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ASSISTED REPRODUCTION TECHNOLOGIES

Early cumulus cell removal could reduce the available embryo rate in human IVF

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Abstract
Purpose This study was designed to compare cumulus cell removal at different times and to evaluate their effects on embryo developmental potential and the outcomes of in vitro fertilization (IVF).
Methods We retrieved 606 IVF cycles with standard long down regulation protocol from January 2010 to December 2010. These cycles were divided into two groups: group A, 364 cycles with high risk of fertilization failure, whose cumulus cells were removed 4 h after short gamete coincubation; group B, 242 cycles as control, whose cumulus cells removal were performed 17 to 18 h after insemination. The epidemiological, clinical, laboratory factors and the outcomes of these cycles were analyzed.
Results The available embryo rate of group A was significantly lower (P=0.002). There were no significant differences in other laboratory parameters and outcomes between the two groups (P>0.05).
Conclusions For patients with high risk of fertilization failure, there could be a risk of compromising the rate of available embryos, if fertilization is judged by the presence of 2 PB by cumulus cell removal only 4 h post-insemination. Therefore, this strategy is not recommended to all IVF cycles and future studies are needed to confirm its reliability.

Keywords Short gamete coincubation · Fertilization · Cumulus cell · Available embryo rate · In vitro fertilization

Capsule Evaluate the effects of early cumulus cell removal on embryo developmental potential and the outcomes of *in vitro* fertilization (IVF).

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Introduction

After brief coincubation of gametes, early fertilization is judged by the presence of the second polar body (2 PB) in daily laboratory practice. Cumulus cells are required to be removed before early fertilization judgment, and rescue intracytoplasmic sperm injection (R-ICSI) is preferable in particular circumstances, e.g. low fertilization rate or total fertilization failure [1]. The early fertilization judgment strategy and R-ICSI is favorable for a portion of patients with high risk of fertilization failure, whereas among a major portion of the patients cumulus cell removal reveals normal fertilization. Therefore, one important issue remains to be defined, whether early cumulus cell removal for early fertilization judgment has potential detrimental effects on embryo developmental potential and IVF outcomes. Here, we retrospectively retrieved 606 cycles, which are distributed into two groups whose cumulus cells are removed at different times. Through this study, we tried to clarify if early cumulus cell removal could affect embryo developmental potential and outcomes. We hope this result could be of value for future IVF practice.

Materials and methods

A total of 774 IVF cycles with long down regulation protocol were enrolled in the Reproductive Medical Center of Henan Provincial People's Hospital from January 2010 to December 2010. Within the 774 cycles, 19 cycles were canceled, and 76 of them cryopreserved all the embryos and R-ICSI were performed in 73 cycles. The remaining 606 cycles with normal fertilization were distributed into two groups according to the risk of fertilization failure, which is inferred from their medical history: for 364 cycles in group A with high risk of fertilization failure, cumulus cell removal was performed only 4 h post-insemination; for 242 cycles in group B with low fertilization failure risk, cumulus cell was removed 17 to 18 h post-insemination. In our IVF center, high risk of fertilization failure was defined as: male or female factor primary infertility, over 5 years secondary infertility, unexplained infertility, a total number of motile sperm between 5×10^6 and 10×10^6, and fertilization rate around 30% in previous cycles. One cycle would be considered to be of high risk of fertilization failure if either of the criteria above was satisfied.

Controlled ovarian stimulation (COS)

All patients underwent standard long down regulation protocol. Gonadotropin releasing hormone (GnRH) agonist was given for down-regulation in the middle of luteal phase of menstrual cycle, and follicle-stimulating hormone (FSH) was added after 14 days. Follicular development was monitored by ultrasound scanning from the 4th day of menstrual cycle. 6000–10 000 IU of human chorionic gonadotrophin (hCG) was administrated when there were one or more follicles with diameter >18 mm. 36–38 h after hCG administration, oocytes retrieval was performed by ultrasound-guiding transvaginal follicle aspiration.

Sperm preparation

Semen samples were collected in sterile containers by masturbation after 3–5 days of sexual abstinence and liquefied 30 min at room temperature. The samples were placed on a 40%/80% density gradient and centrifuged for 20 min at 300 G. The supernatant was removed and the sperm pellet was resuspended in 3 ml of HEPES wash buffer, then mixed well and followed by centrifugation at 350 G for 5 min. The supernatant was removed again and the sperm pellet was resuspended in 2 ml of fertilization medium, then mixed well and followed by centrifugation at 350 G for 5 min. The supernatant was discarded and the sperm pellet was placed at the bottom of fresh fertilization medium so that the swim-up method could be carried out. Post-upstream sperm reached the criterion of IVF, and the sample was incubated at 37°C until use.

Insemination

Ordinary micro-drop insemination was performed by adding 1–2 oocytes corona cumulus complexes (OCCC) into 50 μl fertilization droplets ($3–5\times10^4$ sperms per oocyte). Incubated was carried out at 37°C and 6% CO_2.

Cumulus cells removal and 2 PB observation

The oocytes were aspirated in and out of a pipette of inner diameter slightly smaller than the oocyte (inner diameter 120 μm) and cumulus cells were removed. In group A, the cumulus cells were removed at 4 h post-insemination. The oocytes were observed under 200× stereomicroscope and the states of 2 PB from the meiosis II (MII) oocytes were recorded. Oocytes with ≥70% showing 2 PB were considered as the normal fertilization. Cases with lower fertilization rate or even total fertilization failure were excluded in this study. In group B, the oocytes were transferred to cleavage medium from the fertilization medium after 4 h of incubation, and the cumulus cells were removed next morning.

Assessment of fertilization and cleavage

Fertilization was confirmed by the presence of two pronuclei (2PN) using an inverted microscope 16–18 h after insemination. Embryo cleavage check and score were carried out after further culture for another 24 h and 48 h respectively. The embryos were graded on a scale of one to four grades: grade 1, even sized, symmetrical blastomeres with no obvious fragmentation; grade 2, uneven sized blastomeres, or the total cytoplasmic mass contained <10% fragmentation; grade 3, 10–50% cytoplasm fragmentation; grade 4, >50% cytoplasmic fragmentation [2]. Embryos transferred were selected from grade 1 or grade 2 ones.

Table 1 Characteristics of group A and group B

Groups	cycles	Age(Y)	Oocytes	Maturation rate (%)
Group A	364	31.21±4.58	12.57±5.59	85.74(3913/4564)
Group B	242	31.67±4.10	12.84±5.63	87.10(2707/3108)

Table 2 Laboratory parameters of group A and group B

Groups	2PN rate (%)	Cleavage rate (%)	Polyspermy rate (%)	1PN rate (%)
Group A	56.86 (2595/4564)	98.38 (2553/2595)	12.01 (548/4564)	3.81 (174/4564)
Group B	56.37 (1752/3108)	98.34 (1723/1752)	12.22 (380/3108)	4.02 (125/3108)

1PN=one pronuclei; 2PN=two pronuclei

Statistical analyses

All statistical analysis was performed according to intention to treat. For independent samples, student's t-test was employed to analyze normally distributed quantitative variables, while the chi-square test was used to analyze nominal variables. Continuous variables were summarized with descriptive statistics (n, mean, standard deviation). Categorical variables were described with counts and percentages. All tests were two-sided, and a P-value less than 0.05 are considered statistically significant for all statistical tests. All analyses were performed using SPSS software, version 10.0.

Results

Between the 364 cycles in group A and the 242 cycles in group B,no significant differences were observed in female mean age and number of retrieved oocytes (31.2±4.6 vs. 31.6±4.1, and 12.6±5.6 vs. 12.8±5.6, respectively. $P>0.05$). Oocyte maturation rate in group A (85.7%) was lower than group B (87.1%), which might be the result of early cumulus cell removal in group A. However, this rate does not differ significantly between the two groups ($P>0.05$). See Table 1.

The 2PN rate in group A was 56.9%, whilst it was 56.4% in group B. The cleavage rate and polyspermy rate were similar between group A and group B (98.4% vs. and 98.3% and 12.0% vs. 12.2%, respectively). One pronuclei (1 PN) rate was 3.8% in group A and 4.0% in group B. There were no significant differences in 2PN fertilization rate, 2PN cleavage rate, polyspermy rate, and 1PN rate between the two groups ($P>0.05$). See Table 2.

Available embryo rate in group A was significantly lower than in group B (64.4% vs. 69.0%, $P=0.002$). Compared to the implantation rate in group A, the rate in group B was of no difference (35.8% vs. 36.2%, $P>0.05$). In group A, the clinical pregnancy rate was 52.7% (192/364), while in group B it represented a slightly raised clinical pregnancy rate of 53.7% (130/242). The spontaneous abortion rate in group A (6.9%) was lower than in group B (8.3%). However, there were no significant differences in these two rates between the two groups ($P>0.05$). See Table 3.

Discussion

Along with the improvement of artificial reproductive technique (ART), pregnancy rate has raised considerably. However, in a portion of cycles, low fertilization rate or fertilization failure could result in consequences like few available embryos, or in some rare circumstances, cycle cancellation, which is a tremendous psychological, physical and economical blow to the patients. Meanwhile, in front of ART clinicians and embryologists, there is an urgent need to avoid these unfavorable circumstances. Fertilization is a delicately programmed process comprised of numerous cellular and molecular events of sequence. The fertilization process in human involves sperm and oocyte binding, acrosome reaction, oocyte activation, the oocyte and sperm pronuleus and genetic material fusion [3]. During this process, any changes in the extracellular environment would influence fertilization and the development of embryos. According to statistic, the total fertilization failure rate in conventional IVF is 2–3%, and the lower fertilization rate (<30%) in conventional IVF is 10–20% [4, 5]. We practiced half-ICSI for patients who are prone to have fertilization failure in the past [6]. The half-ICSI strategy could ensure fertilization of a portion of the oocytes but excessively extended the application of ICSI.

The ICSI bypasses some critical cellular and molecular events of the fertilization. During ICSI practice, several manipulations could be potentially detrimental to embryo developmental potential, e.g. the spermatozoa subjectively selected by embryologists, mechanical puncture to penetrate the zona pellucida and oolemma and the polyvinyl pyrrolidone (PVP) injected into the oocyte cytoplasm [7, 8]. Ever since the advent of ICSI, this technique has been controversial because it may potentially increase birth

Table 3 Pregnancy outcomes of group A and group B

Groups	Available embryos rate (%)	Implantation rate (%)	Clinical pregnancy rate (%)	Spontaneous abortion rate (%)
Group A	64.42 (1684/2614)	35.76 (260/727)	52.74 (192/364)	6.87 (25/365)
Group B	69.03 (1228/1779)	36.22 (184/508)	53.72 (130/242)	8.26 (20/242)

defect. In the history of ART, it was no more than 19 years before the first ICSI baby was born [9]. More studies and long-term follow-ups are required to prove the safety of this technology. It is preferable to adopt one protocol that closely mimics the spontaneous insemination in our daily IVF practice.

Chen et al. [10] recommended a short insemination protocol: early sperm media coincubation, early cumulus cell removal, judgment of fertilization according to the early manifestation under microscopes and early performance of "rescue" ICSI to retrieve satisfying outcomes. In recent years, a fair number of reproduction centers have chosen the short insemination protocol and achieved satisfying results. But there is an ongoing debate on the application of short insemination due to its early cumulus cell removal. For the patients whose fertilization was satisfying after early cumulus cell removal, there was no report on whether the outcomes would be compromised by this procedure.

In this study, cumulus cells were removed 4 h post-insemination, the time during which activated oocytes finish meiosis II and released the 2 PB [11]. It was harder to remove the cumulus cells 4 h post-insemination and more repeated aspirations are required compared to cumulus cells removal the next morning. Moreover, the early observation under microscopes would result in possible temperature and pH fluctuations during fertilization. We were concerned that the above factors would influence the formation of female-male pronucleus and embryo developmental potential.

In this study, there were no significant differences in clinical pregnancy rate and early abortion rate between group A and group B, indicating that short insemination had no influence on the outcomes of IVF-ET. Therefore, removing the cumulus cells 4 h post-insemination would not jeopardize the results of the patients with normal fertilization rate. On the other hand, it rescued the cycles with lower fertilization rate or even total fertilization failure. Available embryo rate in group A was significantly lower than that in group B, and the difference was statistically significant ($P<0.01$) (Table 3). This suggested the compromised quality of embryos might be the consequence of the early cumulus cell removal; it could also be the result of the patients with poor prognosis in group A. For patients with high risk of fertilization failure, there was a risk of decreasing the rate of available embryos, if fertilization was judged by the presence of 2 PB through cumulus cells removal only 4 h post-insemination. Therefore, it is not recommended to all IVF cycles and future studies are needed to further confirm its reliability.

This study is a retrospective analysis, and the sources of the patients are not identical between the two groups, which may result in bias. In the future, prospective researches could be done among patients of high fertilization failure risk, among which, in each cycle, randomly, half of oocytes are observed 4 h post-insemination and the other half of oocytes are observed next morning. The principles and optimal conditions for in vitro embryo development are to mimic the spontaneous reproductive events and try to avoid manual interventions as far as possible, that is also the laws should be followed in in vitro insemination.

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