Relationship of the Human Cumulus-Free Oocyte Maturation Profile with In Vitro Outcome Parameters After Intracytoplasmic Sperm Injection

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Purpose: We investigated whether the human oocyte maturation profile at the removal of cumulus/corona cells affects the fertilization rate and subsequent embryo quality after intracytoplasmic sperm injection.

Methods: A total of 1011 oocytes from 150 cycles was included in this retrospective analysis. Cumulus-free oocytes that were in prophase or metaphase I of meiosis at the removal of cumulus/corona cells were incubated in vitro until they reached metaphase II (in vitro-matured oocytes) and were then immediately injected with a single spermatozoa. Oocytes that were in metaphase II at the removal of cumulus/corona cells (MII oocytes) received sperm injection after 3–4 hr of preinjection incubation.

Results: The fertilization rate of the MII oocytes was significantly higher than that of in vitro-matured oocytes (81 vs 62%; $P < 0.001$). The cleavage rates were similar in the two groups (MII oocytes, 94%; in vitro-matured oocytes, 91%). However, MII oocytes had significantly higher percentages of good-quality embryos (grade 1–3 embryos, 87 vs 58%, $P < 0.001$) and embryos with high cumulative embryo scores (score 10–32 embryos, 62 vs 33%, $P < 0.001$). The mean cumulative embryo score of MII oocytes after fertilization was also higher than that of in vitro-matured oocytes (12.1 ± 3.8 vs 8.8 ± 3.4 ; $P = 0.014$).

Conclusions: MII oocytes that extruded the first polar body at the removal of cumulus/corona cells had better fertilization rates and embryo morphology than in vitro-matured oocytes that extruded the first polar body following the removal of cumulus/corona cells and in vitro culture.

KEY WORDS: embryo quality; fertilization rate; in vitro maturation; intracytoplasmic sperm injection; oocyte maturity.

INTRODUCTION

Nuclear maturity, i.e., resumption and progression of meiosis to stage II metaphase (MII), cannot be used as a sole determinant of an oocyte's developmental competence (1). Extensive changes in protein synthesis and posttranslational modifications take place simultaneously with nuclear maturation (2). 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.

While direct intracytoplasmic sperm injection (ICSI) obviates the need for spermatozoa to penetrate the oocyte, not all oocytes become fertilized even when the spermatozoa have been successfully placed in the ooplasm (3). Dominko and First (4) reported that even among successfully inseminated bovine oocytes reaching MII in vitro, not all became fertilized. They suggested that the timing of sperm insemination of oocytes matured in vitro plays an equally important role.

In in vitro fertilization (IVF) programs, the oocyte–cumulus complex is assessed to determine the oocyte maturity. Prior to 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. In this study, we retrospectively compared the fertilization rates, cleavage rates, and embryo quality after ICSI between cumulus-free oocytes that were in metaphase II at the removal of cumulus/corona cells (MII oocytes) and oocytes that were in prophase I or metaphase I at the removal of

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cumulus/corona cells but later reached metaphase II after in vitro culture (in vitro-matured oocytes).

MATERIALS AND METHODS

We reviewed the records of 150 cycles of the ICSI program performed at our department during a 12-month period. All couples presented with severe sperm deficiency or had at least one previous cycle of failed IVF. Ovulation induction and oocyte retrieval were performed as described elsewhere (5). Ovarian stimulation was performed using a long protocol of gonadotropin releasing hormone (GnRH) agonist (Luproid acetate; Takeda, Tokyo), with follicle-stimulating hormone (FSH; Metrodin; Serono Laboratories Inc., Randolph, MA) and human menopausal gonadotropin (hMG; Pergonal; Serono Laboratories Inc.) or, alternatively, hMG only. Human chorionic gonadotropin (hCG; Pregnyl; N.V. Organon, Oss, The Netherlands), usually 10,000 IU, was administered intramuscularly when ultrasound revealed that the two largest follicles had a mean diameter of ≥ 16 mm and adequate estradiol (E_2) levels. Oocytes were retrieved by transvaginal aspiration under ultrasound guidance 35–37 hr after hCG injection.

Following retrieval, the oocytes were exposed to 80 IU/ml hyaluronidase (type VIII; Sigma Chemical Co., St Louis, MO) for 5 to 6 sec. The surrounding cumulus cells were stripped from oocytes by aspiration 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 M2 culture medium (Medicult, Copenhagen, Denmark) for 3–8 hr. Group I oocytes were injected with sperm 3–4 hr after retrieval. Group II oocytes were examined every 2 hr, and ICSI was performed immediately after the appearance of the first polar body (MII stage), which occurred between 4 and 8 hr after oocyte retrieval. Oocytes that did not develop to MII within 8 hr of incubation were discarded.

Sperm concentration and motility were evaluated according to the recommendations of the World Health Organization (6). Kruger's strict criteria were used for assessment of sperm morphology (7). Semen analysis was done at least once before the treatment cycle to

evaluate whether enough spermatozoa were present in the ejaculate to perform ICSI. The specimens were prepared using the Percoll gradient method (8). Patients with azoospermia underwent testicular sperm extraction (TESE) according to the protocol described by Silber *et al.* (9). The patients underwent karyotyping and molecular genetic diagnosis to screen the underlying chromosome anomaly and the deletion of the azoospermia factor region on the Y_q chromosome (10). Only those who had spermatozoa within their testes at the time of TESE or exhibited severe oligozoospermia with a normal chromosomal screen were included. ICSI procedures were performed with thawed testicular sperm in 32 cycles and fresh ejaculated sperm in 118 cycles. The method for cryopreservation of sperm extracted from the testicles was as follows: 2 ml of test yolk buffer containing glycerol (12%; Irvine Scientific, Santa Ana, CA) was slowly added to the sperm suspension. The mixture was then reloaded into several cryotubes (Nunc, Roskilde, Denmark) and homogenized again. The loaded cryotubes were placed in a 37°C incubator for 15 min, then incubated consecutively at 4°C for 15 min, –20°C for 15 min, and –60°C for 15 min. The tubes were finally stored at –196°C in liquid nitrogen. On the day of oocyte retrieval, one of the tubes was removed from the tank of liquid nitrogen and placed in a 37°C water bath to thaw the suspension. The thawed specimens were then prepared with the Percoll gradient method.

The sperm injection procedure was based on the protocol described by Palermo *et al.* (11), although the preparation of sperm droplets was modified. To prepare sperm droplets, 1 μ l of prepared sperm suspension was added to 2–4 μ l of M2 medium (Medicult, Copenhagen, Denmark) without polyvinylpyrrolidone (PVP).

After the ICSI procedure, the oocytes were cultured according to our standardized IVF procedure (5). They were assessed for the presence of pronuclei after 16–18 hr of incubation. Fertilization was considered normal when two clear pronuclei were present. If one pronucleus was observed, a second evaluation was carried out 4 hr later to see whether the pronuclear status had changed. The developmental competence of zygotes with two pronuclei was evaluated after a further 24 hr of in vitro culture.

The embryos were classified according to the morphologic grading system described by Veeck (12). The morphologic grade of embryos was rated as 1 to 5, with lower grades indicating better morphology. The embryo grade was then converted to an embryo score of 0 to 4, with grade 1 corresponding to a score of 4.

The quality of the embryos was evaluated based on the embryo grade and an embryo score. Grade 1 to 3 embryos were defined as good-quality embryos, while grade 4 and 5 embryos were considered poor-quality embryos. The cumulative embryo score per embryo was calculated by multiplying the embryo score by the number of blastomeres within the embryo.

The clinical data on 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 test. The mean embryo score was analyzed with Student's *t* test. A *P* value of <0.05 was considered statistically significant.

RESULTS

ICSI was performed in 150 cycles for couples with severe male infertility or previous IVF failure. Overall, 1011 oocytes were collected. Of these, 726 were in MII at the removal of cumulus/corona cells (group I), and ICSI was performed on 720 (90%). In group II, 285 oocytes were recovered, of which ICSI was performed on 239 (84%). The fertilization rate was significantly higher in group I than in group II (Table I). The cleavage rates were similar between the two groups, as well as the distribution of various embryo stages after 48 hr of in vitro culture (Table II).

The morphologic quality of the embryos differed significantly between the two groups (Table III). The rates of good-quality embryos (grade 1–3 embryos) and embryos with higher cumulative embryo scores (score, 10–32) were significantly higher in group I embryos. Furthermore, the mean cumulative embryo score of the cleavage stage was also significantly higher in group I than in group II ($P = 0.014$).

Table I. Comparison of Fertilization Rates Between the Two Groups of Cumulus-Free Oocytes^a

	Group I	Group II
No. of oocytes collected	726	285
No. of mature oocytes injected	720 (99)	239 (84)
No. of oocytes fertilized	582 (81)	147 (62)*

^a Group I: oocytes in metaphase II at the removal of cumulus/corona cells. These were sperm-injected after 3–4 hr of in vitro culture. Group II: oocytes in prophase or metaphase I at the removal of cumulus/corona cells. These were sperm-injected immediately after developing to metaphase II in in vitro culture. Values in parentheses are percentages.

* $P < 0.001$.

Table II. Comparison of Early Embryo Development Between the Two Groups of Cumulus-Free Oocytes^a

	Group I	Group II
No. of oocytes fertilized	582	147
No. of zygotes cleaved	548 (94)	133 (90)
No. of blastomeres		
≤3	170 (31)	47 (36)
4–7	372 (68)	84 (63)
≥8	6 (1)	2 (1)

^a See Table I, footnote *a*.

DISCUSSION

ICSI treatment in couples with severe male infertility provide an opportunity for an accurate evaluation of oocyte maturity after cumulus cell removal. In cycles comprising GnRH analogue desensitization and stimulation with gonadotropins, a certain proportion of oocytes is 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 84% of the cases in this study. Nevertheless, the fertilization rate with ICSI and the viability of embryo development in such oocytes remain 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.

In cumulus-free oocytes, certain factors are required for the progression of the germinal vesicle stage to the metaphase II stage, which can be supplied either by the culture medium or by a coculture system (13). Sirard *et al.* (14) demonstrated that, in a bovine model, a certain amount of protein synthesis is required for progression of oocytes from metaphase I to metaphase II. These findings indicate the importance of certain in vitro factors in the completion of nuclear and cytoplasmic maturation. In our study, cumulus-free oocytes that reached the MII stage after in vitro culture still did not acquire fertilization competence similar to that of MII oocytes that had reached M II upon removal of the cumulus/corona (regardless of the zona pellucida). We propose, on the basis of these results, that in cumulus-free oocytes, cytoplasmic maturation lags behind nuclear maturation.

Table III. Comparison of Early Embryo Quality Between the Two Groups of Cumulus-Free Oocytes^a

	Group I	Group II
No. of cleaved embryos	548	133
Morphological grading ^b		
No. of good-quality embryos	478 (87)	77 (58)*
No. of poor-quality embryos	70 (13)	56 (42)*
Cumulative embryo score per embryo (mean $\pm$ SD)	12.1 $\pm$ 3.8	8.8 $\pm$ 3.4*
No. of embryos with score 32–10	339 (61)	44 (33)*
No. of embryos with score 9–1	209 (38)	89 (67)*

^a See Table I, footnote a.^b Good-quality embryos: grades 1–3. Poor-quality embryos: grades 4–5.* $P < 0.05$.

There were several differences between the two groups of cumulus-free oocytes in our study. First, the in vitro-matured oocytes reached nuclear maturation 6–7 hr later than the MII oocytes. Second, the in vitro-matured oocytes received ICSI immediately after reaching the MII stage, whereas the MII oocytes were cultured in vitro for 3–4 hr before ICSI. To our knowledge, only a few studies have examined the relationship between the duration of MII arrest and insemination in mammalian oocytes. Kubiak (15) observed that when mouse oocytes are activated soon after MII arrest, the majority is unable to conduct anaphase chromatin movement and progress to the first zygotic interphase. He suggested that the oocytes' ability to support fertilization and early embryo development is not acquired at the time of MII arrest and that this ability might develop gradually after completion of nuclear maturation. In our study, the proportion of oocytes injected with sperm immediately after MII arrest and developing to the pronuclear stage (62%) was significantly lower than in oocytes injected 3–4 hr after MII arrest (81%). In Dominko and First's study (4), early-maturing bovine oocytes (16 hr of culture) had a better fertilization rate than late-maturing oocytes (24 hr of culture). Bovine oocytes allowed to remain in MII arrest for 8 hr before being inseminated developed to pronuclear stages at a higher frequency than MII oocytes inseminated immediately. However, in Kito and Bavister's study (16), cumulus-free metaphase II oocytes which were incubated with hamster oviductal fluid for 3 hr still had a low rate of male pronuclear formation (<40%), and a certain proportion of oocytes matured in vitro was morphologically abnormal, exhibiting elongated meiotic spindles and unaligned chromosomes.

In summary, in vitro-matured oocytes that extruded the first polar body following in vitro culture had a poorer outcome than MII oocytes upon the removal of cumulus/corona cells. In our study, sperm injection was performed on MII oocytes 3–4 hr following oocyte

retrieval, but immediately after the metaphase II stage was reached in in vitro-matured oocytes. We speculate that both the process of in vitro maturation and the timing of sperm injection after oocyte nuclear maturation are responsible for the differences in outcome between the two groups of embryos. Further investigation is necessary to elucidate which factor plays a more relevant role in determining the ideal timing of sperm injection for in-vitro matured oocytes.

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