Human Oocyte Maturity In Vivo Determines the Outcome of Blastocyst Development In Vitro

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Purpose: To date, the impact of oocyte maturity at aspiration on the blastocyst formation in vitro has not been fully evaluated. This study was undertaken to assess the influence of oocyte maturity in patients undergoing in vitro fertilization and blastocyst transfer program.

Methods: A total of 1278 oocytes derived from 147-IVF cycles were retrospectively analyzed. Oocyte maturity was graded on a scale from 1 to 5 based on the morphology of the ooplasm, cumulus mass, corona radiata, and membrana granulosa cells.

Results: Mature oocytes yielded the highest fertilization rates. Although the cleavage rates were similar in both groups, the percentage of poor morphology, day-3 embryos from the nonmature-oocyte group was significantly higher than from the mature-oocyte group (54.7% vs. 15.5%, $P < 0.001$). Although good morphology, day-3 embryos were collected from nonmature oocytes, the incidence of these embryos developing to the blastocyst stage was significantly less than from mature oocytes (33.3% vs. 71.2%, $P < 0.001$). Although blastocyst stage embryos were collected from nonmature oocytes, the incidence of these embryos developing to the top-scoring blastocysts was significantly less than from mature oocytes (58.3% vs. 89.5%, $P < 0.001$).

Conclusions: These phenomena suggest that oocyte maturity produced in vivo determine the fertilization potential and subsequent blastocyst quality in vitro.

KEY WORDS: Blastocyst development; corona radiata; oocyte maturity; zona pellucida.

INTRODUCTION

Multiple factors are important in influencing the pregnancy rate of patients undergoing assisted reproductive technology (ART) procedures. These factors include the etiology of infertility, age of the patient, type of ovarian stimulation, follicular phase estradiol (E2) levels, number of oocytes collected, and number

of oocytes fertilized (1–3). These factors affect the number of embryos that develop and, subsequently, the number of embryos transferred. The number and quality of embryos transferred appears to be the most important factor influencing the pregnancy rate.

Multiple investigators have examined various stimulation protocols to determine which protocol is superior. Differences in the number of follicles developing, number of oocytes collected, rates of fertilization, cleavage, and pregnancy are reported (4–8). Several investigators also tried to predict the outcome of ART before or during the controlled ovarian hyperstimulation (9–15). There were also many investigations that tried to increase the embryo quality in vitro irrespective of the oocyte maturity. These included

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immature oocyte culture with supplements and extended embryo culture for the embryo (16–19). Regardless of the method, the final purpose was to get more mature oocytes and, subsequently, good embryo development in vitro for embryo transfer.

To date, the impact of oocyte maturity at aspiration undergoing in vitro fertilization (IVF) procedures on in vitro blastocyst formation has not been fully evaluated. The purpose of this study was to analyze survival of human oocytes, fertilization potential, and subsequent embryo development, using extended embryo culture and assisted hatching, with respect to different maturities of oocytes in the IVF programs.

MATERIALS AND METHODS

One hundred forty-seven consecutive patients admitted to the IVF program at Chang Gung Medical Center in Kaohsiung, between March 1999 and June 2001, who subsequently underwent blastocyst transfer, were included in this study. In IVF program, infertility was due to tubal factor (48%), endometriosis (13%), unexplained (23%), and multiple factors (16%). The ages of the patients ranged from 20 to 43 years.

Sperm concentration and motility were evaluated according to the recommendations of the World Health Organization (20). Kruger's strict criteria were used for the assessment of sperm morphology (21). Semen analysis was done at least once before the treatment cycle to determine whether enough spermatozoa were present in the ejaculate to perform IVF.

Ovarian stimulation was performed as previously described (22). Briefly, all patients underwent a long stimulation protocol of GnRH agonist therapy followed by follicle stimulating hormone (FSH) administration and transvaginal oocyte retrieval. Once adequate follicular maturation was obtained, human chorionic gonadotropin (10,000 IU; pregnyl; N.V. Organon, Oss, Netherlands) was administered, and oocyte retrieval was performed 35–37 h later by transvaginal aspiration with ultrasound guidance. Follicular fluids and follicular washes were examined microscopically with a dissecting stereo microscope situated inside an isolette with a controlled, humidified atmosphere of 37°C and 5% CO₂.

Oocytes were graded for maturity (from 1 to 5) on the basis of the morphological characteristics of the cumulus mass, corona radiata, ooplasm, and detached membrana granulosa cells. Grade 1 (mature or

preovulatory) oocytes exhibited a very expanded cumulus and a very radiant corona, revealing a distinct zona pellucida. The ooplasm was clear and the membrana granulosa cells were expanded and well aggregated. Grade 2 (approximately mature) oocytes exhibited an expanded cumulus mass and a slightly compact corona radiata. The membrana granulosa cells were expanded and well aggregated. Grade 3 (immature) oocytes exhibited a dense, compact cumulus, if present, and a very adherent, compact layer of corona cells. The ooplasm, if visible, revealed the presence of the germinal vesicle. The membrana granulosa cells were compact and nonaggregated. Grade 4 (postmature) oocytes exhibited a very expanded cumulus often having clumps. The corona radiata was radiant, yet often clumped and irregular or incomplete. The zona pellucida was very visible and the ooplasm either slightly granular or dark. The membrana granulosa cells were small and relatively nonaggregated. Grade 5 (atretic) oocytes rarely had an associated cumulus mass. The corona radiata, if present, was clumped and very irregular. The ooplasm was dark and frequently misshapen and the zona pellucida very visible. The membrana granulosa cells were very small clumps of cells. For the outcome analysis in the study, the oocytes were classified into two groups. Grade 1 oocytes were included in Group I (mature oocyte). Group II (nonmature oocyte) consisted of grade 2–5 oocytes.

The procedure of sperm insemination in IVF cycles was performed according to our routine protocol. In brief, the oocyte–corona–cumulus complexes were cultured in IVF medium (Medicult, Denmark) 4–6 h after retrieval in the four-well multidishes (Nunc, Roskilde, Denmark) before insemination. Following the incubation, oocytes were inseminated with spermatozoa to a concentration of 2×10^5 motile spermatozoa/mL in 1 mL of IVF medium. Then, oocytes were withdrawn from the insemination medium after a 2 h exposure to spermatozoa. Each oocyte was cultured in fresh IVF medium in 4-well culture dish again.

After completion of the insemination procedures, the oocytes were cultured according to our standardized IVF procedure (22). They were assessed for the presence of pronuclei after 16–18 h of incubation. Fertilization was considered have occurred when two clear pronuclei were present. If only one pronucleus was observed, a second evaluation was performed 4 h later to determine whether the pronuclear status had changed. The developmental competence of zygotes with two-pronuclei was evaluated after a further 96 h of in vitro culture.

Embryos were cultured in a similar fashion to the standard IVF protocol with the primary exception of the type of media and the duration of culture. From Day 1 postfertilization the embryos were placed in G1.2 medium (IVF Science, Gothenburg, Sweden), followed by G2.2 (IVF Science) for days 3–6. The cleaved embryos were classified according to the morphologic grading system described by Veeck (23). The blastocysts were assigned a score according to the system of Gardner and Schoolcraft (24). In brief, blastocysts were given a numerical score from 1 to 6 according to their degree of expanding and hatching status: 1, an early blastocyst with a blastocoele that is less than half of the volume of the whole embryo; 2, a blastocyst with a blastocoele that is half or greater than half of the volume of the whole embryo; 3, a full blastocyst with a blastocoele that completely fills the whole embryo; 4, an expanded blastocyst with a blastocoele volume larger than that of the early blastocyst, with a thinning zona pellucida; 5, a hatching blastocyst with the trophoctoderm starting to herniate through the zona pellucida; and 6, a hatched blastocyst, in which the embryo has completely escaped from the zona pellucida. For blastocysts scored as 3–6, the development of the inner cell mass was assessed as: A, tightly packed, many cells; B, loosely grouped, several cells; or C, very few cells. The trophoctoderm was assessed as: A, many cells forming a cohesive epithelium; B, few cells forming a loose epithelium; or C, very few large cells forming an epithelium. Top-scoring blastocysts mean blastocysts with scores of 3AA, 4AA, or 5AA.

The zona pellucida was thinning and opened with a 1.48 μm (Model RD001) diode laser system (Fertilase; MTM Medical Technologies Montreux SA, Clarens, Switzerland) coupled with an inverted microscope (Zeiss, Oberkochen, Germany) that was further equipped with a 40 $\times$ Hofman modulation contrast objective (Leica, Greenvale, New York) and a heated microscope stage. For the laser treatment of

eight-cell embryos, we used a single laser pulse of 5–10 ms that resulted in the one-third thinning around the circle of the embryo and gave an opening of 10–15 μm (Model RD001) in diameter that was drilled at a position within the thinning area of the zona pellucida (25).

The clinical data of the two groups, including fertilization rate, percentage of embryos in the various cell stages, cleavage rate, and grading of embryos were compared by chi-square test, Fisher's exact test, or Mantel-Maenzel test. A *P* value of <0.05 was considered statistically significant.

RESULTS

Overall, 1278 oocytes in 147-IVF cycles were collected. There were 15 immature oocytes, two post-mature oocytes, and one atretic oocyte in Group II. The fertilization ability and embryo development in vitro following fertilization are shown in Tables I and II respectively. The oocytes with normal fertilization were all approximately mature oocytes in Group II, whereas, the oocytes with abnormal fertilization included 35 approximately mature and 3 immature oocytes.

The normal fertilization rate was significantly higher in Group I than in Group II (98.3% vs. 65.7%, *P* < 0.001). However, the cleavage rates were similar between the two groups (97.6% vs. 96.8%, *P* = 0.43). Embryo development in vitro for the two groups is compared in Table II. Although the cleavage rate between the two groups was similar, the distribution of different-grade morphology of cleaving day-3 embryos were significantly different (*P* < 0.001). The percentage of good morphology (grades 1 and 2) embryos was significantly fewer in Group II oocytes (45.3% vs. 84.5%, *P* < 0.001). Furthermore, the number of blastocysts developed from the good morphology day-3 embryos in Group II oocytes (33.3%) was

Table I. Comparison of Fertilization Rates Between the Two Groups of Oocyte Maturities in IVF Patients

	Group I	Group II	<i>P</i>
No. of oocytes collected	809	469	
No. of oocytes with normal fertilization (%)	795 (98.3)	308 (65.7)	<0.001
No. of oocytes with abnormal fertilization (%)	1 (0.1)	36 (7.7)	
No. of oocytes with nonfertilization (%)	13 (1.6)	125 (26.6)	

Note. Group I: Oocytes that were mature at oocyte retrieval. Group II: Oocytes that were not mature at oocyte retrieval.

P value: by Fisher's exact test.

Table II. Comparison of Embryo Development In Vitro Between the Two Groups of Oocyte Maturities in IVF Patients

	Group I	Group II	P
No. of oocytes fertilized	795	308	
No. of zygotes cleaved following 3-day culture (%)	776 (97.6)	298 (96.8)	0.43
Embryo morphology of day-3 embryos			
No. of grade 1 embryos (%)	498 (64.2)	46 (15.4)	<0.001
No. of grade 2 embryos (%)	158 (20.3)	89 (29.9)	
No. of poor embryos (%)	120 (15.5)	163 (54.7)	
Blastocyst formation following 6-day culture			
No. of blastocysts from day-3 embryos (%)	484 (62.4)	48 (16.1)	<0.001
No. of blastocysts from grade 1 embryos (%)	418 (83.9)	19 (41.3)	<0.001
No. of blastocysts from grade 2 embryos (%)	49 (31.0)	26 (29.2)	0.77
No. of blastocysts from poor embryos (%)	17(14.2)	3 (1.8)	<0.001
Morphological grading of blastocysts			
No. of top-scoring blastocyst(%)	433/484 (89.5)	28/48 (58.3)	<0.001
No. of other-scoring blastocyst(%)	51/484 (10.5)	20/48 (41.7)	

Note. Group I/II: see Table I, footnote. Grade 1 embryos: ≥ 8 -cell embryos without fragments. Grade 2 embryos: ≥ 8 -cell embryos with less than 20% fragments. Poor embryos: day-3 embryos except grade 1 or 2 embryos. Top-scoring blastocyst: 3AA, 4AA, or 5AA by using the system of Gardner and Schoolcraft. P value: by chi-square test or Fisher's exact test.

also significantly fewer ($P < 0.001$) than in Group I oocytes (71.2%) following extended culture for 5 or 6 days. The distribution of morphologic quality of the blastocysts differed significantly between the two groups. The percentage of top-scoring blastocysts (3AA, 4AA, or 5AA) was significantly higher in Group I embryos (89.5% vs. 58.3%, $P < 0.001$).

DISCUSSION

Our data demonstrate that the maturity of the oocyte at aspiration affects the subsequent embryo development in vitro in terms of both oocyte fertilization and embryo grading. Although the cleavage rates were similar in both groups, the percentage of good morphology, day-3 embryos in the nonmature-oocyte group was significantly less than in the mature-oocyte group. Although the good morphology, day-3 embryos developed from fertilization of nonmature oocytes, the number of these embryos, developing to the blastocyst-stage was significantly fewer than those developing from fertilized mature oocytes. Although the good morphology, day-3 embryos developed from fertilization of immature oocytes, the number of these embryos, developing to the blastocyst stage was significantly fewer than those developing from fertilized mature oocytes. Although the blastocysts developed from fertilization of nonmature oocytes, the number of these embryos developing to the stage of top-scoring blastocyst was significantly fewer than those developing from fertilized mature oocytes. These

phenomena suggest that oocyte maturity in vivo determines the fertilization potential of oocyte and subsequent embryo quality in vitro.

ART is a complex and costly technique with stringent indications. Providing infertile couples with accurate information about their chances of pregnancy is a priority in all ART programs. Although many factors influence the success of ART, there is a general consensus that the key role is played by ovarian response to stimulation. The reproductive potential of a woman decreases with age and ovarian reserve (9). Assessing a woman's ovarian reserve before undertaking an ART protocol aids in predicting treatment outcome, provides important information for patients to decide whether to undergo infertility treatment, and allows physicians to individualize the treatment plan. Multiple investigators (4–8) examined various stimulation protocols to determine which protocol is superior. The purpose of a protocol is to get the most number of mature oocytes from controlled ovarian hyperstimulation. The more mature oocytes collected, the more good embryos develop for transfer. Nonetheless, there is still a portion of nonmature oocytes collected at aspiration following idealized controlled ovarian hyperstimulation (22,26–27). In Khan's study, 9% postmature, 90% mature (including slightly immature), and only 1% immature oocytes were retrieved in GnRH agonist/HMG stimulated cycles (28). In our study, 0.2% postmature, 98.4 mature (including approximately mature), and 1.4% immature oocytes were collected following the GnRH agonist/FSH stimulation. Khan *et al.* also demonstrated

that a delay of 2, 4, or 6 h in the insemination did not affect the fertilization and cleavage rates. Furthermore, the mature or slightly immature oocytes fertilized well when spermatozoa were added 2, 4, or 6 h after retrieval. However, the fertilization rate in the mature oocytes was different from the nonmature oocytes in our study. There were several differences in the methodologies between the two studies. First, the exposure time of sperm–oocyte interaction when insemination was different in the two studies, which were 2 h in our lab and 12–18 h in the Khan's study. Second, the protocol of controlled ovarian hyperstimulation was different, which was a long protocol of GnRH agonist/FSH stimulation in our study than in Khan's study. Third, in Khan's study, different maturation time (2, 4, or 6 h) of oocyte in vitro was used to compare the effect of maturation time on the fertilization rate and embryo outcome but in this study, maturation time (4–6 h) was used in all types of oocytes to investigate the effect of oocyte maturity in vivo on oocyte fertilization and subsequent embryo outcome in vitro. Maturation time (1–7 h) of oocyte in vitro does not affect the ART outcome in Jacobs's studies with a more than 12 h of sperm–oocyte interaction (29), which is comparable to Khan's results. Some studies have also shown that FSH is a better gonadotropin than HMG in ovarian stimulation, in terms of the number of oocytes in metaphase II (30–31). According to Gianaroli's studies (32), prolonged exposure time (16 h) of sperm–oocyte interaction may be detrimental in decreasing fertilization rate, human embryo viability, and subsequent pregnancy rate/implantation rate. These detrimental effects following the prolonged exposure of sperm–oocyte interaction may be due to reactive oxygen excess (ROS) (33). In the present study, reduced exposure time (2 h) of sperm–oocyte interaction was used, which was different from that (12–18 h) in Khan's study. Thus, the possible risk of ROS in the present study was less. Because of these different methodologies, we got a higher fertilization rate of mature oocytes and subsequent better embryo quality than other studies. However, the fertilization rate (~70%) of nonmature oocytes was not different in these studies, irrespective of different exposure time of sperm–oocyte interaction. In the summary of these studies, exposure time of sperm–oocyte interaction in insemination seems to be a major factor resulting in the different results in these studies. Excess ROS seems to affect the fertilization rate of mature oocytes but it does not change the outcome of nonmature oocytes in vitro. Whatever, it

still needs a further prospective study to demonstrate more clearly.

Improvements in oocyte culture and extended embryo culture could result in more mature oocytes and good embryos for transfer. The need to culture human embryos for longer periods of their development is becoming increasingly clear. Extended in vitro culture increases the degree of embryo selection, thus maximizing the choice of the best quality embryos for transfer and minimizing the need for transfer of greater numbers of embryos (17). However, blastocyst formation itself does not fully reflect the viability of the embryo, and not all blastocysts are of same quality (34). Experience in defining better blastocyst quality in terms of morphology and development rate is growing (35). Close attention to the cell count in the trophectoderm and inner cell mass, rate of blastocyst formation, and historical data on pronuclear and cleavage status in each embryo are all useful in discerning late-stage preimplantation embryo quality (36–38).

Various factors affect blastocyst formation and quality, including culture conditions, number of oocytes, maternal age, and male factor infertility (34). In addition, the quality of early-stage embryos can substantially influence rates of blastocyst formation (37). There are several studies regarding oocyte maturity in ART (34,39–41). In our previous studies, the maturity of human cumulus-free oocytes determines the potential viability of embryo in vitro (36,42). Nonetheless, none of these studies fully demonstrate the influence of oocyte maturity at aspiration in relation to blastocyst formation and quality. In the study, the maturity of oocytes at aspiration influenced the fertilization rate, early-stage embryo development, and subsequently, late-stage embryo development.

Selection of good quality blastocysts for embryo transfer is the key role in extended embryo culture. Currently, several predictors of blastocyst quality are used to select good quality blastocysts for transfer in clinical practice. These include blastocyst scoring, day-3 embryo morphology, cell number of day-3 embryos, and the pronuclear status of a blastocyst (36–38). Our previous studies have also shown that the Z-score alone or in combination with day-3 embryo morphology is useful in determining the most suitable number of embryos for transfer, and achieving the optimal chance of conception while reducing the risk of high-order multiple pregnancy (43). The maturity of oocytes is determined by nuclear and cytoplasmic maturation. Both nuclear and cytoplasmic maturation play important roles in achieving successful

fertilization and subsequent development (44–45). In cumulus-surrounding or cumulus-free oocytes, cytoplasmic maturation lags behind nuclear maturation (18–19,26). This means that maturity of the oocyte at aspiration does not equal nuclear and cytoplasmic maturation, and the difference between oocyte maturation at aspiration and oocyte maturation after aspiration is the difference in the degree of cytoplasmic maturation. Although the nonmature oocytes could reach maturation and subsequent fertilization in vitro culture, the cytoplasmic maturation seems to lag behind the mature oocytes at aspiration. According to the present data, good-score blastocysts can be developed from oocytes that are nonmature at aspiration. The quality of good-score blastocysts from the nonmature oocytes seems not be equal to the same score blastocysts from the mature oocytes. Thus, the criterion for blastocyst selection seems not to depend on only one predictor. Oocyte maturity at aspiration also needs to be considered for blastocyst quality and viability in clinical practice.

In conclusion, the maturity of the oocyte at aspiration is important for determining subsequent embryo quality. Although extended embryo culture in vitro can be used for collecting good-score blastocytes irrespective to oocyte maturity. Oocyte maturity at aspiration is an important factor for prediction of blastocyst quality and viability.

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