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Optimal ICSI timing after the first polar body extrusion in *in vitro* matured human oocytes

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BACKGROUND: This study was conducted to investigate the fertilization and embryo development of human oocytes injected at different time intervals after extrusion of the first polar body (PB) following *in vitro* maturation (IVM) in IVM cycles. Also, we evaluated whether spindle imaging could serve as a tool to determine the optimal ICSI time. METHODS: Oocytes were collected from 43 women with polycystic ovary syndrome. Metaphase I (MI) oocytes after *in vitro* culture for 24 h from germinal vesicle stage were subjected to ICSI according to time after first PB extrusion. The intervals were: within 1 h ($n = 38$); 1–2 h ($n = 30$); 2–4 h ($n = 26$);4–6 ($n = 28$) and 6–8 h ($n = 40$). In some MI oocytes, viable spindle location was evaluated using Polscope microscopy at different time intervals after first PB extrusion. RESULTS: Fertilization rate of the MI oocytes injected within 1 h after first PB extrusion was low (15.8; 6/38) ($P < 0.01$ versus all other times). In contrast, the fertilization rate was 80, 92.3, 82.1 and 85% for oocytes injected 1–2, 2–4, 4–6 and 6–8 h after first PB extrusion, respectively. Development of good-quality embryos was not significantly different among all the groups. Interestingly, all the oocytes injected within 1 h after first PB extrusion were in Telophase I. CONCLUSIONS: Human oocytes matured *in vitro* needed at least 1 h after first PB extrusion to complete nuclear maturation. Use of a live spindle imaging system can help to decide the timing of ICSI for oocytes matured *in vitro*.

Key words: in vitro maturation; ICSI timing; first polar body; spindle imaging

Introduction

The recovery of immature oocytes followed by *in vitro* maturation (IVM) is an attractive alternative to conventional controlled ovarian hyperstimulation (COH), with advantages as the avoidance of the risk of ovarian hyperstimulation syndrome, the reduced cost and the less complicated treatment. A considerable asynchrony of immature oocytes from IVM cycles has been observed. Significant numbers of oocytes are observed at metaphase I (MI) stage when maturity is assessed for ICSI after 24 h culture and some of them can reach maturity between 24 and 32 h on the same day (Son *et al.*, 2005). Therefore, it is important to determine optimal ICSI timing to induce successful fertilization and embryo development when MI oocytes became MII on the same day, especially for patients who have few MII oocytes after culture.

Oocyte nuclear maturation implies the reinitiation and completion of the first meiotic division from germinal vesicle (GV) stage to MII. Beyond these nuclear aspects of oocyte maturation, cytoplasmic maturation occurs and seems to be important for the fertilization and developmental ability of the oocyte. (Eppig *et al.*, 1996). However, these two processes are not completely independent and the timing of maturational events in the nucleus and in the cytoplasm appears to be tightly regulated (Eppig, 1996; Moor *et al.*, 1998; Trounson *et al.*, 2001; Albertini, 2003).

Recently, a new polarization light microscope, the LC Polscope, has been used to assist ICSI procedures in some IVF clinics (Wang *et al.*, 2001; Eichenlaub-Ritter *et al.*, 2002; Moon *et al.*, 2003). In contrast to fluorescence microscopy, the LC Polscope imaging does not require invasive preparative techniques, such as fixation and staining, and the spindle can be imaged in living oocytes (Wang and Keefe, 2002), thereby enabling a better assessment of the oocyte meiotic stage.

Although there is some evidence available with respect to the time period needed for insemination in human COH cycles, to date there is no report on ICSI timing after IVM in unstimulated IVM cycles. Therefore, this study was conducted to evaluate the fertilization and developmental potential of human oocytes subjected to ICSI in relation to the time that has elapsed after the first polar body (PB) extrusion following IVM in IVM cycles. We also examined whether the appropriate timing for ICSI after first PB extrusion can be determined by spindle location imaged with the Polscope.

Materials and Methods

Approval for the IVM programme was obtained from the Institutional Review Board of the Maria Infertility Hospital.

Patients

Our study included 43 polycystic ovary syndrome patients (mean age: 32.1 ± 3.2 years) who underwent a HCG-primed IVM cycle. Patient selection was based on no oocytes matured *in vivo* on the collection day, the number of oocytes collected (≥20), and the presence of mature oocytes (≥8) and immature MI oocytes (≥6) capable of IVM after denudation at 24 h of culture. The patients received an i.m. injection of progesterone (Progest; Samil Pharmacology, Seoul, Korea). Withdrawal bleeding occurred within 3 days after the last progesterone injection.

Oocyte collection

The oocytes were collected between cycle days 9 and 16 based on endometrial thickness of >6 mm. A transvaginal ultrasound machine (Aloka, Tokyo, Japan) with 19-gauge aspiration needle (Cook, Eight Mile Plains, Queensland, Australia) was used to aspirate follicles. A portable aspiration pump was used with a pressure between 80 and 100 mmHg. The aspirates were collected in tubes with prewarmed heparinized Ham's F-10 medium that contained HEPES buffers supplemented with 0.3% human serum albumin. Follicular aspirates were filtered using a 70-μm mesh (Falcon, Becton Dickinson & company, NJ, USA), washed three times, and the oocytes were isolated under a stereomicroscope.

in vitro maturation

The collected immature oocytes (only oocytes at GV stage) were cultured in IVM medium consisting of YS medium (Yoon *et al.*, 2001a, b) supplemented with 1 IU/ml FSH, 10 IU/ml HCG and 10 ng/ml recombinant human epidermal growth factor (Daewoong Pharmaceutical Co., Korea) (Son *et al.*, 2002). After 24 h culture, the oocytes were denuded of cumulus cells with 20 IU/ml hyaluronidase (Sigma chemical Co, St. Louis, MO, USA) and mechanical pipetting. Following examination, the immature oocytes that reached the MII stage after 24 h of *in vitro* culture were used as controls and ICSI was performed 1 h after denudation. The immature oocytes that had already undergone the GV breakdown but had not extruded the first PB were further cultured in fresh medium and the meiotic status was re-examined every 30 min for the progression of meiotic maturation. These oocytes were injected at five different time intervals after the extrusion of the first PB. The intervals were as follows: within 1 h ($n = 38$); 1–2 h ($n = 30$); 2–4 h ($n = 26$); 4–6 h ($n = 28$) and 6–8 h ($n = 40$).

In some MI oocytes that displayed a meiotic spindle near the cortex ($n = 20$), we examined the spindle location after the extrusion of the first PB with Polscope and thus checked on the nuclear status within the oocyte cytoplasm.

IVF and in vitro development

Matured oocytes were inseminated by ICSI using husbands' sperm. Fertilization was assessed 17–19 h after insemination by the appearance of two distinct pronuclei and two PBs. The zygotes were co-cultured with cumulus cells in 10 μl YS medium supplemented with 10% human follicular fluid (Yoon *et al.*, 2001b). The cumulus cells for co-culture were prepared as described previously (Yoon *et al.*, 2001b). Embryonic development was assessed on day 3 of culture according to the regularity of blastomeres, the percentage and pattern of anucleate fragments, and all dysmorphic characteristics of the embryos. For this study, we defined embryos as good quality if they had at least six blastomeres, <20% anucleate fragments and no apparent morphologic abnormalities. Embryos showing blastomere multi-nucleation, poor cell adhesion, uneven cell division and cytoplasmic abnormalities were defined as low quality.

Embryos were transferred on day 4 after oocyte retrieval by the transcervical route in standard fashion. Before transfer, all embryos for each patient were pooled and selected for transfer.

Statistical analysis

Differences between treatment groups were compared with χ^2 test using the Statistical Analysis System (SAS Institute, Cary, NC, USA) software package.

Results

A total of 901 oocytes were collected from 43 IVM patients. Overall, 389 of the retrieved oocytes (43.2%) were mature to the MII stage after 24 h culture (control group) and 265 oocytes remained at the MI stage of the first meiotic division at the time of removal of the corona-cumulus cells. Among 265 immature MI oocytes, 162 oocytes (61.1%) underwent maturation and extruded the first PB during *in vitro* culture by 32 h (day 1). In each cycle, both IVM oocytes after 24 h culture (control group) and their siblings (IVM MII oocytes after culture for >24 h) were injected with sperm from the patient's husband. The fertilization rates are shown in Table 1. No significant difference was observed in the fertilization rate among groups of oocytes left for >1 h after the first PB extrusion compared to control group (82.0%, 319/389). The following fertilization rates were recorded: 80% at 1–2 h after the first PB extrusion, 92.3% at 2–4 h, 82.1% at 4–6 h and 85% at 6–8 h. However, only 15.8% (6/38) of the oocytes that were injected within 1 h after the first PB extrusion were fertilized. After fertilization, there was no significant difference in embryo development between groups (Table 1).

To examine whether appropriate ICSI timing for human oocytes can be determined by spindle location viewed using the Polscope, spindle location was assessed according to the time lapsed after the first PB extrusion of MI stage oocytes ($n = 20$) matured *in vitro*. Figure 1 illustrates the spindle location in oocytes during the IVM process. Figure 1A showed an oocyte at MI stage after 24 h culture. Figure 1B

Table 1: The fertilization and good embryo[a] development rates after ICSI of IVM MI → first PB and IVM (control) human oocytes in HCG-primed IVM cycles

Oocytes	ICSI time post-first PB extrusion	Total no. of oocytes	2PN (%)	No. of good quality embryos (%)
MI → first PB	<1 h	38	6 (15.8)*	2 (33.3)
	1–2 h	30	24 (80.0)	8 (33.3)
	2–4 h	26	24 (92.3)	12 (50.0)
	4–6 h	28	23 (82.1)	9 (39.1)
	6–8 h	40	34 (85.0)	14 (38.9)
Total	0–8 h	162	113 (69.8)	45 (39.8)
Control IVM MII at 24 h		389	319 (82.0)	137 (42.9)

*Significantly different at $P < 0.01$ compared with the other groups; [a]we defined embryos as good quality if they had at least six blastomeres, <20% anucleate fragments and no apparent morphologic abnormalities; PN, pronuclei.

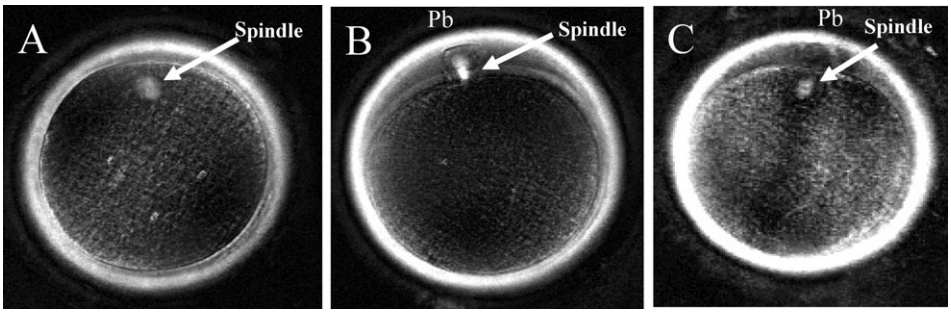

Figure 1: Bi-refringent spindles in living human oocytes imaged at maturation stage with the Polscope following IVM (**A**) MI oocyte had the spindle. (**B**) TI had the spindle located between cytoplasm and the PB. (**C**) MII oocyte had the spindle located under the first PB. Original magnification, ×200

and C illustrated oocytes that extruded the first PB. In Fig. 1B, there was a morphologically MII stage oocyte with the first PB, but the real maturational stage was that at telophase I (TI) since the spindle was located between cytoplasm and the first PB. Figure 1C was an oocyte at real MII stage that had the spindle beneath the first PB. All the oocytes ($n = 20$) that were observed within 1 h after the first PB extrusion showed spindle location similar to the one in Fig. 1B. After 1 h of the extrusion of the first PB, all the oocytes ($n = 20$) became real MII stage as shown in Fig. 1C.

A total of 150 embryos were transferred in 43 cycles, and 15 clinical pregnancies (34.9%, 15/43) and a 12.7% implantation rate (19/150) were established (including 4 twin pregnancies).

Discussion

The results of the present study indicate that human oocytes matured *in vitro* needed at least 1 h incubation after the first PB extrusion to obtain good fertilization. Live spindle imaging system can help decide the ICSI timing in oocytes matured *in vitro*.

When we analysed 311 HCG-primed IVM cycles that were performed over 2 years in our IVF center, the clinical pregnancy rate was significantly correlated with the number of normal fertilized embryos as follows: <6 (8.4%, 11/131), 6–10 (30.7%, 31/101) and >10 (54.4%, 43/79) (unpublished data). This could be due to the possibility of being able to choose better quality embryos to transfer in those cycles with higher numbers of embryos. Therefore, the number of embryos fertilized is an important factor to increase the chance of successful pregnancy in an IVM program as occurs for COH. Currently, ICSI has been considered as the best alternative to increase fertilization of IVM oocytes even in conditions where sperm parameters were considered normal, probably due to altered characteristics of the zona pellucida as a result of the longer culture time before insemination (Nagy *et al.*, 1996). By using ICSI, the fertilization rate of human IVM oocytes has been reported to be 70–80% (Mikkelsen *et al.*, 2000; Son *et al.*, 2006).

One of the characteristics in the human IVM cycles is that asynchronous maturation of the immature oocytes is observed following *in vitro* culture probably because of differences in follicle sizes at the time of collection. In the present study, 43.2% (389/901) of cultured oocytes were mature after 24 h and 18.0% more were able to mature on that same day. Thus, 61.2% of the oocytes were at the MII stage by the end of the second day. In our previous study, the quality of embryos produced from matured oocytes on day 1 (after 24–32 h culture) was significantly higher than that of embryos obtained from day 2 (after 48–52 h culture) (Son *et al.*, 2005). However, the capacity for normal development of embryos fertilized from oocytes that were matured at different times in the same day has not been well documented. The present study indicates that IVM MI oocytes (24–32 h), if fertilized, display a similar developmental potential to their sibling MII oocytes at denudation after 24 h culture. Therefore, it is important to increase the number of embryos from oocytes that were matured from MI stage at denudation after 24 h culture on day 1 so that the number of embryos that are available for transfer can be increased, which may potentially increase the pregnancy rate.

The fertilization and good-quality embryo rates of human oocytes were considerably enhanced when insemination was delayed for >3 h after oocyte retrieval in COH cycles (Rienzi *et al*, 1998). Furthermore, experiments on immature oocytes obtained from COH cycles indicated that the IVM oocytes needed at least 3 h before insemination to obtain reasonable fertilization rates (61%) in comparison to those recorded for the oocytes matured *in vivo* (77%) (Balakier *et al.*, 2004). Based on those results, they have reported that the cytoplasm of freshly matured oocytes remained immature and required additional changes to support fertilization and further development.

Meanwhile, it was shown that IVM oocytes generated from COH cycles were sensitive to post-maturation aging, and delayed sperm injection resulted in a high incidence of one pronucleus, pronucleus size asynchrony and cleavage failure (Goud *et al.*, 1999). Thus, the authors suggested that the timing for ICSI is critical for optimum fertilization of IVM oocytes. Nevertheless, in some early studies, oocytes were inseminated 48 or 56 h after maturation in human IVM cycles (Trounson *et al.*, 1994; Russell *et al.*, 1997; Cha *et al.*, 2005). Thus, the oocytes would be arrested at MII stage for 20–30 h before insemination if we consider that oocytes were matured on day 1. Therefore, the developmental competence of oocytes arrested for a long time may be compromised, resulting in low success. Thus, defining the optimal interval between the first PB extrusion and ICSI is crucial to increase

fertilization and embryo development rates especially in human IVM program.

Our detailed analysis of the data regarding the time interval needed between the first PB extrusion and performance of ICSI in our IVM culture system shows that the vast majority of IVM MI oocytes injected within 1 h after PB extrusion remained unfertilized (84.2%), whereas 84.7% (105/124) fertilization was obtained when oocytes were injected after 1 h of extrusion of the first PB, which was comparable with those recorded for the control group of oocytes (82.0%). Our results are different from those reported by Balakier *et al*. (2004), where the authors examined the fertilization and development according to ICSI timing in oocytes matured *in vivo* or *in vitro* in COH cycles, as discussed before. They concluded that the human oocytes progressively develop the ability for full activation and normal development during the MII arrest stage and a minimum of 3 h is needed to obtain reasonable fertilization and development rates. This difference in the results could be due to the nature of the oocytes used in each study. Balakier *et al*. (2004) studied the MI oocytes generated from COH cycles, whereas we used immature oocytes from unstimulated IVM program. In COH cycles, the maturation process is initiated *in vivo* and completed *in vitro*, whereas in unstimulated cycles the whole maturation process takes place *in vitro*. There seems to be an important difference between the capabilities of immature oocytes that have been recovered following an *in vivo* stimulus for maturation and those that have truly been matured *in vitro*. In addition, oocytes which are at MII stage at retrieval in COH cycles can produce better quality embryos than sibling immature oocytes. Another possible explanation could be the different culture conditions used for obtaining the mature oocytes.

As the oocytes progress into MI, a meiotic spindle is formed and chromosomes are aligned at the equator of the spindle. After that, the oocytes undergo anaphase I and TI stages, and finally the first PB with a set of chromosomes is released from the oocytes and the oocytes progress to MII stage. The MI spindle usually forms centrally in the oocytes and then migrates to cortex just before PB extrusion while the MII spindle is located at the cortex of the oocytes (Wassarman and Fujiwara, 1978). Until now, most scientists believed that nuclear maturation of the oocytes could easily be evaluated by the presence of the first PB and an additional period could be needed for cytoplasmic maturation. However, analysis of our data on spindle location obtained from IVM oocytes using Polscope provides good evidence that nuclear maturation was not complete even within 1 h after the first PB extrusion. All oocytes within 1 h after the first PB extrusion showed the presence of meiotic spindle located between cytoplasm and first PB (Fig. 1b), implying that the real nuclear stage of the oocyte was TI, not MII. Using Polscope microscopy, several authors demonstrated the presence of a spindle bridge between the first PB and the cytoplasm in oocytes collected from COH cycles. (Eichenlaub-Ritter *et al.*, 2002; De Santis *et al.*, 2005; Montag *et al.*, 2006). It is well documented that oocytes at TI stage are just in a transition from MI to MII. In the present study, all the oocytes at TI stage had the first PB with no morphological difference to those at MII stage although these TI oocytes formed an MII spindle after 1 more hour in culture. Therefore, based on current data, completion of nuclear maturation is also important to achieve fertilization and embryo development successfully and it needs a minimum of 1 h after the first PB extrusion under our IVM culture conditions. In contrast, Montag *et al*. (2006) reported that formation of the MII meiotic spindle that appeared underneath the first PB took place ~115–150 min after the first PB extrusion in three MI oocytes collected from COH cycles (Montag *et al.*, 2006). This discrepancy with our results could be due to the nature of the oocytes and/or different culture conditions that were used for studies. It is important to note that completion of nuclear maturation after the first PB extrusion will depend on the source of immature oocytes as well as on each IVM culture system.

We observed that the optimal time for ICSI was 2–4 h after the first PB extrusion, based on fertilization and embryo quality, although the numbers of oocytes in this study were too small to detect any possible significant differences. The explanation for this observation could be that IVM oocytes need more time to achieve cytoplasmic maturation even when nuclear maturation is completed. Our observations are important, especially when few oocytes are available, in order to increase the number of embryos available for transfer or cryopreservation.

In conclusion, final nuclear maturation of human first PB-extruded oocytes is crucial for the acquisition of the ability to undergo normal fertilization and embryo development. In our culture system, human IVM oocytes need at least 1 h after the first PB extrusion to complete nuclear maturation. Live spindle imaging by Polscope in living human oocytes can predict exact nuclear stage that can be used to determine the correct timing to perform ICSI in IVM oocytes in IVM cycles. It is important to continue to find ways to improve both clinical and laboratory procedures to make the use of immature oocytes more efficient.

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