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Melatonin maintains mitochondrial membrane potential and decreases excessive intracellular Ca^{2+} levels in immature human oocytes

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ABSTRACT

Aims: Previous reports have demonstrated that melatonin exists in multiple extrapineal sites, and higher amounts of melatonin are present in human follicular fluid than in serum, which indicates that it might play key roles in human oocyte maturation and subsequent embryonic development. Melatonin has been shown to be a potent antioxidant and might be beneficial to human oocytes during *in vitro* maturation (IVM). However, the underlying mechanisms of melatonin action during IVM have not been thoroughly investigated.

Main methods: Immunofluorescence staining, western blotting, and ELISA were applied to investigate whether melatoninergic components are expressed in the cultured human ovarian cumulus cells. TMRE staining and Fluo-4 AM staining were performed to detect the mitochondrial membrane potential and intracellular Ca^{2+} levels of immature human oocytes respectively.

Key findings: First, cultured human ovary cumulus cells synthesized melatonin *in vitro*, and it expressed serotonin (the precursor of melatonin) and the two key enzymes, *i.e. N*-acetyltransferase (NAT) and hydroxyindole-*O*-methyltransferase (HIOMT). Additionally, the results suggest that melatonin maintains the mitochondrial membrane potential and decrease excessive Ca^{2+} levels in immature human oocytes during IVM.

Significance: In conclusion, we provide evidence that the melatoninergic components were expressed in cultured human ovarian cumulus cells, and melatonin might reduce oxidative stress of human oocytes by ameliorating mitochondrial function. In view of the significant clinical value that immature human oocytes have in assisted reproductive technology (ART), our findings highlight a potential treatment strategy of using melatonin to improve mitochondrial function and to enhance the quality of human oocytes during IVM.

1. Introduction

Previous studies have reported that higher amounts of melatonin were present in human follicular fluid than in serum, and the melatonin concentration in mature ovarian follicles is higher than it is in immature follicles [1]. Recently, long-term melatonin treatment has been shown to delay ovarian aging *via* multiple mechanisms [31]. Furthermore, the melatonin levels in the follicular fluid could act as predictors of ovarian reserve and *in vitro* fertilization (IVF) outcomes [26,35]. Thus, melatonin present in human follicular fluid plays key roles in human oocytes maturation, the formation of high-quality oocytes, and the subsequent embryonic development.

The biosynthetic pathway of pineal melatonin has been thoroughly studied [33]. A growing number of studies have demonstrated that the presence of melatonin has been detected in multiple extrapineal sites, such as the ovary [8,9], testis [6], platelets [16] and lymphocytes [4] and so on. Our group reported that cultured rat cortical astrocytes, and glioma C6 cells synthesized melatonin [13]. In addition, the apolipoprotein E genotype influenced melatonin biosynthesis by regulating NAT and MAOA expression in C6 cells [12]. Furthermore, it was reported that melatonin receptors exist in ovaries [30], lymphocytes [19], platelets [27], astrocytes [2] and cumulus cells [17]. Such a wide

Abbreviations: Serotonin *N*-acetyltransferase, 1(NAT); monoamine oxidase, A(MAOA); hydroxyindole-*O*-methyltransferase, (HIOMT); acetylserotonin methyltransferase, (ASMT); 5-hydroxyindoleacetic acid, (5-HIAA); Medium 199, (M199); N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid, (HEPES); Tris Buffered Saline, (TBS); Serum substitute supplement, (SSS); *in vitro* maturation, (IVM); Melatonin, (MT)

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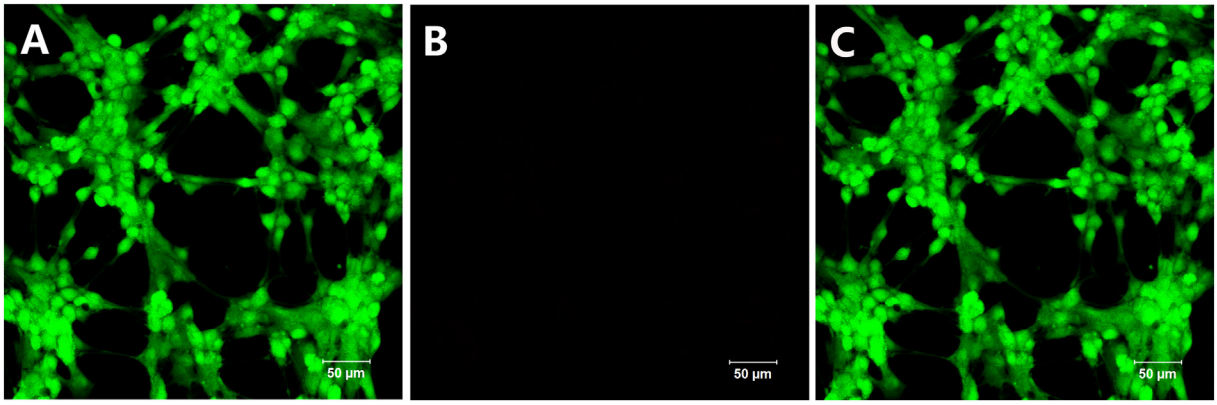

Fig. 1. Measurement of human ovarian cumulus cell viability by Live-dead assay. The Live-dead assay showed the cell viability in the cultured human ovarian cumulus cells. The green colour represents living cells and no dead (red) cells were present. Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

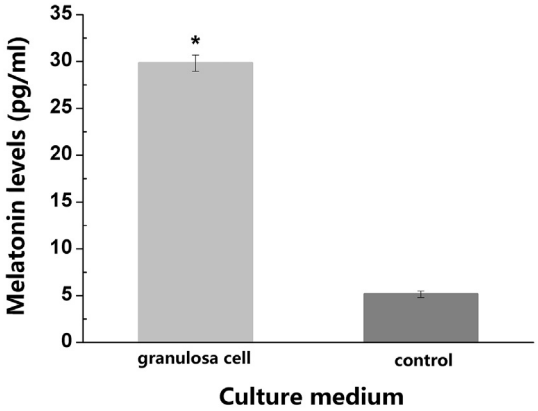

Fig. 2. Melatonin levels in the supernatant of human ovarian cumulus cells. Melatonin was determined in the human ovarian cumulus cell culture medium. All the cultured human ovarian cumulus cells were incubated upto a number to 2×10^5 *in vitro*. There was a very low amount of melatonin in the negative control medium. Values were expressed as mean ± S.E.M., * $p < 0.001$ ($n = 10$).

distribution suggests a local effect of melatonin in these tissues and cells [25]. Melatonin acts as a powerful free radical scavenger that protects many cells, including oocytes, from oxidative stress [3,20,21].

It was reported that melatonin is important for female reproduction [21]. In addition, melatonin was reported to improve the quality of mouse oocytes under *in vitro* conditions [7,18]. Kim et al. reported an amazing clinical pregnancy rate of 60% following routine IVM therapy [11]; however, they did not explore the mechanisms underlying this effect.

Since melatonin contributes to oocyte maturation and embryo development, and since the mitochondrial membrane potential and intracellular Ca^{2+} levels are important to oocyte quality [22], it was of interest to investigate whether melatoninergic components are expressed in cultured human ovarian cumulus cells; it was of further interest to investigate the effect of melatonin on the mitochondrial membrane potential and intracellular Ca^{2+} levels of immature human oocytes.

2. Materials and methods

2.1. Materials

Medium 199 (M199), HEPES (N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid), melatonin, L-glutamine, penicillin, streptomycin, rabbit anti-serotonin antibody, and anti- serotonin *N*-acetyltransferase N-terminal antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). Serum substitute supplement (SSS) was from Irvine Scientific Corporation (Santa Ana, CA, USA). Hyaluronidase was purchased from CooperSurgical Company (Trumbull, CT, USA). Anti-ASMT antibody was from Abcam Corporation (Cambridge, MA, USA). Follicle-stimulating hormone receptor (FSHR) polyclonal antibody was from Protintech Corporation (Rosemont, IL, USA). FITC-goat anti-rabbit IgG were supplied by Santa Cruze Bio-technology Corporation (Dallas, TX, USA). HRP conjugated goat anti-rabbit IgG was purchased from Promega Corporation (Madison, WI, USA). Melatonin ELISA kit was from Cloud-Clone Corporation (Katy, TX, USA). The ECL chemiluminescence detection kit for western blots was offered by Amersham Life science (Arlington Hts, IL, USA). MitoTracker Red, tetramethylrhodamine, ethyl ester perchlorate (TMRE) and Fluo-4 AM were obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Culture of human ovarian cumulus cells

Primary human ovarian cumulus cells were collected from follicular fluids of *in vitro* fertilization and embryo transfer (IVF-ET) patients, and they were obtained with informed patient consent and approval of ethical committees at the First Affiliated Hospital of Anhui Medical University. Briefly, after cumulus-oocyte complex retrieval, the cumulus-oocyte complex was then washed three times with gamete buffer. Subsequently, the cumulus cells were mechanically separated from the cumulus-oocyte complex using a sharp needle [29]. Next, the cumulus cells were carefully transferred into prechilled phosphate-buffered saline (PBS) under sterile conditions. After washing with PBS, the cells were resuspended in 2 ml of 40 U/ml hyaluronidase solution. After 1 min of incubation at 37 °C, 20% serum substitute supplement (SSS) was added to stop the action of the hyaluronidase solution. The cells were dispersed gently and centrifuged at 1000 rpm for 2 min. The cell pellets were then resuspended in Medium 199 supplemented with 20% SSS, 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at a density of 1×10^5 cells/ml. Cells were seeded onto 35 mm dishes and cultured in a humidified 6% CO_2 incubator at 37 °C.

2.3. Live-dead assay

Cell viability of cultured human ovarian cumulus cells were measured by live-dead assay. Samples were processed according to the manufacturer's recommendations. Briefly, the cells were incubated with 500 μl combined live/dead assay reagents for 30 min in a 37 °C incubator after washing with warm phosphate-buffered saline (PBS). After another wash with PBS, the labeled cells were studied under the fluorescence microscope.

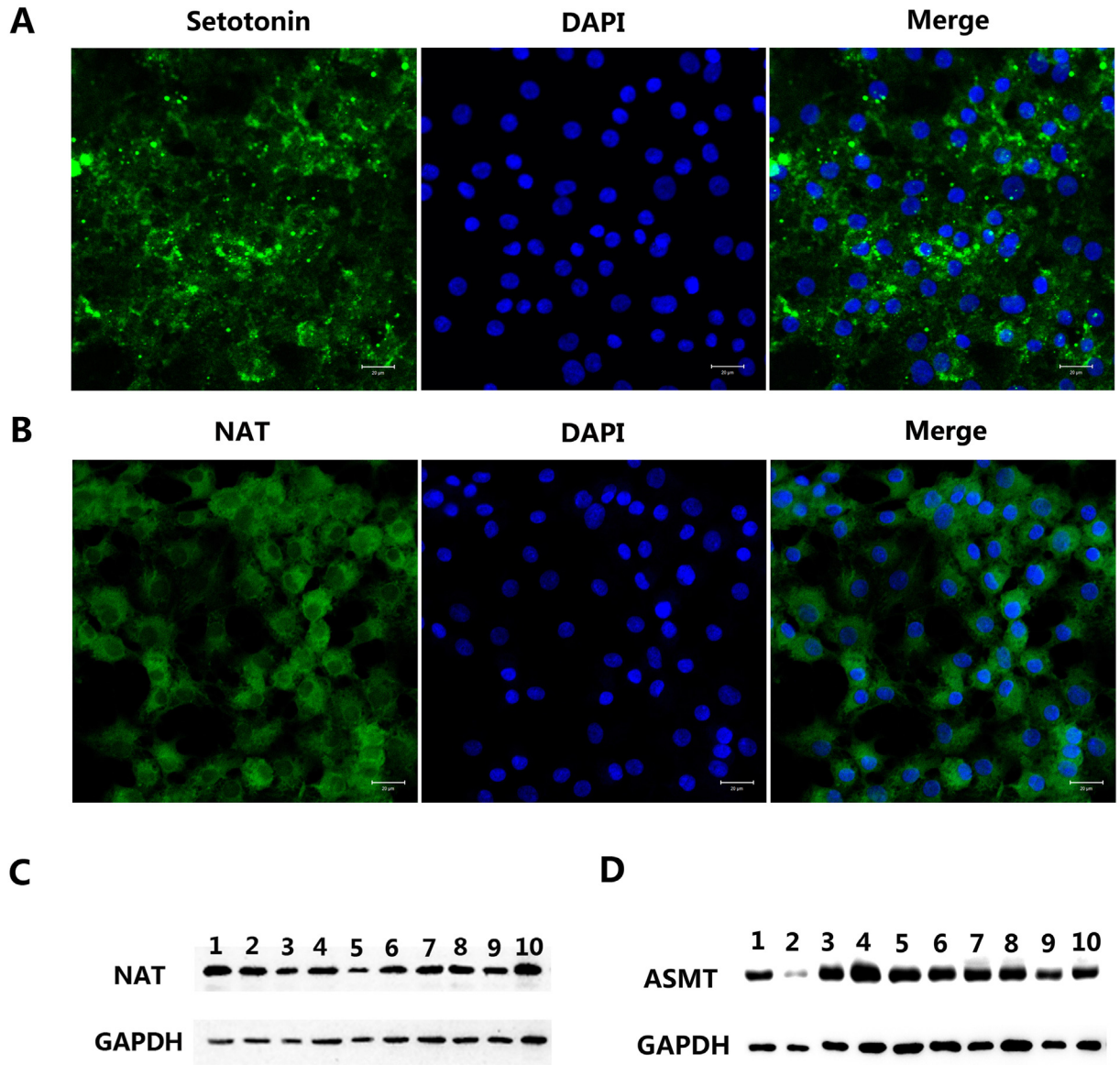

Fig. 3. The precursor to melatonin and the key enzymes involved in its synthesis pathway in cultured human ovarian cumulus cells. (A) Immunofluorescence staining was used to identify whether cultured human ovarian cumulus cells expressed the melatonin precursor serotonin. The results showed that serotonin immunoreactivity (green) in the cultured human ovarian cumulus cells was clearly present. Scale bar = 20 μm. (B) Immunofluorescence staining results indicated that the key enzymein the melatonin synthesis pathway, NAT, was also present in cultured human ovarian cumulus cells. Scale bar = 20 μm. Western blotting analysis showed the protein expression of NAT (C, n = 10) and ASMT (D, n = 10), the key enzymes involved in melatonin production, in cultured human ovarian cumulus cells. GAPDH was used as an internal control to measure the quality of protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4. Immunofluorescence staining

Immunofluorescence staining was performed as previously described [5]. The human ovarian cumulus cells were fixed with 4% paraformaldehyde in TBS (Tris Buffered Saline: 0.05 M Tris, 0.9% NaCl, pH 7.6) for 15 min at room temperature, rinsed in TBS, and treated with 0.3% hydrogen peroxide in TBS for 30 min to quench endogenous peroxidase activity. Subsequently, the human ovarian cumulus cells were incubated with FSHR polyclonal antibody (1:1000), anti-serotonin antibody (1:1000), and anti-serotonin *N*-acetyltransferase N-terminal antibody (1:1000). Confocal microscopy was carried out and the data were processed as described previously [5].

2.5. Western blotting

The protocols used for the preparation of the cell lysate and western blotting have been described previously [12]. For Western blotting, cells were cultured upto cell number to 2×10^5 in 35 mm dishes and harvested, lysed in prechilled lysis buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Nonidet p-40, 0.5% sodium deoxycholate, and a mixture of protease inhibitors) for 30 min and centrifuged at 12,000 rpm for 15 min. The supernatant was collected as the total protein of cells, which was mixed with the same amount of sample buffer. After boiling for 5 min, the protein sample was resolved on 12% SDS-PAGE gels and subsequently transferred onto nitrocellulose membranes and detected by anti-serotonin *N*-acetyltransferase N-terminal antibody (1:2000), and anti-ASMT (1:1000) antibody. The experiment was repeated independently 3 times.

2.6. Culture medium collection

The culture media used for melatonin determination in the human

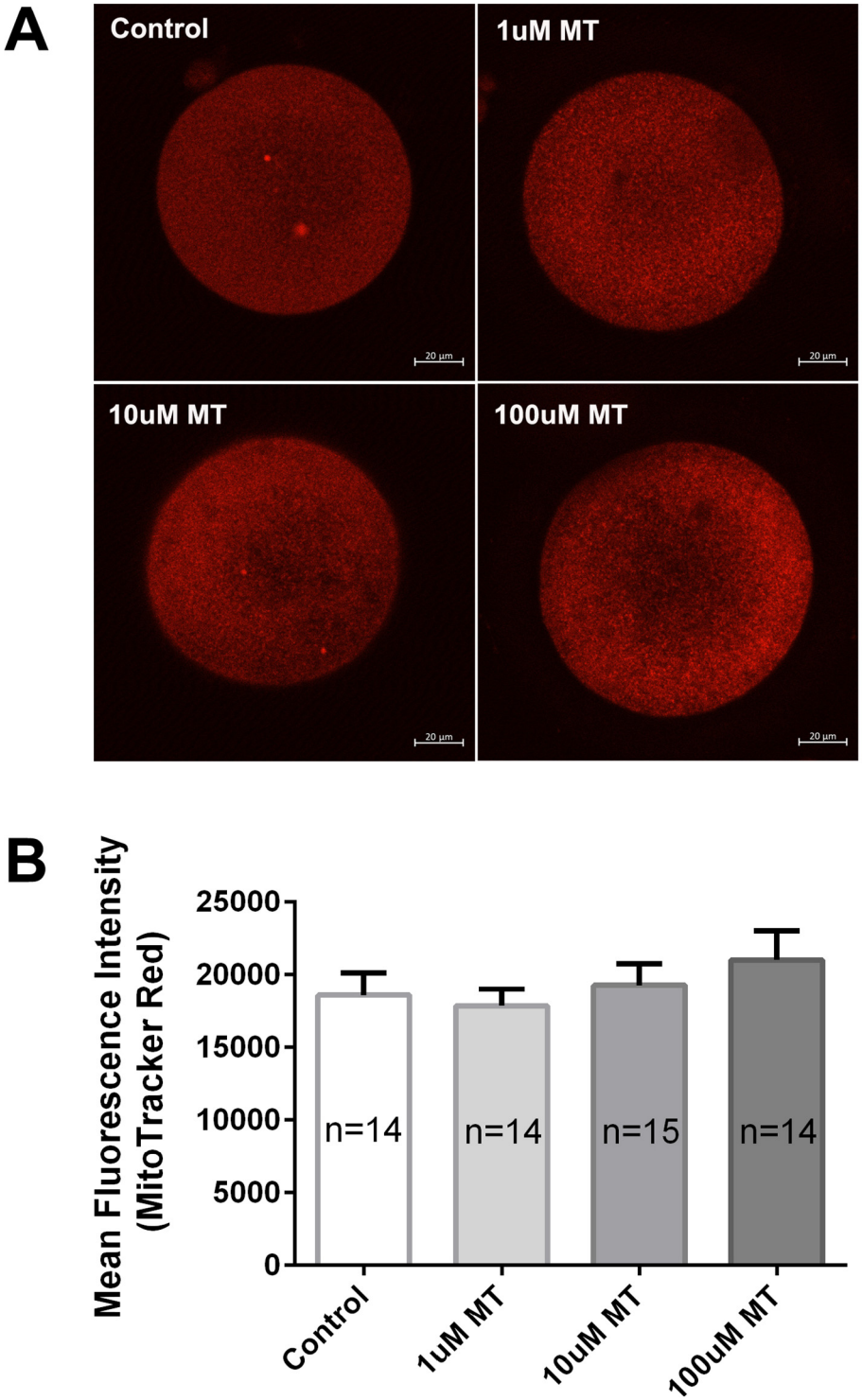

Fig. 4. Effect of different concentrations of melatonin on mitochondrial expression in human oocytes. (A) MitoTracker staining was performed to test the mitochondrial expression and was detected using confocal microscopy. Scale bar = 20 μm. (B) Data are expressed as the mean ± S.E.M; no significant differences were found between the control and melatonin-treated groups(1 uM MT,10 uM MT,100 uM MT) ($p > 0.05$).

ovarian cumulus cells were collected when the cells were cultured up to a cell number of 2×10^5. SSS–supplemented medium was used to negative control. Light was avoided when the cell culture medium was collected and the time for collecting the cell culture medium was < 3 min. Subsequently, the medium was centrifuged for 10 min at 12,000 rpm at 4 °C. The supernatant was collected and kept at −80 °C until assayed.

2.7. Melatonin determination (ELISA)

The melatonin concentrations in the human ovarian cumulus cells culture medium and negative controls were measured by ELISA kit. The assay was conducted according to the procedure of the commercial kit. The standard of melatonin levels in the melatonin research RIA Kit range from 0 pg/ml to 1000 pg/ml. The standard curve of melatonin assay was highly reproducible, with an average correlation coefficients

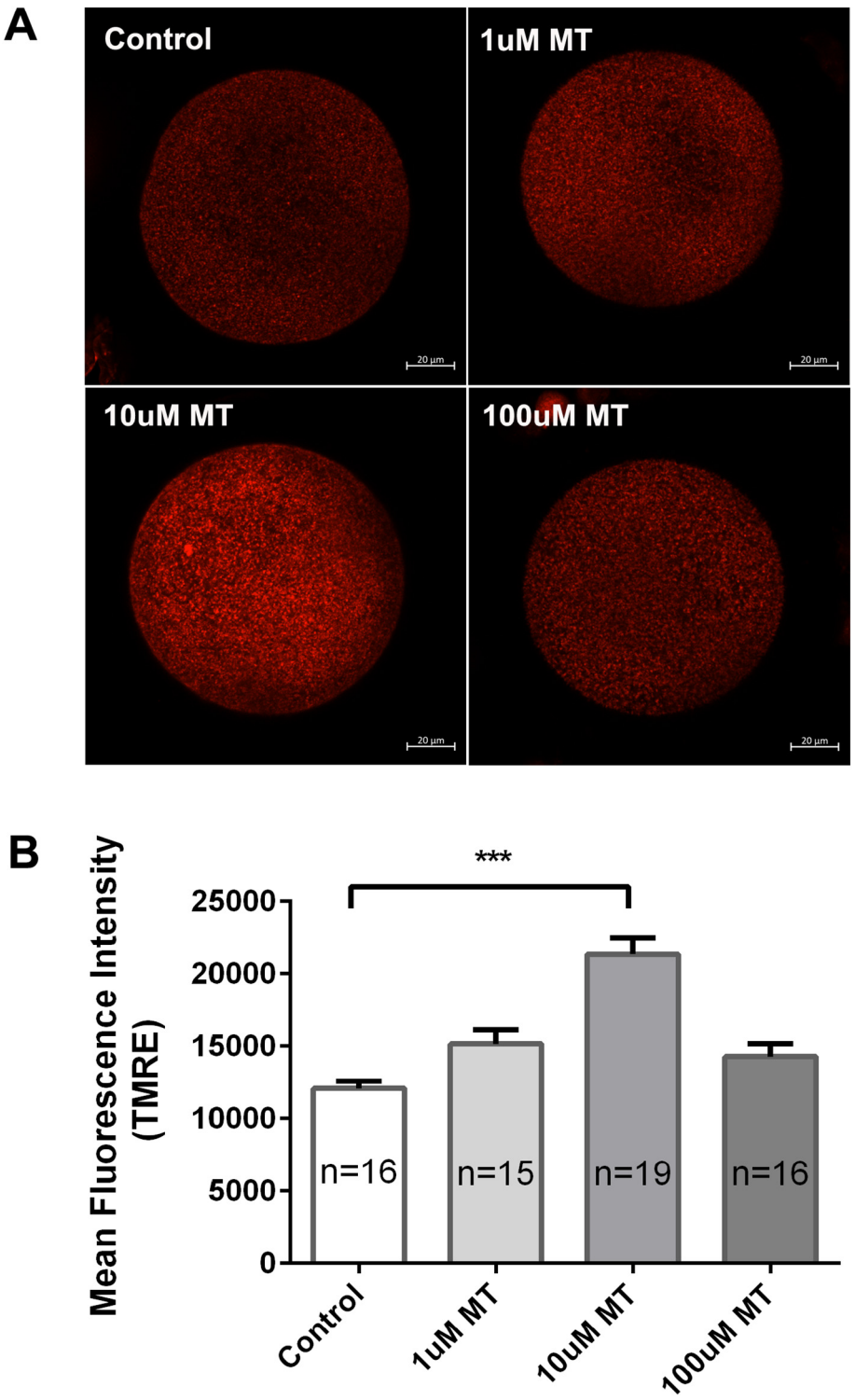

Fig. 5. Effect of different concentrations of melatonin on mitochondrial membrane potential in human oocytes. (A)TMRE staining was performed to measure the effect of different concentrations of melatonin (1 μM , 10 μM , 100 μM) on the mitochondrial membrane potential, and it was detected using confocal microscopy. Scale bar = 20 μm; (B) the data are expressed as the mean ± S.E.M; "***" indicates significant differences, $p < 0.001$.

of 0.99. The average intra- and inter-assay coefficient of variation (CV) was < 10% and < 12%, respectively. The sensitivity of the assay is 5.17 pg/ml.

2.8. Immature oocyte collection

All oocytes were collected from patients younger than 35 years of age who received intracytoplasmic single sperm injection (ICSI) therapy at the Reproductive Medicine Center of the First Affiliated Hospital of Anhui Medical University. This study was approved in advance by the Ethical and Scientific Committee of the First Affiliated Hospital of Anhui Medical University, and all patients provided written informed consent. All matured metaphase II (MII) oocytes were used in the patients' treatments, and the discarded and immature germinal vesicle

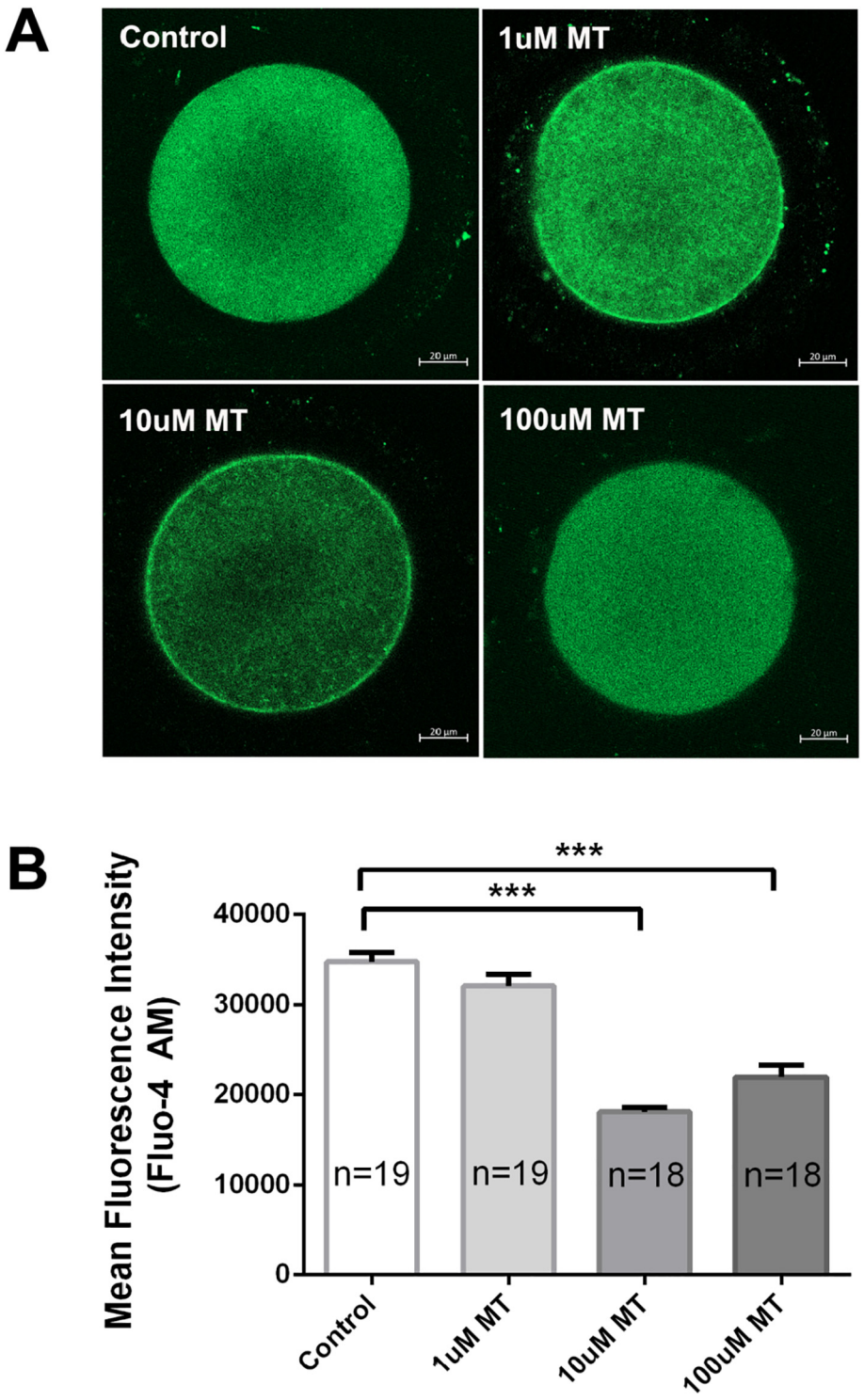

Fig. 6. Effect of different concentrations of melatonin on calcium levels in human oocytes. (A) Fluo-4 AM staining was performed to measure the effect of different concentrations of melatonin (1 μM , 10 μM , 100 μM) on the calcium levels in the human oocytes and was detected by confocal microscopy. Scale bar = 20 μm. (B) Data are expressed as the mean ± S.E.M. "***" indicates significant differences, $p < 0.001$.

(GV) and metaphase I (MI) oocytes were used in the following experiments.

The immature oocytes were collected for the IVM culture and randomly divided into the melatonin-treated group (1 μM , 10 μM , 100 μM) and control group. After 36 h of culture in a humidified atmosphere containing 6% CO_2 and 5% O_2 at 37 °C, the *in vitro* matured oocytes (MII, showing the presence of the first polar body; PB) were used in this study.

2.9. Detection of mitochondrial expression in human oocytes

MitoTracker Red staining was performed to detect mitochondrial expression in human oocytes. Briefly, human oocytes from each group were exposed to 5 μM MitoTracker Red at 37 °C for 30 min. The human oocytes were then washed with PBS to remove the surface fluorescence and analyzed using laser scanning microscopy (LSM 800, Zeiss, Germany). The intensity of the fluorescence in the whole oocyte was

measured, and the parameters used for the image acquisition were similar for all examined oocytes. The experiment was repeated independently 3 times.

2.10. Mitochondrial membrane potential assay in human oocytes

Mitochondrial membrane potential of the human oocytes was measured using TMRE staining. Briefly, human oocytes in each group were exposed to 100 nM TMRE at 37 °C for 20 min and then washed with PBS to remove the surface fluorescence. The intensity of the fluorescence in the whole oocyte was measured, and laser scanning microscopy (LSM 800, Zeiss, Germany) was performed. The parameters used for the image acquisition were similar for all examined oocytes. The experiments were repeated independently 3 times.

2.11. Detection of calcium level in human oocytes

Calcium level in the human oocytes was measured using the Ca^{2+}-sensitive fluorescent probe Fluo-4 AM. Briefly, the human oocytes in each group were loaded with 5 μM Fluo-4 AM at 37 °C for 40 min and then washed with PBS to remove the surface fluorescence. The intensity of the fluorescence in the whole oocytes was measured by laser scanning microscopy (LSM 800, Zeiss, Germany). The parameters used for the image acquisition were similar for all examined oocytes. The experiments were repeated independently 3 times.

2.12. Statistical analysis

Statistical data were analyzed using a *t*-test, which was conducted with GraphPad Prism software (GraphPad Company, San Diego, CA, USA) for Windows. Values are expressed as the mean ± S.E.M. Statistical significance is defined as $p < 0.05$.

3. Results

3.1. The cell viability of cultured human ovarian cumulus cells

The Live-dead staining was used to investigate cell viability in cultured human ovarian cumulus cells. The cultured human ovarian cumulus cells appeared to be nearly 100% alive (green-stained), while dead cells (red-stained) were not observed (Fig. 1). This result suggested that cells grew well after 3 days of culture when the human ovarian cumulus cell culture medium was collected.

3.2. Melatonin synthesis in cultured human ovarian cumulus cells

The melatonin in the culture supernatants of human ovarian cumulus cells was determined by ELISA to be at a low concentration (Fig. 2). The melatonin levels measured by ELISA were 29.83 ± 0.86 pg/ml in the human ovarian cumulus cell culture medium. A very low amount of melatonin was detected in SSS–supplemented medium (negative control, 5.15 ± 0.34 pg/ml). The results suggested that the melatonin levels in human ovarian cumulus cells are much higher than the levels measured in the negative control ($p < 0.001$).

3.3. The expression of precursor to melatonin and the key enzymes involved in its synthesis pathway in cultured human ovarian cumulus cells

The synthesis of melatonin requires the presence of the precursor serotonin and the enzymes involved in its synthesis pathway. Thus, we investigated the presence of serotonin, the key enzyme NAT, and the key enzyme HIOMT (also known as acetylserotonin methyltransferase, ASMT) involved in the melatonin pathway in the human ovarian cumulus cells. Immunofluorescence staining results showed that there were many serotonin containing cells in the human ovarian cumulus cells (Fig. 3A), and there was NAT expression in the human ovarian cumulus cells (Fig. 3B). Western blotting analysis indicated that both NAT (Fig. 3C) and ASMT (Fig. 3D) protein existed in the human ovarian cumulus cells.

3.4. Effect of melatonin on mitochondrial function in human oocytes

To explore the possible mechanism by which melatonin improves the developmental potential of immature human oocytes, we measured the effect of different concentrations of melatonin (1 μM, 10 μM , 100 μM) on mitochondrial function. First, mitochondrial expression and mitochondrial membrane potential were measured in the human oocytes. As shown in Fig. 4, no significant differences were found in the mitochondrial expression in the human oocytes between the control and melatonin-treated groups (1 μM , 10 μM , 100 μM) ($p > 0.05$).

Additionally, the 1 μM and 100 μM concentrations in the melatonin-treated group showed no significant differences in the mitochondrial membrane potential in the human oocytes compared to the control group ($p > 0.05$). Interestingly, the mitochondrial membrane potential in the 10 μM concentration of melatonin-treated group was significantly higher than that in the control group ($p < 0.001$) (Fig. 5). The above findings indicated that melatonin maintained the mitochondrial membrane potential and played an important role in improving mitochondrial function in human oocytes.

3.5. Effect of melatonin on calcium levels in human oocytes

To further explore the mechanism by which melatonin regulates mitochondrial function in human oocytes, the effect of different concentrations of melatonin (1 μM , 10 μM , 100 μM) on the calcium level in human oocytes was tested. As shown in Fig. 6, although there were no significant differences in the calcium level between the 1 μM concentration of melatonin-treated group and the control group, the data showed that the calcium level in the 10 μM and 100 μM concentration of melatonin-treated group was much lower than the values of the control group ($p < 0.001$) (Fig. 6). These data suggest that melatonin improved mitochondrial function in human oocytes by decreased excessive Ca^{2+} levels.

4. Discussion

In this study, melatonin was confirmed to be present in cultures of human ovarian cumulus cells. Our results showed that human ovarian cumulus cells grew well and almost all of the cells expressed FSHR after 3 days of *in vitro* growth under our culture conditions. The immunofluorescence staining results suggested that many serotonin immunoreactive positive cells were found in the cultured human ovarian cumulus cells. Serotonin is catalyzed by NAT to produce *N*-acetyl-5-hydroxytryptamine, which is in turn catalytically converted by HIOMT (also known as ASMT) into melatonin. The presence of the melatonin precursor serotonin and the key enzymes NAT and ASMT in the human ovarian cumulus cells shows that human ovarian cumulus cells have the ability to synthesize melatonin through the traditional synthetic pathway.

Melatonin and its metabolites are potent free radical scavengers and antioxidants [32,33]. As a powerful antioxidant and antiapoptotic agent, melatonin may provide a local protective role or another role by an autocrine or paracrine mechanism. Thus, the possible function of melatonin from human ovarian cumulus cells might be to provide nutrients and support to the oocytes by paracrine signaling. Therefore, the effect of different concentrations of melatonin on the developmental potential of human oocytes was investigated in this study.

To explore the possible mechanism by which melatonin improves the developmental potential of immature human oocytes, we assessed the effect of different concentrations of melatonin (1 μM , 10 μM , 100 μM) on mitochondrial function. The quality of oocytes plays a key

role in successful embryonic development during *in vitro* processing and in producing a healthy live birth [14,15]. As the powerhouses of the cell, mitochondria are the major source of free radical generation. In addition, mitochondria are able to store Ca^{2+}, which contributes to calcium homeostasis in oocytes. Mitochondrial dysfunction results in the extensive generation of reactive oxygen species, which has a negative impact on human oocytes and early embryonic development [28]. Melatonin preserves optimal mitochondrial function by reducing mitochondrial oxidative stress in mouse embryos [10,23]. Although melatonin has been reported to improve the developmental potential of vitrified bovine oocytes and to improve the quality of murine oocytes under *in vitro* conditions [7,34], our study is the first to demonstrate that the 10 μM and 100 μM melatonin treatment decreases excessive intracellular Ca^{2+} and restores mitochondrial function in human oocytes. Additionally, although no significant differences were found in the mitochondrial expression in the human oocytes between the control and different concentrations of melatonin-treated groups (1 μM , 10 μM , 100 μM), the mitochondrial membrane potential in the 10 μM melatonin-treated group was significantly higher than that it was in the control group. These results indicate that the human oocytes that matured in the medium without melatonin suffered from stress under the *in vitro* conditions; however, 10 μM melatonin decreased the excessive intracellular Ca^{2+}, maintained the mitochondrial membrane potential of the human oocytes, and provided protection from exposure to stress.

This study has some limitations. First, only three different concentrations of melatonin (1 μM, 10 μM, 100 μM) were applied because the resource of immature human oocytes is precious and scarce. Additionally, as previously mentioned, long-term melatonin treatment delays ovarian aging *via* multiple mechanisms [24]. The present study demonstrated that only 10 uM melatonin maintained the mitochondrial membrane potential and decreased excessive intracellular Ca^{2+} levels in human oocytes. However, it is possible that melatonin improves the developmental potential of immature human oocytes *via* multiple mechanisms, including epigenetic regulation, autophagy, and receptor-mediated mechanisms; these mechanisms are our research interests in the future studies. Furthermore, the sample size should be increased in the future studies.

5. Conclusion

Taken together, these data provide definitive evidence regarding the capability of human ovarian cumulus cells to metabolize serotonin to melatonin through the traditional synthetic pathway. Furthermore, 10 uM melatonin treatment of oocytes was shown to maintain the mitochondrial membrane potential and decrease excessive intracellular Ca^{2+} levels in human oocytes. Melatonin might reduce oxidative stress injury of human oocytes by improving mitochondrial function. Considering the significant clinical value of immature human oocytes in ART, our findings highlight the value of melatonin as a potential treatment for ameliorating mitochondrial function and improving the quality of human oocytes during IVM .

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Authors' contributions

Ya-Jing Liu and Dong-Mei Ji contributed equally to this work by conducting the experiments and drafting the manuscript. Zhen-bang Liu assisted in the use of the laser scanning microscopy. Tian-Juan Wang and Fen-fen Xie assisted in the Live-Dead staining. Zhi-Guo Zhang, Zhao-Lian Wei, and Ping Zhou revised the manuscript. Yun-xia Cao and Ya-Jing Liu designed the experiments and revised the manuscript.

References

[1] D. Acuna-Castroviejo, G. Escames, C. Venegas, M.E. Diaz-Casado, E. Lima-Cabello, L.C. Lopez, et al., Extrapineal melatonin: sources, regulation, and potential functions, Cell. Mol. Life Sci. 71 (2014) 2997–3025.
[2] A. Adachi, A.K. Natesan, M.G. Whitfield-Rucker, S.E. Weigum, V.M. Cassone, Functional melatonin receptors and metabolic coupling in cultured chick astrocytes, Glia 39 (2002) 268–278.
[3] R.S. Barberino, V.G. Menezes, A. Ribeiro, R.C. Palheta Jr., X. Jiang, J.E.J. Smitz, et al., Melatonin protects against cisplatin-induced ovarian damage in mice via the MT1 receptor and antioxidant activity, Biol. Reprod. 96 (2017) 1244–1255.
[4] A. Carrillo-Vico, J.R. Calvo, P. Abreu, P.J. Lardone, S. Garcia-Maurino, R.J. Reiter, et al., Evidence of melatonin synthesis by human lymphocytes and its physiological significance: possible role as intracrine, autocrine, and/or paracrine substance, FASEB J. 18 (2004) 537–539.
[5] E.H. Ernst, S. Franks, K. Hardy, P. Villesen, K. Lykke-Hartmann, Granulosa cells from human primordial and primary follicles show differential global gene expression profiles, Hum. Reprod. 33 (2018) 666–679.
[6] M. Gonzalez-Arto, T.R. Hamilton, M. Gallego, E. Gaspar-Torrubia, D. Aguilar, E. Serrano-Blesa, et al., Evidence of melatonin synthesis in the ram reproductive tract, Andrology 4 (2016) 163–171.
[7] C. He, J. Wang, Z. Zhang, M. Yang, Y. Li, X. Tian, et al., Mitochondria synthesize melatonin to ameliorate its function and improve mice oocyte's quality under in vitro conditions, Int. J. Mol. Sci. 17 (6) (2016), https://doi.org/10.3390/ijms17060939 pii: E939.
[8] M.T. Itoh, B. Ishizuka, Y. Kudo, S. Fusama, A. Amemiya, Y. Sumi, Detection of melatonin and serotonin N-acetyltransferase and hydroxyindole-O-methyltransferase activities in rat ovary, Mol. Cell. Endocrinol. 136 (1997) 7–13.
[9] M.T. Itoh, B. Ishizuka, Y. Kuribayashi, A. Amemiya, Y. Sumi, Melatonin, its precursors, and synthesizing enzyme activities in the human ovary, Mol. Hum. Reprod. 5 (1999) 402–408.
[10] M.J. Jou, T.I. Peng, P.Z. Yu, S.B. Jou, R.J. Reiter, J.Y. Chen, et al., Melatonin protects against common deletion of mitochondrial DNA-augmented mitochondrial oxidative stress and apoptosis, J. Pineal Res. 43 (2007) 389–403.
[11] M.K. Kim, E.A. Park, H.J. Kim, W.Y. Choi, J.H. Cho, W.S. Lee, et al., Does supplementation of in-vitro culture medium with melatonin improve IVF outcome in PCOS? Reprod. BioMed. Online 26 (2013) 22–29.
[12] Y.J. Liu, F.T. Meng, L.L. Wang, L.F. Zhang, X.P. Cheng, J.N. Zhou, Apolipoprotein E influences melatonin biosynthesis by regulating NAT and MAOA expression in C6 cells, J. Pineal Res. 52 (2012) 397–402.
[13] Y.J. Liu, J. Zhuang, H.Y. Zhu, Y.X. Shen, Z.L. Tan, J.N. Zhou, Cultured rat cortical astrocytes synthesize melatonin: absence of a diurnal rhythm, J. Pineal Res. 43 (2007) 232–238.
[14] T. Lord, R.J. Aitken, Oxidative stress and ageing of the post-ovulatory oocyte, Reproduction 146 (2013) R217–R227.
[15] G. Marteil, L. Richard-Parpaillon, J.Z. Kubiak, Role of oocyte quality in meiotic maturation and embryonic development, Reprod. Biol. 9 (2009) 203–224.
[16] A.L. Morera, P. Abreu, Existence of melatonin in human platelets, J. Pineal Res. 39 (2005) 432–433.
[17] L.P. Niles, J. Wang, L. Shen, D.K. Lobb, E.V. Younglai, Melatonin receptor mRNA expression in human granulosa cells, Mol. Cell. Endocrinol. 156 (1999) 107–110.
[18] Y.W. Pang, X.L. Jiang, Y.C. Wang, Y.Y. Wang, H.S. Hao, S.J. Zhao, et al., Melatonin protects against paraquat-induced damage during in vitro maturation of bovine oocytes, J. Pineal Res. 66 (2019) e12532.
[19] D. Pozo, M. Delgado, J.M. Fernandez-Santos, J.R. Calvo, R.P. Gomariz, I. Martin-Lacave, et al., Expression of the Mel1a-melatonin receptor mRNA in T and B subsets of lymphocytes from rat thymus and spleen, FASEB J. 11 (1997) 466–473.
[20] R.J. Reiter, S. Rosales-Corral, D.X. Tan, M.J. Jou, A. Galano, B. Xu, Melatonin as a mitochondria-targeted antioxidant: one of evolution's best ideas, Cell. Mol. Life Sci 74 (2017) 3863–3881.
[21] R.J. Reiter, H. Tamura, D.X. Tan, X.Y. Xu, Melatonin and the circadian system: contributions to successful female reproduction, Fertil. Steril. 102 (2014) 321–328.
[22] R.J. Reiter, D.X. Tan, A. Korkmaz, S.A. Rosales-Corral, Melatonin and stable circadian rhythms optimize maternal, placental and fetal physiology, Hum. Reprod. Update 20 (2014) 293–307.
[23] L. Ren, Z. Wang, L. An, Z. Zhang, K. Tan, K. Miao, et al., Dynamic comparisons of high-resolution expression profiles highlighting mitochondria-related genes between in vivo and in vitro fertilized early mouse embryos, Hum. Reprod. 30 (2015) 2892–2911.
[24] H. Tamura, M. Kawamoto, S. Sato, I. Tamura, R. Maekawa, T. Taketani, et al., Long-term melatonin treatment delays ovarian aging, J. Pineal Res. 62 (2017), https://doi.org/10.1111/jpi.12381.
[25] D.X. Tan, L.C. Manchester, R. Hardeland, S. Lopez-Burillo, J.C. Mayo, R.M. Sainz,

et al., Melatonin: a hormone, a tissue factor, an autocoid, a paracoid, and an antioxidant vitamin, J. Pineal Res. 34 (2003) 75–78.
[26] J. Tong, S. Sheng, Y. Sun, H. Li, W.P. Li, C. Zhang, et al., Melatonin levels in follicular fluid as markers for IVF outcomes and predicting ovarian reserve, Reproduction 153 (2017) 443–451.
[27] M.I. Vacas, M.M. Del Zar, M. Martinuzzo, D.P. Cardinali, Binding sites for [3H]-melatonin in human platelets, J. Pineal Res. 13 (1992) 60–65.
[28] J. Van Blerkom, P. Davis, V. Mathwig, S. Alexander, Domains of high-polarized and low-polarized mitochondria may occur in mouse and human oocytes and early embryos, Hum. Reprod. 17 (2002) 393–406.
[29] T. Wang, Y. Liu, M. Lv, Q. Xing, Z. Zhang, X. He, et al., miR-323-3p regulates the steroidogenesis and cell apoptosis in polycystic ovary syndrome (PCOS) by targeting IGF-1, Gene 683 (2019) 87–100.
[30] P.A. Witt-Enderby, M.I. Masana, M.L. Dubocovich, Physiological exposure to melatonin supersensitizes the cyclic adenosine 3′,5′-monophosphate-dependent signal transduction cascade in Chinese hamster ovary cells expressing the human mt1 melatonin receptor, Endocrinology 139 (1998) 3064–3071.
[31] Y. Yang, H.H. Cheung, C. Zhang, J. Wu, W.Y. Chan, Melatonin as potential targets for delaying ovarian aging, Curr. Drug Targets 20 (1) (2019) 16–28.
[32] H. Yilmaz, T. Ertekin, E. Atay, M. Nisari, H. Susar Guler, O. Al, et al., Antioxidant role of melatonin against nicotine's teratogenic effects on embryonic bone development, Iran. J. Basic Med. Sci. 21 (2018) 787–793.
[33] H.M. Zhang, Y. Zhang, Melatonin: a well-documented antioxidant with conditional pro-oxidant actions, J. Pineal Res. 57 (2014) 131–146.
[34] X.M. Zhao, H.S. Hao, W.H. Du, S.J. Zhao, H.Y. Wang, N. Wang, et al., Melatonin inhibits apoptosis and improves the developmental potential of vitrified bovine oocytes, J. Pineal Res. 60 (2016) 132–141.
[35] M. Zheng, J. Tong, W.P. Li, Z.J. Chen, C. Zhang, Melatonin concentration in follicular fluid is correlated with antral follicle count (AFC) and in vitro fertilization (IVF) outcomes in women undergoing assisted reproductive technology (ART) procedures, Gynecol. Endocrinol. 34 (2018) 446–450.