The Maturity of Human Cumulus-Free Oocytes is Positively Related to Blastocyst Development and Viability

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Purpose: We investigated whether the human oocyte maturity at the removal of cumulus/corona cells affects the embryo outcome in vitro.

Methods: A total of 620 oocytes, which subsequently underwent blastocyst culture, were included in this analysis. Oocytes that were in prophase or Metaphase I of meiosis at the removal of cumulus/corona cells were in Group I. Oocytes that were in Metaphase II at the removal of cumulus/corona cells were in Group II.

Results: Group I oocytes yielded the highest fertilization rates (96.3% vs. 77.1%, $P < 0.001$). The incidence of Group II oocytes developing to the blastocyst stage was significantly less than from Group I oocytes (38.1% vs. 86.1%, $P < 0.001$). The percentage of top-scoring blastocysts from Group I oocytes was higher than that of Group II oocytes (95.4% vs. 76.2%, $P < 0.001$).

Conclusions: Oocyte maturity at the removal of cumulus/corona cells needs to be considered in selecting good quality blastocysts for embryo transfer.

KEY WORDS: Blastocyst development; cumulus-free oocytes; fertilization rate; intracytoplasmic sperm injection.

INTRODUCTION

Multiple investigators have examined various stimulation protocols to determine which protocol might be superior. Differences in the number of follicles developing, the number of oocytes collected, the rate of fertilization, of cleavage, and of ET have been reported (1–4). Several investigators have also tried to predict the outcome of ART before or during the controlled ovarian hyperstimulation (5–8). There were also many investigations, which tried to increase the

embryo quality in vitro irrespective of the oocyte maturity. These included immature oocyte culture with supplements and extended embryo culture for the embryo (9–12). Whatever, the final purpose was to get more mature oocytes, and subsequent good embryo development in vitro to transfer.

In in-vitro fertilization (IVF) programs, the oocyte–cumulus complex is assessed to determine the oocyte maturity. Prior to intracytoplasmic sperm injection (ICSI), oocytes must be separated from their surrounding cumulus/corona cells within a few hours after retrieval, and this creates an opportunity to study in detail the meiotic progression of cumulus-free oocytes in vitro. The timing of meiotic progression of oocytes can be assessed more accurately, and the mechanisms of maturation and early embryo development can be studied in greater detail through these cumulus-free oocytes. However, nuclear maturity, i.e., resumption and progression of meiosis to stage II metaphase (MII), cannot be used as a sole

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determinant of an oocyte's developmental competence (13). Extensive changes in protein synthesis and posttranslational modifications take place simultaneously with nuclear maturation (14). These changes have been referred to as cytoplasmic maturation and are thought to determine an oocyte's developmental competence, although they have not been elucidated in any species.

To date, the impact of oocyte maturity at the removal of cumulus/corona cells on the blastocyst development in vitro has not been fully evaluated. The purpose of this study was designed to analyze survival of human cumulus-free oocyte, fertilization potential, and subsequent blastocyst development, using the extended embryo culture with respect to different maturity of oocytes in ICSI programs.

MATERIALS AND METHODS

Seventy-four consecutive patients admitted to the intracytoplasmic sperm injection (ICSI) program at Chang Gung Medical Center in Kaohsiung, between March 1999 and June 2001, who subsequently underwent blastocyst transfer, were included in this study. There were 100 cycles in ICSI program. They included male factor (85%) and nonmale factor (15%). The age of these patients ranged from 20 to 43 years. Sperm concentration and motility were evaluated according to the recommendations of the World Health Organization (15). Kruger's strict criteria were used for the assessment of sperm morphology (16). Semen analysis was done at least once before the treatment cycle in order to determine whether enough spermatozoa were present in the ejaculate to perform ICSI or in-vitro fertilization. Patients with azoospermia underwent testicular sperm extraction (TESE) according to the protocol described previously (17,18). The method for cryopreservation of sperm extracted from the testicles was as described previously (17).

Ovarian stimulation was performed as previously described (8). Briefly, all patients underwent a long stimulation protocol of GnRH agonist therapy followed by FSH or r-FSH administration and transvaginal oocyte retrieval. Once adequate follicular maturation had been obtained, human chorionic gonadotropin (10,000 IU; Organon) 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 having a controlled, humidified atmosphere of 37°C

and 5% CO₂. In ICSI program, the oocytes were exposed to 80 IU/mL hyaluronidase (Type VIII; Sigma Chemical, St Louis, MO, USA) for 5–6 s. The surrounding cumulus cells were stripped from oocytes by aspirating through a series of pipettes with decreasing inner diameters (220, 200, 180, and 160 μ m), in 100- μ L droplets of human tubal fluid (HTF) medium. The denuded oocytes were classified into two groups according to the developmental stage. Group I consisted of oocytes that were in Metaphase II (MII) at the removal of cumulus/corona cells, while Group II consisted of oocytes that were in prophase or Metaphase I (MI). The denuded oocytes of both groups were cultured in MII culture medium (Medicult, Copenhagen, Denmark) for 3–8 h. The Group I oocytes were injected with sperm 3–4 h after retrieval. Group II oocytes were examined every 2 h, and ICSI was performed immediately after appearance of the first polar body (MII Stage), which occurred between 4 and 8 h after oocyte retrieval. Oocytes that did not develop to MII within 8 h of incubation were discarded. The procedure of sperm-injection in the ICSI cycle was based on a modified protocol as previously described (19).

After completion of the ICSI procedures, the oocytes were cultured according to our standardized in-vitro-culture procedure (20). 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 are cultured in a similar fashion to the standard protocol with the primary exception of the type of media and the duration of culture. Briefly, from Day 1 postfertilization the embryos are placed in G1.2TM media, followed by G2.2TM for Days 3–6. The cleaved embryos were classified according to the morphologic grading system described by Veeck (21). The blastocysts were assigned a score according to the system of Gardner *et al.* (22). In brief, blastocysts were given a numerical score from 1 to 6 according their degree of expanding and hatching status, as follows: 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

Table I. Comparison of Fertilization Rates and Embryo Development In Vitro Between the Two Groups of Cumulus-Free Oocytes in ICSI Patients

	Group I	Group II	<i>P</i>
No. of oocytes collected	434	186	
No. of mature oocytes injected (%)	431 (99.3)	153 (82.3)	<0.001
Total no. of injected sperm	431	186	
No. of fresh ejaculated sperm (%)	317 (73.5)	135 (72.6)	0.79
No. of thawed testicular sperm (%)	114 (26.5)	51 (27.4)	
No. of oocytes with normal fertilization (%)	415 (96.3)	118 (77.1)	<0.001
No. of oocytes with abnormal fertilization (%)	0 (0)	3 (2.0)	
No. of oocytes with nonfertilization (%)	16 (3.7)	32 (20.9)	
No. of oocytes with normal fertilization	415	118	
No. of zygotes cleaved following 3-day culture (%)	403 (97.1)	115 (97.5)	0.99
Embryo grading			
No. of embryos with Grade 1 embryos (%)	223 (55.4)	21 (18.3)	<0.001
No. of embryos with Grade 2 embryos (%)	88 (21.8)	40 (34.8)	
No. of embryos with poor embryos (%)	92 (22.8)	53 (46.9)	
Blastocyst formation following 6-day culture			
No. of blastocysts from Day-3 embryos (%)	219 (54.3)	21 (18.4)	<0.001
No. of blastocysts from Grade 1 embryos (%)	192 (86.1)	8 (38.1)	<0.001
No. of blastocysts from Grade 2 embryos (%)	23 (26.1)	11 (27.5)	0.87
No. of blastocysts from poor embryos (%)	4 (4.4)	2 (3.7)	0.99

Note. Group I: Oocytes in Metaphase II at the removal of cumulus/corona cells; Group II: Oocytes in prophase or Metaphase I at the removal of cumulus/corona cells; 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; *P* value: By chi-square test or Fisher's exact test.

larger than that of the early blastocyst, with a thinning zona pellucida; 5) a hatching blastocyst with the trophectoderm 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 follows: A) tightly packed, many cells; B) loosely grouped, several cells; or C) very few cells. the trophectoderm was assessed as follows: 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 clinical data of the two groups, including fertilization rate, percentage of embryos in the various cell stages, cleavage rate, grading of embryos, and percentage of different scores of embryos were compared by χ^2 tests or Fisher's exact test. A *P* value of <0.05 was considered statistically significant.

RESULTS

ICSI was performed in 100 cycles for couples with severe male infertility or previous in-vitro fertilization failure. Overall, 620 oocytes were collected. Of these, 434 were in MII at the removal of cumulus/corona cells (Group I), and ICSI was performed on 431

(99.3%). In Group II, 186 oocytes were recovered, of which ICSI was performed on 153 (82.3%). The fertilization rate was significantly higher in Group I than in Group II (Table I). The cleavage rates were similar between the two groups.

In vitro embryo development for the two ICSI groups is compared in Table I. Although the cleavage rate between the two groups was similar ($P = 0.99$), the grade of cleaving Day-3 embryos was significantly different between the two groups ($P < 0.001$). The number of good-morphology (Grades 1 and 2) embryos was significantly fewer in Group II ($P < 0.001$). Furthermore, the number of blastocysts developed from the good-morphology Day-3 embryos in Group II (38.1%) was significantly fewer ($P < 0.001$) than in Group I (86.1%) following extended culture for 5 or 6 days.

The morphologic quality of the blastocysts differed significantly between the two groups (Table II). The rates of top-scoring blastocysts (3AA, 4AA, or 5AA) was significantly higher in Group I embryos ($P < 0.001$).

DISCUSSION

The data presented in this study clearly demonstrate that the maturity of the oocyte at the removal of cumulus/corona cells affects the subsequent embryo

Table II. Comparison of Blastocyst In Vitro Between the Two Groups of Cumulus-Free Oocytes in ICSI Patients

	Group I	Group II	P
No. of blastocysts from good Day-3 embryos	219	21	
Morphological grading			
No. of top-scoring blastocysts (%)	209 (95.4)	16 (76.2)	<0.001
No. of other-scoring blastocysts (%)	10 (4.6)	5 (23.8)	

Note. Group I: Oocytes in Metaphase II at the removal of cumulus/corona cells; Group II: Oocytes in prophase or Metaphase I at the removal of cumulus/corona cells; Good embryos: Day-3 embryo with Grade 1 or 2; Top-scoring blastocyst: 3AA, 4AA, or 5AA by using the system of Gardner and Schoolcraft; P value: By chi-square test.

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-grading Day-3 embryo from immature-oocyte group was significantly less than from mature-oocyte group. Although the good-grading Day-3 embryos were collected from the immature oocytes, the number of these embryos, developing to the blastocyst-stage embryo was still significantly fewer than from the mature oocytes. The percentage of top-scoring blastocysts from the immature oocytes was also lower than from the mature oocytes. These phenomena suggest the oocyte maturity at the removal of cumulus/corona cells determines the fertilization potential and subsequent blastocyst quality in vitro.

ICSI treatment in couples with severe male infertility provides an opportunity for an accurate evaluation of oocyte maturity after cumulus-cell removal. In cycles comprising GnRH analog desensitization and stimulation with gonadotropins, a certain proportion of oocytes are still immature (in prophase or Metaphase I stage) or degenerative. These oocytes can achieve meiosis to reach Metaphase II in vitro, as was observed in 82.3% of the cases in this study. Nevertheless, the fertilization rate with ICSI and the viability of embryo development in such oocytes remains lower than in oocytes that have achieved MII stage at the removal of cumulus/corona cells. It is possible that during the final stages of nuclear and cytoplasmic maturation, the oocyte acquires cytoplasmic mechanisms that are necessary not only to support fertilization but also for embryo development. In other words, the quality of cytoplasmic maturation is the probable cause for the difference in fertilization and embryo quality and influences the developmental competence

of these two groups of oocytes. The maturity of oocyte consists of the nuclear and cytoplasmic maturation. Both nuclear and cytoplasmic maturation play important roles in achieving successful fertilization and subsequent development (23,24). On the basis of several results, in cumulus-surrounding or cumulus-free oocytes, cytoplasmic maturation lags behind nuclear maturation (11,12,20). It means that maturity of oocyte at the removal of cumulus/corona cells does not equal to nuclear and cytoplasmic maturation and the difference between oocyte maturation at the removal of cumulus/corona cells and oocyte maturation after the removal of cumulus/corona cells is the different degree of cytoplasmic maturation.

Improvements in the oocyte and extended embryo culture are made to get 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 (9). However, blastocyst formation itself does not fully reflect the viability of the embryo, and not all blastocysts are of equal quality (25). Experience in defining better blastocyst quality in terms of morphology and development rate is growing. 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 (26–28).

Various factors affect blastocyst formation and quality, including culture conditions, number of oocytes, maternal age, and male factor infertility (25). In addition, the quality of early-stage embryos can substantially influence rates of blastocyst formation (28). There were several studies regarding to oocyte maturity in assisted reproductive technology (20,29–31). However, no literatures fully demonstrated the influence of oocyte maturity at the removal of cumulus/corona cells in the blastocyst formation and quality. In this study, the maturity of oocyte influence the fertilization rate, the 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 (26–28). According to our data, good score blastocysts can be developed from oocytes that are immature at the removal of cumulus/corona cells. Our data further indicates that a good-score for a blastocyst does not equate to blastocyst quality and viability. Thus, the criterion for blastocyst selection seems not to depend on only one predictor. Oocyte maturity at the removal of cumulus/corona cells also needs to be considered for blastocyst quality and viability in clinical practice.

In conclusion, the maturity of the oocyte at the removal of cumulus/corona cells is important for determining subsequent embryo quality. Although extended embryo culture in vitro was used for some embryos, they did not influence embryo viability with respect to oocyte maturity. Oocyte maturity at the removal of cumulus/corona cells is an important factor for prediction of blastocyst quality and viability.

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