Observational Study

**In vitro** maturation of human oocytes maintaining good development potential for rescue intracytoplasmic sperm injection with fresh sperm

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**Abstract**

**BACKGROUND**

The outcomes of the use of commercial **in vitro** maturation (IVM) medium to culture immature oocytes obtained from conventional ovulation induction, followed by rescue intracytoplasmic sperm injection (RICSI), are not ideal. It is thus difficult to widely adopt this approach in clinical practice. Therefore, it is necessary to explore methods for improving the clinical outcome of IVM.

**AIM**

To study the effect of sperm on the developmental potential of in vitro-matured oocytes in conventional culture.

**METHODS**

This was a retrospective study of patients whose immature oocytes were harvested from conventional oocyte stimulation cycles and underwent ICSI at our hospital between June 2018 and August 2020. RICSI was performed using sperm collected on the day of oocyte harvest (old) and sperm collected on the day of RICSI (fresh) and oocytes matured **in vitro** after 24 h of culture in conventional medium. The rates of **in vitro** oocyte maturation, normal fertilization, normal cleavage, day-3 top-quality embryos, and useful blastocyst formation were compared between the two groups.

**RESULTS**

In total, 102 germinal vesicle (GV)-stage immature oocytes were cultured in the old sperm group. In the fresh sperm group, 122 GV-stage immature oocytes were collected and cultured **in vitro** for 24 h. There were no significant differences in the general conditions of males and females between the two groups (P > 0.05). The oocyte maturation, normal fertilization, and normal cleavage rates of the old and
fresh groups were 51.0% vs 55.7%, 61.5% vs 64.7%, and 93.8% vs 93.2%, respectively. None of the rates differed significantly ($P > 0.05$) between the two groups. However, the day-3 top-quality embryo and useful blastocyst rates of the old and fresh sperm groups were 16.6% vs 63.4%; 6.67% vs 34.6%, respectively. The day-3 top-quality embryos and useful blastocyst rates of the old sperm group were significantly lower than those of the fresh group ($P < 0.05$).

CONCLUSION

In vitro maturation with conventional culture medium combined with the use of fresh sperm collected on the day of RICSI is an easy-to-implement strategy for patients whose oocytes are completely or mostly immature.

Key Words: In vitro oocyte maturation; Sperm injections; Intracytoplasmic; Semen analysis; In vitro fertilization; Human oocyte

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Core Tip: Owing to the low implantation rate, in vitro maturation (IVM) is difficult to widely adopt in clinical practice. Therefore, there is an urgent need to explore methods for improving the clinical outcome of IVM. We studied the effect of sperm on early embryo development from in vitro-matured oocytes. Our results show that IVM with conventional culture medium combined with the use of fresh sperm collected on the day of rescue intracytoplasmic sperm injection is an easy-to-implement strategy for patients whose oocytes are completely or mostly immature.

Citation: Dong YQ, Chen CQ, Huang YQ, Liu D, Zhang XQ, Liu FH. In vitro maturation of human oocytes maintaining good development potential for rescue intracytoplasmic sperm injection with fresh sperm. World J Clin Cases 2022; 10(7): 2166-2173
URL: https://www.wjgnet.com/2307-8960/full/v10/i7/2166.htm
DOI: https://dx.doi.org/10.12998/wjcc.v10.i7.2166

INTRODUCTION

Early in 2014, over 5 million babies throughout the world were born with the assistance of in vitro fertilization and embryo transfer (IVF-ET)[1]. IVF-ET has made great progress as an important treatment for infertility. The continuous improvement of controlled ovarian stimulation (COS) is an important factor [2-4]. In the process of COS, the threshold of the follicle-stimulating hormone (FSH) window is artificially increased by using exogenous gonadotropin (GN), and the time of the FSH window was prolonged so that the follicles that were about to atresia in the physiological state continued to grow. However, due to the different thresholds of FSH required by each follicle in the follicle cluster, the development of follicles is often out of sync. Ovulation induction drugs amplify the out of sync of follicle development. Therefore, there are many immature oocytes in COS cycles[5]. On the other hand, due to differences in sensitivity to drugs or other unknown reasons in some patients, the obtained oocytes are sometimes completely or mostly immature. Generally, such immature oocytes are considered to have no clinical application value and are discarded, which imposes heavy financial and psychological burdens on patients.

In 1983, Veeck et al[6] first reported the in vitro maturation (IVM) of immature oocytes obtained in COS cycles and achieved clinical pregnancy. In 1999, De Vos[7] reported that the IVM of metaphase I oocytes obtained from COS cycles cultured for 4 h with intracytoplasmic sperm injection (ICSI) resulted in a healthy baby. Subsequently, different authors reported the value of IVM-ICSI[8-10]. These previous studies demonstrated that even immature oocytes from either partially or completely immature oocyte cycles could fertilize and divide into embryos after appropriate in vitro culture. However, several studies examined the use of commercial IVM medium to culture immature oocytes obtained after conventional ovulation induction, followed by rescue ICSI (RICSI), but the outcomes were not ideal[11-13]. Owing to the low implantation rate compared to conventional in vitro fertilization (IVF), IVM is difficult to be widely adopted in clinical practice, and optimization is still necessary. Thus, for patients with non-genetically derived in vivo delayed oocyte maturation, improving the IVM clinical outcome has major significance.

In early clinical work, we found that immature oocytes can achieve a better clinical outcome after being cultured in vitro and fertilized with fresh sperm. Therefore, in this study, we wanted to further confirm this result and compare the outcome of in vitro matured eggs fertilized with fresh and old sperm.
MATERIALS AND METHODS

Participants
Germlinal vesicle (GV)-stage oocytes were donated by 28 patients who underwent ICSI treatment due to male factors at the reproductive center of Guangdong Women and Children’s Hospital between June 2018 and August 2020. The age of the females was less than 35 years. Patients underwent the mid-luteal long protocol. Each patient has at least five GV eggs, and the patients had requested their reuse.

Ethical approval
The study was approved by the Institutional Review Board of Guangdong Women and Children’s Hospital (Approval No. 202001158). Each participant in the study provided written informed consent. The embryos in this study were limited to the patient and were not used for any other reproductive purpose.

Oocyte collection
Follicles were punctured under ultrasound guidance 36 h after the administration of 10000 IU human chorionic GN (hCG, Merck Serono, Switzerland). The corona-cumulus cells were removed by 80 IU/ML hyaluronidase (Irvine Scientiﬁc, United States), and the meiotic status of human oocytes was then assessed. Only Metaphase-II (MII) stage oocytes were fertilized by ICSI. GV-stage oocytes with a discernable germinal vesicle were donated and collected for this study.

Semen treatment
Semen samples were obtained from the man who came to our center for ART. Fresh semen was processed on a discontinuous density gradient (90/45, Spermient, COOK), followed by centrifugation at 250 g for 20 min. After centrifugation, the pellet was washed once in G-MOPS-PLUS (Vitrolife, Sweden) by centrifugation at 250 g, 5 min, and concentrated to the appropriate final concentration.

ICSI and IVM
A total of 224 GV-stage oocytes obtained from 28 patients were randomly distributed to the old and fresh sperm groups. They were cultured for 24 h in the G-IVF-PLUS (Vitrolife, Sweden) medium. The criterion for nuclear maturation was the extrusion of the rst polar body. Then, the number of MII-stage oocytes was subjected to RICSI using old or fresh sperm. RICSI was carried out under an inverted microscope (Nikon, Japan), and all procedures employed sequential culture media supplied by G-IVF-PLUS, G1-PLUS, and G2-PLUS, (Vitrolife, Sweden).

Embryo culture and outcomes
Sixteen to eighteen hours after RICSI, fertilization was evaluated, and oocytes with normal fertilization, which had two pronuclear (2PN) oocytes, were counted. day-3 embryos were assessed 72 h after ICSI. The cell morphology was evaluated as follows: (1) Grade I, the cells showed even size, regular morphology, and homogeneous, clear cytoplasm with no granulation; (2) Grade II, the cells were slightly uneven in size, the morphology was slightly irregular, and granulation could be found in the cytoplasm; (3) Grade III, the cells were substantially uneven in size, irregular morphology was evident, and granulation could be found in the cytoplasm; or (4) Grade IV, the cells were severely uneven in size, and evident granulation could be found in the cyto-plasm. Cell fragmentation was evaluated as follows: (1) Grade I, 0-10% fragmentation; (2) Grade II, 21%-50% fragmentation; or (3) Grade III, > 50% fragmentation. At 120 to 144 h after ICSI, the number of blastocysts was determined. All embryos were incubated up to the blastocyst stage and assessed according to Gardner’s criteria. In this study, the oocyte maturation rate was calculated by the number of MII oocytes divided by the number of cultured oocytes. The rate of normal fertilization was calculated by the number of 2PN divided by the number of MII oocytes. The normal cleavage rate was calculated as the number of 2PN cleavages divided by the 2PN oocyte number. The day-3 top embryo rate was calculated as the number of embryos ≥ 7-9c/II. I in 2PN cleavage divided by the 2PN cleavage number. The useful blastocyst rate was the number of blastocysts above B/3bc divided by the 2PN cleavage.

Statistical analysis
SPSS 21.0 (IBM, Armonk, NY, United States) was used for statistical analysis. Continuous data are described as the means ± SD and were analyzed using one-way analysis of variance, followed by the least significant difference test to compare different groups. Categorical data are described as percentages (%) and were analyzed using the chi-square test or Fisher’s exact test. P < 0.05 was considered statistically significant.
RESULTS

**Characteristics of the female patients**
The general characteristics of the two groups are shown in Table 1. There were no differences in female age ($P = 0.175$), infertility duration ($P = 0.546$), average GN days ($P = 0.324$), average GN consumption ($P = 0.532$), body mass index ($P = 0.301$), or average number of oocytes ($P = 0.326$).

**Characteristics of the male patients**
There were no differences in male age ($P = 0.324$) or infertility duration ($P = 0.546$). There were also no differences in the total number of motile sperm before treatment ($P = 0.245$) between the two groups. The general characteristics of the two groups are shown in Table 2.

**Maturation rates of IVM in conventional culture medium for 24 h**
Immature oocytes (102 GV) were cultured in the old sperm group. The number of mature oocytes was 52 after 24 h of in vitro culture (oocyte maturation rate was 51.0%). For the patients in the fresh sperm group, there were 122 GV immature oocytes. The number of mature oocytes was 68 after 24 h of in vitro culture (oocyte maturation rate was 55.7%). There was no significant difference between the two groups ($P = 0.477$) (Table 3).

**Sperm may have an effect on early embryo development**
In the old sperm group, the number of normally fertilized oocytes was 32 (normal fertilization rate of 61.5%, 32/52). The number of normal cleavages was 30 (cleavage rate of 93.8%, 30/32). The number of day-3 top embryos was 5 (day-3 top embryo rate of 16.6%, 5/30), and the number of useful blastocysts was only 2 (blastocyst rate was 6.67%, 2/30). For the patients in the fresh sperm group. The corresponding rates of normal fertilization, normal cleavages, day-3 top embryos, and useful blastocysts were 64.7% (44/68), 93.2% (41/44), 63.4% (26/41), and 34.6% (9/26), respectively. The day-3 top embryo rate and useful blastocyst rate were significantly different between the two groups ($P < 0.05$), while the normal fertilization rate and cleavage rate were not significantly different ($P > 0.05$) (Table 4).

**Clinical outcomes of frozen-thawed embryo transfer cycles from RICSI of immature oocytes**
There were a total of 11 patients whose oocytes were all immature. Two patients underwent RICSI with old sperm, and each received two useful blastocysts. However, no clinical pregnancy was achieved after frozen-thawed embryo transfer (FET). For the other nine patients, fresh sperm was used for RICSI. Eight of the nine patients yielded transferrable blastocysts, and only one patient yielded no useful blastocysts. FET was performed on the eight patients, resulting in six successful clinical pregnancies, for a clinical pregnancy rate of 75.0%. In addition, all of these patients exhibited healthy birth. At the same time, the clinical pregnancy rate of conventional FET was 55%.

DISCUSSION

Generally, immature oocytes after conventional ovulation induction are considered to have no clinical application value and are thus discarded. Several researchers have investigated the application value of immature oocytes obtained after conventional ovulation induction after culture in commercial IVM medium, but the findings showed that although immature oocytes still have the potential for further development, the rate of day-3-top-quality embryos is low, and the clinical outcomes are poor.[6,7] Nevertheless, because of its short shelf life and low frequency of use, it is not routinely stored for most centers. Therefore, it is necessary to improve the outcome of IVM under conventional culture conditions.

The results strongly suggest that IVM with conventional culture medium combined with the use of fresh sperm collected on the day of RICSI is a recommendable method. The findings of this study showed that after being cultured in conventional medium for 24 h, approximately half of the GV-stage oocytes obtained from conventional ovulation induction matured. Although the maturity rate was still relatively lower than that of normal cycles, the normal fertilization rate, cleavage rate, and day-3 top embryo rate reached the level of conventional cycles. We only observed the oocyte maturation rate under culture for 24 h because it was not convenient to require the man to extract sperm repeatedly in the fresh sperm group. The findings of this study showed that the day-3 top embryo rate and useful blastocyst rate were significantly different between the two groups. The difference between the two groups was that the sperm were either obtained 1 day before ICSI (i.e., on the day of oocyte retrieval) or freshly obtained on the day of RICSI, meaning that the sperm in the old sperm group were cultured in vitro for over 24 h before use. The advantages of using freshly obtained sperm include avoiding the increased level of reactive oxygen species (ROS) in the culture medium induced by long culture. In general, there is a
Table 1 Comparison of the characteristics of the patients (female)

| Variables                  | Old sperm group (n = 15) | Fresh sperm group (n = 13) | P value |
|----------------------------|--------------------------|---------------------------|---------|
| Age (yr)                   | 29.5 ± 2.1               | 30.6 ± 2.3                | 0.175   |
| Infertility duration (yr)  | 3.4 ± 1.9                | 3.1 ± 2.0                 | 0.546   |
| Average Gn days            | 9.9 ± 3.2                | 11.1 ± 1.5                | 0.324   |
| Average Gn consumption (IU)| 2591.2 ± 1453.7          | 2076.6 ± 1267.3           | 0.532   |
| BMI                        | 21.2 ± 2.4               | 21.6 ± 2.7                | 0.301   |
| Average oocyte number      | 12.1 ± 4.7               | 13.5 ± 4.9                | 0.326   |

Gn: Gonadotropin; BMI: Body mass index.

Table 2 Comparison of the characteristics of the patients (male)

| Variables                  | Old sperm group(n = 15) | Fresh sperm group(n = 13) | P value |
|----------------------------|--------------------------|---------------------------|---------|
| Age (yr)                   | 32.5 ± 2.2               | 31.6 ± 2.3                | 0.324   |
| Infertility duration (yr)  | 3.4 ± 1.9                | 3.1 ± 2.0                 | 0.546   |
| Motile sperm number (× 10^6)| 3.2                      | 2.9                       | 0.245   |

Table 3 Comparison of the 24 h in vitro maturation rate of the two groups

| Groups                   | No. of GV oocytes | No. of MII oocytes (24 h) | Maturation rate (%) |
|--------------------------|-------------------|---------------------------|---------------------|
| Old sperm group          | 102               | 52                        | 51.0                |
| Fresh sperm group        | 122               | 68                        | 55.7*               |

*P > 0.05 between the two groups.
GV: Germinal vesicle; MII: Metaphase-II.

Table 4 Comparison of in vitro maturation outcomes between the two groups

| Variables                  | Old sperm Group | Fresh sperm Group | P value |
|----------------------------|-----------------|-------------------|---------|
| 2PN rate (%)               | 61.5 (32/52)    | 64.7 (44/68)      | 0.130   |
| Cleavage rate (%)          | 93.8 (30/32)    | 93.2 (41/44)      | 1.00    |
| day-3 top embryo rate (%)  | 16.6 (5/30)     | 63.4 (26/41)      | 0.001*  |
| Useful blastocyst rate (%) | 6.67 (2/30)     | 34.6 (9/26)       | 0.009*  |

*P < 0.05 between the two groups.
2PN: Two pronuclear.

balance between the antioxidative system and oxidative stress in semen. Nevertheless, with the sperm increased storage time in culture, the increase in ROS in the environment can induce double-strand deoxyribonucleic acid (DNA) breaks, base modification, and abnormal chromatin crosslinking, consequently damaging the integrity of the nucleic chromatin of sperm[15,16]. On the other hand, the shortened time of abstinence could also improve semen quality and significantly reduce the DNA fragmentation rate. Most studies have demonstrated that a long period of abstinence may lead to lower normal morphology and DNA quality rates of sperm, while increased DNA fragmentation can reduce the rate of top embryos, induce embryo development arrest, and even lead to abortion[17,18]. Since the sperm to be used for ICSI are selected randomly, in patients with an increased DNA fragmentation rate, the possibility of selecting sperm with DNA breakage is also increased.
CONCLUSION

This study has some limitations. The sample sizes of the two groups were both small. The clinical outcomes require further observation in a large sample. In conclusion, non-genetic oocyte immaturity after retrieval from conventional ovulation induction maintains high application value after culture in conventional medium. It is recommended to retrieve sperm freshly on the day of RICSI. Finally, for patients with poor oocyte maturity due to unknown reasons, the ICSI strategy could be recommended to be modified in the next cycle to provide a chance of the maturation of immature oocytes in culture in vitro.

ARTICLE HIGHLIGHTS

Research background
Some patients suffer from complete immaturity of all eggs from conventional ovulation induction. The outcomes of the use of commercial in vitro maturation (IVM) medium to culture these immature oocytes followed by rescue intracytoplasmic sperm injection (RICSI) are not ideal. It is thus difficult to widely adopt this approach in clinical practice. Therefore, it is necessary to explore methods for improving the clinical outcome of IVM.

Research motivation
In early clinical work, we found that immature oocytes can achieve a better clinical outcome after being cultured in vitro and fertilized with fresh sperm. Therefore, in this study, we wanted to further confirm this result and compare the outcome of in vitro matured eggs fertilized with fresh and old sperm.

Research objectives
To study the effect of sperm on the developmental potential of in vitro-matured oocytes in conventional culture.

Research methods
Participants: The germinal vesicle (GV)-stage oocytes were donated by 28 patients who underwent intracytoplasmic sperm injection (ICSI) treatment due to male factors. Oocytes collection: Those GV-stage oocytes with a discernable germinal vesicle were donated and collected for this study. ICSI and IVM: GV-stage oocytes were randomly distributed to the old and fresh sperm groups. They were cultured for 24 h in the G-IVF-PLUS medium. The criterion of nuclear maturation was the extrusion of the first polar body. Then the number of MII-stage oocytes were conducted rescue intracytoplasmic sperm injection (RICSI) using old or fresh sperm.

Research results
None of the oocyte maturation, normal fertilization, and normal cleavage rates differed significantly between the two groups. The day-3 top-quality embryos and useful blastocyst rates of the old sperm group were significantly lower than those of the fresh group.

Research conclusions
In vitro maturation with conventional culture medium combined with the use of fresh sperm collected on the day of RICSI is an easy-to-implement strategy for patients whose oocytes are completely or mostly immature.

Research perspectives
For patients with poor oocyte maturity due to unknown reasons, the ICSI strategy could be recommended to be modified in the next cycle to provide a chance of the maturation of immature oocytes in culture in vitro.

FOOTNOTES

Author contributions: Dong YQ and Liu FH contribute to conceptualization; Dong YQ and Liu D contribute to methodology; Dong YQ, Chen CQ, and Huang YQ contribute to formal analysis and investigation; Dong YQ, Liu FH, and Zhang XQ writing - original draft preparation; Dong YQ and Chen CQ writing - review and editing; Liu FH funding acquisition; Dong YQ and Zhang XQ resources; Liu FH and Zhang XQ supervision; all authors read and approved the final manuscript.

Supported by Science and Technology Collaborative Innovation Project of Guangzhou, No. 201704020217.
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