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Fertilization, cleavage and blastocyst development according to the maturation timing of oocytes in *in vitro* maturation cycles*

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BACKGROUND: This study was to examine the developmental capacity of oocytes collected from an *in vitro* maturation (IVM) programme according to their maturation time. METHODS: The study included 47 IVM cycles that underwent blastocyst transfer. The patients ($n = 38$) were primed with 10 000 IU HCG 36 h before their oocyte retrieval. The oocytes were classified into three groups: group 1 ($n = 139$) where oocytes were matured on day of oocyte collection; group 2 ($n = 627$) where oocytes were matured on day 1 after IVM; group 3 ($n = 163$) where oocytes matured on day 2 after IVM. Fertilization, cleavage and blastocyst formation were compared between three groups. RESULTS: Rates of cleavage and blastocyst development in group 3 (72.2%, 96/133; 19.0%, 15/133) were significantly lower than those of group 1 (100%, 108/108; 58.3%, 63/108) and group 2 (91.5%, 487/532; 50.4%, 268/532) respectively ($P < 0.01$). The number of freezable good quality blastocysts among blastocysts developed from group 1 (52.4%, 33/63) was significantly higher than those from group 2 (35.4%, 95/268) and group 3 (6.7%, 1/15) ($P < 0.01$). There were 24 clinical pregnancies (51.1%, 24/47) after transfer of the blastocysts and 29 healthy babies were delivered. CONCLUSION: These results suggest that oocytes reaching metaphase II faster in an IVM programme have better embryonic developmental competence.

Key words: blastocyst/HCG/immature oocytes/IVM/maturation time

Introduction

Controlled ovarian stimulation (COS) is used to achieve multifollicular recruitment, enabling an increased number of embryos to be transferred. However, there are disadvantages associated with COS. In comparison with COS, the major benefits of *in vitro* maturation (IVM) treatment include avoidance of the risk of ovarian hyperstimulation syndrome (OHSS), reduced cost, and less complicated treatment. Recently, knowledge regarding IVM of immature human oocytes and its clinical application has accumulated. However, the quality of maturation appears to be suboptimal, frequently showing retarded cleavage and blockage of the development of the *in vitro*-matured oocytes (Barnes *et al*., 1996; Trounson *et al*., 1998), which may be related to the poor pregnancy outcomes.

To overcome these problems, several authors have attempted IVM of oocytes retrieved from ovaries exposed to gonadotrophin stimulation prior to oocyte collection. Chian *et al*. (2000) reported that higher rates of oocyte maturation and pregnancies were achieved in patients with PCOS by HCG priming. They also observed that the oocyte maturation was hastened by HCG priming. In addition, Son *et al*. (2002a,b) observed that if mature oocytes could be collected at the time of oocyte collection by the HCG priming in IVM cycles, clinical pregnancy could be established by the transfer of blastocysts derived from these mature oocytes.

Previous studies in humans reported that ~80% of immature oocytes show nuclear maturation (extrusion of a polar body) and will be at metaphase II (MII) by 48–54 h of culture (Trounson *et al*., 1994; Russell *et al*., 1997). However, a considerable asynchrony of maturation has been observed, and our IVM study without hormonal priming showed that ≥40% of the oocytes will be at MII after 24 h culture (Yoon *et al*., 2001a). However, the developmental capacity of oocytes according to the IVM time required to reach MII stage in an IVM cycle has not been clearly analysed. Therefore, this study was performed to compare the fertilization, cleavage, and the embryonic development to the blastocyst stage between oocytes matured *in vivo* and oocytes matured after culture in HCG-primed IVM cycles.

Materials and methods

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

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Patients

This study was conducted from June 2001 to June 2002. During this period, a total of 178 women went through 200 cycles with immature oocyte retrieval. Only those patients who had a risk of ovarian hyperstimulation in previous IVF cycles were recruited. Out of 200 IVM cycles, 57 cycles were transferred at the blastocyst stage during this study. Of these, 10 cycles were not included in this study because there were no MII stage oocytes on the day of oocyte recovery. A total of 38 patients (mean age: 33.3±2.8 years) underwent 47 cycles in which immature oocytes were recovered and transferred at the blastocyst stage. Patients had the following types of infertility: polycystic ovary (PCO) (n = 23), unexplained (n = 4), male (n = 7), and tubal factor (n = 4).

Oocyte recovery

The oocytes were collected between cycle days 7 and 16 based on the patient's cycle length and endometrium thickness of >6 mm. The patients were given 10 000 IU of HCG (IVF-C, LG Chemical, Korea) 36 h before oocyte retrieval. A transvaginal ultrasound machine 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 containing heparinized Ham's F-10 medium that contained bicarbonate and HEPES buffers. Follicular aspirates were filtered (70 mm mesh size, Falcon 1060; Life Technologies) and washed by the addition of copious medium to filtrate. The filtrate was further washed with medium by vigorous pipetting using 10 ml serological pipette (Becton Dickinson & Co., NJ, USA) to remove erythrocytes and small cellular debris. The retained cells were then resuspended in the medium. The oocytes were isolated under a stereomicroscope and washed twice in the same medium.

In vitro *maturation*

After collection, oocyte maturity was evaluated under the microscope with high magnification using the sliding method, and the oocytes that did not have a germinal vesicle (GV) were checked for maturity by denuding the cumulus cells with hyaluronidase. Immature oocytes were cultured in maturation medium, consisting of YS medium with 30% human follicular fluid (hFF) supplemented with 1 IU/ml FSH, 10 IU/ml HCG and 10 ng/ml rhEGF (Daewoong Pharmaceutical Co., Korea) (Son *et al.*, 2002a). The hFF was prepared using the method reported by Chi *et al*. (1998). The oocytes were cultured in IVM medium at 37°C in 5% CO_2, 5% O_2 and 90% N_2. Oocytes that reached the MII stage were classified into three groups according to the culture time needed for maturation: group 1 contained oocytes that were at the MII stage on the day of oocyte collection (*in vivo*-matured); group 2 contained oocytes that matured *in vitro* on day 1 (after 24–30 h culture); group 3 included oocytes that reached the MII stage on day 2 (after 48–52 h culture).

IVF, blastocyst development and embryo transfer

ICSI was used to fertilize the mature oocytes in each group. Fertilization was assessed 17–19 h after insemination for the appearance of two distinct pronuclei and two polar bodies. The zygotes were co-cultured with cumulus cells in 10 µl YS medium supplemented with 10% hFF (Yoon *et al.*, 2001b). The cumulus cells for co-culture were retrieved from matured oocytes at the time of collection and prepared as described previously (Yoon *et al.*, 2001b). Embryos were transferred at the blastocyst stage on day 6 after oocyte retrieval. Blastocyst transfers were performed in patients (aged <40 years) who had more than seven zygotes and three or more good quality embryos on day 3 following oocyte collection. The remaining patients were allotted to day 4 transfer due to the possibility of not producing blastocyst stage embryos *in vitro*. The blastocyst development was evaluated in embryos derived from the three groups until day 6 following ICSI. The developed blastocysts were classified according to their degree of expansion reported previously by Cho *et al*. (2002). Briefly, early blastocyst (ErB) is <140 µm in diameter; early expanding blastocyst (EEB) is 140–160 µm in diameter; middle expanding blastocyst (MEB) is 161–180 µm in diameter: expanded blastocyst (EdB) is >180 µm in diameter. The blastocysts were assigned one of four grades: grade A, a clear inner cell mass (ICM) and trophectoderm cells; grade B, a clear ICM but poor trophectoderm development; grade C, a poor ICM but good trophectoderm cells; grade D, a poor ICM and poor trophectoderm cells. Before transfer, all embryos for each patient were pooled and selected for transfer. After the blastocyst transfer, surplus embryos were cultured, and only the embryos that developed to the expanded blastocyst stage (diameter is >160 µm and grade A, B) were cryopreserved by vitrification on electron microscope grids after artificial shrinkage (Son *et al*., 2003).

Endometrium preparation

For the preparation of the endometrium, estradiol valerate (Progynova; Schering, Berlin, Germany) 6 mg and Progest 100 mg were administered daily from the day after oocyte retrieval. Both medications were continued until either a negative pregnancy test or 9–10 weeks of pregnancy.

Statistical analysis

Differences between treatment groups in each experiment were compared using χ^2-test (Statistical Analysis System; SAS Institute, Cary, NC, USA).

Results

The total number of oocytes collected in the 47 IVM blastocyst transfer cycles was 1195. Table I compares the number of MII oocytes, fertilization, cleavage and blastocyst formation rates in the three groups. A total of 139 (11.6%) oocytes were mature on the day of oocyte aspiration (group 1). Fifty-two per cent (627/1195) of oocytes were mature on day 1 after IVM (group 2), and 14% (163/1195) of oocytes were mature on day 2

Table I. Comparison of fertilization, cleavage and blastocyst development in oocytes derived from three groups

Variable	Group 1	Group 2	Group 3
No. of oocytes matured (%)	139 (11.6)	627 (52.5)	163 (13.6)
No. of oocytes fertilized (%)	108 (77.7)	532 (84.8)	133 (81.6)
No. of oocytes cleaved (%)	108 (100)	487 (91.5)	96 (72.2)*
No. of embryos developed to blastocyst from 2PN (%)	63 (58.3)	268 (50.4)	15 (11.3)*

Group 1: oocytes matured by oocyte collection day; group 2: oocytes matured by day 1 of culture; group 3: oocytes matured by day 2 of culture.
*P < 0.01 compared with group 1 and 2.
2PN = two-pronucleus.

(group 3). The fertilization rate was similar between the three groups (group 1 = 77.7%; group 2 = 84.8%; group 3 = 81.6%). However, the cleavage rate in group 3 (72.2%, 96/133) was significantly lower than those of group 1 (100%, 108/108) and group 2 (91.5%, 487/532) ($P < 0.01$). The blastocyst formation rate in group 3 (11.3%, 15/133) was also significantly lower than those of group I (58.3%, 63/108) and group 2 (50.4%, 268/532) ($P < 0.01$). There were no significant differences in the fertilization, cleavage and blastocyst formation rates between groups 1 and 2. Table II summarizes the expansion degree and quality of the blastocyts formed from each group in more detail. The number of freezable good quality blastocysts that were >160 μm diameter and grade A, B among blastocysts developed from group 1 (52.4%, 33/63) was significantly higher than those from group 2 (35.4%, 95/268) and group 3 (6.7%, 1/15) ($P < 0.01$). Table III summarizes the clinical results. The mean number of oocytes collected from 47 cycles was 25.4 ± 10.8. The total maturation, fertilization, cleavage and blastocyst rates were 78.0% (929/1195), 83.2% (773/929), 89.4% (691/773) and 44.8 (346/773) respectively. After transfer of blastocysts, 24 clinical pregnancies (51.1%, 24/47) were established, which included four miscarriages and 20 term deliveries (including nine twin pregnancies). Birthweights of the infants were within the range of 1850–3500 g, and all delivered infants had a normal physical profile up to the present.

Table II. Detail in comparison of blastocyst formation derived from oocytes in groups 1, 2 and 3

Degree of blastocyst	Grade of blastocyst	Group 1	Group 2	Group 3
ErB	A, B (%)	2 (3.2)	13 (4.9)	1 (6.7)
	C, D (%)	3 (4.7)	19 (7.1)	3 (20.0)
EEB	A, B (%)	2 (3.2)	20 (7.5)	1 (6.7)
	C, D (%)	5 (7.9)	33 (12.3)	5 (33.3)
MEB	A, B (%)[a]	6 (9.5)*	37 (13.8)	1 (6.7)
	C, D (%)	9 (14.3)	26 (9.7)	1 (6.7)
EDB	A, B (%)[a]	27 (42.9)*	58 (21.6)	0 (0.0)
	C, D (%)	9 (14.3)	62 (23.1)	3 (20.0)
No. of blastocysts		63/108	268/532	15/133

[a]Freezable blastocysts which had >160 mm diameter and good quality.
*Significantly different at $P < 0.01$ compared with groups 1 and 2.
Group 1: oocytes matured by oocyte collection day; group 2: oocytes matured by day 1 of culture; group 3: oocytes matured by day 2 of culture.
Grade A: a clear inner cell mass (ICM) and trophectoderm cells; grade B: a clear ICM but poor trophectoderm; grade C: a poor ICM but good trophectoderm cells; grade D: a poor ICM and poor trophectoderm cells.
ErB = early blastocyst (<140 μm); EEB = early expanding blastocyst (140–160 μm); MEB = middle expanding blastocyst (161–180 μm); EdB = expanded blastocyst (>180 μm).

Table III. Clinical results for patients receiving blastocysts developed from oocytes retrieved from *in vitro* maturation cycles

Variable	Value
No. of cycles	47
No. of oocytes (mean ± SD)	1195 (25.4 ± 10.8)
No. of oocytes matured (%)	929 (78.0)
No. of oocytes fertilized (%)	773 (83.2)
No. of oocytes cleaved (%)	691 (89.4)
No. of embryos developed to blastocyst (%)	346 (44.8)
No. of blastocysts transferred (mean)	136 (2.9)
No. of implantations (%)	36 (26.4)
No. of clinical pregnancies (%)	24 (51.1)

Discussion

This study demonstrates that developmental capacity of oocytes collected following HCG-priming in an IVM programme is correlated with their maturation time.

Among several factors affecting the success of IVM until now, the number of oocytes retrieved is the most important for pregnancy success (Paulson *et al*., 1994; Barnes *et al*., 1996; Russell *et al*., 1997; Child *et al*., 2001). Therefore, the success rate of pregnancy following IVM in women with a regular menstrual cycle has been low in general (Paulson *et al*., 1994). We also previously obtained only a non-satisfactory clinical pregnancy rate of 17.6% from IVM cycles of women with regular menstrual cycle (Yoon *et al*., 2001a). Therefore, we have performed IVM cycles selectively in women with high risk of OHSS. In this study, the matured oocytes were divided into three groups according to the length of time needed to reach MII stage and we have compared the developmental capacity between embryos derived from these oocytes. Because the ability to develop to blastocyst stage is a good indicator of developmental capacity of oocytes, we only assessed the blastocyst transfer cases in our IVM cycles. However, we did not include typical anovulatory PCOS patients in this study because the patients rarely have a leading follicle of >9 mm in diameter at the time of immature oocyte recovery. Consequently it was hard to obtain an MII stage oocyte on the day of oocyte collection.

A significant relationship was observed between embryo developmental potential and the length of time to reach MII. The oocytes matured late *in vitro* (day 2) had a significantly higher blockage of cleavage at the pronuclear (PN) stage compared with oocytes matured *in vivo* and on day 1. In addition, the rate of blastocyst formation from 2PN in oocytes matured on day of oocyte retrieval (day 0) and day 1 was significantly higher than that of oocytes that matured late (day 2) (day 0 = 58.3%; day 1 = 50.4%; day 2 = 11.3%). There was no significant difference in blastocyst development between group 1 and group 2. These results imply that the immature oocytes retrieved from cohort small follicles have viability even though mature oocytes were collected from leading follicles. Actually, of the five patients that had two blastocysts transferred from groups 1 and 2, each had a twin pregnancy. Three patients were transferred blastocysts derived from only group 2 and had a singleton pregnancy. Russell (1998) reported a marked decrease in the rates of maturation, fertilization and transfer among cycles in which immature oocytes were retrieved when a dominant follicle of ≥14 mm was present at the time of retrieval. In our study, the leading follicles were 11–13 mm in diameter at the time of oocyte collection. This study therefore demonstrates that the developmental competence of immature oocytes may not be detrimentally affected by the presence of <14 mm dominant follicles during the follicular phase.

However, the rate of freezable good quality blastocyts in group 1 (52.4%, 33/63) was higher than that of group 2 (35.4%,

95/268). These results indicate that the *in vitro* culture system adequately supports nuclear maturation in human oocytes following IVM but is still incomplete to produce oocytes with cytoplasmic competence, thereby resulting in embryos with reduced developmental potential in oocytes which had matured *in vitro*, especially late matured oocytes. Cleavage and development will depend on the establishment of M-phase promoting factor and associated cyclins in the correct sequence of activation for syngamy, cleavage, and mitosis (Barnes *et al*., 1996). Therefore, low developmental competence of embryos derived from oocytes matured slowly might be due to the loss of M-phase promoting factor activity, cyclin production, and other proteins controlling the cell cycle. Another possible explanation could be a wide variation in M-phase promoting factor stability in *in vitro*-matured human oocytes by the time of maturation. Therefore, the decreased blastocyst development of the zygotes derived from late matured oocytes in this study may reflect abnormalities of cytoplasmic maturation.

The asynchrony in maturation time *in vitro* may be due to intrinsic differences in oocytes recovered from various sized follicles *in vivo*. Follicle size is known to have an influence on the developmental competence of mice and cattle oocytes (Eppig *et al*., 1992; Pavlok *et al*., 1992; Lonergan *et al*., 1994). Also human oocytes appear to have a follicle size-dependent ability to resume meiosis and complete maturation in unstimulated oocytes (Durinzi *et al*., 1995). Tsuji *et al*. (1985) reported that maturation rate of oocytes from small follicles (3–4 mm) was decreased compared with that from larger follicles (9–15 mm). Embryo cleavage rates were reported to be significantly decreased or not significantly different for oocytes obtained from follicles <12 mm in diameter (Haines and Emes, 1991; Wittmaack *et al*., 1994). Although we were unable to compare the maturation rate between oocytes retrieved from various sizes of follicles because we did not know which oocyte was from which follicle, embryo cleavage rate in embryos derived from oocytes that matured late (day 2) was significantly lower, implying that the late-maturing oocytes were from small follicles. Therefore, it could be speculated that the various sizes of follicles were presented in ovaries of patients undergoing IVM cycles and the recovery of oocytes from smaller follicles may provide slow maturation and incomplete developmental competence. Further studies to clarify the correlation of follicular size, maturation and developmental capacity of oocytes in IVM programmes are necessary but probably will only be undertaken satisfactorily if the follicles were dissected from ovariectomy specimens, to be certain of the follicular origins of oocytes recovered.

In conclusion, our results suggest that the maturation time of oocytes plays a predictive role in the cleavage and blastocyst development of the oocytes recovered in HCG-stimulated IVM cycles, and may be a relevant parameter in the advances in technology for oocyte development.

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