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Melatonin promotes the development of immature oocytes from the COH cycle into healthy offspring by protecting mitochondrial function

Huijuan Zou[1,2,3] | **Beili Chen**[1,2,3] | **Ding Ding**[1,2,3] | **Ming Gao**[2,3,4] | **Dawei Chen**[1,5,6] | **Yajing Liu**[1,5,6] | **Yan Hao**[1,5,6] | **Weiwei Zou**[1,5,6] | **Dongmei Ji**[1,5,6] | **Ping Zhou**[1,5,6] | **Zhaolian Wei**[1,5,6] | **Yunxia Cao**[1,2,3] | **Zhiguo Zhang**[1,2,3]

[1]Department of Obstetrics and Gynecology, Reproductive Medicine Center, The First Affiliated Hospital of Anhui Medical University, Hefei, China

[2]NHC Key Laboratory of study on abnormal gametes and reproductive tract (Anhui Medical University), Hefei, China

[3]Key Laboratory of Population Health Across Life Cycle (Anhui Medical University), Ministry of Education of the People's Republic of China, Hefei, China

[4]Department of Obstetrics and Gynecology, The Second Affiliated Hospital of Anhui Medical University, Hefei, China

[5]Anhui Province Key Laboratory of Reproductive Health and Genetics, Hefei, China

[6]Biopreservation and Artificial Organs, Anhui Provincial Engineering Research Center, Anhui Medical University, Hefei, China

Correspondence
Zhiguo Zhang and Yunxia Cao, Department of Obstetrics and Gynecology, Reproductive Medicine Center, The First Affiliated Hospital of Anhui Medical University, 218 Jixi Road, Hefei 230022, China.
Emails: zzg_100@163.com (ZZ); caoyunxia6@126.com (YC)

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Abstract

Melatonin (MT) regulates reproductive performance as a potent antioxidant; however, its beneficial effects on oocyte development remain largely unknown, especially in human oocytes. The collected 193 immature oocytes from the controlled ovarian hyperstimulation (COH) cycle underwent in vitro maturation (IVM) in IVM medium contained 10 μmol/L MT (n = 105, M group) and no MT (n = 88, NM group), followed by insemination and embryo culture. The results showed that the high-quality blastocyst formation rate in the M group (28.4%) was significantly higher than that in the NM group (2.0%) ($P = .0001$), and the aneuploidy rate was low (15.8%). In the subsequent clinical trials, three healthy infants were delivered. Next, single-cell RNA-seq data revealed 1026 differentially expressed genes (DEGs) between the two groups, KEGG enrichment analysis revealed that the majority of DEGs involved in oxidative phosphorylation pathway, which associated with ATP generation, was upregulated in the M group. Finally, confocal fluorescence staining results revealed that the mitochondrial membrane potential in the oocytes significantly increased and intracellular ROS and Ca^{2+} levels greatly decreased in the M group. Melatonin can promote the development of immature human oocytes retrieved from the COH cycle into healthy offspring by protecting mitochondrial function.

KEYWORDS

immature human oocyte, in vitro maturation, melatonin, mitochondrial function, offspring, oxidative phosphorylation

Zou, Chen, Ding and Gao contributed equally to this work.

1 | INTRODUCTION

Melatonin (MT) is an indoleamine produced in a variety of tissues, including the pineal gland and female reproductive system.[1,2] Initially, MT was identified as a hormone primarily involved in the regulation of circadian rhythms of various physiological and neuroendocrine functions. However, MT has been recently shown to have multiple functions, especially in the reproductive system, by acting as a powerful free radical scavenger to protect the cells from oxidative stress damage,[1-4] delay ovarian aging,[5] regulate the production of progesterone,[6] predict ovarian reserve, and in vitro fertilization (IVF) outcomes,[7] as well as promote embryonic development.[8]

Spermatozoon initiates fertilization of the oocyte; however, subsequent events associated with embryogenesis are controlled by the maternal material present in the oocyte.[9] Therefore, high-quality oocytes are critical to the proper development of an embryo. Compared with the routine IVF treatment, the pregnancy rate of the in vitro maturation (IVM) treatment using immature oocytes is relatively low, mainly because only a few high-quality embryos are obtained. The fundamental reason for that is that immature oocytes rarely develop into high-quality mature oocytes during IVM culture. In the animal studies, attempts to optimize the IVM culture by supplementing the IVM media with MT have produced relatively desirable results.[10-13] However, only a few similar studies have been performed in humans.[8]

Clinical studies have shown that approximately 15% of oocytes collected following ovulation induction using controlled ovarian hyperstimulation (COH) are immature.[14] Most of these immature oocytes are derived from relatively small ovarian follicles, whereas the mature oocytes are derived from the dominant follicles. During follicular development, hormones and factors secreted by the dominant follicles suppress the development of the small follicles by inducing apoptotic processes.[15] Therefore, the immature oocytes obtained from the COH cycle are usually discarded because of their poor developmental potential. However, pioneering studies performed by Tucker et al and De Vos et al resulted in successful pregnancies and live births through IVM of immature oocytes obtained from the COH cycle followed by intracytoplasmic single sperm injection (ICSI) and early embryo culture (the entire process was designated as IVM program).[16,17]

Therefore, the immature oocytes obtained from the COH cycle are rarely used for IVF treatment. In 2013, Kim et al reported an amazing clinical pregnancy rate of 60% by using the medium containing 10 μmol/L of MT for IVM of immature oocytes collected in a routine IVM cycle; this rate was significantly higher than that reported previously (28% ~ 35%).[18] The results showed that MT (10 μmol/L) supplementation significantly improved IVM outcome, suggesting that MT may ameliorate the development of oocytes.[18] However, in that study, the immature oocytes were acquired from the routine IVM cycle and not from the COH cycle. In general, the immature oocytes obtained from the routine IVM cycle (inducing ovulation with a low dose of a drug or without any drugs) have superior developmental potential than those obtained in the COH cycle because the former does not experience growth suppression, while the latter does.

In this study, we sought to assess whether MT supplementation can improve the developmental potential of immature human oocytes retrieved from the COH cycle and to explore the mechanism of action of MT and the clinical value of these "special" oocytes.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

MT and the other reagents, unless otherwise specified, were purchased from Sigma Chemical Co. (Additional details are in Appendix S1).

2.2 | Immature oocytes collection

After COH for the patients enrolled in the present study, an ultrasound-guided ovum retrieval was conducted; the high-quality immature oocytes (germinal vesical: GV or metaphase I: MI oocyte) were collected for the subsequent IVM culture. Additional details are in Supplementary Immature oocyte collection.

2.3 | Study design

This study included the following three experimental groups. In experiment I, immature oocytes collected from the COH cycle were randomly assigned to the MT group (M group, n = 105) and the non-MT group (NM group, n = 88) for IVM culture. This was followed by ICSI insemination of the in vitro-matured oocytes (IVM-metaphase II [MII] oocytes) and 4 ~ 5 days of early embryo culture of the fertilized oocytes, during which the generated high-quality blastocysts were frozen by vitrification for subsequent examination of aneuploidy by array comparative genomic hybridization (CGH). There was no significant difference in the baseline data of the patients between the two groups (Table S1). In experiment II, two independent clinical trials were performed; two special patients received the treatment based on the IVM program and the thawed embryos transfer (T-ET). In experiment III, we used single-cell sequencing and confocal fluorescence staining technologies

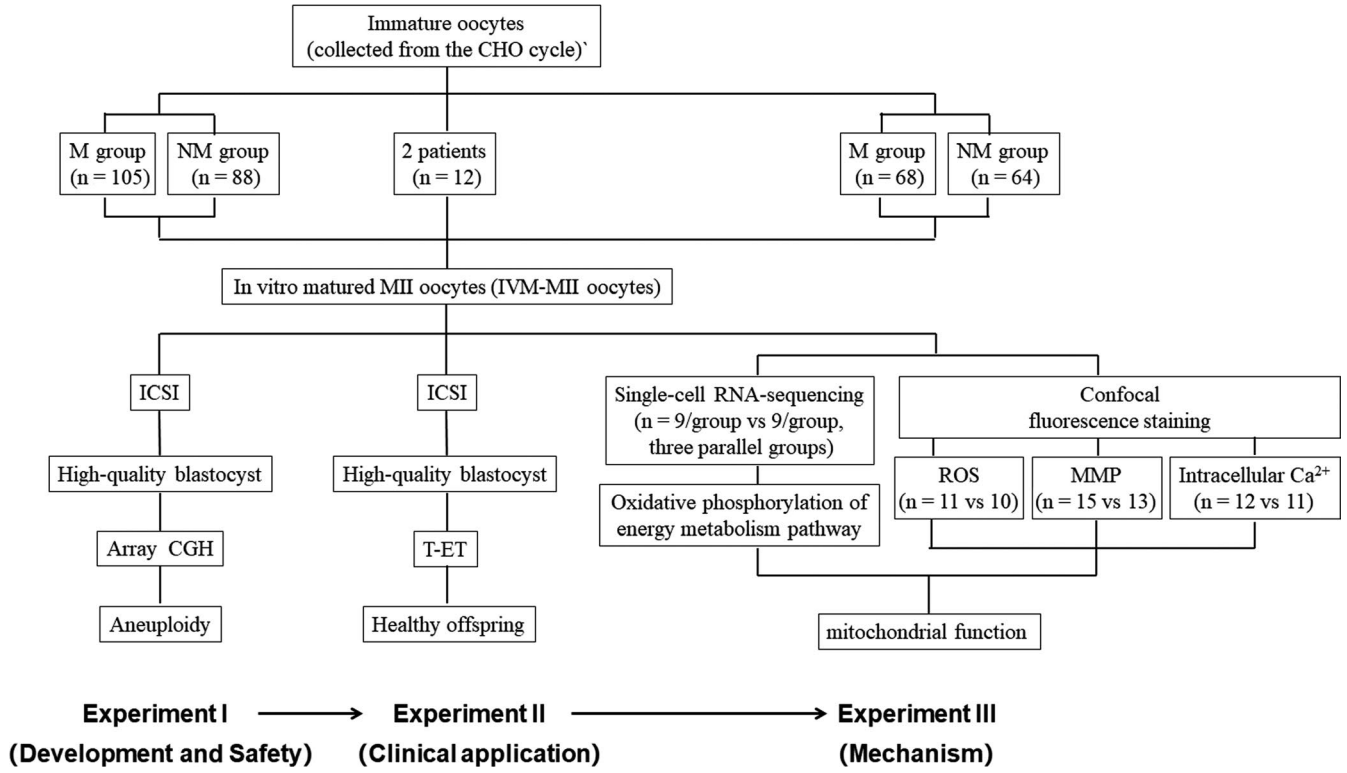


FIGURE 1 A flowchart of the experimental design

to detect and analyze the transcriptome and indicators related to mitochondrial function in IVM-MII oocytes. The experimental flowchart is shown in Figure 1.

2.4 | Ethics statement

This study was conducted at the First Affiliated Hospital of Anhui Medical University Reproductive Medicine Center and was approved by the Committee of Medical Ethics. All patients in this study had written their informed consent.

2.5 | Establishment of the MT-IVM program

In our previous studies, we showed that the number of high-quality blastocysts was significantly higher ($P < .05$) in the 10^{-5} mol/L (10 μmol/L) melatonin-treated group compared with that in the others groups (0, 10^{-11}, 10^{-9}, 10^{-7} mol/L).[19] Therefore, we used 10 μmol/L of MT supplementation in the basic IVM medium for the IVM culture of human immature oocytes retrieved from the COH cycle and then with subsequent ICSI insemination and 5 ~ 6 days of embryo culture of the fertilized oocytes to form blastocysts. The whole process was defined as the MT-supplemented IVM program (MT-IVM program). (Additional details are in Supplementary "Preparation of IVM medium," "Protocol for IVM, ICSI, and embryo culture," and "Blastocyst grading").

2.6 | Blastocyst vitrification and thawing

All high-quality blastocysts formed in the experiment were vitrified and thawed according to the Kuwayama protocol using a Cryotop as reported elsewhere.[20] (Additional details are in Supplementary "Blastocyst vitrification and thawing").

2.7 | Examination of aneuploidy

All frozen high-quality blastocysts formed in experiment I were warmed and examined for aneuploidy using array CGH. (Additional details are in Supplementary "Array CGH protocol").

2.8 | Clinical application of the MT-IVM program

Two special patients received the treatment according to the MT-IVM program, including blastocyst vitrification and

thawing, T-ET and pregnancy determination. (Additional details are in the Supplementary "Clinical application of the MT-IVM program").

2.9 | Single-cell RNA-sequence analysis

A total of 54 immature oocytes were collected from 14 young infertile women (<35 years old). High-quality immature oocytes were collected for the IVM culture and randomly allocated to two groups, including immature oocytes treated with 10 μmol/L of MT (M group, n = 27) and immature oocytes treated without MT (NM group, n = 27). After culture, 54 MII oocytes were retrieved and washed three times in gamete medium (COOK). A total of nine oocytes in each group were prepared for single-cell RNA-sequence analysis. (Additional details are in Supplementary "Single-cell RNA sequence analysis").

2.10 | Detection of ROS levels in human oocytes

To determine the amount of ROS generation, the examined MII oocytes were loaded with an oxidation-sensitive fluorescent probe DCFH-DA (Beyotime Biotechnology Inc) by incubation at 37°C for 30 minutes in PBS; then, the oocytes were mounted on the glass substrate dishes. Fluorescent signals were measured using a confocal microscope (Zeiss LSM 800). Photographs were analyzed using the ImageJ software (Research Services Branch, National Institute of Mental Health) to measure the brightness of the staining in each oocyte.

2.11 | Mitochondrial function assay in human oocytes

The mitochondrial membrane potential (MMP) of the human oocytes was measured using the JC-1 staining (mitochondrial membrane potential assay kit, Abbkine Scientific Co., Wu Han, China). (Additional details are in Supplementary "Mitochondrial function assay in human oocytes").

2.12 | Detection of calcium levels in human oocytes

The calcium levels in the human oocytes were measured using a Ca^{2+}-sensitive fluorescent probe Fluo-4 AM (Beyotime Biotechnology Inc). (Additional details are in Supplementary "Detection of calcium levels in human oocytes").

TABLE 1 Maturation rates of the immature human oocytes collected from the COH cycles

	M group	NM group
No. of immature oocytes (GV + MI) (n)	105	88
No. of immature oocytes (GV) (n)	85	46
No. of immature oocytes (MI) (n)	20	42
Rate of matured oocytes (GV + MI) (%)	81.9 (86/105)	76.1 (67/88)
Rate of matured oocytes (GV) (%)	80.0 (68/85)	65.2 (30/46)
Rate of matured oocytes (MI) (%)	90.0 (18/20)	88.1 (37/42)

Note: The maturation rate of the immature human oocytes collected from COH cycles was determined according to the ratio of the number of mature oocytes to the number of immature oocytes. There was no significant difference between the M group and the NM group in the maturation rates of immature human oocytes (GV or MI) collected from COH cycles ($P > .05$).

3 | RESULTS

3.1 | MT supplementation enhanced the developmental potential of immature oocytes

A total of 193 immature oocytes, including 131 germinal vesicles (GVs, n = 131) and 62 metaphase I (MI, n = 62), were collected and used in the subsequent IVM experiments. These immature oocytes included 105 oocytes (85 GVs and 20 MIs) obtained from the MT treatment group (M group) and 88 oocytes (46 GVs and 42 MIs) obtained from the non-MT treatment group (NM group, a control group) (Table 1). Figure 2 and Video S1 showed the IVM process of an immature oocyte of the M group. There was no significant difference in the maturation rate of immature oocytes between the M group and the NM groups in the case of the GV ($P = .06$) and MI ($P = 1$) oocytes, respectively (Table 1). The representative images of high-quality blastocysts of the M group are shown in Figure 3. Notably, the blastocyst formation rates and high-quality blastocyst formation rates were higher in the M group than that in the NM group (49.3% [33/67] vs 24.5% [12/49], $P < .01$ and 28.4% [19/67] vs 2.0% [1/49], $P < .01$, respectively) (Table 2).

To investigate the possible differential effects of MT on the fertilization rate, cleavage, blastocyst formation, and high-quality blastocyst formation in immature human oocytes obtained during different maturation stages, the immature human oocytes of each group were subdivided into 2 subgroups (NM-MI and NM-GV; M-MI and M-GV). As shown in Table 3, there was a significant difference in the maturation rates between the NM-GV and the NM-MI groups [65.2% (30/46) vs 88.1% (37/42), respectively] ($P < .05$).

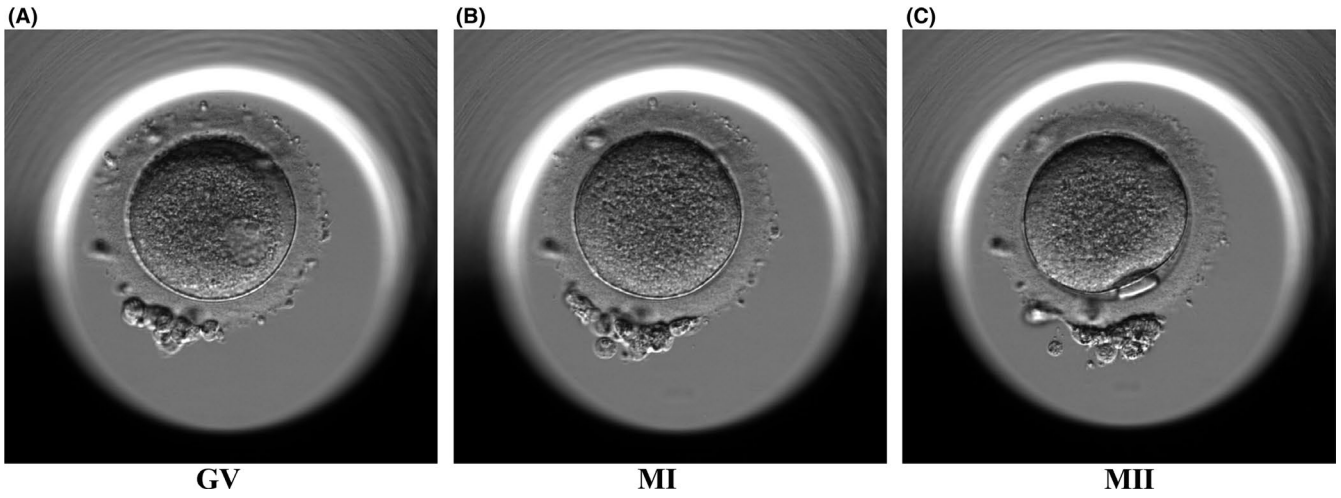


FIGURE 2 Images on IVM of immature oocyte from GV to MII stage. (A) Oocyte at GV stage; (B) oocyte at MI stage; and (C) matured oocyte treated with MT in vitro. GV, germinal vesicle; MI, metaphase I; MII, metaphase II

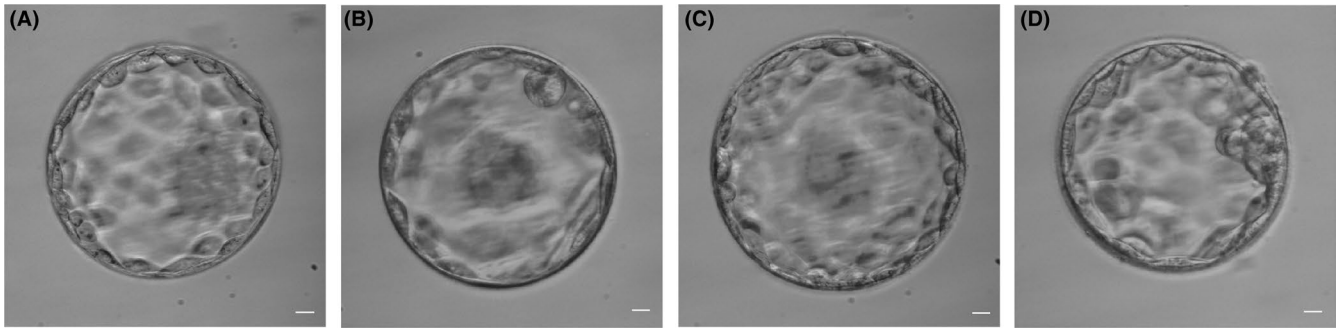


FIGURE 3 Representative images of high-quality blastocysts of the M group. (A) High-quality blastocyst 4AA; (B) high-quality blastocyst 4AB; (C) high-quality blastocyst 4BA; (D) high-quality blastocyst 4BB [note: 4 represents an expanded blastocyst; the first capital letter (A or B) corresponds to the grade assigned to the inner cell mass (ICM); grade A ICM had numerous tightly packed cells, whereas grade B ICM had several loosely grouped cells; the second capital letter (A or B) corresponds to the grade assigned to the trophectoderm (TM): grade A TE had a cohesive epithelium composed of numerous cells, whereas grade B TE had a loose epithelium composed of a few cells. Blastocysts were considered high-quality if they had a grade 3 or 4 blastocoel, a grade A or B ICM, and a grade A or B TE on days 5 or 6 after insemination; scale bar = 10 μm

3.2 | Effects of MT supplementation on the incidence rate of chromosomal anomalies

A total of 20 high-quality blastocysts (19 from the M group and 1 from the NM group) were thawed and used to detect aneuploidy using array CGH. The results of array CGH detection showed that 16 blastocysts (84.2%) were euploid, while the remaining three blastocysts (15.8%) were aneuploid. The chromosomal aneuploidies of these three blastocysts from the M group were trisomy, monosomic chromosomes, and complex chromosomal abnormalities. However, the incidence of aneuploidy in the high-quality blastocysts in the M group was low (15.8%, 3/19). In addition, one embryo from the NM group was aneuploid. The representative images of the array CGH results are shown in Figure 4. Therefore, we believe that an effective IVM program was established.

3.3 | Clinical application of the MT-IVM program

To further confirm the clinical application of this novel IVM program, two infertile women received the transfer of high-quality blastocysts derived from immature oocytes obtained from the COH cycles in our subsequent clinical studies. Notably, both women achieved successful clinical pregnancies, including one singleton and one twins. The fetuses were delivered by scheduled cesarean delivery at an estimated gestational age of 37 weeks. Three newborns were healthy following delivery, including 2 females and 1 male, respectively. The Apgar scores are 10, 8, and 9; the body weights are 2.6, 1.4, and 1.4 kg, and the body lengths are 51, 40, and 40 cm, respectively. To date, the physical and mental developments of these infants are normal on their regularly postnatal follow-up.

3.4 | Global gene expression characteristics of oocytes in the control and the MT supplementation groups

To explore the mechanisms of the effect of MT on IVM of immature oocytes from the COH cycle, as well as on subsequent embryonic development, single-cell RNA-sequencing was performed for the transcriptome in the formed IVM-MII oocytes to analyze the differences in gene expression between the control and MT groups. Cluster analysis of gene expression levels (FPKM) in each sample showed the differences in the profiles, which revealed that the RNA sequencing data of this study met the conditions for differential expression analysis (Figure 5A).

Significantly differentially expressed genes (DEGs) were considered if the genes with FPKM > 2 and adjusted *P*-value < 0.05. Generally, 1026 genes were differentially expressed in the MII oocytes of the control and MT groups. These DEGs included 726 upregulated genes and 300 downregulated genes (Figure 5B). Gene Ontology enrichment analysis was conducted to identify the distribution of the expression of the MT-related differentially expressed genes between various biological functions (Figure 5C). The results showed that "cellular process" was the most significant enrichment term in the biological process category; "cell part" was the most significant enrichment term in the cellular components category; and "binding" was the most significant enrichment term in the molecular function category. Kyoto Encyclopedia of Genes and Genomes enrichment analysis was performed to identify the distribution of the expression of the MT-related differential genes between various biological pathways (Figure 5D). The results showed that "Transport and catabolism" was the most significant enrichment term in the cellular processes category, which included 203 DEGs. Kyoto Encyclopedia of Genes and Genomes enrichment analysis revealed that the majority of DEGs involved in mitochondrial oxidative phosphorylation of the energy metabolism pathway was upregulated in the M group compared with that in the NM group (Figure 5E, Table S2). The majority of these DEGs were encoded by the mitochondrial genome and participated in the mitochondrial respiratory chain by encoding mitochondrial genome-encoded nicotine-amide adenine dinucleotide (NADH) dehydrogenase (mt-Nd), the key protein subunit of complex I. The DEGs included mt-Ndufc2 (Figure 5E). The genes encoding the subunits of the complex III and complex V of the respiratory chain

TABLE 2 Comparison of the rates of fertilization, cleavage, blastocyst formation, and high-quality blastocyst formation in the NM group and M group

	M group	NM group
No. of immature oocytes (GV + MI) (n)	105	88
No. of mature oocytes (GV + MI) (n)	86	67
Rate of fertilized oocytes (GV + MI) (%)	79.1 (68/86)	76.1 (51/67)
Rate of cleaved oocytes (GV + MI) (%)	98.5 (67/68)	96.1 (49/51)
Rate of blastocysts (GV + MI) (%)	49.3 (33/67)[a]	24.5 (12/49)
Rate of high-quality blastocysts (GV + MI) (%)	28.4 (19/67)[b]	2.0 (1/49)

Note: The cleavage rate was calculated as the ratio of cleaved embryos to fertilized oocytes. The formation rate of blastocysts was determined according to the ratio of the number of blastocysts to the number of cleaved embryos. The formation rate of high-quality blastocysts was determined according to the ratio of the number of blastocysts with >3BB grade to the number of cleaved embryos.

[a]$P < 0.01$ compared with the NM group.

[b]$P < 0.01$ compared with the NM group.

TABLE 3 Comparison of the rates of fertilization, cleavage, blastocyst formation, and high-quality blastocyst formation between the NM-GV, M-GV, NM-MI, and M-MI groups

	GV Group		MI Group	
	NM-GV	M-GV	NM-MI	M-MI
No. of immature oocytes (n)	46	85	42	20
No. of mature oocytes (n)	30	68	37	18
No. of matured oocytes (%)	65.2 (30/46)	80.0 (68/85)	88.1 (37/42)[a]	90.0 (18/20)
No. of fertilized oocytes (%)	73.3 (22/30)	77.9 (53/68)	78.4 (29/37)	83.3 (15/18)
No. of cleaved oocytes (%)	95.5 (21/22)	98.1 (52/53)	96.6 (28/29)	100 (15/15)
No. of blastocysts (%)	38.1 (8/21)	51.9 (27/52)	14.3 (4/28)	40.0 (6/15)
No. of high-quality blastocysts (%)	0(0/21)	32.7 (17/52)[b]	3.6 (1/28)	13.3 (2/15)

Note: The maturation rate of the oocytes was determined according to the ratio of the number of mature oocytes to the number of immature oocytes. The cleavage rate was calculated as the ratio of cleaved embryos to fertilized oocytes. The formation rate of the blastocysts was determined according to the ratio of the number of blastocysts to the number of cleaved embryos. The formation rate of high-quality blastocysts was determined according to the ratio of the number of blastocysts with >3BB grade to the number of cleaved embryos.

[a]$P < 0.05$ compared with the NM-GV group.

[b]$P < 0.01$ compared with the NM-GV group.

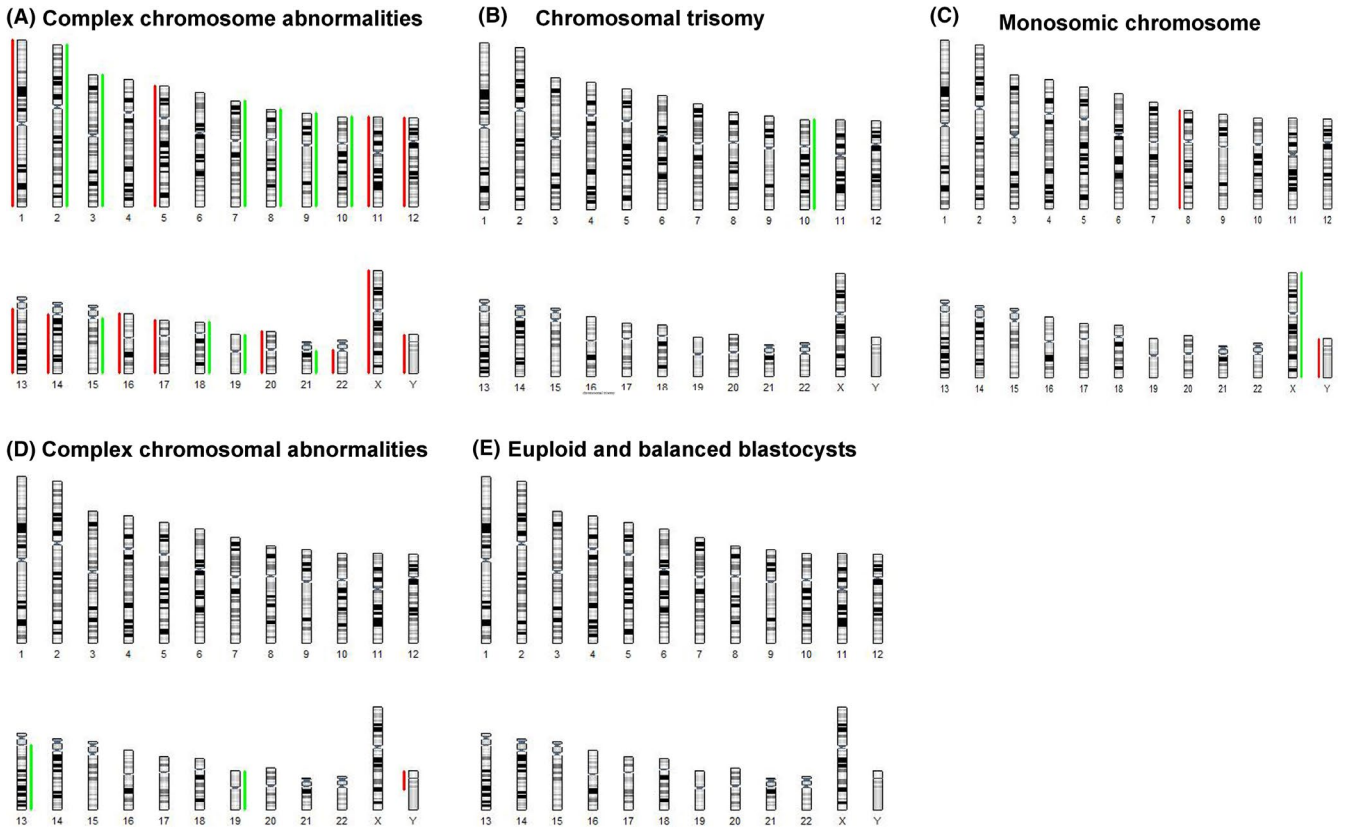


FIGURE 4 Representative images of array CGH results from high-quality blastocysts. In total, 20 high-quality blastocysts (19 from the M group and 1 from the NM group) were used for array CGH detection. The results showed that of the 19 high-quality blastocysts from the M group, 16 were normal; the other three blastocysts were diagnosed as aneuploid. The three blastocysts in the M group that were diagnosed as aneuploid showed chromosomal trisomy, a monosomic chromosome, and complex chromosomal abnormalities. The results showed that one blastocyst in the NM group was aneuploid. (A) Blastocyst from the NM group with complex chromosome abnormalities. (B) Blastocyst from the M group with chromosomal trisomy. (C) Blastocyst from the M group with a monosomic chromosome; (D) Blastocyst from the M group with complex chromosomal abnormalities. (E) representative image of euploid and balanced blastocysts from the M group. Green indicates chromosomal duplication, and red indicates chromosomal deletion. Images of the other euploid and balanced blastocysts from the M group are not shown. The results indicate that the incidence rate of aneuploidies in the high-quality blastocysts in the M group was very low (15.79%, 3/19)

including, cytochrome C oxidase subunit 17 (COX17), and V-type proton ATPase subunit G 2 (ATP6V1G2) were found to be significantly upregulated in the M group.

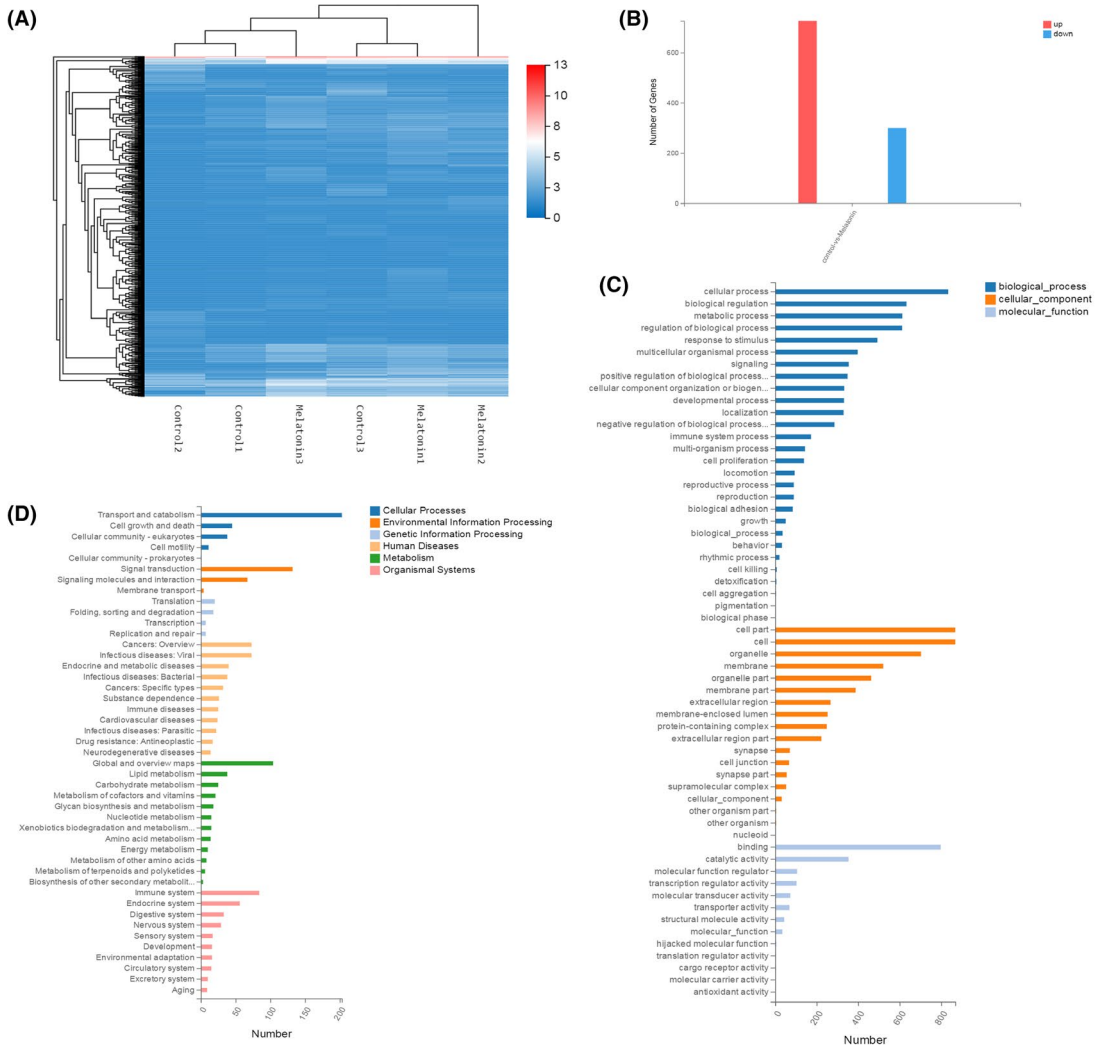
3.5 | Effects of MT supplementation on oxidative stress

Oxidative phosphorylation of the energy metabolism pathway exists in mitochondria. It has been also reported that the impairment of oocyte developmental competence involves the alterations in mitochondrial function.[21-24] Therefore, we further assess the mitochondrial function in the IVM-MII oocytes by detecting indicators related to mitochondrial function.

ROS is one of the important products derived from the oxidative phosphorylation[25,26]; however, excessive ROS production triggers oxidative stress, impairs mitochondrial function, and impedes oocyte development.[27] As shown in Figure 6A, MT treatment resulted in a dramatic decrease in ROS generation in human oocytes compared with that in the control NM group. The mean fluorescence intensity of ROS was significantly lower in the MT treatment group (n = 11) than in the control group oocytes (n = 10) ($P < .01$) (Figure 6B), indicating that a decrease in oxidative stress occurred after the MT treatment.

3.6 | Effects of MT supplementation on mitochondrial membrane potential (MMP)

Mitochondrial membrane potential is one of the important indicators reflecting mitochondrial function. To examine the mitochondrial function, we specifically focused on the MMP of oocytes cultured in the IVM medium with or without MT. At the inner MMP ($\Delta\Psi m$) <100 mV, JC-1 remains a monomer and emits green fluorescence in the FITC channel (low polarized mitochondria), whereas at $\Delta\Psi m > 140$ mV, JC-1 forms J-aggregates and emits red fluorescence (highly polarized mitochondria). We investigated high polarization of mitochondria


(A)
control2
control1
Melatonin3
control3
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(B)
up
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(D)
Cellular Processes
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Human Diseases
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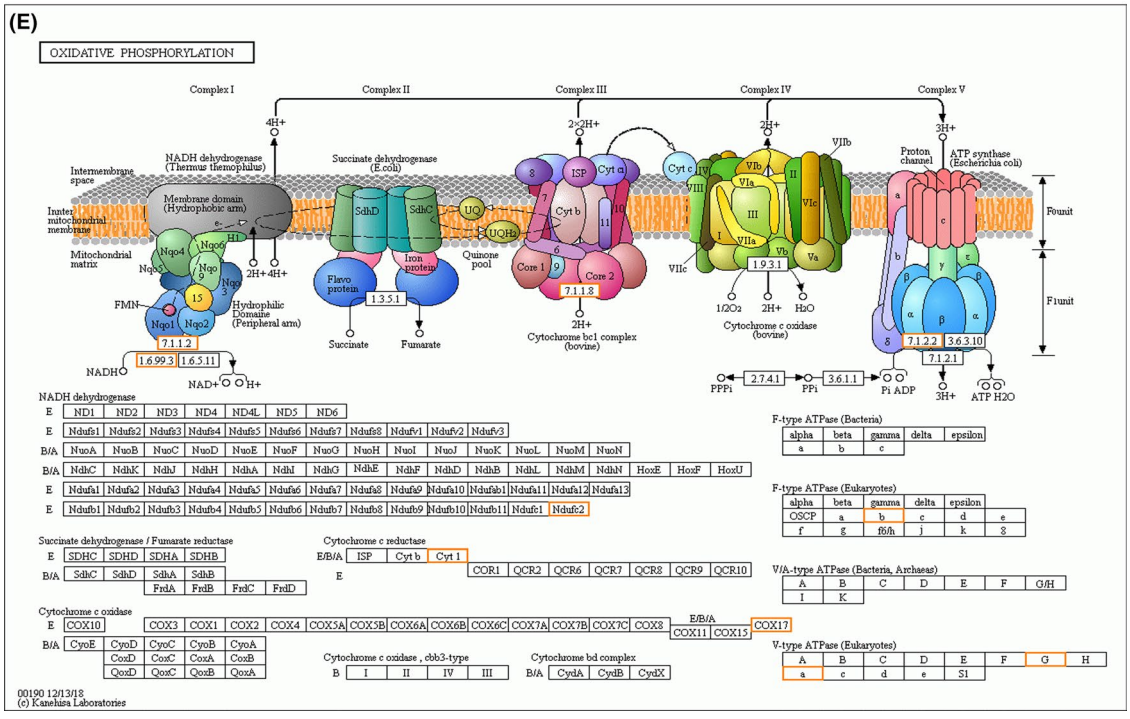
(E)
OXIDATIVE PHOSPHORYLATION
Complex I
Complex II
Complex III
Complex IV
Complex V
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Succinate dehydrogenase (E.coli)
Cytochrome bc1 complex (bovine)
Cytochrome c oxidase (bovine)
ATP synthase (Escherichia coli)
Intermembrane space
Inner mitochondrial membrane
Mitochondrial matrix
Membrane domain (Hydrophobic arm)
Hydrophilic Domain (Peripheral arm)
Quinone pool
Iron protein
Flavo protein
Succinate
Fumarate
NADH
NAD+
FMN
Proton channel
Fo unit
F1 unit
NADH dehydrogenase
Succinate dehydrogenase / Fumarate reductase
Cytochrome c reductase
Cytochrome c oxidase
Cytochrome c oxidase , cbb3-type
Cytochrome bd complex
F-type ATPase (Bacteria)
F-type ATPase (Eukaryotes)
V/A-type ATPase (Bacteria, Archaeas)
V-type ATPase (Eukaryotes)
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FIGURE 5 Gene expression levels of the IVM-MII oocytes in the M group and NM group. (A) The heat map shows the clustering analysis of the gene expression levels (FPKM) in each sample. (B) The histogram shows the differentially expressed genes (DEGs) in the oocytes of the MT and control groups. Red dots in the volcano plot refer to upregulated DEGs, and blue dots in the volcano plot refer to downregulated DEGs. Gray dots represent the genes that were not differentially expressed in the MT and control groups. (C) GO (Gene Ontology, http://www.geneontology.org/) enrichment analysis on DEGs. Blue bars refer to the terms related to biological processes; orange bars refer to the terms related to cellular components; and gray bars refer to the terms related to molecular function. (D) KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways enriched by DEGs. Blue bars refer to the terms related to cellular processes; orange bars refer to the terms related to environmental information processing; gray bars refer to the terms related to genetic information processing; light orange bars represent the terms related to human diseases; green bars represent the terms related to metabolism; and pink bars represent the terms related to organismal systems. (E) The KEGG pathway of oxidative phosphorylation pathway responds to IVM-MII oocyte, and the genes highlighted in orange are enriched and significantly upregulated in oocytes from the M group

in MII oocytes by examining the relative levels of red to green fluorescence emission. Relative inner membrane potential in MII oocytes from the M group (n = 15) was substantially increased compared with that in the NM group (n = 13) ($P < .01$) (Figure 7A,B) indicating an increase in $\Delta\Psi m$.

3.7 | Effects of MT supplementation on calcium concentration

The level of intracellular Ca^{2+} is another manifestation of mitochondrial function. MMP and calcium storage are tightly linked to a number of mitochondrial processes that maintain cellular homeostasis.[28] We investigated the functional role of the mitochondria in the MT-induced improvement of the developmental potential of human oocytes by measuring the calcium concentrations in the cytoplasm. Intriguingly, the data obtained in 23 oocytes showed that the calcium levels in the oocytes of the M group (n = 12) were significantly lower than those of the control group (n = 11) ($P < .01$) (Figure 8A,B).

4 | DISCUSSION

Melatonin and its metabolites are potent free radical scavengers and antioxidants in many organisms.[1,29] It has been reported that MT can improve oocyte maturation in vitro[10,30] and promote early embryo development[8,17,31] in an animal model and even in humans; however, there is insufficient information on whether MT improves the developmental potential of immature human oocytes collected from the COH cycle and on the mechanisms of the effect.

Based on the optimal IVM clinical outcome of Kim et al,[32] the present study was designed to explore whether the MT-supplemented IVM program can improve the developmental potential of immature human oocytes retrieved after COH for the purpose of ICSI. In the process of follicular development, dominant follicles inhibit the growth of small follicles and eventually induce their apoptosis; hence, it has been widely accepted that immature human oocytes retrieved from the COH cycle have a very low chance to develop into offspring and have no reusable value. Thus, in routine IVF and ICSI therapies, immature human oocytes collected from the COH cycle are typically discarded. However, the ability to efficiently use a "special" oocyte may represent a major breakthrough in the theory and technology of assisted reproduction. In addition, these "special" oocytes can be used by patients to increase the cumulative pregnancy rate or to donate to an egg bank or for scientific research as a precious but scarce material.

The results of the present study confirm that immature human oocytes collected from the COH cycle can develop

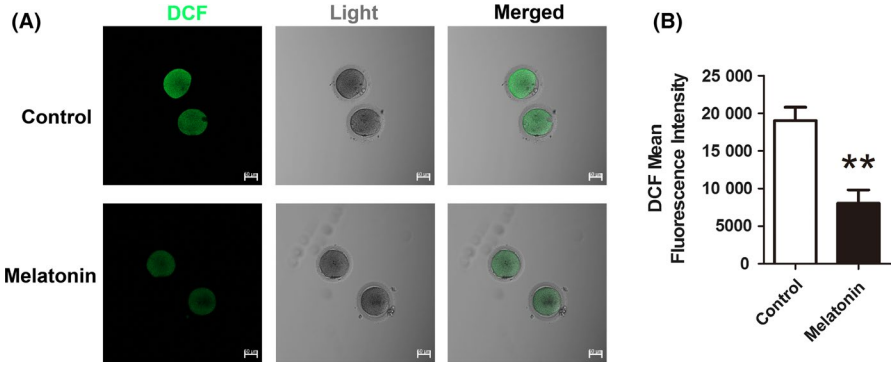

FIGURE 6 ROS levels in the IVM-MII oocytes from the M group and NM group. (A) DCFH-DA staining was performed to measure the concentrations of reactive oxygen species in the human oocytes, and the staining was detected using confocal microscopy. Scale bar = 50 μm. (B) Data are expressed as the mean ± SEM (n = 10 vs n = 11). "**" indicates significant differences, $P < .01$

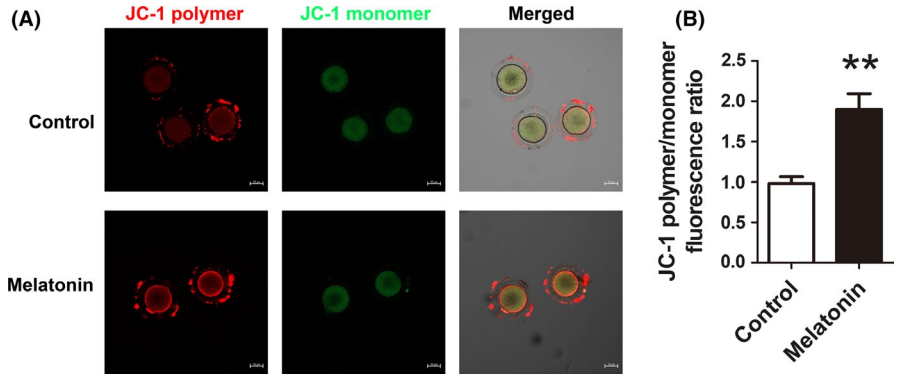

FIGURE 7 MMP in the IVM-MII oocytes from the M group and NM group. (A) JC-1 staining was performed to measure the mitochondrial membrane potential, and the staining was detected using confocal microscopy. Scale bar = 50 μm. (B) Data are expressed as the mean ± SEM (n = 14 vs n = 14); "**" indicates significant differences, $P < .01$

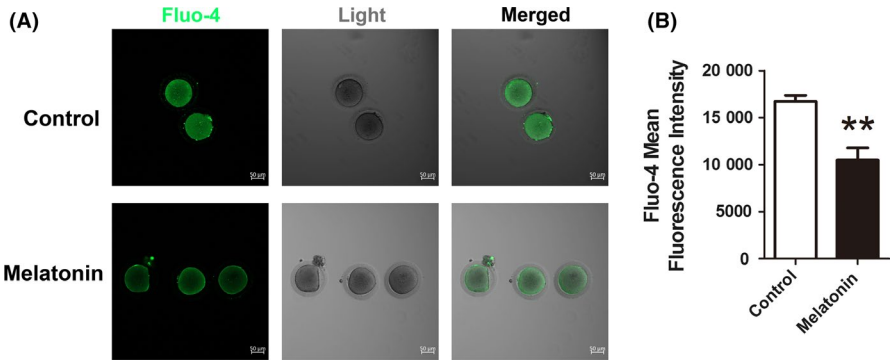

FIGURE 8 Calcium levels in the IVM-MII oocytes from the M group and NM group. (A) Fluo-4 AM staining was performed to measure the calcium concentrations in the human oocytes, and the staining was detected using confocal microscopy. Scale bar = 50 μm. (B) Data are expressed as the mean ± SEM (n = 11 vs n = 12). "**" indicates significant differences, $P < .01$

into high-quality blastocysts through our MT-IVM program. However, a high-efficiency IVM medium must be developed in order to achieve an ideal IVM outcome with these "special" oocytes. In the present study, a total of 105 oocytes were placed into the IVM medium containing 10 μmol/L of MT for the IVM culture. This process was followed by ICSI with subsequent culture of cleaved embryo and blastocysts. Finally, 33 blastocysts formed, including 19 high-quality blastocysts. However, a total of 88 oocytes were subjected to IVM culture using a general IVM medium[33] in the absence of MT and only one high-quality blastocyst formed. In the present study, the rate of high-quality blastocyst formation in the M group was significantly higher than that in the NM group. As we know, high-quality oocytes are needed for the formation of well-developed embryos. Our results imply that MT plays a crucial role in the development of immature oocytes into the competent mature oocytes during IVM culture. Subsequently, all 20 high-quality blastocysts were subjected to aneuploidy detection via array CGH. Array CGH is a useful tool to screen the chromosome-normal embryos for subsequent transfer.[34] Array CGH results showed that the incidence of aneuploidy in the high-quality blastocysts of the M group was very low (15.8%, 3/19). The results indicate that MT supplementation into the IVM medium for the IVM culture of immature human oocytes retrieved from the COH cycle can promote the development of "special" oocytes into high-quality mature oocytes, thus greatly enhancing their reusable value. The above results also indicate that a safe and effective MT-IVM program has been established for the reuse of these "special" oocytes.

Based on the results of experiment I, the MT-IVM program was subsequently applied to clinical IVM therapy in two patients. Fortunately, both patients achieved pregnancy and eventually gave birth to three healthy offspring. This result provides further evidence that antioxidant MT can substantially improve the developmental potential of immature human oocytes obtained from the COH cycle. In addition, in experiment II, the granulosa cells of the "special" oocytes were completely removed during IVM culture, representing a departure from the routine IVM program,[32,33] in which immature oocytes are surrounded by granulosa cells during IVM culture. Therefore, we propose that immature oocytes without granulosa cells can be subjected to the IVM culture and can achieve an ideal outcome of the MT-IVM program. This result also implies that MT can, in part, substitute the function of the granulosa cells in the process of IVM of immature oocytes in the absence of the granulosa cells.

Reproductive performance is sensitive to variable stress exposure, including environments, thermal stress, and food

toxins.[35] In vitro maturation is a serious environmental stress for immature oocytes, leading to the low-quality mature oocytes and poor-quality embryos, expressed by the reduced maturation and developmental competence, as a consequence of the low IVM efficacy.[36] In vitro environment may induce aberrant expression of a number of genes; however, a compensatory mechanism can prevent oocytes from failure of maturation or development.[37] Stress-induced impairment of oocyte developmental competence involves the alterations in mitochondrial function.[21-24] Within the oocyte, mitochondria are contributed to ATP generation,[38] ROS production,[27] calcium homeostasis,[39] cytoplasmic oxidation-reduction regulation, signal transduction, and apoptosis.[40] Stress impairs the expression of mitochondria-associated genes, including oxidative phosphorylation complex genes encoding ATP.[41]

In experiment III, to elucidate the mechanism of MT promotion in oocyte development, single-cell RNA-sequencing was performed for the transcriptome in the IVM-MII oocytes to analyze the differences in gene expression between the control and MT groups. The data revealed that there were 1026 DEGs between the two groups. In the M group, 726 DEGs upregulated while 300 DEGs downregulated. Interestingly, the KEGG pathway analysis revealed that six DEGs present in the mitochondrial oxidative phosphorylation pathway significantly upregulated in the M group and each of them was associated with ATP generation, indicating that ATP production of the IVM-MII oocytes in the M group is promoted. It has been also reported that reduction of ATP levels in the oocyte below the required threshold compromises subsequent embryonic development.[42] In the present study, the high-quality blastocysts formation rate in the M group was significantly higher than that in the NM group ($P < .05$), as well as such blastocysts also developed into healthy offspring. Therefore, the results imply that MT application can well maintain the mitochondrial oxidative phosphorylation function in the oocytes by effectively inhibiting environmental stress during IVM culture, thereby providing sufficient ATP for subsequent embryo development. Therefore, targeting the production of a sufficient energy supply in the mitochondria is a promising approach to ameliorate the poor outcome of human IVM oocytes.[37]

ROS is another important product derived from the oxidative phosphorylation.[25,26] Under physiological conditions, ROS are essential for nuclear maturation.[36] However, excessive ROS production triggers oxidative stress, impairs mitochondrial function, and impedes oocyte development.[27] Equilibrium between ROS production and antioxidative

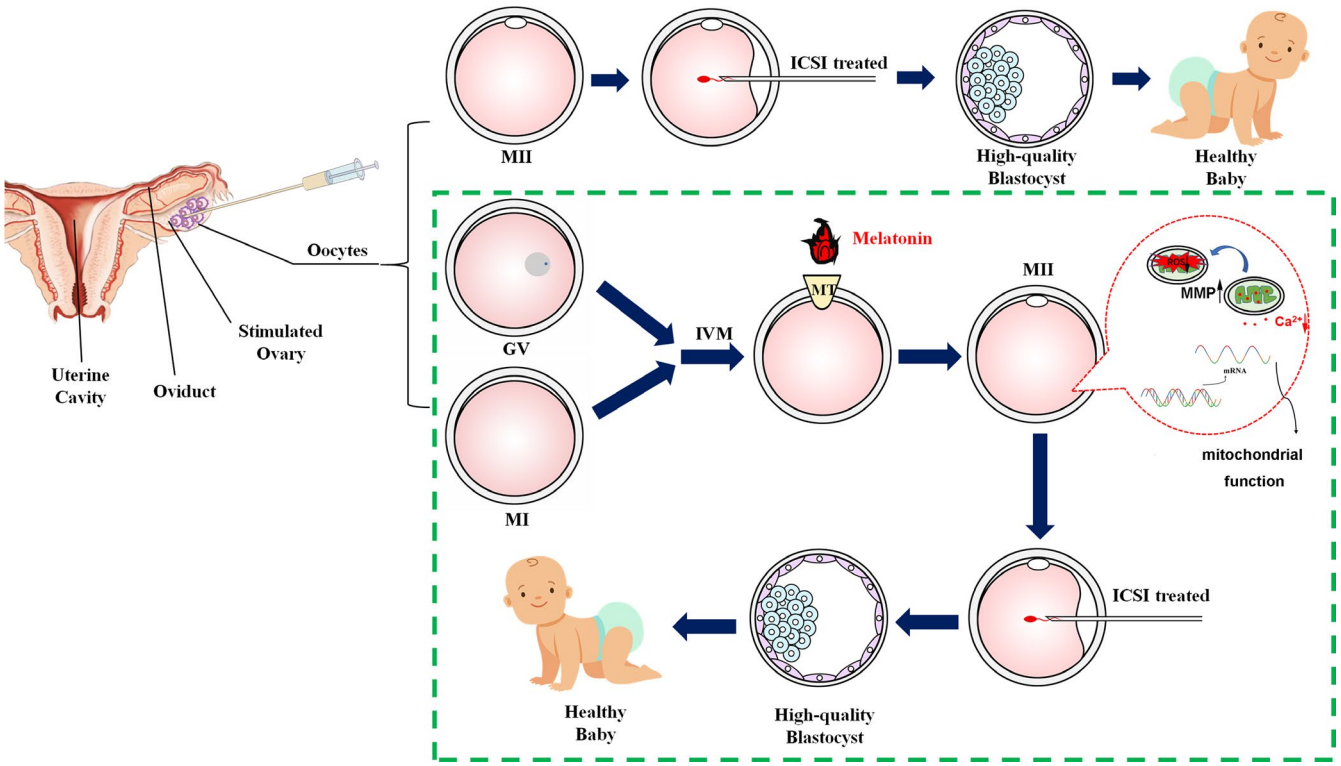

FIGURE 9 Reuse of immature oocytes retrieved from the COH cycle through MT-IVM program. MT supplementation into IVM medium for the IVM culture of GV or MI oocytes retrieved from the COH cycle, followed by ICSI insemination for the IVM-MII oocytes, embryo culture for the fertilized oocytes, vitrification and thawing for the high-quality blastocysts formed, T-ET, pregnancy determination and delivery of health offspring (as shown in the green line box), in which MT protected the oxidative phosphorylation pathway with six upregulated DEGs, as well as the mitochondrial function with an increased MMP, reduced intracellular ROS and Ca^{2+} levels in the IVM-MII oocytes (as shown in the red circle)

capacity is critical to maintaining the mitochondrial function.[27] In the experiment III, we used confocal fluorescence staining to detect the mitochondrial function of the IVM-MII oocytes in the two groups. It was found that the MMP of the oocytes in the M group was higher than that in the NM group; intracellular ROS and Ca^{2+} levels were lower than those in the NM group. MMP is one of the important indicators reflecting mitochondrial function. The level of intracellular Ca^{2+} is another manifestation of mitochondrial function. Mitochondria can store Ca^{2+} and maintain calcium homeostasis in an oocyte. If intracellular Ca^{2+} concentration increases, mitochondrial function is impaired. The above results indicated that the mitochondrial function of the oocytes in the M group was well protected during IVM culture, which was attributed to the addition of MT. As a high-effective antioxidant, the addition of 10 μmol/L of MT effectively maintains the equilibrium between ROS production and antioxidant capacity, which also means that 10 μmol/L is an ideal additive concentration for the IVM culture of immature oocytes from the COH cycle.

Collectively, the results of this study suggest that melatonin can improve the developmental potential of immature human oocytes retrieved from the COH cycle by protecting mitochondrial function and ultimately achieve the birth of healthy offspring. (Figure 9).

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Huijuan Zou, Beili Chen, Ding Ding, and Ming Gao contributed equally to this work by conducting the experiments and drafting the manuscript. Dawei Chen and Yan Hao completed the array CGH examination. Yajing Liu, Weiwei Zou, and Dongmei Ji performed the IVM experiments. Ping Zhou and Zhaolian Wei supervised the research. Zhiguo Zhang and Yunxia Cao designed the experiments and revised the manuscript. The authors have no conflict of interest with the contents of this article.

ORCID

Zhiguo Zhang https://orcid.org/0000-0003-1483-3321

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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