Incomplete denudation of oocytes prior to ICSI enhances embryo quality and blastocyst development

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BACKGROUND: Granulosa cells are essential mediators of oocyte maturation and fertilization. Because of the denudation of oocytes in preparation for ICSI, any potential positive effect of surplus cumulus cells (CCs) on further development would be unable to exert further effect. In order to evaluate the actual influence of adhering cumulus cells on further preimplantation development, this prospective study was carried out. METHODS: Sibling cumulus–oocyte complexes for 57 ICSI patients were split into a study group (incomplete denudation, n = 314) and a control group (complete denudation, n = 336). According to the cumulus cell pattern after partial denudation, mature gametes from the study group were further subdivided into type A oocytes, which showed several prominent CC clusters (n = 202), and type B (n = 75), which showed a more homogeneous pattern with CC covering the whole surface of the gamete. RESULTS: In immature oocytes, presence of adhered CCs led to a significant increase in resumption of meiosis (P < 0.01). Fertilization rate (P < 0.05) and ability to cleave (P < 0.01) was impaired in the study group, because of difficulties in ICSI of type B oocytes. By contrast, embryo morphology on days 2 (P < 0.01) and 3 (P < 0.05), as well as blastocyst formation, was better (P < 0.05) in the study group (55 blastocysts out of 88 zygotes) as compared to that in the control group (49/105). CONCLUSION: These data indicate that co-culture of oocytes with attached CCs may enhance preimplantation development.

Key words: blastocyst formation/cumulus cells/embryo quality/ICSI/oocyte maturation

Introduction

Recent data strongly indicate that after conventional IVF, embryos reach blastocyst stage more frequently than after ICSI (Shoukir et al., 1998; Dumoulin et al., 2000; Griffiths et al., 2000; Miller and Smith, 2001).

Although it is suspected that blastocyst development rather reflects the competence of the oocyte itself and is relatively independent of a paternal genomic effect (Banerjee et al., 1995; Sakkas et al., 2000, 2002) consider injection of abnormal spermatozoa to be the most likely reason for this observed discrepancy in blastocyst formation rate. While this may indeed be true for severe cases of male infertility in patients with milder forms, additional factors may also exert an influence.

In this regard, a theoretical impact of cumulus cells (CCs) may be raised. These somatic cells are mediators (Sutton et al., 2003) of oocyte maturation, development and fertilization (McKenzie et al., 2004) and are, in turn, regulated by oocyte factors (Albertini et al., 2001; Eppig, 2001; Matzuk et al., 2002). Therefore, in ICSI, where oocytes are denuded before injection, any potential positive effect of surplus CCs on further development cannot exert further influence. This might result in lower numbers of blastocysts as compared to conventional IVF, in which CCs are not removed until the day of fertilization. Thus, attached cumulus tissue on ICSI oocytes could somehow mimic the suggested effect of co-culture with homologous granulosa (Dirnfeld et al., 1997; Fabbri et al., 2000) or CCs (Quinn and Margalit, 1995; Saito et al., 1995; Carrell et al., 1999) on further outcome.

To support this hypothesis, in the present study, we prospectively split sibling oocytes into two groups; in the first cohort, gametes were denuded completely, and in the second, numerous CCs were left attached to the zona pellucida (ZP) before ICSI. Thus, any impact of those CCs on preimplantation development and pregnancy outcome could be evaluated.

Materials and methods

During the 6-month study period, all ICSI couples who had a good prognosis in terms of ovarian response (e.g. more than eight oocytes collected) and who gave written consent to the study protocol were included in this prospective analysis (n = 57). Because it was guaranteed that at least half of the oocytes were injected with our routine ICSI method (Ebner et al., 2001) and, on the contrary, certain publications suggest a benefit from co-culture with CCs (Mansour et al., 1994; Carrell et al., 1999), this work was accepted by the Institutional Review Board. The experimental design is schematically shown in Figure 1.
According to the study design, women who showed normal FSH values (6.9 ± 2.3 IU/ml) which led to adequate levels of estradiol (1639 ± 630 pg/ml) and a sufficient number of oocytes (11.1 ± 3.2) were split into two groups. All couples were treated for either male factor infertility (n = 53) or previously failed fertilization during an IVF cycle (n = 4). No patients with severe forms of male factor infertility were included in this study; e.g. all men had a sperm count of >8 × 10^6/ml and at least 10% morphologically normal spermatozoa.

In this prospective approach, two routine stimulation protocols were applied. Cycle monitoring consisted of ovarian ultrasonography cycle day 1–3 to exclude functional cysts and further ovarian ultrasonography and serum E2, progesterone and LH measurement from stimulation day 5 onwards with monitoring frequency based on patient response.

Less than half of the patients (n = 20) were stimulated using a long protocol. After down-regulation of the pituitary with the GnRH agonist buserelin (Suprecur®, Aventis Pharma, Vienna, Austria), stimulation was initiated with human menopausal gonadotrophin (hMG, Menogon®, Ferring, Kiel, Germany).

In the GnRH-antagonist protocol (n = 37), hMG (Menogen®) was started on day 2 of the cycle. In addition, a GnRH-antagonist (Orgalutran®; Organon, Vienna, Austria) was administered after 5–6 days of stimulation, depending on the presence of a 12-13-mm follicle in the ultrasound scan.

In all patients, ovulation was induced with 5000–10 000 IU human chorionic gonadotrophin (hCG, Pregnyl®, Organon). Routinely, oocyte retrieval was carried out transvaginally under ultrasound guidance 36 h after hCG administration.

After collection, all cumulus–oocyte complexes (COCs) were cultured in vitro in BM1 medium (NMS Bio-Medical, Praroman, Switzerland) for 2–3 h (37°C, 6.5% CO2). After this resting period, assignment to the treatment (partial denudation) or control group (complete denudation) was made prospectively prior to denudation in order to minimize any deviation in denudation characteristics of individual COCs (Carrell et al., 1999).

At the beginning of the denudation process, both groups of COCs were treated the same, for example, a 30-s exposure to hyaluronidase (80 IU/ml; Medicult, Copenagen, Denmark). However, further procedure slightly differed between the two groups. In the control group, all CCs were completely removed from the ZP using hand-drawn glass pipettes of an appropriate diameter. Because special care was taken not to harm the oocyte mechanically (e.g. distortion, polar body dislocation), the final denudation step could take up to 1 min. In case several CCs remained attached to the zona, they were removed immediately before ICSI using the holding as well as injection pipettes.

Because, in the study group, the aim was to keep most of the CCs attached to the zona, hand-drawn glass tools had to have a larger diameter compared with those in the control group. This procedure was continued until the CC tissue no longer interfered with ICSI or hindered further evaluation of oocyte morphology (e.g. polar body location), which was performed according to our internal guidelines (Ebner et al., 2006). It has to be mentioned that the degree of denudation could not be standardized. In detail, two major patterns of CC attachment occurred with either several prominent CC clusters (type A, Figure 2a) or a more homogeneous pattern with CC involving the whole surface of the gamete (type B, Figure 2b). However, in both subtypes, it was guaranteed that a minimum of 300–500 cells per oocyte were not removed.

ICSI procedure in the control group was always performed at the 3 o’clock position with the polar body being held at the 6 o’clock position. The same was planned for the study group; however, individual denudation performance of COCs (e.g. no gap within CCs at 3 o’clock position) made it necessary to inject approximately one-quarter of the partly denuded oocytes (all type B) at alternative sites. This is expected to have no significant influence on fertilization and cleavage (Blake et al., 2000) as long as the first polar body is not held at the 9 o’clock position.

All gametes found to be at prophase or metaphase I after complete or incomplete denudation were cultured for another 24 h to see whether final maturation occurred. MII oocytes were checked for morphological normality, for example, whether they showed a clear, moderately granulate cytoplasm, a small perivitelline space, an intact zona and complete denudation (control group) or partial denudation (study group) was made prospectively prior to denudation in order to minimize any deviation in denudation characteristics of individual COCs (Carrell et al., 1999).

In case of blastocyst transfer, medium was changed from Blastassist System Medium 1 to Blastassist System Medium 2 (MediCult). Although mechanical removal of CC remnants was carefully avoided during zygote handling, a minor portion of CCs sometimes lost their contact to the zona. These single cells were not transferred further.

Zygote morphology (Ebner et al., 2003) and cleavage behaviour (days 2–3) were evaluated in all embryos (number and shape of blastomeres, degree of fragmentation, multinucleation), whereas signs of compaction (day 4) and blastocyst morphology (day 5) were only scored if blastocyst transfer was scheduled. Blastocyst quality was evaluated according to the work of Gardner and Schoolcraft (1999). In detail, according to the degree of expansion, the blastocysts were scored using Roman numbers in ascending order from grade I (blastocoele less than half of the volume of the embryo) to VI (completely hatched blastocyst). Beginning with full blastocyst stage (grade III), additional assessment of inner cell mass (ICM) and trophoectoderm (TE) was performed (based on cell number) in order to predict developmental competence.

In case of blastocyst transfer, medium was changed from Blastassist System Medium 1 to Blastassist System Medium 2 (MediCult) on
day 3. Documentation was performed using an imaging and archival software (Octax EyeWare®, MTG, Altdorf, Germany).

On the basis of the number of day 3 embryos (Racowsky et al., 2000) and/or patient request, transfer was scheduled either on the same day if the number was less than 3 (n = 35) or on day 5 (n = 22). Because these results did not differ from each other, the data were pooled. According to the guidelines of our Internal Review Board, the decision as to which embryos to transfer was solely based on embryo or blastocyst morphology.

Fisher’s exact test, Mann–Whitney U-test and chi-square test were used to analyse variables in the form of frequency tables. All tests were two-tailed with a significance of 95% (P < 0.05).

Results

During this study, a total of 650 gametes were collected, of which 575 (88.5%) were found to be at metaphase II. In detail, 24 oocytes showed a germinal vesicle and 51 were at metaphase I. Within 24 h, immature gametes finished maturation more frequently (P < 0.01) in the study group than in the control group (Table I) mainly because of an increased resumption of meiosis in MI oocytes compared to PI.

Table I further indicates that surplus CC impaired fertilization (P < 0.05) and the ability to cleave (P < 0.01) compared with the control oocytes. By contrast, quality of the embryos on days 2 (P < 0.01) and 3 (P < 0.05) turned out to be improved in the presence of abundant CCs.

Further subdivision of the study group according to the individual pattern of CC attachment (Figures 2a,b) after incomplete denudation (Table II) revealed that it is mostly the type B pattern that interferes with ICSI results because significantly more damaged oocytes were observed (P < 0.05). In addition, rates of fertilization (P < 0.05) and cleavage (P < 0.001) were decreased in this type compared to pattern A. On the contrary, the CC appearance covering the whole surface (type B) was associated with a better embryo morphology on day 2 (P < 0.05) as indicated in Table II.

Prolonged culture to day 5 (n = 193) led to an overall blastocyst formation rate of 53.9% (104/193); however, only 38 of 104 (36.5%) were of optimal quality as assessed by the morphology of the ICM and TE. While the rate of blastocyst formation was significantly increased in the study group (P < 0.05), the rate of top quality blastocysts was not affected. Focusing on the CC types of the study group, it was found that further preimplantation development was comparable between both subgroups, although blastocyst quality showed a trend towards higher quality blastocysts in type B (P = 0.07).

A mean number of 1.9 (±0.3) embryos or blastocysts was transferred. Of 57 patients, 22 achieved clinical pregnancy (38.6%) corresponding to an implantation rate of 21.3% (23/108). Because of our strategy to choose transferable embryos exclusively based on their morphology, 21 patients had so-called mixed transfers, for example, one embryo from the completely denuded oocyte group and one from the study group (6 of 21 patients achieved clinical pregnancy). Where it was possible to analyise homogeneous transfers, the clinical pregnancy rate did not differ between those patients who got only embryos from the study cohort (10/19; 52.6%) and those whose embryos derived from control oocytes (6/17; 35.3%).

Discussion

A recent review (Sutton et al., 2003) emphasizes that intercellular communication between oocytes and granulosa cells (CCs and mural granulosa cells) is essential for normal follicular differentiation and oocyte developmental competence.

In detail, two separate ways of signalling between gametes and somatic cells occur, either paracrine or via gap junctions, with both forms being essential for normal oogenesis (Dong et al., 1996; Albertini et al., 2001). Several key molecules produced by the oocyte (e.g. growth differentiation factor 9 or bone morphogenic protein 15) play an important role in granulosa cell function and differentiation (Galloway et al., 2000; Li et al., 2000). In addition, gametes promote their own development via metabolic cooperativity with CCs, e.g. inducing amino acid uptake (Eppig et al., 2005) or glycolysis (Sugiura et al., 2005).
By contrast, CCs play an important role during early maturational steps and after resumption of meiosis, although at this time transzonal cytoplasmic processes are mostly withdrawn, because paracrine communication compensates for the loss of gap junctional connection. This is supported by the finding that IVF results are compromised if the CCs are removed prior to IVF compared to post-IVF (Fatehi et al., 2002; Wongsrikeao et al., 2005).

In order not to lose any beneficial effect of CC mass on in vitro development of early embryos, several authors introduced co-culture with either mural granulosa cells (Dirnfeld et al., 1997; Fabbri et al., 2000) or CCs (Quinn and Margalit, 1995; Saito et al., 1995; Carrell et al., 1999). The mechanisms which contribute to better embryonic development in co-culture with homologous granulosa cells are still unclear; however, it can be hypothesized that that is the result of detoxifying and/or embryotrophic factors.

Of course, the more physiological approach would be to leave the majority of the cumulus oophorus intact 24 h past IVF which has successfully been used in order to increase embryo quality and pregnancy rate (Mansour et al., 1994; Carrell et al., 1999; Khamsi et al., 1999). In ICSI, however, evidence of any effect of attached CCs on further competence of the ovum is scarce. Actually, to date, only one ICSI article exists, and this deals with partly denuded rabbit oocytes (Zheng et al., 2004). These authors did not find any benefit in terms of fertilization, cleavage or blastocyst formation. However, they removed all remaining CCs as early as 8 h after injection in order to guarantee adequate pronuclear evaluation. Thus, any possible long-term effect of the additional presence of CC on further developmental competence of the conceptus is reduced.

During the study, a methodological problem had to be faced first described by Carrell et al. (1999). Unfortunately, individual COCs show different patterns of CC dispersal after partial denudation. To exclude any bias, for example, assigning all gametes that were easy to denude to the control cohort, group assignment was made prior to initiation of the denudation process. Thus, it has to be emphasized that it is not possible to denude all COCs in a standardized manner. However, because

<table>
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<tr>
<th>Table I. Comparison between study and control groups in terms of maturation, fertilization and development to the blastocyst stage</th>
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<tbody>
<tr>
<td>Control group (complete denudation)</td>
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<tr>
<td>Number of COC</td>
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<td>PI</td>
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<tr>
<td>MI</td>
</tr>
<tr>
<td>MII</td>
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<tr>
<td>Normal oocytes</td>
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<tr>
<td>Resumption of meiosis*</td>
</tr>
<tr>
<td>PI</td>
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<tr>
<td>MI</td>
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<tr>
<td>2PN</td>
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<tr>
<td>Optimal pronuclear pattern</td>
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<tr>
<td>Halo formation</td>
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<td>Degeneration after ICSI</td>
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<tr>
<td>Cleavage on day 2</td>
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<td>n good embryos day 2</td>
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<td>n good embryos day 3</td>
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<td>n 2PN for blastocyst culture</td>
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<td>Compacting day 4</td>
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<tr>
<td>Blastocyst formation</td>
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<tr>
<td>Top quality blastocyst</td>
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</tbody>
</table>

COC, cumulus–oocyte complex; MI, metaphase I; MII, metaphase II; PI, prophase I; PN, pronuclei.
Values in parentheses are percentages.
*Resumption of meiosis within 24 h.

<table>
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<tr>
<th>Table II. Comparison of ICSI outcome between CC pattern showing distinct clusters (type A) and a more homogeneous appearance (type B) in the study group (( n = 277 ))</th>
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<td>Type</td>
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<td>n ICSI</td>
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<tr>
<td>2PN</td>
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<tr>
<td>Degeneration after ICSI</td>
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<tr>
<td>Cleavage on day 2</td>
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<tr>
<td>Number of good embryos day 2</td>
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<td>Number of good embryos day 3</td>
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<tr>
<td>Number of 2PN for blastocyst culture</td>
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<tr>
<td>Blastocyst formation</td>
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<td>Top quality blastocyst</td>
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PN, pronuclei.
Values in parentheses are percentages.
we guaranteed presence of an adequate number of cells on the ZP comparable with those in previous articles (Carrell et al., 1999; Zheng et al., 2004), the predictive value of the present data is not likely to have been influenced. Indeed, we tried to solve this problem by further subdividing the CC appearance after incomplete denudation to see whether there was a difference in outcome between the CC pattern showing several cell clusters (type A) and the more homogeneous one (type B).

To our knowledge, this article is the first ICSI study, which prospectively analyses the actual effect of co-culture using homologous CCs in situ. These data support previous publications, suggesting that the presence of CCs is associated with an enhanced rate of in vitro maturation in oocytes that were immature at the time of denudation (Goud et al., 1998; Yamazaki et al., 2001). This indicates that nuclear as well as cytoplasmic maturation is superior in the presence of attached CCs (Goud et al., 1998).

Interestingly, these data revealed a negative impact of the attached CC remnants on fertilization and the ability to cleave. Taking into consideration the statistically significant differences in ICSI outcome between the two subclasses of the study group (Table II), it may be concluded that the above-mentioned phenomenon is mostly caused by the more homogeneous CC pattern B. Two possible explanations may account for this interpretation. Firstly, gametes with CCs covering the whole surface did not regularly show a gap within the cumulus tissue at the planned site of injection. Thus, sites other than the 3 o'clock position had to be chosen for ICSI, which might have influenced fertilization, although this has not been shown to be the case (Blake et al., 2000). Secondly, a higher rate of degenerated oocytes in type B oocytes support this theory; it could be that CCs through attached CCs might have either harmed the oocytes mechanically or raised the possibility that foreign somatic coronal DNA could enter the ovum (Stanger et al., 2001).

Interestingly, once the oocytes were fertilized and cleaved, the above effects were reversed, that is the quality of the embryos at the 4- and 8-cell stage was significantly superior in oocytes that were incompletely denuded (irrespective of the CC pattern). In these cases, additional CC might gain direct influence on embryonic metabolism, for example, via increased amino acid transport. Biol Reprod 73,351–357.


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