturation rate of immature oocytes retrieved from PCOS patients is significantly lower than those recovered from normal menstrual cycle patients (K.Y.Cha *et al*., unpublished data). The results indicate that 60.0% (499/832) of oocytes cultured *in vitro* matured to metaphase II and that 73.0% (364/499) of the oocytes fertilized by ICSI and 89.9% (187/208) of the cultured pronuclear oocytes cleaved to 2- and 4-cell stage (Cha *et al*., 1996). Up to now, a total of 16 pregnancies have been achieved from PCOS patients in our centre (Table V). These indicate that immature oocyte recovery from women with infertility due to PCOS can be used with this therapy for assisted reproduction.

Cryopreservation of immature oocytes

Successful cryopreservation of the immature oocytes would widen the possibility of establishing an ovum bank. Oocyte cryopreservation has the potential to overcome many of the legal and ethical problems associated with embryo cryopreservation and provides more options for patient treatment. A clinical application of cryo-preservation of immature oocytes would be to store oocytes from patients who risk the loss of ovarian function. Oocytes are very large cells with well-defined structures which do not withstand temperature alterations without impairment of their fertilizing and developmental capacity. During freezing and thawing, the oocyte changes with rearrangement of the cytoskeleton and of the meiotic spindle, clumping and migration of the cortical granules and hardening of the zona pellucida (Vincent and Johnson, 1992; George *et al*., 1994; Van Blerkom and Davis, 1994). Although successful pregnancies after cryopreservation of metaphase II oocytes have been reported (Chen, 1986; Al-Hasani *et al*., 1987; Van Uem *et al*., 1987), the success rate is still low. It has been suggested that the microtubular spindle of metaphase II oocytes is sensitive to temperature changes (Van der Elst *et al*., 1988). Thus, chromatid non-disjunction may occur during cooling and it would result in aneuploidy after fertilization (Pickering *et al*., 1990). Freezing of immature oocytes may solve these problems. The advantage of freezing at prophase I is that the procedure can be achieved before spindle formation in the oocytes.

Production of live young after immature oocyte freezing has been successful in mice (Candy *et al*., 1994). Human germinal vesicle oocytes can survive and mature *in vitro* after freeze–thawing (Mandelbaum *et al*., 1988; Toth *et al*., 1994a,b; Son *et al*., 1996; Park *et al*., 1997): 55.1% (54/98) of oocytes survived and 59.3% (32/54) of oocytes matured to metaphase II following freeze–thawing, 42.9% (6/14) of oocytes fertilized and 16.7% (1/6) of fertilized oocytes cleaved (Table VI), suggesting that immature human oocytes are capable of meiotic maturation, fertilization and development following cryopreservation (Son *et al*., 1996; Park *et al*., 1997). However, the freeze–thawing procedures have detrimental effects on the maturation of human oocytes and subsequent early developmental capacity. It has been indicated that human oocytes matured *in vitro* after cryopreservation at the germinal vesicle stage also showed an increased incidence of chromosomal and spindle abnormalities, suggesting that these abnormalities may impair the capacity for further development of embryos derived from frozen–thawed oocytes (Park *et al*., 1997). Furthermore, when in-vitro matured germinal vesicle oocytes after cryopreservation were inseminated by either conventional IVF or ICSI, the fertilization rate was higher in the ICSI group (64.3%) than the conventional IVF group (27.3%) (Park *et al*., 1996). This result suggests that the reduced rate of fertilization after freeze–thawing may be due to hardening of the zona pellucida.

Table V. Pregnancy outcome of immature oocytes derived from PCOS patients[a]

No. of cases	No. of oocytes retrieved	No. of oocytes cultured	No. of oocytes matured (%)	No. of oocytes fertilized (%)[b]	No. of embryos transferred (%)[c]	Pregnancies (%)[d]
72	910	832	499 (60.0)	400 (80.2)	306 (76.5)	16 (25.0)

[a]Data modified from Cha *et al.* (1996).
[b]Abnormally fertilized oocytes (n = 36) were included.
[c]No. of embryos transferred/no. of fertilized oocytes cultured = 187/208. Mean no. of embryos transferred was 4.7 per patient.
[d]No. of pregnant patients/no. of patients who had embryos transferred = 16/64; one of these pregnancies was a twin pregnancy, whilst the rest were singletons.

Table VI. Comparison of maturation, fertilization and cleavage rates between cryopreserved immature human oocytes and control[a]

	Control	Frozen–thawed at germinal vesicle stage[b]
No. of oocytes	82	98
No. surviving (%)	82 (100)	54 (55.1)
No. undergoing maturation (%)	63 (76.8)	32 (59.3)
No. undergoing insemination	21	14
No. fertilized (%)	19 (90.5)	6 (42.9)
No. cleaved (%)	18 (94.7)	1 (16.7)

[a]Data modified from Son *et al.* (1996).
[b]Significantly different from control ($P < 0.01$).

Early studies indicated that restoration of fertility could be obtained by reimplantation of frozen–thawed ovarian tissue (Parrott, 1960) and that only primordial follicles survived and allowed in-vitro development following the cryopreservation process (Deanesly, 1954; Green *et al*., 1956; Parkes, 1958). Since these studies, cryopreservation techniques have been much improved. Recently the technique of reimplantation of frozen–thawed ovarian tissue has been applied to several species resulting in live offspring (Carroll and Gosden, 1993; Gosden *et al*., 1994). On the other hand, it has been known that immature oocytes from early preantral follicles from prepubertal mice matured, fertilized, and the embryos developed *in vitro* in a simplified culture system (Cortvindt *et al*., 1996a). They also indicated that cryopreserved whole early preantral follicles matured *in vitro* and responded to stimulation with HCG as the control group and that these cryopreserved, in-vitro matured oocytes had the potential to fertilize and develop to hatched blastocysts (Cortvrindt *et al*., 1996b). These experiments suggest that freezing of whole early follicles may circumvent potential injuries to chromosomal material, cytoplasmic constituents and to the sperm penetration potential of the zona and oolemma. Cryopreserving isolated whole follicles and maturing the oocytes in the follicles *in vitro* may provide more options for patient treatment.

Conclusions

The cytoplasm of the oocyte is of key interest in oocyte maturation. The best way to improve embryo quality is to improve oocyte quality. Addition of gonadotrophins (FSH, LH) to the maturation medium is beneficial to cytoplasmic maturation of immature human oocytes. Oocytes derived from stimulated and unstimulated ovaries can be matured and fertilized *in vitro* even though the fertilization rates are different. Immature oocytes retrieved from PCOS patients also can be matured and fertilized *in vitro*. This indicates that immature oocytes recovered from women with infertility due to PCOS can be used with this therapy for assisted reproduction. Immature germinal vesicle oocytes are also capable of meiotic maturation, fertilization and development *in vitro* following cryopreservation.

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