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

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Summary

To explore whether embryo culture with melatonin (MT) can improve the embryonic development and clinical outcome of patients with repeated cycles after *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) failure, immature oocytes from controlled ovarian superovulation cycles were collected for *in vitro* maturation (IVM) and ICSI. The obtained embryos were cultured in 0, 10^{-11}, 10^{-9}, 10^{-7} and 10^{-5} M MT medium respectively, and 10^{-9} M was screened out as the optimal concentration. Subsequently, 140 patients who underwent failed IVF/ICSI cycles received 140 cycles of embryo culture *in vitro* with a medium containing 10^{-9} M MT, these 140 MT culture cycles were designated as the experimental group (10^{-9} M group), and the control group was the previous failed cycles of patients (0 M group). The results showed that 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 ($P < 0.01$; $P < 0.01$; $P < 0.0001$; $P < 0.0001$; $P < 0.0001$). To date, in total, 50 vitrified-warmed cycle transfers have been 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 (all $P < 0.0001$). Two healthy infants were delivered successfully and the other 18 women who achieved clinical pregnancy also had good examination indexes. Therefore the application of 10^{-9} M MT to embryo cultures *in vitro* improved embryonic development in patients with repeated cycles after failed IVF/ICSI cycles and had good clinical outcomes.
Trial registration: ChiCTR2100045552.

Introduction

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 (Esteves *et al.*, 2019). 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 (Liu *et al.*, 2016), 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 rhythmically released during the dark hours at night by the pineal gland (Olcese, 2020). The circadian rhythm of MT plays major roles in the secretion of reproductively active hormones (Reiter *et al.*, 2014). MT, which is both fat and water soluble, is distinctive in that it can easily pass though cell membranes and easily reaches nuclei and mitochondria, where it accumulates in high concentrations (Acuña-Castroviejo *et al.*, 2014). MT is a direct free radical scavenger with a more powerful antioxidant effect than conventional antioxidants such as

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vitamins C and E, glutathione (GSH), and mannitol (Reiter, 1995). MT and its metabolites can directly remove reactive oxygen species (ROS) in cells, activate antioxidant enzymes, increase the expression level and activity of GSH, and inhibit pro-oxidant enzymes to reduce cell oxidation injury (Manchester *et al.*, 2015). Growing evidence of reproductive medicine supports the role of melatonin in human reproduction (Rafael *et al.*, 2019). It is possible that *in vivo* administration may improve the quality of oocytes and *in vitro* incubation may improve oocytes *in vitro* maturation (IVM) and embryonic development (Manchester *et al.*, 2015). 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 (Lin *et al.*, 2018; An *et al.*, 2019; Barros *et al.*, 2020a). Through research on human IVF and IVM, Li *et al.* (2021) found that the addition of MT to the embryo culture medium can improve the clinical outcomes of IVF and IVM. Zou *et al.* (2020) 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. 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

Research patients

In total, 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 the genetic factors of both the 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 five 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 4–5 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 $\geq$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 statuses of each subgroup in the 10^{-9} M and 0 M groups were compared and analyzed.

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 5 or 6 days of embryo culture *in vitro*. The detailed process has been reported previously in the published literature (Sacha *et al.*, 2018).

Preparation of the embryo culture medium

Firstly, MT (Sigma, USA) was fully dissolved in absolute ethanol and diluted to a MT concentration of 10^{-2} M (stock solution). Subsequently, the stock solution was distributed into centrifuge tubes, 10–20 μl each tube, and finally frozen at −20°C; On the day before each experiment, one tube or more of the stock solution was warmed and gradually diluted with embryo culture medium (cleavage or blastocyst, Cook, Sydney, Australia) through the dilution multiple method to reach an appropriate MT concentration for the embryo culture medium (10^{-11}, 10^{-9}, 10^{-7} or 10^{-5} M). Next, 6–8 droplets (30 μl/drop) were placed in a dish with a diameter of 60 mm and covered with 2 ml 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 fluid 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 *in vitro*, followed by ICSI or IVF insemination. The process of ICSI insemination has been described in detail in previously publications (Ding *et al.*, 2020). Following ICSI, the inseminated oocytes were transferred directly into cleavage culture medium containing MT for embryo culture *in vitro*. For IVF insemination, the COC, which underwent 4–6 h of culture *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 cumulus cells around the

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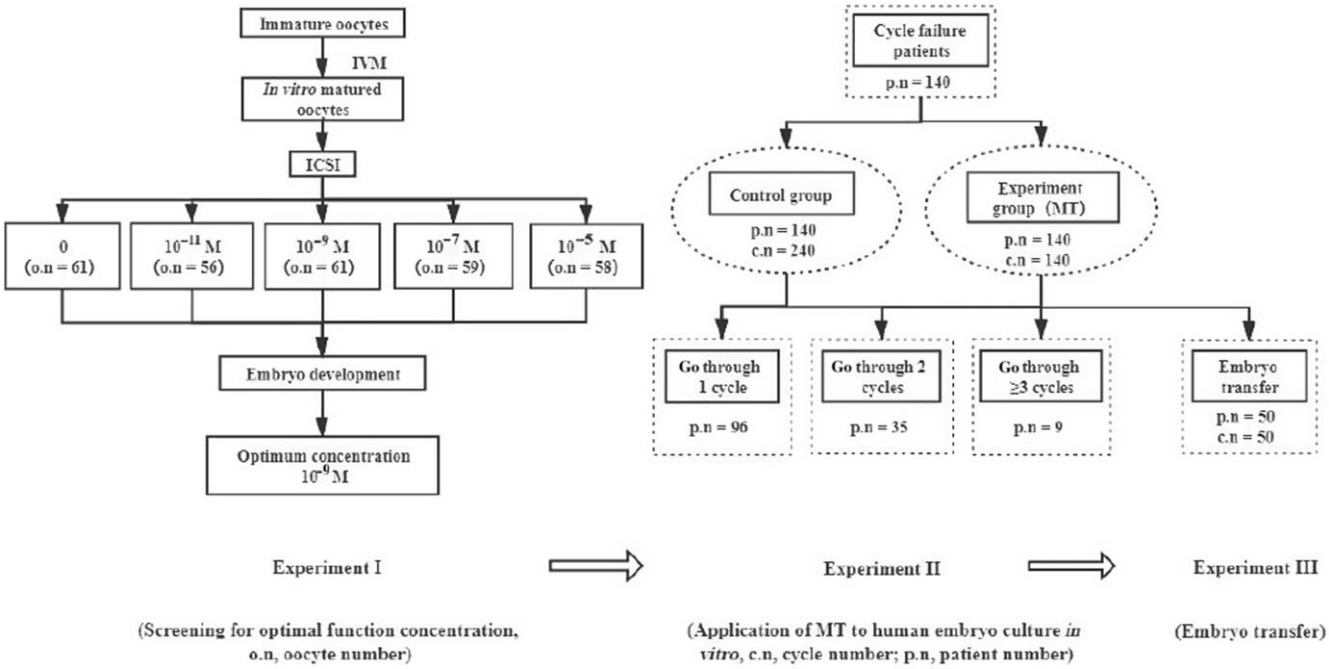

Figure 1. Experimental flow chart. c.n, cycle number; o.n, oocyte number; p.n, patient number.

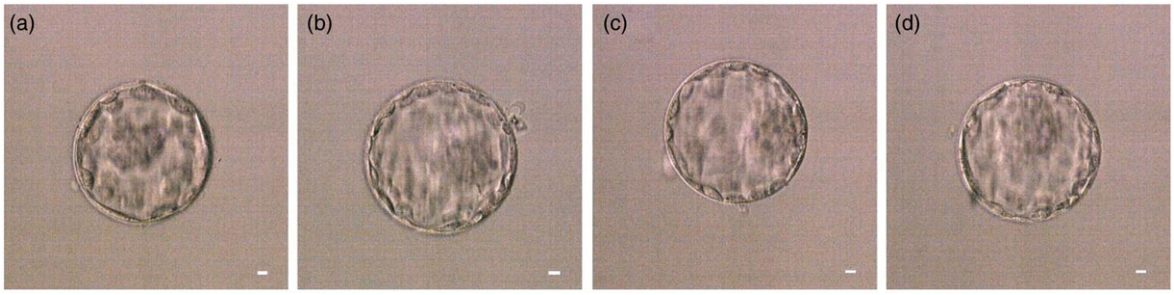

Figure 2. Representative images of the high-quality blastocysts. (a) High-quality blastocyst 4AA. (b) High-quality blastocyst 4AA. (c) High-quality blastocyst 4AB. (d) High-quality blastocyst 4BA. Scale bars, 10 μM.

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 *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 blastocyte culture droplets (one to three embryos/drop) for an additional 2 or 3 days of blastocyst culture. Finally, the obtained high-quality blastocysts (refer to 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 Tomás *et al.* (1998) and Gardner and Schoolcraft (1999) 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 (≥35 years old, two embryos per transfer cycle, <35 years old, one embryo per transfer cycle). 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 7 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 (Camargos *et al.*, 2019).

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 analyzed by one-way analysis of variance (ANOVA). Values are presented as mean ± standard deviation. Categorical data on developmental competence (rates of fertilization, cleavage, high-quality cleavage embryo, and high-quality blastocyst) in each group were analyzed using the chi-squared test or Fisher's exact test. *P*-values < 0.05 were considered to be statistically significant.

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Table 1. 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 (52/61)	78.6 (44/56)	90.2 (55/61)[a]	83.1 (49/59)	87.9 (51/58)
Cleavage rate (%)	82.7 (43/52)	84.1 (37/44)	98.2 (54/55)[a]	85.7 (42/49)	88.2 (45/51)
Blastocyst rate (%)	16.3 (7/43)	16.2 (6/37)	42.6 (23/54)[a,c,d]	21.4 (9/42)	11.1 (5/45)
High-quality blastocyst rate (%)	4.7 (2/43)	2.7 (1/37)	24.1 (13/54)[a,b,d]	9.5 (4/42)	4.4 (2/45)

Data were analyzed using chi-squared or Fisher's 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 2. Comparison of baseline data of the five groups of patients

	0 M	10^{-11} M	10^{-9} M	10^{-7} M	10^{-5} M	*P*-value
Male age (year)	31.18 ± 3.343	30.85 ± 3.890	30.95 ± 4.214	30.89 ± 3.748	30.24 ± 3.494	>0.05
Female age (year)	30.15 ± 3.549	29.33 ± 2.787	29.79 ± 3.361	30.14 ± 2.987	29.17 ± 3.011	>0.05
Infertility period (year)	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 (mIU/ml)	6.663 ± 2.259	7.034 ± 1.911	7.111 ± 1.428	6.465 ± 1.901	7.055 ± 2.362	>0.05
LH (mIU/ml)	5.700 ± 2.679	4.959 ± 2.582	5.721 ± 5.465	5.334 ± 3.286	4.965 ± 3.158	>0.05
E2 (pmol/l)	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
AMH (ng/ml)	3.842 ± 2.934	4.067 ± 3.503	3.650 ± 3.368	4.305 ± 4.435	4.263 ± 4.178	>0.05

Data were analyzed using one-way analysis of variance (ANOVA). Values are presented as mean ± standard deviation.

Results

Screening the optimal MT concentration for embryo culture medium

In total, 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 *in vitro* were performed, and the detailed results are shown in Table 1. There were no significant differences in age, FSH, LH, oestradiol (E2), BMI, years of infertility, or number of oocytes obtained in each group (refer to Table 2).

As shown in Table 1, 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 from 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 *in vitro* development of the fertilized embryo, and that 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), in total, 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 subsequently added MT (10^{-9} M group), in total, 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

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)[a]	1147/1372 (83.6)
Cleavage rate (%)	1062/1129 (94.1)[a]	1038/1147 (90.5)
High-quality embryo rate (%)	619/1062 (58.3)[b]	455/1038 (43.8)
Blastocyst rate (%)	543/1062 (51.1)[b]	434/1038 (41.8)
High-quality blastocyst rate (%)	461/1062 (43.4)[b]	238/1038 (22.9)

Data were 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]$P < 0.01$, compared with the 0 MT group.
[b]$P < 0.0001$, compared with the 0 MT group.

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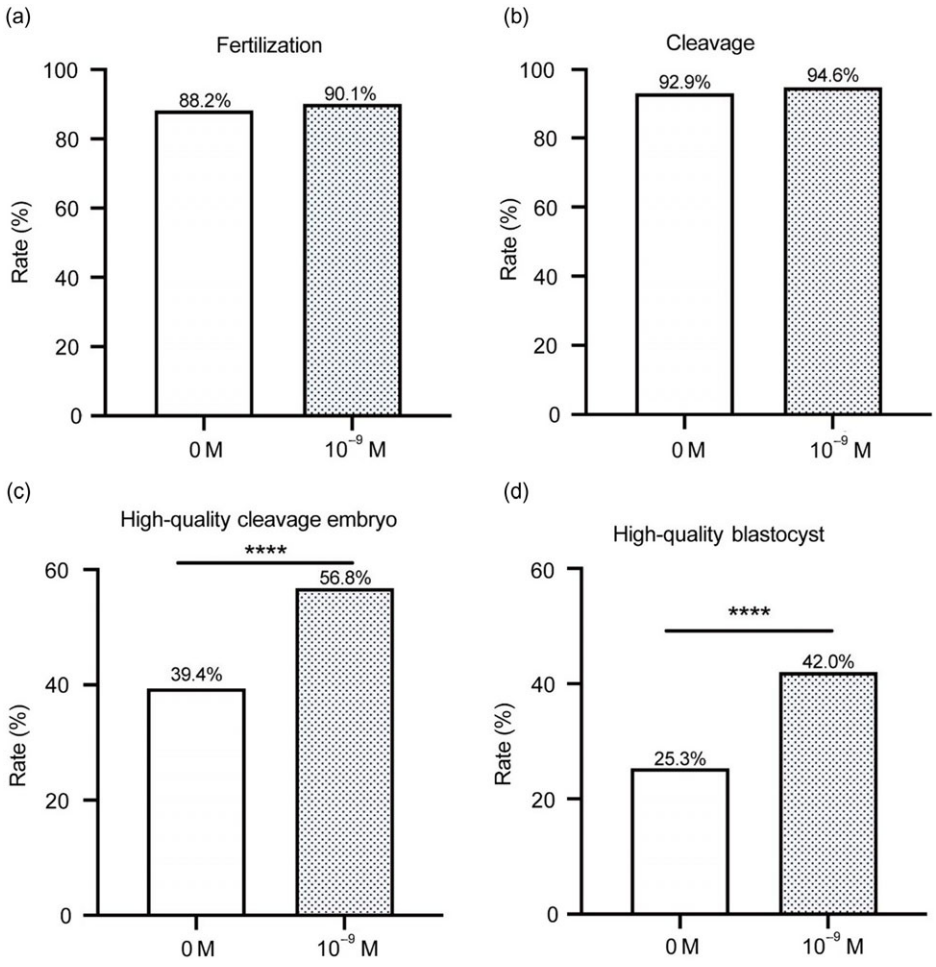

Figure 3. Status of fertilization and early embryo development in patients with one failed IVF/ICSI cycle. (a) fertilization; (b) cleavage; (c) high-quality embryo; and (d) high-quality blastocyst (****$P < 0.0001$).

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 one 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 $P < 0.0001$).

Result of oocyte fertilization and subsequent embryo development in the 10^{-9} M and 0 M groups of patients who experienced two failed IVF/ICSI cycles

As shown in Fig 4, there were, in total, 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%, $P < 0.0001$).

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, in total, 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. In total, 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

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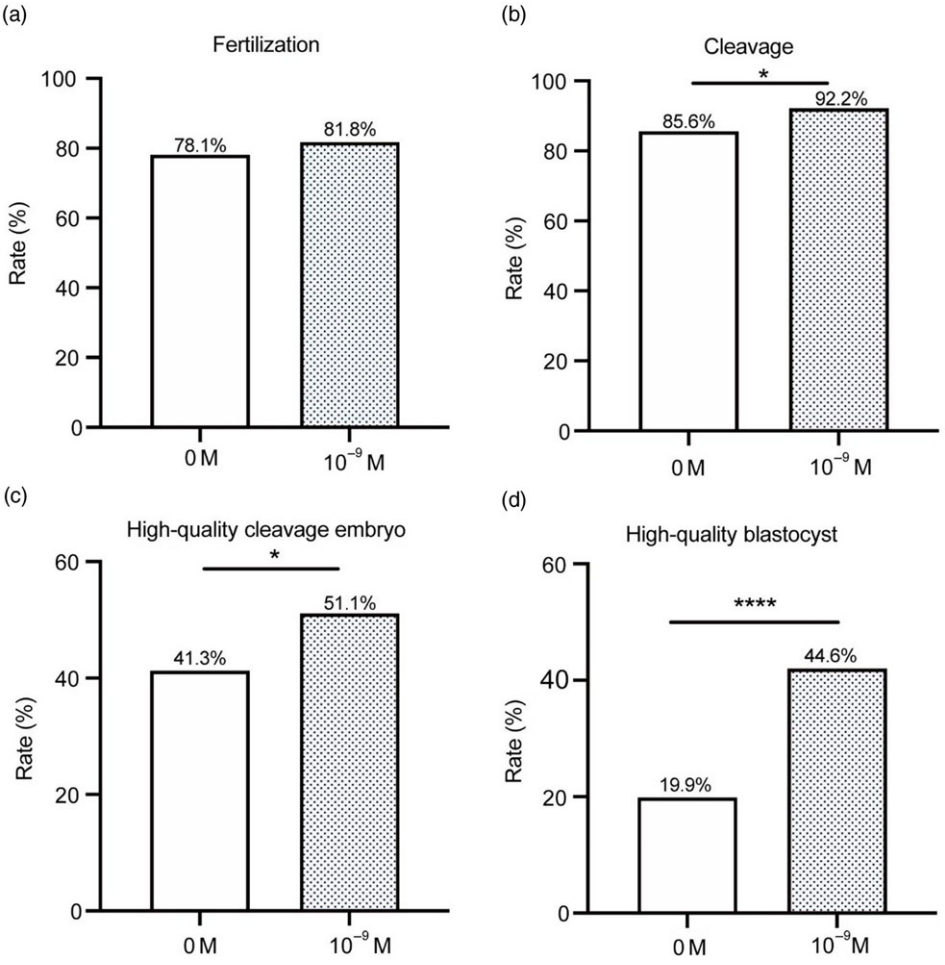

Figure 4. Status of fertilization and early embryo development in patients with two failed IVF/ICSI cycles. (a) fertilization; (b) cleavage; (c) high-quality embryo; and (d) high-quality blastocyst (*$P < 0.05$; ****$P < 0.0001$).

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 newborns, including a boy and a girl, 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 (Tarahomi *et al.*, 2019), CO_2 concentration (Brom-De-Luna *et al.*, 2019), temperature (Brom-De-Luna *et al.*, 2019), *in vitro* operations (Imesch *et al.*, 2013; Lu *et al.*, 2019), and sperm and oocyte quality (Keefe *et al.*, 2015; Ribas-Maynou and Benet, 2019). 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 (Miyara *et al.*, 2003) and, ultimately, the cycle outcome of ART treatment. Therefore, 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 effect on embryo development.

According to research reports, in the process of *in vitro* culture, embryo transfer, fertilization and developmental observation need to be performed in a high-oxygen environment. This inevitable high-oxygen environment will cause a certain degree of oxidative stress damage to embryo development. In addition, frequent opening of the incubator during embryo culture will result in an unstable O_2 concentration within the chamber, which will also cause different degrees of oxidative stress damage to embryo development, therefore affecting the quality of oocytes and outcome of embryo development *in vitro* (An *et al.*, 2019; Hiroshi *et al.*, 2020). 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 (Wang *et al.*, 2014). In 2020, we 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; 10^{-5} M was found to be the optimal concentration (Zou *et al.*, 2020). 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

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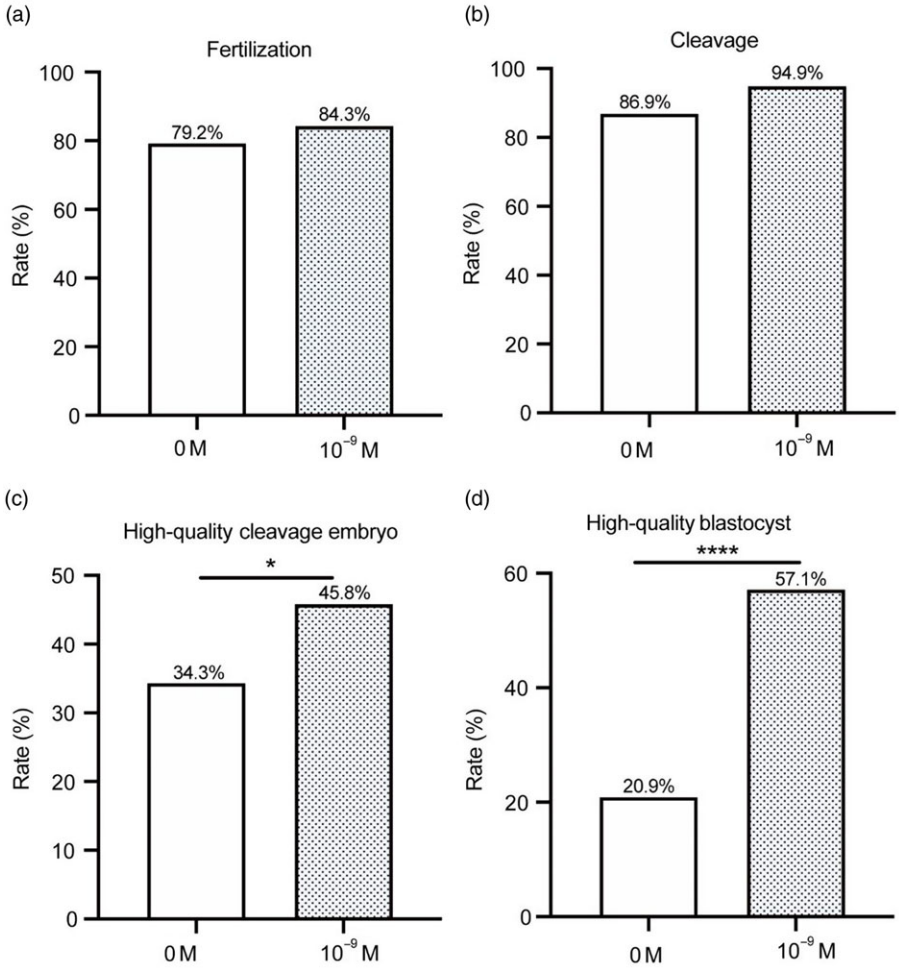

Figure 5. Status of fertilization and early embryo development in patients with three failed IVF/ICSI cycles. (a) fertilization; (b) cleavage; (c) high-quality embryo; and (d) high-quality blastocyst (*$P < 0.05$; ****$P < 0.0001$).

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. A systematic comparative analysis was performed of the fertilization of oocytes and early embryo development in each group. 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 (Lord *et al.*, 2013). Wang *et al.* (2014) 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. 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. Large numbers 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 (Kang *et al.*, 2009; Zhao *et al.*, 2016; Barros *et al.*, 2020a, 2020b). Nakano *et al.* (2012) confirmed that the addition of MT during embryo culture *in vitro* could 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. Moreover, some studies have also demonstrated that MT can improve mitochondrial function (Martín *et al.*, 2000; Rodríguez-Varela and Labarta, 2020). Mitochondria are a major source of

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Table 4. Comparison of clinical outcomes between 10^{-9} M group and 0 M group

	10^{-9} M	0 M
Average number of transfers	1.2	1.2
Number of gestational sac	40	28
Implantation rate (%)	40/61 (65.6)[a]	28/288 (9.7)
Biochemical pregnancy rate (%)	32/50 (64.0)[a]	30/240 (12.5)
Clinical pregnancy (%)	20/50 (40.0)[a]	28/240 (11.7)
Live birth (*n*)	2	0
Ongoing pregnancy (*n*)	18	–

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.

ROS production and they need additional protection from oxidative stress (Izyumov *et al.*, 2010). Mitochondria provide ATP for most cellular energy-requiring processes through the oxidative phosphorylation pathway in early embryos. Niu *et al.* (2020) found that MT could compensate for the mitochondrial depletion and energy deficiency due to toxin exposure by reducing ROS production and promoting mitochondrial biogenesis, which rescued impairment of early porcine embryo development. Yang *et al.* (2017) found that, in inferior bovine oocytes, MT treatment significantly normalized the mitochondrial distribution and improved their function to produce more ATP, which promoted the cytoplasmic maturation of oocytes and their embryos development. In our previous research, it has been found that MT can significantly increase the maturation rate and developmental potential of human immature oocytes by directly scavenging ROS and inhibiting oxidative stress (Zou *et al.*, 2020). Therefore, the above evidence indicated that MT can effectively inhibit oxidative stress not only by scavenging free radical, but also by protecting mitochondrial function, thereby improving gamete quality and embryo development both in human and animals during ART treatment. Further clinical studies indicated that MT greatly ameliorated clinical outcome, and successfully delivered healthy newborns, which strongly proved the feasibility of MT in clinical treatment (Zou *et al.*, 2020; Li *et al.*, 2021). 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 newborns 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 confidence in treating such patients and thereby increases the overall success rate of ART treatment.

In conclusion, the addition 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.

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Competing interests. The authors declare that there are no conflicts of interest.

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.

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

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