The effect of human cumulus cells on the maturation and developmental potential of immature oocytes in ICSI cycles

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Abstract

Purpose To investigate the effect of human cumulus cells on the maturation and developmental potential of immature oocytes in ICSI cycles.

Methods Immature oocytes were randomly divided into two groups: the cumulus-denuded oocyte group (group A) and the cumulus-intact oocyte group (group B). Only oocytes that reached metaphase II (MII) stage after in vitro maturation were used in the ICSI procedure. In vivo mature sibling MII oocytes served as the control group. Maturation rate, fertilization rate, embryo quality and developmental potential were examined. **Results** There was no significant difference in maturation rate between group A (68.16%) and group B (70.49%; $P>0.05$). The total fertilization rate among the three groups was comparable ($P>0.05$), while the zygotes with two pronuclei in group A (74.59%) or group B (75.97%) were significantly lower than those in control group (84.29%; $P<0.05$). The

available embryo rate in group A (11.49%) was markedly lower than that in group B (27.66%; $P<0.05$), and both of them were significantly lower than that in control group (62.38%; $P<0.05$). The proportion of ≥ 6 -cell embryos in group B (45.74%) was notably higher than in group A (26.44%; $P<0.05$), and both were markedly lower than in control group (65.92%; $P<0.05$). The proportion of embryos with $<10\%$ fragmentation in group A (13.79%) was significantly lower than in group B (29.79%; $P<0.05$), and both were notably lower than in control group (42.98%; $P<0.05$). **Conclusions** The presence of cumulus cells surrounding the immature oocytes during IVM before ICSI had no influence on nuclear maturation and fertilization, but leads to better subsequent embryonic development. This is perhaps mediated by an improvement in cytoplasmic maturation.

Keywords Cumulus cells · In vitro maturation · Intracytoplasmic sperm injection · Cytoplasmic maturation

Capsule Cytoplasmic but not nuclear maturation is improved when IVM and ICSI are carried out with cumulus cells attached to human oocytes.

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Introduction

Controlled ovarian stimulation (COS) is usually used to increase the number of available oocytes and the outcome of in vitro fertilization-embryo transfer (IVF-ET). However, on account of the desynchronized development of the oocytes in stimulated cycles, approximately 10%–20% of the retrieved human oocytes, either at metaphase I (MI) or the germinal-vesicle (GV) stage, are immature [2,32,41]. In intracytoplasmic sperm injection (ICSI) cycles, these oocytes are usually discarded, thus, resulting in a serious waste of oocyte resources. It is of great importance to explore the optimal use of immature oocytes, as the use of immature oocytes not only increases the number of available embryos to improve the

chance of pregnancy [33,34,41], but also opens a new horizon for the source of embryonic stem cells.

Many research groups have investigated the best use of immature oocytes. It has been reported that these in vitro matured oocytes lead to lower fertilization rates [1,9,34], abnormal embryonic development [1,4,34], and lower implantation rates [9] compared to oocytes that mature in vivo. In these studies, the removal of cumulus cells from oocytes was performed after their retrieval before in vitro culture [1,6,9,27,32,34,35,41]. Some authors [16,17] also reported the developmental potential of immature oocytes that were not denuded of their cumulus cells (CCs) on the day of retrieval. However, only GV oocytes were involved in these studies. Furthermore, the conclusions as to the role of CCs in the in vitro maturation of GV oocytes were inconsistent between these studies. Some authors reported that oocyte developmental competence was affected by follicle size [19], which indicates that the developmental competence of MI oocytes may be different from that of GV oocytes. To date, the developmental potential of MI oocytes derived from COS cycles, denuded cumulus cells or not on the retrieval day, remains unknown. In addition, after collection, it is difficult to distinguish MI oocytes from GV oocytes if cumulus cells are not denuded. Therefore, the evaluation of oocyte maturity has been based on the diameter of follicles [29]. In order to improve the utilization of oocytes in COS cycles, it is necessary to systematically study the effect of cumulus cells on the developmental potential of immature oocytes during ICSI cycles.

The purpose of the present study was to investigate the effect of cumulus cells on the maturation rate, fertilization rate, and developmental potential of immature human oocytes derived from small follicles during ICSI cycles.

Materials and methods

Patients and oocyte recovery

This study was approved by the Ethical Committee of our hospital. The study group comprised of 241 consenting women who each underwent an ICSI cycle ($n=241$) between May 2010 and June 2011 in our IVF center. The inclusion criteria were: i) aged ≤ 35 years old; ii) basal FSH <10 mIU/ml; iii) undergoing their first treatment ICSI cycle with the long luteal gonadotropin-releasing hormone (GnRH) downregulation protocol; and iv) more than five oocytes were retrieved. Patients with PCOS were excluded. For each cycle, at least one immature oocyte was retrieved. The identification of immature oocytes was based on the method used by Russell et al. [29] with slight modifications. If the diameter of the follicles was smaller than or equal to 10 mm, the oocyte was considered immature.

A long stimulation protocol was used. In all patients, down-regulation of the pituitary was carried out by the administration of a GnRH analog (GnRHa; triptorelin acetate; Ferring, Germany) during the middle luteal phase. When the suppressive effects were obtained ($E_2 < 50$ pg/ml, with no cysts or ultrasound follicles >1.0 cm across the maximum diameter), the control of ovarian hyperstimulation was achieved by administering 150–300 IU of rFSH (Gonal-F; Serono, Switzerland; 75 IU FSH per ampoule) per day and human menopausal gonadotrophin (HMG; Lizhu, China). The women had a normal response to controlled ovarian hyperstimulation (COH). Human chorionic gonadotropin was administered when the leading follicle was at least 20 mm in diameter. Transvaginal oocyte retrieval was performed 34–36 h after the administration of HCG (Profasi; Serono).

In vitro maturation, fertilization and embryo culture

According to whether or not the immature oocytes were denuded on the oocyte retrieval day, the oocytes were randomly divided into two groups. In group A, the denudation of cumulus cells from immature oocytes was performed 1 h after retrieval and the denuded oocytes were cultured for 24 h prior to ICSI. In group B, the immature oocyte-cumulus complexes (OCCs) were cultured for 24 h and then were denuded prior to ICSI. HTF medium (Irvine Scientific, USA) with 10% synthetic serum substitute (SSS, Irvine Scientific, USA) which served as the fertilization medium in conventional IVF, was used to culture immature oocytes. Fertilized oocytes were cultured in an incubator at 37°C under an atmosphere of 5% CO_2 with saturated humidity. Hyaluronidase (80 IU/mL, Sigma, USA) was used for oocyte denudation in this study.

Only oocytes that reached metaphase II after in vitro maturation were used for the ICSI procedure. The sperm samples were prepared by centrifugation with isolate sperm separation medium (Irvine Scientific, USA). Spermatozoa were injected according to the method described by Van Steirteghem et al. [39]. After ICSI, all of the injected oocytes were cultured in HTF medium with 10% SSS, covered with oil (Irvine Scientific, USA) in a humidified incubator with 5% CO_2 at 37°C. Each oocyte was examined for structural integrity and fertilization 16–18 h after injection. Normal fertilization was confirmed if two distinct pronuclei and two polar bodies were observed under the inverted microscope. Then, the zygotes were transferred into P-1 medium (Irvine Scientific, USA) with 10% SSS covered with oil. The embryo quality was evaluated 68–72 h after injection according to the criteria described by Puissant F et al. [26] with small modifications. The number of blastomeres and the extent of fragmentation were recorded. Embryos were classified and scored according to the number of blastomeres, symmetry of the blastomeres, and the amount of the detached anuclear fragments. The number of blastomeres were scored as follows: '4', 8–10

cells; '3', >10 or 6–7 cells; '2', 5 cells; and '0', 2–4 cells. To score the symmetry of the blastomeres, '0' was given for asymmetry and '1' was given for symmetry or low levels of asymmetry. The amount of detached anuclear fragments were scored as follows: '4', 0–5%; '3', 6%–10%; '2', 11–25%; '1', 26–50%; and '0', >50%. The final score of an embryo was the sum of the scores of all three parameters. For example, the final score of an 8-cell (scored 4) embryo with symmetrical blastomeres (scored 1) and <5% fragmentation (scored 4) was 9. Available embryos were those scored as ≥ 5 ; Unavailable embryos were those scored as <4. Top-quality embryos were those scored as 9 or 10. In the control group, the available embryos might be used to transfer or cryopreservation and the unavailable embryos would be discarded on day three.

Statistical analysis

The Chi-square test was applied for the comparison of in vitro maturation, fertilization, cleavage and early development. Data regarding age and number of oocytes collected were reported as means $\pm$ standard error (SEM). The level of significance was set at $P < 0.05$ for all the tests.

Results

In total, 241 ICSI cycles performed in our reproductive medical center from May 2010 and June 2011 were included in this study. According to whether or not the immature oocytes were denuded on the oocyte retrieval day, the oocytes were randomly divided into two groups: the cumulus-denuded oocyte group (A) and cumulus-intact oocyte group (B).

Data for the rates of maturation with or without cumulus cells are shown in Table 1. The number of ICSI cycles, the mean age of the patients and number of oocytes recovered between both groups were all comparable. In total, 179 and 183 immature oocytes were collected in group A and group B, respectively. The number of GV stage and MI stage oocytes, in group A on the retrieval day, was 62 and 117, respectively. The proportion of the oocytes that reached metaphase II (MII)

was comparable between groups (68.16% versus 70.49%, $P > 0.05$). In vivo matured sibling MII oocytes, retrieved during the same treatment cycles, served as the control group. In total, 3,035 oocytes were collected.

The pronuclei (PN) formation of MII-stage oocytes according to maturation condition after ICSI is shown in Table 2. Of 122 MII oocytes in group A, 82.79% had PN (91 oocytes with 2PN, and 10 oocytes with 1PN or 3PN). Of 129 MII oocytes in group B, 82.95% had PN (98 oocytes with 2PN, and 9 oocytes with 1PN or 3PN). Of 2,673 MII oocytes in control group, 87.92% had PN (2 253 oocytes with 2PN, and 97 oocytes with 1PN or 3PN). There was no significant difference in the total fertilization rate among the three groups. The proportion of oocytes with 2PN in group A and B was not significantly different ($P > 0.05$), but both were notably lower than that in control group ($P < 0.05$). Abnormal fertilization rate (1PN + 3PN), significantly higher in group A than in control group ($P < 0.05$), was comparable between group A and group B, as well as between group B and control group ($P > 0.05$).

Table 3 compares the developmental potential of 2PN embryos among the three groups. The cleavage rate was similar among three groups ($P > 0.05$), but the available embryo rate in group A was markedly lower than that in group B ($P < 0.05$), and both were significantly lower than in control group ($P < 0.05$). The top quality embryo rate in group B was higher than in group A, and lower than in control group, but the difference was not significant, respectively ($P > 0.05$). The top quality embryo rate was markedly lower in group A than in control group ($P < 0.05$); the proportion of ≥ 6 -cell embryos in group B was notably higher than in group A ($P < 0.05$), and both of them were markedly lower than in control group ($P < 0.05$). The proportion of embryos with <10% fragmentation in group A was significantly lower than in group B ($P < 0.05$), and both were notably lower than in control group ($P < 0.05$). The proportion of oocytes with $\geq 50\%$ fragmentation was not significantly different between group A and group B ($P > 0.05$); however, both were significantly higher than in control group ($P < 0.05$). Moreover, the blastocyst formation rate in group A was significantly lower than in group B ($P < 0.05$), but not compared with the control group for embryo transfers which were performed routinely on day three in our center.

Table 1 Comparison of in vitro maturation rate according to maturation conditions

	Group A	Group B
No. of cycle	120	121
Mean age	29.78 $\pm$ 3.27	29.23 $\pm$ 4.63
Mean No. of oocytes collected	12.51 $\pm$ 6.16	12.67 $\pm$ 5.33
No. of immature oocytes	179	183
No. of mature oocyte after 24 h (%)	122 (68.16%) ^a	129 (70.49%) ^a

^a $p > 0.05$

Discussion

Cumulus cells (CCs) are of great importance in oocyte maturation, fertilization and subsequent early embryonic development in many species both in vivo and in vitro [11,36,37,40,43], including in humans [14,16,17]. In vitro maturation followed by fertilization opens a new avenue to make full use of the immature oocytes derived from controlled ovarian stimulation cycles. Several studies [1,6,9,12,17,27,32,34,35,41] have reported how to use the

Table 2 Comparison of fertilization rate according to maturation conditions

	Group A	Group B	Control group
No. of MII oocytes	122	129	2673
Total fertilization(%)	101 (82.79%) ^a	107 (82.95%) ^a	2350 (87.92%) ^a
2PN(%)	91 (74.59%) ^{b, c}	98 (75.97%) ^{b, d}	2253 (84.29%) ^{c, d}
1PN and 3PN(%)	10 (8.20%) ^{e, f}	9 (6.98%) ^{e, g}	97 (3.63%) ^{f, g}

a, b, c, g $p > 0.05$; c, d, f $p < 0.05$

immature oocytes; however, the detailed influence of CCs on the developmental potential of human immature oocytes has not been extensively examined. In this study, our results indicated that, although there was no significant difference in the proportion of oocytes that reached metaphase II with the presence or absence of cumulus cells, it was noteworthy that an improvement in developmental potential was observed in the immature oocytes with CCs.

We investigated two groups of immature oocytes that had undergone denudation of their CCs or not on the oocyte retrieval day, in order to determine whether CCs could improve the maturation and developmental potential of these oocytes. Our data showed that culture with CCs did not alter the nuclear maturation rate and both groups of these immature oocytes matured at a high rate. Our findings were similar to those of Johnson et al., who reported that co-culture with CCs did not alter the maturation rate between metaphase I (MI) and metaphase II (MII) oocytes [16]. Kim et al. also demonstrated that the maturation rate of GV oocytes from follicles primed with gonadotropin was not affected by the presence or absence of CCs [17], whereas Goud et al. demonstrated that the overall nuclear maturation rates and cleavage rates were higher in cumulus-intact oocytes compared to cumulus-denuded human GV-stage oocytes [12].

In agreement with previous studies [27,32], we found that total fertilization rates were similar between 24 h IVM (with or without CCs) and in vivo matured sibling oocytes. In contrast to these results, several studies showed consistently lower

fertilization rates of in vitro matured oocytes compared to sibling in vivo matured oocytes [1,9,34,41]. This discrepancy among different studies may be attributed to the timing of oocyte denudation, the period of in vitro culture, the IVM time interval between the first polar body extrusion and sperm injection, stimulation protocol, culture medium and laboratory protocols.

Our results showed that denudation cumulus cells of immature oocytes on the retrieval day would result in a delayed embryo development and an increased rate of fragmentation, which would compromise embryo quality and developmental competence. A highly significant improvement in available quality embryo rate after the IVM of oocytes was observed in the presence of cumulus cells for 24 h of culture before ICSI. We also observed that the proportion of blastocysts derived from the oocytes with CCs was significantly higher than that from the oocytes without CCs.

The mechanisms responsible for the effect of CCs on early embryonic development were undefined as yet. Based upon our observations that the nuclear maturation rates did not show significant difference between CCs denuded group and intact group, we suggest that this effect may be mediated by improved cytoplasmic maturation [42]. It has been shown that the cumulus cells are very important to complete oocyte cytoplasmic maturation in mouse, sheep, rats, cattle and pigs [11,36,37,40,43]. Many oocytes are mature in nucleus but immature in ooplasm after IVM. As a consequence, this asynchronization could compromise on the

Table 3 Comparison of embryos with 2PN among the three groups

	Group A	Group B	Control group
Embryo cleavage (%) *	87 (95.60%) ^a	94 (95.92%) ^a	2201 (97.69%) ^a
Available embryo (%) **	10 (11.49%) ^b	26 (27.66%) ^b	1373 (62.38%) ^b
top- quality embryo (%) **	7 (8.05%) ^{c, e}	16 (17.02%) ^{c, d}	535 (24.31%) ^{d, e}
No. of ≥ 6 -cell embryo (%) **	23 (26.44%) ^f	43 (45.74%) ^f	1451 (65.92%) ^f
No. of fragment <10% embryo (%) **	12 (13.79%) ^g	28 (29.79%) ^g	946 (42.98%) ^g
No. of fragment $\geq 50\%$ embryo ** (%)	41 (47.13%) ^{h, i}	34 (36.17%) ^{h, j}	397 (18.03%) ^{i, j}
No. of blastocyst (%) ***	6 (6.90%) ^k	19 (20.21%) ^k	—

* on D₂; ** on D₃; *** on D₅

a, c, d, h $p > 0.05$; b, e, f, g, i, j, k $p < 0.05$

developmental potential [37]. It has been demonstrated that cumulus cells co-culture starting at various IVF stages has no effect on fertilization and cleavage development but significantly improves rates of embryo development to morula or blastocyst stages [44]. It is further illustrated that, in the rhesus monkey, successful IVM has only been achieved with cumulus-enclosed oocytes, indicating that it is crucial for IVM to remain COCs intact. [24].

The surrounding CCs might secrete paracrine growth factors or produce adhesion molecules on their surface membranes which might play a role in the oocyte cytoplasmic maturation [38]. It has been demonstrated that cumulus cells can produce EGF-like factors, including amphiregulin, epiregulin, and betacellulin, which play an important role probably through the maximal activation of the EGFR signaling pathway during the in vitro maturation of primate oocytes [24]. One of the routes by which the factors are transmitted from cumulus cells to the oocyte is gap junctional communication (GJC)[22]. Gap junctions help to mediate the transport of molecules less than 1000 Da such as ions, second messenger, nucleotides, and amino acids between cumulus cells and the oocytes, all of which are necessary for oocyte metabolism and cytoplasmic maturation [22,31]. Taken together, we consider that CCs improve embryo quality through their influence on the cytoplasmic maturation of in vitro-matured oocytes.

Culture media components and culture conditions can affect and even modulate the meiotic regulation of mammalian oocytes [10,18]. Many studies have been conducted and are currently ongoing to develop an optimal maturation medium for IVM. For the in vitro maturation of human oocytes, the media has been supplemented with HMG [25], pregnant mare serum gonadotropin [3], HCG [3,21], FSH and LH [15,21], and E2 [3,21]. However, the receptors for many of these molecules are on the CCs [5,20], which implies that the supplements may not play a full role for cumulus-free oocytes. Based on this opinion, HTF medium supplemented with 10% SSS, without additional supplements, was used to culture immature oocytes. We found that the maturation and fertilization rate were comparable with previous studies, in which the culture medium, supplemented with hormones, was used. The proportion of available embryos derived from the in vitro-matured oocytes was higher than that of Johnson et al., who reported that the available embryo rate was approximately 13%. In their study, immature oocytes were co-cultured with granulosa cells, and HTF, without additional hormone, was also used as the culture medium [16]. We consider that the discrepancy may be attributable to severing the communication between oocytes and their surrounding CCs [22].

However, our data also showed that both 2PN zygotes and available embryos in group A or group B were significantly lower than those of control group. Previous studies showed that successful embryonic development relied not only upon nuclear maturation, but also on cytoplasmic maturation [27,32]. The

poor oocyte cytoplasmic maturation might cause the defect of embryonic development [23,30]. Cytoplasmic maturation involves complicated processes that prepare the oocyte cytoplasm for activation, fertilization and development [2]. During this process, RNA molecules, proteins and imprinted genes accumulate in the cytoplasm to regulate oocyte meiosis and development [8]. In vitro fertilization cycles, premature chromosome condensation (PCC) of the sperm chromatin and abnormal dispersion of oocyte chromosomes reflect cytoplasmic immaturity and cytoskeletal anomalies in the oocyte, which affects the normal zygote and embryo formation [45]. Glutathione has been identified in a number of species as a critical component of fertilization, and the depletion of oocyte GSH results in the failure of male pronucleus [7]. Most of trippronuclei zygotes are triploid and result from the retention of the second polar body [13] or the zygotes on the pronuclear stage undergo premature irregular cytokinesis [28]. Cumow et al. reported that supplementation of the IVM medium with GSH-OEt promoted better maturation and normal fertilization of macaque oocytes compared with non-supplemented medium [7]. These studies reflect that the depletion of one or some factors related to fertilization or embryo development in oocytes will result in abnormal fertilization or poor embryo quality. From this viewpoint, we consider that, even though retention of the attached CCs helps to improve cytoplasmic maturation, the in vitro-matured oocytes are in suboptimal constitutions for in vitro fertilization and subsequent embryonic development. It implies that, besides the genetic factors of immature oocytes, the current culture system may be suboptimal for oocyte maturation in vitro.

In conclusion, we have demonstrated that culturing oocytes with cumulus cells prior to ICSI had no influence on the nuclear maturation of immature oocytes, but it has a better effect on embryonic development. Our findings suggest that the low developmental competence of premature denuded oocytes can be attributed, at least in part, to incomplete or delayed oocyte cytoplasmic maturation rather than to nuclear maturation. In order to the optimal use of oocytes in COS cycles, immature oocytes derived from small follicles during in vitro fertilization cycles may have better outcomes if they are cultured in vitro with cumulus cells. Further studies are required to investigate the factors that affect the in vitro maturation of the oocyte, as well as the safety of the embryos derived from in vitro-matured oocytes.

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