In Vitro Maturation and Fertilization of Immature Oocytes: A Comparative Study of Fertilization Techniques

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Purpose: Our purpose was to investigate the factors influencing maturation and fertilization of immature oocytes.

Methods: Immature oocytes were obtained from women undergoing cesarean section. They were cultured in the maturation medium either with or without cumulus cells. After maturation to metaphase II, they were randomly fertilized by in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI).

Results: After incubation for 48 hr, 441 oocytes (42.8%) reached metaphase II. Among them, 56.6% of cumulus-enclosed oocytes, but only 29.2% of denuded oocytes, reached metaphase II. Of the 289 cumulus-enclosed oocytes, the fertilization rates by IVF and ICSI were 56.3 and 84.1%, respectively ($P < 0.01$). Of the 152 denuded oocytes, the fertilization rates by IVF and ICSI were 39.5 and 84.5%, respectively ($P < 0.01$). The cleavage rates, however, were similar.

Conclusions: Cumulus cells are beneficial in the maturation of human oocytes in vitro and that ICSI increases the fertilization rate for the in vitro matured oocytes. The developmental potential of the fertilized oocytes, however, is similar irrespective of the fertilization method or the presence or absence of cumulus cells.

KEY WORDS: immature oocytes; in vitro maturation; cumulus cells; fertilization; intracytoplasmic sperm injection.

INTRODUCTION

After birth, human oocytes remain in the dictyate stage until they are stimulated by gonadotropin to resume meiosis at the time of ovulation. They then enter meta-

phase II until fertilization occurs. Mammalian follicular oocytes have been demonstrated to be able to mature in vitro with an appropriate culture medium (1). Cha *et al.* first reported that immature oocytes obtained from unstimulated ovaries could be matured in vitro and fertilized (2). A woman with premature ovarian failure delivered triplets from donated immature oocytes. However, the clinical use of immature oocytes is still limited by their low rates of fertilization and pregnancy.

Recently, intracytoplasmic sperm injection (ICSI) has become the most promising technique in assisted reproductive technologies. Used initially for couples with male factor infertility, ICSI has been applied to other fields of infertility to increase the rate of fertilization even with normal sperm parameters. Trounson *et al.* first reported that ICSI increased the fertilization rate of in vitro matured human oocytes from unstimulated PCOD patients (3). There are, however, no controlled studies to compare the fertilization methods. In order to investigate whether ICSI increases the fertilization rate of the in vitro matured primary oocytes, these oocytes were fertilized by either in vitro fertilization (IVF) or ICSI. Their fertilization rates and cleavage rates were compared.

Intercellular communication between the oocyte and the cumulus cells is necessary for oocyte growth in vitro (4). In the rat, cumulus cells have an essential role for promoting the cytoplasmic maturation of oocytes and the subsequent development of fertilized oocytes (5). However, cumulus cells are not necessary for the nuclear maturation and fertilization of in vitro matured bovine oocytes (6). The effect of corona and cumulus cells on the maturation and development of human oocytes was first reported by Barnes *et al.* (7). They compared the oocytes with and without the cover of cumulus cells and they found that the frequency of development was not significantly different. In the

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second part of this study, we compared the maturation rates and subsequent development of embryos of immature oocytes both with and without cumulus cells.

MATERIALS AND METHODS

One thousand thirty-one immature oocytes were collected from 234 consenting women undergoing cesarean section. The mean age of the women were 30.1 years old with a mean gestation weeks of 37.9. The oocytes were aspirated by puncturing follicles which were 2 to 5 mm in diameter with a 22-gauge needle attached to a syringe filled with HEPES-buffered human tubal fluid medium (HTF; Irvine Scientific, Santa Ana, CA). After collection, cumulus-oocyte complexes were randomly divided into two groups: cumulus-enclosed and cumulus-denuded. Those immature oocytes that were assigned to the cumulus-denuded group were removed of cumulus cells by repeatedly passing them through a fine pipette gently. The atretic oocytes, in which the cumulus and corona layers contained dark atretic bodies or the ooplasm was darkish, were discarded and were not included in this study. The maturation medium we used was Tissue Culture Medium 199 (Sigma Chemical Co., St. Louis, MO) supplemented with 20% bovine serum albumin (BSA), 10 IU/ml pregnant mare serum gonadotropin (PMSG; Sigma), and 10 IU/ml human chorionic gonadotropin (hCG). The oocytes were transferred into four-well dishes (Nunc, Copenhagen, Denmark) containing maturation medium and cultured for 48 hr at 37°C in an atmosphere of 5% CO₂ in air. After incubation they were observed under an inverted microscope to determine maturity. The oocytes with cumulus were denuded of cumulus cells before observation. Only those oocytes with extrusion of the first polar body were considered mature (metaphase II or MII stage) and went on for further experimentation.

Fertilization Techniques

Those oocytes which matured to the MII stage were randomly fertilized by IVF or ICSI using donated

semen from fertile men. For IVF, the oocytes were transferred into four-well dishes containing HTF supplemented with 10% synthetic serum substitute (Irvine Scientific) and then inseminated with 100,000 motile sperm per oocyte. The ICSI procedure was the same as that described by Palermo *et al.* (8). Sixteen to 18 hours after fertilization the oocytes were observed for the presence of two pronuclei. The fertilized oocytes were cultured for an additional 2 to 3 days. The final stage of the embryos was recorded.

The results were analyzed using the Yates corrected chi-square test. A *P* value less than 0.05 was considered significant.

RESULTS

Altogether, 1031 immature oocytes were collected from 234 patients. After incubation in the culture medium for 48 hr, 441 oocytes reached metaphase II, with a maturation rate of 42.8%. The maturation rate of oocytes with cumulus cells was 56.6%, which was significantly higher than that of denuded oocytes (29.2%) (*P* < 0.01; Table I). The damaged oocytes comprised oocytes that were ruptured during pipetting and oocytes that displayed darkened ooplasm after culture. The cumulus-denuded oocytes had a higher rate of damage during in vitro culture (Table I). Of the 289 oocytes matured from oocytes with cumulus cells, 138 oocytes were fertilized by IVF and 151 oocytes were fertilized by ICSI. The fertilization rate was significantly higher with ICSI than with IVF (84.1 vs 56.5%; *P* < 0.01; Table II). For oocytes matured from denuded oocytes, ICSI also yielded a higher fertilization rate than IVF (84.5 vs 39.5%; *P* < 0.01; Table III). Interestingly, for oocytes with and without cumulus cells, the cleavage rates of embryos fertilized by IVF or ICSI were similar (Tables II and III). When these embryos were cultured further, almost all the embryos degenerated beyond the eight-cell stage.

Of oocytes fertilized by IVF, cumulus-enclosed oocytes had a higher fertilization rate than cumulus-denuded oocytes (56.5 vs. 39.5%; *P* < 0.05; Table

Table I. Comparison of Maturation Rates of Immature Oocytes^a

Oocytes	No. eggs	MI I (%)	GVBD (%)	GV (%)	Damaged (%)
Cumulus-enclosed	511	289 (56.6) ^b	98 (19.2)	6 (1.2)	118 (23.1) ^c
Cumulus-denuded	520	152 (29.2) ^b	83 (15.9)	37 (7.1)	248 (47.6) ^c

^a MII, metaphase II; GVBD, germinal vesicle breakdown; GV, germinal vesicle.

^b *P* < 0.01, Yates corrected chi-square test.

^c *P* < 0.01, Yates corrected chi-square test.

Table II. Comparison of Fertilization Rates of MII Oocytes (Obtained from Cumulus-Enclosed Oocytes) by ICSI or IVF

Fertilization	No. MII oocytes	Fertilization (%)	2-cell/4-cell (%)
IVF	138	78 (56.5) ^a	63 (80.8)
ICSI	151	127 (84.1) ^a	118 (92.9)

^a $P < 0.01$, Yates corrected chi-square test.

IV). On the contrary, ICSI gave comparable fertilization rates for oocytes with and without cumulus cells (84.1 and 84.5%; Table V). The cleavage rates of these oocytes were also similar (Tables IV and V).

DISCUSSION

This study demonstrates that immature oocytes obtained from unstimulated ovaries can be matured in vitro, fertilize, and develop to embryos. For in vitro matured human oocytes, ICSI is a better method of fertilization than IVF. Cumulus cells had a beneficial effect on the maturation and fertilization of immature oocytes. In vitro matured denuded oocytes had a lower fertilization rate with IVF, which can be overcome by ICSI.

It has been demonstrated that immature oocytes obtained from unstimulated ovaries can be matured in vitro (2,3,7,9). Excluding the denuded oocytes, the maturation rate of in vitro matured oocytes after 48 hr in this study was 56.6%, which was similar to the rate reported by Cha *et al.* (2), but lower than that reported by Trounson *et al.* (9). Because these authors used different sources of immature oocytes and maturation media, it is difficult to explain the difference in maturation rates. Until now, there is little information about the best culture condition required for human immature oocytes. Whether the medium used by Trounson *et al.* is better demands further study.

The exact role of cumulus cells in the maturation of oocytes remains unclear. A beneficial effect of cumulus cells on the maturation of oocytes has been reported

Table IV. Comparison of Fertilization Rates of MII Oocytes by IVF from Cumulus-Denuded or Cumulus-Enclosed In Vitro Matured Oocytes

Oocytes	No.	Fertilization (%)	2-cell/4-cell (%)
Cumulus-denuded	81	32 (39.5) ^a	27 (84.3)
Cumulus-enclosed	138	78 (56.5) ^a	63 (80.8)

^a $P < 0.05$, Yates corrected chi-square test.

for mouse (5), ovine (10), bovine (6), and human oocytes (11). In the rat, cumulus cells have an essential role for promoting cytoplasmic maturation of oocytes and for pronuclear formation and subsequent development of penetrated oocytes (5). Mouse oocytes matured with cumulus cells have greater fertilizability than those matured without cumulus cells (12). Sheep oocytes matured without cumulus cells fail to undergo cleavage after fertilization (13). Chian *et al.* demonstrated that cumulus cells were not necessary for nuclear maturation and fertilization of in vitro matured bovine oocytes but that development beyond the 8- to 32-cell stage was significantly lower in cumulus-free than in cumulus-intact oocytes (6). In the present study, there was significant difference in the maturation rate between the oocytes with and those without cumulus cells (56.6 vs. 29.2%), suggesting that cumulus cells play a role in the maturation of human immature oocytes. It was speculated that oocytes and cumulus cells were coupled by gap junction and granulosa cells control germinal vesicle breakdown (GVBD) via the cumulus cells (14). It is interesting to note that once the human oocytes were matured in vitro and fertilized, their cleavage rates were similar either with or without cumulus cells, indicating that the cumulus cells have no influence on the developmental potential of the embryos. The oocytes matured in vitro, however, had a significantly higher blockage of cleavage at the pronuclear stage compared with oocytes matured in vivo. The embryo development ratio was also lower in oocytes matured in vitro compared with those matured in vivo (7).

Table III. Comparison of Fertilization Rates of MII Oocytes (Obtained from Cumulus-Denuded Oocytes) by ICSI or IVF

Fertilization	No. MII oocytes	Fertilization (%)	2-cell/4-cell (%)
IVF	81	32 (39.5) ^a	27 (84.3)
ICSI	71	60 (84.5) ^a	54 (90)

^a $P < 0.01$, Yates corrected chi-square test.**Table V.** Comparison of Fertilization Rates of MII Oocytes by ICSI from Cumulus-Denuded or Cumulus-Enclosed In Vitro Matured Oocytes

Oocytes	No.	Fertilization (%)	2-cell/4-cell (%)
Cumulus-denuded	71	60 (84.5) ^a	54 (90)
Cumulus-enclosed	151	127 (84.1) ^a	63 (80.8)

^a $P > 0.05$, Yates corrected chi-square test.

The damage rate of denuded oocytes was higher than that of cumulus-enclosed oocytes (47.6 vs. 23.1%). The damaged oocytes comprised oocytes that were ruptured during pipetting and oocytes that displayed darkened ooplasm after maturation *in vitro*. Although the cumulus cells were very sticky and were very difficult to be removed by pipetting, no hyaluronidase was used in order to control other factors. Therefore some immature oocytes were ruptured before culture. The high damage rate in the denuded oocytes may be partly explained by the difficulty in removing the cumulus cells. Another reason might be that without the beneficial effect of cumulus cells, the oocytes were prone to degenerate during maturation *in vitro*.

Compared with oocytes matured *in vivo*, oocytes matured *in vitro* had lower rates of fertilization, cleavage, and development (7,9). This may be due to zona hardening (14), failure of sperm nuclei to decondense (15), or poor potential for embryonic development (17). Cha *et al.* and Trounson *et al.* reported a fertilization rate of about 45% in the *in vitro* matured oocytes that were inseminated conventionally (2,9). Later reports showed that ICSI increased the fertilization rate to 70 or 75% (3,18). It was speculated that zona hardening occurred under the culture condition presently used and ICSI minimized the potential detrimental effects of long-term culture on the zona pellucida (3,19). To our knowledge, this is the first and the largest controlled study to prove the hypothesis. In this study the *in vitro* matured oocytes fertilized by ICSI had a significantly higher fertilization rate. Besides, ICSI also gave comparable fertilization rates to oocytes either with or without cumulus cells. The fertilization rate we achieved using ICSI is higher than those of previous reports (3,18). This may be due to the reason that the oocytes we used were obtained from fertile women undergoing cesarean section; in contrast, previous reports using oocytes either from PCOD patients or infertile women (3,18). Barnes *et al.* reported that oocytes from regular cycling patients matured and fertilized at significantly higher rates than irregular cycling and anovulatory women, although conventional IVF was used in this study (7). Zona hardening not only reduces the fertilization capability by conventional IVF, but also impedes blastocyst hatching. Therefore it may be necessary to perform ICSI and assisted hatching for all *in vitro* matured oocytes in order to achieve successful pregnancy.

When we tried to culture the embryos further, almost all the embryos degenerated beyond the eight-cell stage. Barnes *et al.* also found that these embryos had zygotic block and they speculated that it was due to

abnormalities of cytoplasmic maturation in *in vitro* matured oocytes (7). This is probably one of the reasons why the pregnancy rate with immature oocyte donation has remained very low. Further study is needed to synchronize nuclear maturation with cytoplasmic maturation or new culture medium has to be developed that can support the embryos to the blastocyst stage.

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