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ASSISTED REPRODUCTION TECHNOLOGIES

Effect of cumulus cell removal 4 h post-insemination on fertilization and embryo quality: a prospective randomized sibling-oocyte study

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Abstract
Purpose The study was designed to evaluate whether cumulus cell removal 4 h post-insemination could influence fertilization and embryo quality.
Methods The study included 61couples undergoing standard long down regulation protocol from July 2011 to May 2012. Sibling oocytes of each patient were randomly assigned to either the 4 h group or the 20 group. For the 4 h group, cumulus cells were removed 4 h after gamete coincubation; for the 20 group, cumulus cells removal was performed 20 h after insemination. Fertilization rate, embryo quality, pregnancy rate and implantation rate were assessed.
Results A total of 801 sibling cumulus-oocyte complexes (COCs) were randomized to the 4 h group (421 COCs) or 20 h group (380 COCs). There was no difference in the two pronuclei, one pronucleus and grade 1–2 embryo rate. Three pronuclei rate was significantly higher in the 4 h group compared to the 20 h group (12.6 % vs. 8.2 %, P=0.041). Comparison of embryo transfer cycles in which either embryos from the 4 h group or 20 h group were transferred did not reveal any statistically significant differences in pregnancy or implantation rates.
Conclusion The results of the present study indicate that cumulus cell removal 4 h post-insemination may increase the percentage of tripronuclear zygotes. However, normal fertilization rate, embryo development, clinical pregnancy rate and implantation rates are not influenced by the timing of cumulus cell removal.

Keywords Sibling oocytes · Cumulus cell · Fertilization · Embryo quality

Capsule Cumulus cells removal 4 h post-insemination may increase the percentage of tripronuclear zygotes and does not influence normal fertilization rate, embryo development, clinical pregnancy and implantation rates.

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Introduction

Total fertilization failure remains hard to predicted in in-vitro fertilization (IVF) cycles [13, 14, 17]. Rescue of 1-day-old oocytes by intracytoplasmic sperm injection (ICSI) that have failed to fertilize usually results in very poor clinical outcomes [5, 6, 8, 9, 11–13, 15, 16]. In order to reduce the proportion of failed fertilization cycles, a treatment observing early signs of fertilization after a short time of gamete coincubation has been used in IVF cycles [1, 10, 19, 21]. Those in which the second polar body (2 PB) has extruded are considered to be fertilized. However, only when the cumulus cells are removed completely, can the second polar body be observed under the microscope. In a limited number of studies, there are conflicting data regarding the effect of early cumulus cell removal on fertilization and embryo quality. Wei et al. [19] reported a significant reduction in the rate of available embryo after cumulus cell removal 4 h post-insemination, while others have described an equivalent embryo morphology compared with cumulus cell removal between 18 and 22 h post-insemination [1, 21]. The polyspermy rate in case of early cumulus cell removal was usually unchanged except as reported by Xiong et al. [21], which has described a decreased polyspermy rate. These results, obtained from existing studies so far, are contradictory and suggest that the effects of early cumulus cell removal remain unclear.

Results of previous studies were generally obtained from a cohort of oocytes collected after ovarian stimulation. The difference in oocytes quality between women might influence the final results. Therefore, this study was designed as a sibling study, where oocytes from individual women were randomizedly divided into two groups. By allowing each patient to serve as her own control, we could reduce the interindividual variability observed in multiple cohort studies as well as the technology platform-based variability.

The aim of the present study was to investigate whether the effects of early cumulus cells removal on fertilization and embryo quality using a prospective auto controlled design. As far as we know, this work is the first randomized sibling-oocyte study ever published on this field.

Materials and methods

Design of the study

We performed a prospective randomized trial and compared on sibling oocytes the following outcomes: two pronuclei (2PN), one pronucleus (1PN), three pronuclei (3PN), and grade 1–2 embryos rate. This trial was included 65 patients who underwent IVF-ET treatment in the Reproduction Centre, Sir Run Run Shaw Hospital, China, between July 2011 and May 2012. The inclusion criteria were as following: unexplained infertility, secondary infertility of at least 5 years duration, first IVF treatment, normozoospermic semen according to WHO criteria [20], at least ten oocytes retrieved. No male factor was involved. Within the 65 patients, four patients were excluded due to total fertilization failure. The oocytes were randomly allocated into one of two groups at the time of removing cumulus cells: for 4 h group, cumulus cell was removed only 4 h post-insemination; for 20 h group, cumulus cell removal was performed 20 h post-insemination. When there were uneven numbers of oocytes, the additional oocyte was allocated to 4 h group.

Institutional review board approval was obtained to perform early removal of cumulus cells. All patients were informed that total fertilization failure after conventional IVF was possible. Written informed consent was obtained from each couple after offering them the fertilization method.

Ovarian stimulation

Patients included in the study were treated by routine controlled ovarian hyperstimulation (COH). After mid-luteal pituitary down-regulation with a gonadotrophinreleasing hormone (GnRH) agonist, the ovaries were stimulated with follicle-stimulating hormone (FSH). Follicular development was monitored by ultrasound scanning. Human chorionic gonadotropin (hCG) was administered when at least three follicles measured >18 mm diameter. Transvaginal oocyte retrieval was performed 36 h after hCG injection. The COC_S were collected in IVF medium (G-IVF, Vitrolife Sweden AB, Sweden) containing 10 % serum substitute supplement (SSS, Irvine Scientific, Santa Ana, CA) and incubated at 6%CO_2, 37 °C incubators for insemination.

Semen preparation and in vitro insemination

Semen samples were collected by ejaculation in the morning of the oocyte retrieval day. Isolate (Irvine Scientific, USA) discontinuous concentration gradient was used for isolation of motile spermatozoa. The pellet was transferred into a new centrifuge tube and washed twice in IVF medium. Concentration of spermatozoa was determined by pipetting an aliquot of 5 μl semen into a Makler Counting Chamber (Sefi Medical Instruments, Israel). Finally, spermatozoa were incubated at 6%CO_2, 37 °C until use.

Each COC was inseminated with 50,000–100,000 motile spermatozoa/ml in a 75 μl microdroplet at 38~40 h post-hCG.

Cumulus cells removal

In 4 h group, COCs were coincubated with spermatozoa for 4 h, and the cumulus cells were mechanically removed. The oocytes were gently aspirated in and out of pipette of inner diameter slightly smaller than the oocytes until cumulus cells were completely removed. Fertilization was determined when two polar bodies were present. Those in which a second polar body was evident were considered as fertilized. Fertilization failure was determined when the oocytes were absent of the second polar body. In patients with fertilization failure, rescue ICSI was performed on those oocytes that did not show a second polar body at 6 h of insemination.

In 20 h group, after 20 h of co-incubation, cumulus cells were removed using fine pipettes for fertilization assessment.

Pronuclei and embryo evaluation

Pronuclei evaluation was carried out 20 h after insemination. All zygotes in two groups were transferred to fresh cleavage medium (G-1, Vitrolife Sweden AB, Sweden) with 12 % SSS. Normal fertilization was confirmed by the presence of two pronuclei (2PN). The fertilization rate was expressed as numbers of two pronuclei per numbers of COC. Embryonic development was assessed on Day 3 (70–72 h) after oocyte retrieval with an inverted microscope using routine examination of: [1] the number of blastomeres; [2] the degree of cytoplasmic fragments; [3] the uniformity of blastomeres. Embryos morphology was scored as: grade 1, equal blastomeres with no obvious fragmentation; grade 2, <20 % fragmentation and/or unequal blastomeres; grade 3, 20–50 % cytoplasm fragmentation; grade 4, >50 % cytoplasm fragmentation.

Embryo transfer and pregnancy testing

Embryo transfer took place 3 day after oocytes retrieval under ultrasound guidance. To reduce the risk of high-rank multiple pregnancies, the number of embryos replaced is mostly limited to one or two.

Luteal support (combination of estrogen and progesterone) was initiated on day 1 after oocytes retrieval. Clinical pregnancy rate was defined by the presence of gestational sac with fetal heartbeat after at 7 weeks or later. Implantation rate was defined as the number of gestational sacs per number of embryos transferred.

Statistical analysis

Analyses were performed using the SPSS 17.0 statistical package (SPSS, Inc., Chicago, IL). Comparison of fertilization rates and embryo quality were performed using the Chi square test. Pregnancy rates and implantation rates were compared by using the Fisher's exact test (two-tailed).

The study design involved randomization of oocytes; hence the oocyte was the unit of analysis. Sample size calculation was based on the fertilization rate as the primary outcome. To detect a 10 % difference between the groups with a power of 0.8 (alpha-level 0.05), at least 269 oocytes would be necessary for each group.

Results

In 65 oocyte retrievals, 846 oocytes were collected, of which 444 oocytes were randomly assigned to 4 h group and 402 oocytes were randomly allocated to 20 h group. According to the exclusion criteria of fertilization failure within the two groups, 23 and 22 oocytes were not analyzed in the 4 h and 20 h group, respectively.

Patient demographic data including female age, duration of infertility, infertility diagnosis and number of oocytes retrieved are given in Table 1.

Table 1 Demographic data of patients included in the study

Patients (n)	61
Female age (y)	30.0±3.3
Duration of infertility (y)	4.0±2.6
Aetiology	
Unkonwn factor(unexplained infertility)	10
Tuboperitoneal	10
Ovulatory dysfounction	7
Endometriosis	11
Mixed factor	27
No. of retrieved COCs	801
No. of COCs per patient	13.1±1.9

Patients with fertilization failure were not included

Table 2 shows that the two pronuclei and one pronucleus rate were not different between 4 h and 20 h groups (69.1 % vs. 66.8 %, $P>0.05$; 4.0 % vs. 5.0 %, $P>0.05$). Cumulus cells removal 4 h post-insemination resulted in significantly higher three pronuclei rate (12.6 % vs. 8.2 %, $P=0.041$). There was no different in grade 1–2 embryos rate (52.1 % vs. 49.4 %, $P>0.05$).

A total of 116 embryo transfers were performed. Thirty-six of these were mixed transfers. More embryos from 4 h group were transferred compared with 20 h group (60 vs. 20). In total, 28 patients became pregnant: 13 in the 4 h group (40.6 % per transfer), 5 in the 20 h group (45.5 % per transfer) and 10 in the group with mixed transfers (55.6 % per transfer). These data are given in Table 3. No significant differences were observed with regard to pregnancy rates and implantation rates between the three groups of embryo transfers.

Discussion

Cumulus cells play a major role in regulation of oocyte growth and induction of oocyte maturation, since they might provide growth factors or express adhesion molecules [3, 4, 18]. In this study, there was no significant difference between an early and a late cumulus cells removal on the normal fertilization. Cumulus cells removal after 4 h co-incubation seems to have no effect on normal fertilization rate. In normal fertilization, both nuclear and cytoplasmic maturation of oocytes are required independently [3, 4]. Nuclear maturation refers to the progression of the oocyte nucleus from the germinal vesicle (GV) to the metaphase II stage. Cytoplasmic maturation prepares the oocyte for activation, fertilization. Earlier reports indicated oocytes that are at stage of metaphase-I after retrieval and mature within 4 h in vitro culture in the absence of surrounding cumulus cells, show a tendency of lower fertilization rate, but the cytoplasmic maturity of the metaphase-II oocytes does not appear to be affected by surrounding cumulus cells [18]. It may be assumed that in this study the percentage of cytoplasmic maturity of the metaphase-II oocytes was similar between the two groups. One possible explanation for the results is that the oocytes are well synchronized at the level of nuclear and cytoplasmic maturation using the standardized ovarian simulation with GnRHa/FSH/hMG protocol [7].

Three pronuclei, resulting from failure in the fertilization process [2], were observed in the two groups. A significant higher percentage of three pronuclei (12.6 %) zygotes was obtained in the 4 h group. The high three pronuclei rate appeared to be related to the timing of cumulus cells removal. A similar study has been previously reported by Wei et al. [19], in which polyspermy rate was 12.0 % in the group with removal of cumulus cells 4 h post-insemination [19]. However, other two studies have reported low percentage of tripronuclear. In a study by Chen and Kattera [1], tripronuclear

Table 2 Outcomes in the two study groups

	4 h group	20 h group	*P*-value
No. COCs	421	380	
Two pronuclei (%)	291(69.1)	254(66.8)	NS
One pronucleus (%)	17(4.0)	19(5.0)	NS
Three pronuclei (%)	53(12.6)	31(8.2)	0.041*
Grade 1–2 embryos (%)	147(52.1)	128(49.4)	NS

* Denotes statistical significance

rate was 6.1 % was obtained. 7.5 % polyspermy rate was observed by Xiong et al. [21]. It should be noted that the cumulus cells were removed at 6 h of insemination in the latter two studies. We speculated that the different time to remove cumulus cells may cause different abnormal fertilization rates. As we know, it was harder to remove cumulus cells 4 h post-insemination compared to cumulus cells removal at 6 h of insemination. The repeated aspiration may cause damage to cytoplasmic structures and subsequent fertilization process, leading to multiple sperm penetrations, or inhibition of the second polar body extrusion. In addition, three pronuclear may be correlated with oocyte immaturity [2]. Although the majority of the oocytes are well synchronization on the level of nuclear and cytoplasmic maturation, there is still a small portion of oocytes that have not yet reached full maturity. These oocytes that are in germinal vesicle or metaphase I stage become mature during long-term incubation, resulting in a decreased three pronuclei rate in 20 h group.

This study showed a similar grade 1–2 embryo rate between 4 h group and 20 h group, indicating that cumulus cells removal at 4 h of insemination did not affect embryo quality. This result supports the previous data [1, 21] but is inconsistent with another report by Wei et al. [19], which have shown a decrease in the quantity of available embryo rate. The different study designs could explain these conflicting results. Patients assignment described in the study of Wei et al. [19] associated with non-randomized control. Patients with low risk of fertilization failure was assigned to the control group, whereas patients with high risk of fertilization failure to the experimental group. When analyzing the results, one might question whether a decrease in the available embryo rate was due to early cumulus cells removal. In our study, with random assignment, there is a better chance at detecting if the observed effect is due to early cumulus cells removal. Consequently, we concluded that early cumulus cells removal has no significant detrimental effect concerning embryo quality.

The best quality embryos were transferred regardless of whether they were derived from 4 h group or 20 h group. The aim was to provide the patient with the highest possible chance of pregnancy. This transfer guideline led to three groups of transfer: transfers performed with (1) embryos from 4 h group (group 1, n=32), (2) embryos from 20 h (group 2, n=11) or (3) embryos from both groups (group 3, n=18). We did not observe statistical differences in pregnancy and implantation rates.

The analyze power in this study is limited by a relatively small sample size. In spite of the limitation, this is still a prospective randomized study. Furthermore, the present study has sufficient power to detect differences of fertilization and embryo quality between 4 h and 20 h groups. However, the results of embryo transfer showed no statistical differences in pregnancy and implantation rates. A larger scale work might be needed to validate this finding.

In conclusion, the results of the present study suggest that cumulus cells removal 4 h post-insemination may increase the percentage of three pronuclei zygotes. However, the normal fertilization, grade 1–2 embryo, as well as pregnancy and implantation rates are not influenced by different timing of cumulus cell removal.

Table 3 Clinical results after transfer of embryos chosen only from the 4 h group or only from 20 h group, and transfer of embryos chosen from both groups

	Only from the 4 h group	Only from the 20 h group	Mixed	*P*-value
No. of transfers	32	11	18	
Transferred embryos	60	20	36	
Mean embryos per transfer	1.88	1.82	2.00	NS
Grade 1–2 embryos transferred (%)	48(80.0)	14(70.0)	31(86.1)	NS
Positive hCG	15	5	10	NS
Biochemical pregnancy	0	0	0	NS
Preclinical abortion	0	0	0	NS
Ectopic pregnancy	2	0	0	NS
Clinical pregnancy (% per ET)	13(40.6)	5(45.5)	10(55.6)	NS
Implantation rate (%)	16(20.0)	6(30.0)	14(38.9)	NS

References

1. Chen C, Kattera S. Rescue ICSI of oocytes that failed to extrude the second polar body 6 h post-insemination in conventional IVF. Hum Reprod. 2003;18:2118–21.
2. Feng H, Hershlag A. Fertilization abnormalities following human in vitro fertilization and intracytoplasmic sperm injection. Microsc Res Tech. 2003;61:358–61.
3. Ho JY, Chen MJ, Yi YC, Guu HF, Ho ES. The effect of preincubation period of oocytes on nuclear maturity, fertilization rate, embryo quality, and pregnancy outcome in IVF and ICSI. J Assist Reprod Genet. 2003;20:358–64.
4. Isiklar A, Mercan R, Balaban B, Alatas C, Aksoy S, Urman B. Impact of oocyte pre-incubation time on fertilization, embryo quality and pregnancy rate after intracytoplasmic sperm injection. Reprod Biomed Online. 2004;8:682–6.
5. Kuczynski W, Dhont M, Grygoruk C, Pietrewicz P, Redzko S, Szamatowicz M. Rescue ICSI of unfertilized oocytes after IVF. Hum Reprod. 2002;17:2423–7.
6. Lundin K, Sjogren A, Hamberger L. Reinsemination of one-day-old oocytes by use of intracytoplasmic sperm injection. Fertil Steril. 1996;66:118–21.
7. Macklon NS, Stouffer RL, Giudice LC, Fauser BC. The science behind 25 years of ovarian stimulation for in vitro fertilization. Endocr Rev. 2006;27:170–207.
8. Ming L, Liu P, Qiao J, Lian Y, Zheng X, Ren X, et al. Synchronization between embryo development and endometrium is a contributing factor for rescue ICSI outcome. Reprod Biomed Online. 2012;24:527–31.
9. Morton PC, Yoder CS, Tucker MJ, Wright G, Brockman WD, Kort HI. Reinsemination by intracytoplasmic sperm injection of 1-day-old oocytes after complete conventional fertilization failure. Fertil Steril. 1997;68:488–91.
10. Nagy ZP, Rienzi LF, Ubaldi FM, Greco E, Massey JB, Kort HI. Effect of reduced oocyte aging on the outcome of rescue intracytoplasmic sperm injection. Fertil Steril. 2006;85:901–6.
11. Park KS, Song HB, Chun SS. Late fertilization of unfertilized human oocytes in in vitro fertilization and intracytoplasmic sperm injection cycles: conventional insemination versus ICSI. J Assist Reprod Genet. 2000;17:419–24.
12. Pehlivan T, Rubio C, Ruiz A, Navarro J, Remohi J, Pellicer A, et al. Embryonic chromosomal abnormalities obtained after rescue intracytoplasmic sperm injection of 1-day-old unfertilized oocytes. J Assist Reprod Genet. 2004;21:55–7.
13. Shalom-paz E, Alshalati J, Shehata F, Jimenez L, Son WY, Holzer H, et al. Clinical and economic analysis of rescue intracytoplasmic sperm injection cycles. Gynecol Endocrinol. 2011;27:993–6.
14. Shveiky D, Simon A, Gino H, Safran A, Lewin A, Reubinoff B, et al. Sibling oocyte submission to IVF and ICSI in unexplained infertility patients: a potential assay for gamete quality. Reprod Biomed Online. 2006;12:371–4.
15. Tsirigotis M, Redgment C, Craft I. Late intracytoplasmic sperm injection (ICSI) in in-vitro fertilization (IVF) cycles. Hum Reprod. 1994;9:1359.
16. Tsirigotis M, Nicholson N, Taranissi M, Bennett V, Pelekanos M, Craft I. Late intracytoplasmic sperm injection in unexpected failed fertilization in vitro: diagnostic or therapeutic? Fertil Steril. 1995;63:816–9.
17. van der Westerlaken L, Helmerhorst F, Dieben S, Naaktgeboren N. Intracytoplasmic sperm injection as a treatment for unexplained total fertilization failure or low fertilization after conventional in vitro fertilization. Fertil Steril. 2005;83:612–7.
18. Van de Velde H, De Vos A, Joris H, Nagy ZP, Van Steirteghem AC. Effect of timing of oocyte denudation and micro-injection on survival, fertilization and embryo quality after intracytoplasmic sperm injection. Hum Reprod. 1998;13:3160–4.
19. Wei D, Zhang C, Yin B, Wang P, Xie J, Song X, et al. Early cumulus cell removal could reduce the available embryo rate in human IVF. J Assist Reprod Genet. 2011;28:1213–6.
20. World Health Organization. WHO laboratory manual for the examination of human semen and semen–cervical mucus interaction. 4th ed. Cambridge: Cambridge University Press; 1999.
21. Xiong S, Han W, Liu JX, Zhang XD, Liu WW, Liu H, et al. Effects of cumulus cells removal after 6 h co-incubation of gametes on the outcomes of human IVF. J Assist Reprod Genet. 2011;28:1205–11.