Effect of melatonin on the clinical outcome of patients with repeated cycles after failed cycles of in vitro fertilization and intracytoplasmic sperm injection

Qi Zhu
Anhui Medical University

Kaijuan Wang
Reproductive Medicine Center, Department of Obstetrics and Gynecology, the First Affiliated Hospital of Anhui Medical University, Hefei, China

Chao Zhang
Reproductive Medicine Center, Department of Obstetrics and Gynecology, the First Affiliated Hospital of Anhui Medical University, Hefei, China

Beili Chen
Reproductive Medicine Center, Department of Obstetrics and Gynecology, the First Affiliated Hospital of Anhui Medical University, Hefei, China

Huijuan Zou
Reproductive Medicine Center, Department of Obstetrics and Gynecology, the First Affiliated Hospital of Anhui Medical University, Hefei, China

Weiwei Zou
Reproductive Medicine Center, Department of Obstetrics and Gynecology, the First Affiliated Hospital of Anhui Medical University, Hefei, China

Rufeng Xue
Reproductive Medicine Center, Department of Obstetrics and Gynecology, the First Affiliated Hospital of Anhui Medical University, Hefei, China

Dongmei Ji
Reproductive Medicine Center, Department of Obstetrics and Gynecology, the First Affiliated Hospital of Anhui Medical University, Hefei, China

Zhaojuan Yu
Reproductive Medicine Center, Department of Obstetrics and Gynecology, the First Affiliated Hospital of Anhui Medical University, Hefei, China

Bihua Rao
Reproductive Medicine Center, Department of Obstetrics and Gynecology, the First Affiliated Hospital of Anhui University, Hefei, China

Yunxia Cao
Reproductive Medicine Center, Department of Obstetrics and Gynecology, the First Affiliated Hospital of Anhui Medical University, Hefei, China

Ding Ding
Reproductive Medicine Center, Department of Obstetrics and Gynecology, the First Affiliated Hospital of Anhui Medical University

Zhiguo Zhang (✉ zzg_100@163.com)
Anhui Medical University https://orcid.org/0000-0003-1483-3321

Research

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Abstract

**Background:** Melatonin (MT), a powerful antioxidant, can effectively ameliorate the *in vitro* development of animal embryos, but few studies have been performed on human embryos. Therefore, we investigated whether the application of MT in embryo cultures can improve embryonic development and clinical outcomes of patients with repeated cycles after failed *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) cycles.

**Methods:** Human immature oocytes from controlled ovarian hyperstimulation cycles were collected for *in vitro* maturation culture and ICSI fertilization. The obtained embryos were cultured *in vitro* in medium containing 0, 10^{-11}, 10^{-9}, 10^{-7} or 10^{-5} M MT, and 10^{-9} M was determined to be the optimal concentration. Subsequently, 140 patients who experienced failed IVF/ICSI cycles underwent 140 cycles of embryo culture *in vitro* with medium containing 10^{-9} M MT. High-quality blastocysts were collected and cryopreserved for three months before vitrified-warmed embryo transfer. These culture cycles served as the experimental (10^{-9} M) group. The control (0 M) group comprised previous failed cycles. The patients were further divided into subgroups of 1, 2 or ≥3 failed cycles. The fertilization and embryo development statuses were compared.

**Results:** The fertilization, cleavage, high-quality embryo, blastocyst, and high-quality blastocyst rates of the 10^{-9} M group were significantly higher than those of the 0 M group (87.7% vs. 83.6%, *p* < 0.01; 94.1% vs. 90.5%, *p* < 0.01; 58.3% vs. 43.8%, 51.1% vs. 41.8%, 43.4% vs. 22.9%, all *p* < 0.0001). To date, a total of 50 vitrified-warmed cycle transfers were performed in the 10^{-9} M group and the implantation rate, biochemical pregnancy rate and clinical pregnancy rate were significantly higher than those in the 0 M group (65.6% vs. 9.7%, *p* < 0.0001; 64.0% vs. 12.5%, *p* < 0.0001; 40.0% vs. 11.7%, *p* < 0.0001). Two healthy infants were delivered successfully and the other 18 women who achieved clinical pregnancy also had good examination indexes.

**Conclusion:** The application of MT to embryo cultures *in vitro* improved embryonic development in patients with repeated cycles after failed IVF/ICSI cycles and had good clinical outcomes. The optimal concentration of MT was 10^{-9} M.

**Trial registration:** Name in the registry: Effect of melatonin on the clinical outcome of patients with repeated cycles after failed cycles of *in vitro* fertilization and intracytoplasmic sperm injection; registration number: ChiCTR2100045552; date of registration: April 19, 2021 (retrospectively registered); URL of trial registry record: www.medresman.org.cn.

Background

Assisted reproductive technology (ART) involves the use of medical methods to artificially manipulate oocytes, sperm, fertilized eggs, and embryos to achieve the goal of conception [1]. Among them, *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are the core technologies of ART. IVF refers
to the natural fertilization of eggs and sperm through artificial methods and the subsequent in vitro culture of early embryos, while ICSI refers to the process of injecting a single sperm into the cytoplasm of an egg using micromanipulation technology to achieve fertilization. In vitro fertilization and embryo transfer (IVF-ET) technology has become an important means of infertility treatment since its discovery, but the risk of poor embryo development with the traditional IVF/ICSI treatment cycle is still unavoidable [2], which leads to pregnancy failure due to poor development of the embryos transferred. Therefore, exploring an efficient technology of embryo culture in vitro to obtain high-quality embryos is one of the key features for improving the effect of ART treatment.

Melatonin (MT) is a multifunctional molecule that is produced mainly in the pineal gland [3]. It is a powerful free radical scavenger and antioxidant [4]. MT and its metabolites can directly remove reactive oxygen species (ROS) in cells, activate antioxidant enzymes, increase the expression level and activity of glutathione (GSH), and inhibit pro-oxidant enzymes to reduce cell oxidation injury, which can improve oocyte in vitro maturation (IVM) and embryonic development [5]. It has been reported that adding the appropriate amount of MT to IVM and embryo culture medium can significantly improve the developmental potential of oocytes and the in vitro development of embryos [6-8]. Through research on human IVF and IVM, Li et al. found that the addition of MT to the embryo culture medium can improve the clinical outcomes of IVF and IVM [9]. In 2019, our research team added MT to IVM medium to culture human immature oocytes from a controlled ovarian hyperstimulation (COH) cycle, which significantly reduced the levels of ROS and Ca$^{2+}$ in oocytes during the IVM process, increased mitochondrial membrane potential, and improved embryo development, eventually resulting in healthy offspring [10]. The present study was the first attempt to use embryo culture medium supplemented with MT to culture embryos of patients undergoing repeated cycles after failed IVF/ICSI cycles and aimed to explore whether the application of MT could improve the embryonic development and clinical outcomes of patients with repeated IVF/ICSI cycles.

Materials And Methods

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Anhui Medical University(2015013). Before conducting the study, the partners of all enrolled patients were interviewed and provided signed informed consent.

Research patients

A total of 140 patients with failed cycles who visited the Reproductive Medicine Center of the First Affiliated Hospital of Anhui Medical University from June 2020 to November 2020 were selected. The inclusion criteria were as follows: (1) at least one IVF/ICSI failed cycle and (2) patient age $\leq$ 36 years. After genetic factors of both men and women were excluded, the causes of infertility included 18 cases of male-only factors, involving oligoasthenoteratozoospermia; 77 cases of female-only factors, including 41
cases of sequelae of pelvic inflammatory disease, 26 cases of polycystic ovary syndrome, and 10 cases of endometriosis; 40 cases of combined male and female factors; and 5 cases with unknown causes.

**COH regimen**

All patients in this study were treated with gonadotropin hormone-releasing hormone (GnRH) antagonist (flexible regimen) for ovarian stimulation. Recombinant human follicle-stimulating hormone (Gn, Gonal F; Serino Barueri, SP, Brazil) was given on the second or third day of the menstrual cycle for ovarian hyperstimulation. After four to five days, the dosage of Gn was adjusted according to the follicle size and hormone level. When the dominant follicle reached 12-14 mm, GnRH antagonist (GnRH-A, Cetrotide, Merck Serono SA - Geneva, Switzerland) was added until the day of human chorionic gonadotropin (HCG) injection. When there were two or three follicles with a diameter ≥18 mm, HCG (10,000 U; Pregnyl; AESCA Pharma, Austria) was injected, and oocyte retrieval was completed after 36-38 h under the guidance of a transvaginal ultrasound.

**Experimental design**

**Experiment 1**

Immature human oocytes obtained from the COH cycle were collected for IVM to obtain mature oocytes (IVM-MII) *in vitro* and perform ICSI insemination. Then, embryos were cultured *in vitro* in medium containing 0, 10^{-11}, 10^{-9}, 10^{-7} or 10^{-5} M MT. Ultimately, 10^{-9} M was determined to be the optimal concentration of MT.

**Experiment 2**

For the 140 enrolled patients with failed IVF/ICSI cycles, embryo culture medium containing 10^{-9} M MT was prepared to conduct embryo culture *in vitro* in the subsequent 140 repeated cycles. High-quality blastocysts were collected and cryopreserved. These 140 MT culture cycles were designated as the experimental group (10^{-9} M group), and the control group consisted of the previous failed cycles of the same patients (0 M group). In addition, according to the number of failed cycles, the patients were further divided into subgroups of one, two, or at least three failed cycles, and the fertilization and embryo development status of each subgroup in the 10^{-9} M and 0 M groups were compared and analysed.

**Experiment 3**

After 3 months, vitrified-warmed embryo transfer was performed. The experimental process is shown in Fig. 1.

**IVM of oocytes from COH cycles**

Human immature oocytes with normal morphology from the COH cycle were collected and placed into IVM medium prepared and balanced overnight to culture for 24 h. Subsequently, the IVM-MII oocytes were
selected for ICSI insemination, followed by five or six days of embryo culture \textit{in vitro}. The detailed process has been previously reported in published literature [11].

**Preparation of the embryo culture medium**

An appropriate amount of embryo culture medium (cleavage or blastocyst; Cook, Sydney, Australia) was prepared, and then an appropriate concentration of MT was added. Next, six to eight droplets (30 μl/drop) were made in a dish with a diameter of 60 mm and covered with an appropriate amount of tissue culture oil. Finally, the dish was placed at 37°C with 6% CO$_2$ and saturated humidity for 18 h of equilibration.

**ICSI/IVF insemination**

Under a microscope, the cumulus-oocyte complex (COC) in follicular uid was picked up and placed into balanced fertilization medium (Cook, Sydney, Australia) at 37°C with 6% CO$_2$ and saturated humidity for 4-6 h of culture \textit{in vitro}, followed by ICSI or IVF insemination. The process of ICSI insemination has been described in detail in our previously published literature [12]. Following ICSI, the inseminated oocytes were directly transferred into cleavage culture medium containing MT for embryo culture \textit{in vitro}. For IVF insemination, the COC, which underwent 4-6 h of culture \textit{in vitro}, was transferred into fertilization medium containing 5×10$^5$/ml grade A and grade B sperm for 6 h of culture at 37°C with 6% CO$_2$ and saturated humidity. Next, the granule cells around the oocytes were removed and those with the second polar body, which were considered fertilized oocytes, were selected under an inverted microscope. Finally, all fertilized oocytes were transferred into cleavage culture medium with MT for subsequent embryo culture.

**Embryo culture**

The oocytes undergoing \textit{in vitro} culture in the balanced cleavage culture droplets (one oocyte/drop) were observed based on their fertilization status 14-16 h after insemination, and then the culture was continued at 37°C, 6% CO$_2$, and saturated humidity. Two days later, all of the formed cleavage embryos were transferred into balanced blastocyst culture droplets (one to three embryos/drop) for an additional two or three days of blastocyst culture. Finally, the obtained high-quality blastocysts (see Fig. 2) were selected and cryopreserved in -196°C liquid nitrogen using the vitrification method. In this process, all embryos formed were scored according to the Tomas [13]and Gardner [14]scoring standards.

**Embryo transfer and pregnancy determination**

Three months later, the cryopreserved blastocyst was warmed. According to the patient’s age, one or two warmed blastocysts were transferred into the uterus under B-ultrasound guidance. Two weeks after embryo transfer, serum hCG levels were examined and a biochemical pregnancy was defined as a positive hCG value (≥25 IU/L). At seven weeks following embryo transfer, the presence of a gestational sac identified by an ultrasound scan was referred to as a clinical pregnancy. For the detailed operation process of embryo vitrification and warming, please refer to our previously published literature [15].
**Statistical analysis**

SPSS software version 23.0 was used to perform statistical analysis. The differences in means between continuous variables [female age, male age, basic follicle-stimulating hormone (FSH) level, basic luteinizing hormone (LH) level, basic E2 level, duration infertility, average number of oocytes retrieved and body mass index (BMI)] were expressed as the mean ± SD. Categorical data on developmental competence (rates of fertilization, cleavage, high-quality cleavage embryo, and high-quality blastocyst) in each group were analysed using the chi-squared test or Fisher’s exact test. \( P \)-values < 0.05 were considered statistically significant.

**Results**

**Screening the optimal MT concentration for embryo culture medium**

A total of 400 human immature oocytes of 198 COH cycles from 198 patients were collected for IVM culture, and 295 IVM-MII oocytes were obtained. ICSI insemination and embryo culture \textit{in vitro} were performed, and the detailed results are shown in Table 1. There were no significant differences in age, FSH, luteinizing hormone (LH), oestradiol (E2), body mass index (BMI), years of infertility, or number of oocytes obtained in each group (see Table 1).

As shown in Table 2, after adding \( 10^{-9} \) M MT to the embryo culture medium, the fertilization, cleavage, blastocyst and high-quality blastocyst rates of the \( 10^{-9} \) M group were significantly different those of the 0 M group \( (p < 0.01) \). The blastocyst and high-quality blastocyst rates of the \( 10^{-9} \) M group were significantly different from those of the \( 10^{-5} \) M group \( (p < 0.01, p < 0.001) \), and the blastocyst rate of the \( 10^{-9} \) M group was significantly different from that of the \( 10^{-11} \) M group \( (p < 0.01) \). The above data revealed that the addition of \( 10^{-9} \) M MT to the embryo culture could significantly improve the fertilization and \textit{in vitro} development of the fertilized embryo, and \( 10^{-9} \) M was the optimal concentration of MT.

**Fertilization and subsequent embryo development of oocytes in the \( 10^{-9} \) M and 0 M groups**

Table 3 shows that in the previous cycle without MT (0 M group), a total of 1838 oocytes were collected, of which 1372 MII oocytes were fertilized by IVF/ICSI, and the fertilization rate was 83.6\% (1147/1372). Among the 140 cycles of 140 patients with subsequent added MT (\( 10^{-9} \) M group), a total of 1658 oocytes were collected, including 1288 MII oocytes, and the fertilization rate after insemination was 87.7\% (1129/1288). The fertilization, cleavage, high-quality embryo, blastocyst, and high-quality blastocyst rates in the \( 10^{-9} \) M group were significantly different from those in the 0 M group (87.7\% vs. 83.6\%, \( p < 0.01 \); 94.1\% vs. 90.5\%, \( p < 0.01 \); 58.3\% vs. 43.8\%, \( p < 0.0001 \); 51.1\% vs. 41.8\%, \( p < 0.0001 \); 43.4\% vs. 22.9\%, \( p < 0.0001 \)).

**Oocyte fertilization and subsequent embryo development in the \( 10^{-9} \) M and 0 M groups of patients who experienced 1 failed IVF/ICSI cycle**
As shown in Fig. 3, there were 1116 oocytes in the $10^{-9}$ M group and 1009 oocytes in the 0 M group of patients who experienced a single failed IVF/ICSI cycle. In terms of fertilization rate (90.1% vs. 88.2%) and cleavage rate (94.6% vs. 92.9%), the rates of the $10^{-9}$ M group were higher than those of the 0 M group, but these differences were not significant. The high-quality embryo rate (56.8% vs. 39.4%) and high-quality blastocyst rate (42.0% vs. 25.3%) of the $10^{-9}$ M group were significantly higher than those of the 0 M group (both $p < 0.0001$).

### The result of oocyte fertilization and subsequent embryo development in the $10^{-9}$ M and 0 M groups of patients who experienced 2 failed IVF/ICSI cycles

As shown in Fig. 4, there were a total of 468 oocytes in the $10^{-9}$ M group and 688 oocytes in the 0 M group of patients who experienced two failed IVF/ICSI cycles. The fertilization, cleavage, high-quality embryo and high-quality blastocyst rates of the $10^{-9}$ M group were higher than those of the 0 M group. The cleavage, high-quality embryo and high-quality blastocyst rates of the $10^{-9}$ M group were significantly different from those of the 0 M group (92.2% vs. 85.6%, $p < 0.05$; 51.1% vs. 41.3%, $p < 0.05$, 44.6% vs. 19.9%, $p < 0.0001$).

### The result of oocyte fertilization and subsequent embryo development in the $10^{-9}$ M and 0 M groups of patients who experienced ≥3 failed IVF/ICSI cycles

As shown in Fig. 5, there were a total of 74 oocytes in the $10^{-9}$ M group and 141 oocytes in the 0 M group of patients who experienced three or more failed IVF/ICSI cycles. The fertilization, cleavage, high-quality embryo and high-quality blastocyst rates of the $10^{-9}$ M group were higher than those of the 0 M group. Among these, the high-quality embryo and high-quality blastocyst rates of the $10^{-9}$ M group were significantly different from those of the 0 M group (45.8% vs. 34.3%, $p < 0.05$, 57.1% vs. 20.9%, $p < 0.0001$).

### Clinical transfer outcomes

By the end of this manuscript submission, there had been 50 cycles of vitrified-warmed embryo transfers conducted the experimental group. A total of 61 warmed blastocysts were transferred, and the average number of transfers was 1.22. Thirty-two cases achieved biochemical pregnancy, and clinical pregnancy was confirmed in 20 cases. The implantation rate, biochemical pregnancy rate and clinical pregnancy rate were significantly higher than those of the 0 M group (65.6% vs. 9.7%, $p < 0.0001$; 64.0% vs. 12.5%, $p < 0.0001$; 40.0% vs. 11.7%, $p < 0.0001$) (see Table 4). Notably, to date, two women delivered two healthy new-borns, including a male and a female, by caesarean section at 38 weeks of gestational age. Apgar scores were 10 and 9; the body weights were 2.7 and 2.5 kg, and the body lengths were 53 and 51 cm, respectively. The physical and mental development of these infants was normal at their regular postnatal follow-up and the other 18 women who achieved clinical pregnancy also had good examination indexes.

### Discussion
In the process of ART treatment, patients encounter many cycles of treatment failure due to poor embryo quality. There are many factors affecting embryo development, such as culture medium [16], CO₂ concentration [17], temperature [17], in vitro operations [18, 19], and sperm and oocyte quality [20, 21]. It can be considered that during ART treatment, the quality of oocytes is one of the most critical factors that determine the embryo status and clinical outcome. Any factor affecting the quality of the oocyte will affect its fertilization and subsequent embryo development [22] and, ultimately, the cycle outcome of ART treatment. In this study, an appropriate concentration of MT was added to the embryo culture medium and the embryo development and clinical treatment outcomes of the patients were observed, to understand whether the application of MT has a positive impact on embryo development.

According to research reports, embryos are in a relatively high oxygen environment during in vitro culture, which will cause oxidative stress due to an imbalance, antioxidant defence system, resulting in a large amount of ROS produced by oocytes and embryos during development, thus affecting the quality of oocytes and outcome of embryo development in vitro [23]. MT is a highly effective antioxidant with strong antioxidant activity. Animal studies have shown that the addition of MT to embryo culture medium can improve embryo development in vitro, and it has been confirmed that the improvement is concentration dependent [4]. In 2020, our team found that adding MT to human IVM medium can improve the IVM outcome of human immature oocytes by promoting mitochondrial function and inhibiting damage due to oxidative stress, and 10⁻⁵ M was found to be the optimal concentration [10]. Based on the conclusions of previous studies, two main questions were posed. (1) Can adding MT to the culture medium of human embryos improve the in vitro development and clinical outcome of embryos? (2) Is its effect also correlated with concentration? Therefore, in this study, immature human oocytes in the COH cycle were collected for IVM to obtain IVM-MII oocytes and ICSI insemination was conducted. Next, the fertilized oocytes were cultured in vitro in culture medium containing 0, 10⁻¹¹, 10⁻⁹, 10⁻⁷ or 10⁻⁵ M MT, and a systematic comparative analysis was performed of the fertilization of oocytes and early embryo development in each group was performed. The fertilization, cleavage, blastocyst and high-quality blastocyst rates of the 10⁻⁹ M group were all significantly higher than those of the 0 M group. The blastocyst and high-quality blastocyst rates of the 10⁻⁹ M group were significantly different from those of the 10⁻⁵ M group, and the blastocyst rate was also significantly different from that of the 10⁻¹¹ M group. In addition, the fertilization, cleavage, blastocyst and high-quality blastocyst rates of the 10⁻⁹ M group were higher than those of the 10⁻⁷ M group, but these differences were not significant. It has been reported that the fertilization rate, cleavage rate and total blastocyst cell number of porcine IVF embryos cultured in medium containing 10⁻⁹ M MT were significantly increased [24]. Wang et al. [4] added different concentrations of MT to the culture medium of bovine embryos in vitro and found that 10⁻⁹ M MT could significantly promote embryo development. These results are consistent with the results of the present study. These results showed that the addition of 10⁻⁹ M MT to human embryo culture medium could significantly improve the fertilization of human oocytes and embryo development in vitro obtained after fertilization, and 10⁻⁹ M was the optimal concentration.
Next, $10^{-9}$ M was selected as the MT concentration to be added to the embryo culture medium of patients with failed IVF/ICSI cycles for embryo culture in vitro. The study found that in terms of the fertilization, cleavage, high-quality embryo, blastocyst and high-quality blastocyst rates, the values of the $10^{-9}$ M group were significantly higher than those of the 0 M group. In this study, further group analysis was also carried out according to the number of failed IVF/ICSI cycles and it was discovered that the application of $10^{-9}$ M MT to embryo culture in vitro could significantly improve embryonic development in the repeated cycle of each group. A large number of studies have found that adding MT to the culture medium can reduce the levels of ROS in oocytes and promote embryonic development of cattle, mice, sheep, and pigs [7, 25-27]. Nakano et al. [28] confirmed that the addition of MT during embryo culture in vitro can reduce the level of ROS in parthenogenetic embryos and promote embryo development. These findings are consistent with the results of the current study, suggesting that the improvement of embryonic development in patients with failed IVF/ICSI cycles by MT may be related to the highly effective antioxidant properties of MT. Oxidative stress inhibits oocyte maturation and embryo development, and MT has a strong antioxidant effect, which can resist oxidative stress, maintain the balance of the antioxidant system, reduce ROS content in the embryo, and promote gamete maturation and embryo development. In our previous study, we found that MT can improve the embryo development and clinical outcome of IVM-MII, and successfully delivered healthy new-borns, which proved the feasibility of MT in clinical treatment [9, 10]. In this study, to date, 50 cycles of vitrified-warmed embryo transfers with MT intervention have been performed. The implantation rate, biochemical pregnancy rate, and clinical pregnancy rate of the $10^{-9}$ M group were significantly higher than those of the 0 M group, and two healthy new-borns were successfully delivered. This result shows that the application of $10^{-9}$ M MT to embryo culture medium in vitro can significantly improve the clinical treatment effect of patients with failed IVF/ICSI cycles and ultimately achieve the birth of healthy offspring, which greatly enhances our confidence in treating such patients and thereby increases the overall success rate of ART treatment.

In conclusion, the application of MT to embryo culture medium in vitro can improve embryonic development for patients with repeated cycles after failed IVF/ICSI cycles and lead to good clinical outcomes. The optimal concentration of MT was $10^{-9}$ M.

**Abbreviations**

MT: melatonin; IVF: In vitro fertilization; ICSI: Intracytoplasmic sperm injection; IVM: In vitro maturation; ART: Assisted reproductive technology; IVF-ET: In vitro fertilization and embryo transfer; ROS: Reactive oxygen species; GSH: glutathione; COH: Controlled ovarian hyperstimulation; GnRH: Gonadotropin hormone-releasing hormone; HCG: Human chorionic gonadotropin; GnRH-A: GnRH antagonist; COC: Cumulus-oocyte complex; FSH: Follicle-stimulating hormone; LH: Luteinizing hormone; BMI: Body mass index.

**Declarations**
Ethics approval and consent to participate

This study was approved by the Ethics Committee of Anhui Medical University(2015013). Before conducting the study, the partners of all enrolled patients were interviewed and provided signed informed consent.

Consent for publication

All authors provided final approval of the version to be published and agree to be accountable for all aspects of the work in ensuring that questions.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that there are no conflicts of interest.

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Authors’ contributions

Qi Zhu: Manuscript writing and sample collection; Kaijuan Wang and Chao Zhang: Data collection; Zhaojuan Yu and Bihua Rao: Data analysis; Beili Chen, Huijuan Zou and Weiwei Zou: clinical assessments and experiment conducting; Yunxia Cao: Manuscript editing; Ding Ding and Zhiguo Zhang: Manuscript revising and study design. All authors read and approved the final manuscript.

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Tables

Table 1. Comparison of baseline data of the 5 groups of patients

|                          | 0M          | 10⁻¹¹M      | 10⁻⁹M       | 10⁻⁷M       | 10⁻⁵M       | P-value |
|--------------------------|-------------|-------------|-------------|-------------|-------------|---------|
| Male age (yr)            | 31.18±3.343 | 30.85±3.890 | 30.95±4.214 | 30.89±3.748 | 30.24±3.494 | >0.05   |
| Female age (yr)          | 30.15±3.549 | 29.33±2.787 | 29.79±3.361 | 30.14±2.987 | 29.17±3.011 | >0.05   |
| Infertility period (yr)  | 3.200±1.800 | 3.074±1.796 | 3.000±1.593 | 3.000±1.493 | 3.057±1.598 | >0.05   |
| No. of retrieved oocytes | 18.68±8.404 | 18.44±5.366 | 18.00±7.191 | 18.67±6.533 | 19.13±7.932 | >0.05   |
| FSH                      | 6.663±2.259 | 7.034±1.911 | 7.111±1.428 | 6.465±1.901 | 7.055±2.362 | >0.05   |
| LH                       | 5.700±2.679 | 4.959±2.582 | 5.721±5.465 | 5.334±3.286 | 4.965±3.158 | >0.05   |
| E2                       | 167.9±100.4 | 178.0±94.5  | 162.1±96.43 | 177.7±109.5 | 163.2±94.59 | >0.05   |
| BMI                      | 21.76±2.791 | 21.66±1.920 | 22.76±3.680 | 21.76±2.380 | 21.91±3.117 | >0.05   |

Values are presented as mean ± standard deviation.

Table 2. The effects of different concentrations of MT on embryo development in vitro
|                      | 0 M  | 10^{-11} M | 10^{-9} M | 10^{-7} M | 10^{-5} M |
|----------------------|------|------------|-----------|-----------|-----------|
| IVM-MII (n)          | 61   | 56         | 61        | 59        | 58        |
| Fertilization rate (%)| 85.2 | 78.6       | 90.2      | 83.1      | 87.9      |
|                      | (52/61) | (44/56)   | (55/61)   | (49/59)   | (51/58)   |
| Cleavage rate (%)    | 82.7 | 84.1       | 98.2      | 85.7      | 88.2      |
|                      | (43/52) | (37/44)   | (54/55)   | (42/49)   | (45/51)   |
| Blastocyst rate (%)  | 16.3 | 16.2       | 42.6      | 21.4      | 11.1      |
|                      | (7/43)  | (6/37)     | (23/54)   | (9/42)    | (5/45)    |
| High-quality blastocyst rate (%) | 4.7 | 2.7        | 24.1      | 9.5       | 4.4       |
|                      | (2/43)  | (1/37)     | (13/54)   | (4/42)    | (2/45)    |

Datas analyzed using Chi-square or Fisher Exact test. Different symbols within columns and different letters within columns and within rows indicate significant differences. \(^{a}P<0.01\), compared with 0 M group; \(^{b}P<0.01\), compared with 10^{-5} M group; \(^{c}P<0.001\), compared with 10^{-5} M group; \(^{d}P<0.01\), compared with 10^{-11} M group.

**Table 3. Comparison of oocyte fertilization and embryo development between the 10^{-9} M group and the 0 M group**

|                      | 10^{-9} M | 0 M  |
|----------------------|-----------|------|
| Number of cycles     | 140       | 240  |
| Number of oocytes    | 1658      | 1838 |
| Number of MII        | 1288      | 1372 |
| Fertilization rate (%)| 1129/1288(87.7) | 1147/1372(83.6) |
| Cleavage rate (%)    | 1062/1129(94.1) | 1038/1147(90.5) |
| High-quality embryo rate (%) | 619/1062(58.3) | 455/1038(43.8) |
| Blastocyst rate (%)  | 543/1062(51.1) | 434/1038(41.8) |
| High-quality blastocyst rate (%) | 461/1062(43.4) | 238/1038(22.9) |

Data analyzed using chi-squared test or Fisher’s exact test.

Different symbols within columns and different letters within columns and within rows
Data analyzed using chi-squared test or Fisher’s exact test. Different symbols within columns and different letters within columns and within rows indicate significant difference. $^a P<0.0001$, compared with the 0 MT group.
Figure 1

Experimental flow chart. o.n, oocyte number; c.n, cycle number; p.n, patient number.

Figure 2

Representative images of the high-quality blastocysts. Note: (A) High-quality blastocyst 4AA; (B) high-quality blastocyst 4AA; (C) high-quality blastocyst 4AB; (D) high-quality blastocyst 4BA. Scale bar=10uM.
Status of fertilization and early embryo development in patients with one failed IVF/ICSI cycle. Note: A: fertilization; B: cleavage; C: high-quality embryo and D: high-quality blastocyst. (****P < 0.0001).
Figure 4

Status of fertilization and early embryo development in patients with two failed IVF/ICSI cycles. Note: A: fertilization; B: cleavage; C: high-quality embryo and D: high-quality blastocyst. (*P <0.05; ****P <0.0001).
Figure 5

Status of fertilization and early embryo development in patients with one failed IVF/ICSI cycles. Note: A: fertilization; B: cleavage; C: high-quality embryo and D: high-quality blastocyst. (*P <0.05; ****P <0.0001).