Melatonin protects ovarian function in whole cryopreserved rat ovarian transplantation via the MT1/Nrf2/ARE pathway

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Research Article

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Abstract

Background: Whole ovarian transplantation has the potential to restore fertility in cancer patients, but ovarian ischemia-reperfusion injury following transplantation causes decreased graft function. Melatonin protects against antioxidant damage and has anti-inflammatory effects, but its effects in whole ovarian transplantation have not been investigated.

Objective:This study was aimed to verify the beneficial antioxidant and anti-inflammatory effects of melatonin in whole ovarian transplantation.

Methods: The cryopreserved whole ovaries were allotransplanted in LEWIS rats. Forty rats were randomly divided into 8 groups: control group, sham surgery group, saline group; low-dose (25 mg/kg) melatonin group; high-dose (50 mg/kg) melatonin group; melatonin (50 mg/kg) + ML385 group; melatonin (50 mg/kg) + luzindole group, and melatonin+ 4P-PDOT group. The estrous cycle recovery was evaluated by vaginal exfoliative cell monitoring and serum hormone. Follicle morphology was observed by HE. The levels of eoxidative stress factors, antioxidant factors, and inflammatory factors in both serum and ovarian tissues were measured by ELISA, RT-qPCR, western blot and fenton detection. RT-qPCR, western blot and immunofluorescence assays were used to measure the levels of MT1 and Nrf2.

Results: The rats in high-dose and low-dose melatonin groups recovered estrous cycle faster and lost fewer follicles, and the serum endocrine hormone levels were close to normal. The serum and ovarian tissue antioxidant capacity were significant higher, while the levels of inflammatory factors were significant lower in the high-dose and low-dose melatonin groups. In addition, the melatonin receptor MT1 was found to be involved in antioxidant and anti-inflammatory processes. Melatonin also triggered the Nrf2/ARE pathway activity via receptor MT1. Blocking Nrf2 or MT1 receptors could eliminate the beneficial effects of melatonin on whole transplanted ovaries.

These findings suggest that melatonin can attenuate oxidative stress injury and inflammatory responses in whole transplanted ovaries via the MT1/Nrf2/ARE signaling pathway, thereby effectively protecting whole transplanted ovarian function.

1. Introduction

Anti-cancer treatment has saved the lives of many young women. However, high doses of gonadotoxic drugs during chemotherapy may lead to ovarian failure and infertility[1]. Cryopreservation of whole ovarian tissue for transplantation is considered to be a promising experimental technique[2], as this technique involves extremely low-temperature cryopreservation of the entire ovary with the tip; the ovary can then be thawed and reimplanted using vascular anastomosis techniques and tissue suturing, resulting in immediate revascularization. This shortens the ischemic time of the transplanted ovary and theoretically reduces the ischemic damage to the ovarian tissue due to revascularization. Notably, whole ovarian transplantation preserves all follicular reserves within the ovary. Whole ovarian transplants performed in large animals have achieved satisfactory results in terms of rapid recovery of ovarian function and

successful reproduction[3–5]. Nevertheless, follicle survival in the transplanted ovary is still very low (1.7%-7.6%)[3], which leads to reduced ovarian function, shorter life span, and reduced successful pregnancies. Current studies have identified ischemia-reperfusion injury in transplanted ovarian tissues as the main cause of follicular loss[6], including massive reactive oxygen species production, calcium overload, and impaired cellular function. Therefore, reducing ischemia-reperfusion injury in whole transplanted ovaries has become an urgent problem.

Melatonin is an indoleamine neurohormone secreted mainly by the mammalian pineal gland and is an important endocrine hormone that regulates the circadian rhythm. Previous studies have shown that melatonin has great anti-inflammatory and antioxidative actions. Some studies showed that melatonin may provide the cryopreserved ovarian tissue and promote the quality of ovarian grafts [7,27]. However, whether melatonin can provide protection in whole transplanted ovaries remains unknown. In the present study, we aimed to investigate whether melatonin exerts a protective effect on whole ovarian transplantation and its mechanisms.

2. Materials and methods

2.1 Animals and experimental protocols

The 8-12 weeks-old adult female LEWIS rats were randomly divided into 8 groups, with 5 rats each group: control group, sham surgery group, surgery + saline group, surgery + low-dose (25 mg/kg) melatonin group, surgery + high-dose (50 mg/kg) melatonin group, surgery + melatonin (50 mg/kg) + ML385 50 mg/kg group, surgery + melatonin (50 mg/kg) + luzindole 0.25 mg/kg group, surgery + melatonin + 4P-PDOT 0.25 mg/kg group.

Referring to the surgical protocol of Wang X et al,[12] and perfusion protocol[13], in G1 whole ovaries were obtained, then perfused following vitrification cryopreservation and thawing procedures. After thawing, the whole ovaries were transplanted by end-to-end anastomosis.

2.2 Endocrine function in rats

Vaginal exfoliative cell monitoring was performed starting from the first day after ovarian allograft surgery to observe the estrous cycle in rats.

On the 4th day after transplantation, whole blood specimens from rats were taken at room temperature and then centrifuged. The supernatant was extracted. The ELISA kits for progesterone, FSH, AMH, estradiol, TNF-α and IL-6 were used according to the manufacturer's instructions. The optical density values were determined within 5 min using a 450 nm spectrophotometer.

2.3 The number and morphology of whole ovarian transplanted follicles

Fresh ovaries from the saline group, low-dose melatonin, high-dose melatonin and normal control groups were recovered and fixed in 4% paraformaldehyde for 1 hour, embedded in paraffin blocks and serially sectioned at a thickness of 4–5 μm. Sections were stained with hematoxylin-eosin, mounted and observed under a light microscope (Olympus, Tokyo, Japan) at ×400 magnification to observe the morphological structure of the ovaries as well as to measure the number of follicles at each level.

2.4 Ovarian tissue reactive oxygen species ROS measurement

Weigh an appropriate amount of fresh ovarian tissue, add extract for homogenization on ice, centrifuge at 100×g, 4℃, and take the supernatant for measurement. Add homogenization supernatant, DHE probe in a 96-well plate, and blow with a pipette to mix well. Incubate at 37°C protected from light. Place in a fluorescent enzyme marker, and then detect the fluorescence intensity. Another appropriate amount of supernatant homogenate was taken for protein quantification. The intensity of tissue reactive oxygen species was expressed as fluorescence intensity (RFU)/protein concentration (mg protein).

2.5 Serum reactive oxygen species ROS measurement

Blood samples of recipient rats were taken and centrifuged at 4°C after standing at room temperature, and the upper layer of serum was taken. Serum was incubated with O12 probe 15 min at 37°C and protected from light. The samples were excited at 488 nm, and the fluorescence emission intensity was measured at 520 nm.

2.6 Tissue superoxide dismutase (SOD), glutathione (GSH) and malondialdehyde (MDA) content measurement

An appropriate amount of recipient rat fresh ovaries was cut into 5mm*5mm size pieces, put into tissue grinding tubes and centrifuged at 10,000g for 10 min at 4°C with a tissue homogenizer, and the supernatants were used for SOD, GSH and MDA assays according to the manufacturer's instructions.

2.7 Tissue total antioxidant capacity (TAC) measurement

Blood samples from recipient rats were taken and centrifuged after standing at room temperature, and the upper serum layer was taken. Incubating serum with the Cu2 + working solution for 90 min on a shaker at room temperature.

2.8 Fenton kit for detection of ovarian tissue hydroxyl radical concentration

Weigh an appropriate amount of fresh ovarian tissue, homogenize on ice and centrifuge at 4℃, and take the supernatant for measurement. The reaction reagent was prepared according to the manufacturer's instructions, vortex mixed and incubated at 37℃ for 1 h. 10,000rp, centrifuged at room temperature and supernatant was taken to determine the absorbance at 536nm. The absorbance of the measurement

group, control group, and blank group were denoted At, Ac, and A0, respectively. The hydroxyl radical clearance ratio was calculated by (At − Ac)/(A0 − Ac).

2.9 Ovarian tissue Nrf2 protein nuclear translocation assay

Paraffin slides were rehydrated in a reduced concentration of alcohol and then placed in 10 mM sodium citrate buffer and heated to 95°C for 30 min to repair the antigen. The slides were then permeabilized with 0.5% Triton-X and blocked with 5% BSA for 2 hours. Slides were probed with Nrf2 antibody followed by secondary antibody. The slides were incubated in DAPI for 10 min and then washed. Finally, mount the slides using fade-resistant mounting medium and use a Nikon fluorescence microscope(Carl Zeiss, Oberkochen, German)to collect images.

2.10 Western blot analysis

Fresh ovarian tissue was cut into the smal pieces and extracted for cytoplasmic and cytosolic proteins using the Cytoplasmic Cytosolic Nucleus Extraction Protein Kit. Protein concentration was measured using the BCA Protein Concentration Assay Kit. SDS-PAGE protein electrophoresis was performed, and then the proteins were transferred to PVDF membranes. After being closed with 5% skim milk for 60 min, the membranes were incubated overnight at 4°C with rabbit anti-Nrf2 anti-SOD1 anti-HO-1 anti-IL-6 anti-TNF-α anti-HistoneH3 and rabbit anti-β-Actin monoclonal antibody. After washing, the samples were incubated with goat anti-rabbit IgG (HRP) for 120 min. Color was developed and exposed using ECL color developing solution. The images were analyzed using ImageJ software.

2.11 Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis

A certain weight of ovarian tissue was cut into pieces and total RNA samples and reverse transcription reactions were purified using commercially available kits. PCR conditions were performed at 95°C for 5 min, followed by 40 cycles of 10 s at 95°C and then 30 s at 60°C using primers. List of quantitative real-time PCR primers used in the study can be found in Supplemental Information.

2.12 Immunofluorescence analysis

Ovarian tissue was fixed in 10% formalin for 24 hours, embedded in paraffin, and sectioned at 5 μm. Tissue sections were dewaxed, rehydrated, and then treated with antibodies against Nrf2 (1:400 dilution), MLTR1A (1:400 dilution) at 4°C overnight. Secondary antibodies were coupled to IgG (1:200 dilution) and, 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) was applied. Analyze slides by fluorescence microscopy.

2.13 Statistical Analysis

IBM GraphPad prism 9.4.1 and IBM SPSS Statistics26.0 were used for statistical analysis. Data were expressed as the mean ± standard deviation of three independent experiments and presented in tables and bar graphs. One-way ANOVA and LSD were used to analyze the differences between groups, and results were considered significant when $P < 0.05$.

3. Results

3.1 Melatonin protects the number and morphology of whole ovarian transplanted follicles, endocrine function, and estrous cycle in rats

As shown in Fig. 1, the morphological structure of follicles in the ovarian tissue in the high-dose melatonin group was closest to that in the control group, while a small number of deformed follicles and irregularly arranged granulosa cells were seen in the low-dose melatonin group. Moreover, a large number of deformed and atretic follicles with granulosa cells detached from the follicular membrane were seen in the saline group. The follicle counts at all levels were higher in the high-dose group than in the low-dose melatonin group and the saline group. The lowest number of primordial follicles, anterior sinus follicles, and sinus follicles were observed in the saline group. The follicle counts are shown in Table 1.

The follicles in the control group and sham group were morphologically whole, with uniform distribution of granulosa cells and visible oocyte nuclei. In the saline group, disorganized granulosa cells were seen in the follicles, the number of granulosa cells was sparse and detached from the basement membrane, and the oocyte nuclei disappeared. In the low-dose group, the follicle morphology was more complete, but the granulosa cells were still disorganized and detached from the basement membrane in some areas, and normal oocyte nuclei were visible. In the high-dose group, the follicle morphology was closest to that of the control group, and the follicle morphology, number of granulosa cells, and cell arrangement were more normal. The high-dose group showed the closest follicle morphology to that in the control group.

Serum estradiol levels and AMH were significantly lower in the transplantation groups compared to the control group ($P < 0.001$), and the levels were significantly higher in the groups with melatonin compared to the groups without melatonin, as shown in Figs. 2a and 2c. In addition, estradiol levels were significantly higher in the high-dose group than in the low-dose group. As shown in Fig. 2b, the serum FSH levels showed the opposite trend.

As shown in Fig. 3, the rats in the high-dose group resumed a regular estrous cycle starting from postoperative day 12, and the rats in the low-dose group and the saline group showed an estrous cycle on postoperative day 16 and day 21, respectively. One rat in the saline group did not resume the estrous cycle until 30 days postoperatively, and it was found that dense adhesions in the abdominus. These results suggest that high-dose melatonin treatment could preserve the follicular morphology, follicular numbers at all levels, endocrine hormone levels, and faster recovery of the estrous cycle in the whole ovarian transplanted rats.

3.2 Melatonin attenuates oxidative stress in whole transplanted ovaries of rats

To evaluate whether melatonin can regulate the oxidative-antioxidant balance in rat whole transplanted ovaries, the serum ROS, TAC (Fig. 4a, 4b), and the levels of ROS, hydroxyl radicals, MDA and GSH in ovarian tissue (Fig. 4c-4f) were measured in recipient rats on the fourth postoperative day. The serum and

tissue ROS, TAC, MDA, hydroxyl radical scavenging, and GSH levels were significantly higher in the three transplantation groups than those in the saline and sham-operated groups ($P < 0.01$). Among them, serum ROS was significantly higher in the saline group than in the high-dose melatonin group, and significantly higher in the low-dose group than in the high-dose group. In contrast, TAC was significantly lower in the physiological saline group than in both melatonin groups, and significantly lower in the low-dose group than in the high-dose group. The tissue ROS levels in the saline group were significantly higher than in the high and low-dose melatonin groups; the tissue MDA levels in the saline and low-dose groups were significantly higher than in the high-dose group, and the tissue hydroxyl radical scavenging rate and GSH in the saline group were significantly lower than those in the high and low dose groups. As shown in Fig. 4g-4i, the western blot results of SOD and HO-1 showed that the melatonin group had stronger expression than the control, sham-operated and saline groups. The expression of antioxidant enzymes was proportional to the melatonin concentration. These findings indicate that high-dose melatonin treatment can effectively improve the antioxidant capacity of whole transplanted ovarian tissues and better resist oxidative damage.

3.3 Melatonin modulates the inflammatory response in rat whole transplanted ovaries

Overexpression of inflammatory factors in the recipient may affect the transplantation success. The serum levels of IL-6 and TNF-α were measured by ELISA to investigate whether melatonin inhibits inflammatory factor overexpression in rat whole transplanted ovaries (Fig. 5a-5b), and the mRNA levels of IL-6 and TNF-α in ovarian tissues were evaluated by RT-qPCR (Fig. 5c). Moreover, a western blot assay was performed to detect the protein expression of IL-6 and TNF-α (Fig. 5d-5f). The results showed that the serum levels and mRNA levels of both inflammatory factors in the three transplantation groups were significantly higher than those in the fresh control group ($P < 0.01$). Moreover, the levels of inflammatory factors were significantly higher in the high-dose group than those in the saline and low-dose groups. However, the low-dose serum inflammatory factors were significantly lower than those in the saline group. In addition, western blot results showed that the IL-6 and TNF-α levels were significantly higher in the saline group than in the other groups, while the high-dose melatonin group was significantly lower than the other two transplantation groups. These findings suggest that melatonin, especially high-dose melatonin, can effectively control the inflammation level in whole frozen transplanted ovaries.

3.4 Melatonin exerts anti-oxidative stress and anti-inflammatory effects through MT1 receptors

To verify the involvement of the MT1 receptor in the protective process of whole ovarian transplantation in rats, immunofluorescence assays were performed on whole ovarian tissues of recipient rats. It showed a significantly stronger MT1 fluorescence signal in the melatonin group than in the saline group and the control groups (Fig. 6a), indicating that MT1 was expressed in rat frozen transplanted ovaries and was activated by melatonin.

Subsequently, the non-specific melatonin receptor inhibitor luzindole and the MT2-specific inhibitor 4P-PDOT were administered intraperitoneally to the recipient rats. Immunofluorescence images showed that the fluorescence intensity of the melatonin group was higher than that in the melatonin + luzindole group and the melatonin + 4P-PDOT group. Moreover, it showed a significantly lower fluorescence intensity in the melatonin + luzindole group compared to the melatonin + 4P-PDOT group, as illustrated in Fig. 7a. MT1 receptors were effectively inhibited by the non-selective inhibitor luzindole and were not significantly affected by the MT2-specific inhibitor 4P-PDOT.

As shown in Fig. 8, the levels of serum and tissue ROS in the melatonin group were significantly lower than those in the saline group, but significantly higher than in the control group. Furthermore, the serum and tissue ROS levels in the luzindole group were significantly higher than in the 4P-PDOT group and melatonin groups, with no significant difference between the saline and 4P-PDOT groups (Figs. 8a and 8c). The levels of serum TAC and tissue GSH were significantly higher in the melatonin group than in the control, saline, and luzindole groups, and significantly higher in the 4P-PDOT group than in the luzindole group (Fig. 8b and 8d).

The protein expression of the downstream antioxidant enzymes SOD and HO-1 in ovarian tissues in the melatonin group was significantly higher than that in the other surgical groups. The inflammatory factor protein expression was significantly lower than that in the other surgical groups. The non-specific inhibitor of MT1/MT2 receptors luzindole effectively reversed this regulatory effect. Interestingly, although the 4P-PDOT group selectively blocked the MT2 receptor, the antioxidant and anti-inflammatory effects of melatonin were not significantly affected. The results suggested that receptor MT1 was involved in the antioxidant and anti-inflammatory effects of melatonin on whole frozen transplanted ovaries in rats, while MT2 may not.

3.5 Melatonin protects against oxidative stress and inflammatory damage via the MT1/Nrf2/ARE signaling pathway

As shown in Fig. 9, the expression of sMafg and Nrf2 was significantly higher than that in the saline group, while the expression of KEAP1 showed no significant difference from that of the saline group, but was still significantly higher than that in the control and sham groups.

Immunofluorescence results showed that the levels of Nrf2 protein in the melatonin group shifted from the cytoplasm to the nucleus (Fig. 10a). This change was also observed in the saline group, but the fluorescence signal was significantly weaker than that in the melatonin group. In contrast, the Nrf2 fluorescence signal of the control group remained weak and showed no transfer into the nucleus. The changes in cytoplasmic and cytosolic Nrf2 protein levels were assessed by western blot, as shown in Fig. 10b. The expression of both cytoplasmic and cytosolic Nrf2 levels in the melatonin group was significantly higher than that in the non-melatonin group, which was proportional to the melatonin dose.

After the Nrf2 inhibitor ML385 blocking, it showed that the cytoplasmic cytosolic Nrf2 protein expression was significantly lower than the melatonin group (Fig. 11a). The downstream SOD1, HO-1, and mRNA levels were significantly lower than those in the melatonin group, while IL-6 and TNF-α mRNA expression were significantly higher than in the melatonin group (Fig. 11b). The same trend was found in the WB results (Fig. 11c, 11d). The findings indicated that the antioxidant and anti-inflammatory capacities of melatonin were effectively inhibited.

As shown in Fig. 12a, immunofluorescence results showed that the Nrf2 fluorescence signal in the melatonin + luzindole group was significantly weaker than in the melatonin group, and no nuclear translocation was observed. In contrast, selective inhibition of MT2 with 4P-PDOT still resulted in a strong Nrf2 fluorescence signal in the cytoplasmic nucleus, which was slightly lower than that in the melatonin group, and Nrf2 nuclear translocation was still clearly seen with DAPI. As shown in Fig. 12b, the melatonin + luzindole group appeared to have significantly lower Nrf2 protein expression in both cytoplasmic nuclei than that in the melatonin group, while Nrf2 expression in the melatonin + 4PP-DOT group was almost unaffected. These results suggested that melatonin activates MT1 to induce the Nrf2/ARE signaling pathway in rats with whole transplanted ovarian tissue.

4. Discussion

Whole ovarian transplantation is a promising technique to preserve fertility that allows immediate revascularization after anastomosis. As we know, this is the first study which revealed the beneficial effects of melatonin on preserving the function of whole frozen-thawed transplanted ovaries. The present study further revealed that melatonin interacted with the transmembrane receptor MT1 to transmit signals and activated the Nrf2/ARE signaling pathway to regulate the expression of downstream antioxidant enzymes and inflammatory genes to protect the transplanted ovaries.

Many previous experiments have been successfully performed using human or animal ovarian cortical slices for transplantation[19–27]. However, ovarian tissue without vasculature is exposed to an ischemic period of several days. Based on our previous surgical protocol[13], vascular anastomosis was performed at the same time as the cryopreserved ovarian transplantation, resulting in clear intraoperative changes. The vascular network improved from pale to red, reflecting the reduced ischemic time to only a few hours. Hematologic reconstitution occurs in the initial days after transplantation[14,15], characterized by peak oxidative damage and inflammatory responses[16–18]. Therefore, melatonin was injected during this period to minimize the effects of ischemia-reperfusion injury. As a previous study found a significant difference in the effects of vitrification-frozen homozygous whole ovarian grafts using different concentrations of melatonin[22], two doses of melatonin were tested in this study, and it revealed that high-dose melatonin exerted stronger antioxidant and anti-inflammatory effects.

Melatonin is a powerful free radical scavenger. Previous studies have shown that melatonin exerts a powerful regulatory effect on the female reproductive system[8,9]. In the study, melatonin effectively increased the antioxidant capacity of whole transplanted ovaries, preserving more follicular reserve, and

maintaining endocrine hormone secretion in the melatonin groups, which was in accordance with previous findings[19–27]. Several studies have found that melatonin is also a potent immunomodulator that plays a role in humoral and cellular immune pathways after organ transplantation[28–30]. Similarly, melatonin significantly suppressed TNF-α and IL-6 levels in the serum of recipient rats in the present study, effectively reducing inflammatory damage which lead to early graft failure.

The melatonin membrane receptors MT1 and MT2 are part of the guanine nucleotide-binding protein coupled receptor superfamily[31]. Previous studies confirmed that MT1 and MT2 are widely expressed in animal granulosa cells[10], follicles, and corpus luteum[9] and they regulate ovarian cyclic activity[32], while MT1 receptors are mainly involved in the maintenance of ovarian fertility[8]. In our study, for the first time, melatonin MT1 receptors were found to be involved in the protective effect of melatonin on whole ovarian transplantation, while MT2 receptors showed no apparent involvement. Currently, there is no MT1 receptor-specific inhibitor, so the non-selective receptor inhibitor luzindole and the selective MT2 receptor inhibitor 4P-PDOT were used to block the receptors. Luzindole almost completely blocked the antioxidant and anti-inflammatory effects of melatonin on recipient rat ovarian tissue, whereas 4P-PDOT had little effect.

Nrf2 is a pleiotropic protein that is essential for cellular defense systems. In the cellular stress response, stimulus signal entry into cells promotes Nrf2 uncoupling from the ubiquitinated substrate ligase Keap1, reducing ubiquitination, and inducing cytoplasmic Nrf2 to translocate to the nucleus. Nrf2 then forms heterodimers with sMaf proteins bound to the AREs enhancer region of target genes, and co-stimulatory proteins are recruited to activate the transcription of cellular defense target genes[13]. In line with previous studies[33], the immunofluorescence experiments in our study showed a weaker signal in the saline group and a stronger Nrf2 fluorescence in the melatonin group, in which the Nrf2 fluorescent signal translocated into the nucleus. In addition, the western blot assay revealed enhanced expression of downstream antioxidant and anti-inflammatory proteins. Nrf2 is a defense factor that is automatically activated in response to trauma and stimuli. However, this effect is relatively weak and may not provide much protection against injury; our study confirms that classical defense factors, such as melatonin, can further enhance the activation of the Nrf2/ARE pathway.

However, we found that both cytoplasmic and cytosolic Nrf2 proteins were significantly increased under the influence of melatonin, which is not explained by reduced Nrf2 ubiquitination alone. Therefore, we suspected that melatonin might somehow promote Nrf2 expression. In the present study, mRNA transcription of Nrf2 and protein expression of Nrf2 and sMAFg were significantly upregulated by melatonin, and cytoplasmic Nrf2 protein levels were slightly higher than nuclear, which correlated with active cytoplasmic Nrf2 protein synthesis. After treatment with the inhibitor ML385, the downstream antioxidant and anti-inflammatory protein expression were substantially reduced, as well as Nrf2 expression. ML385 is a specific Nrf2 inhibitor discovered in recent years that blocks NRF2 transcriptional activity by competitively binding to the Neh1 structural domain of Nrf2, which heterodimerizes with small Maf proteins. Furthermore, Nrf2 can modulate its expression in tumor tissues through its own ARE-like

elements[34], and melatonin might contribute to Nrf2 accumulation by promoting this positive feedback mechanism. Interestingly, the expression of Nrf2 under ML385 blockade was still higher than that in the control group, suggesting that the Nrf2 auto-positive feedback was not the only regulatory pathway. Collectively, melatonin can increase Nrf2 accumulation in cells by at least two pathways: reducing Nrf2 ubiquitination and indirectly upregulating NFE2L2 expression, thereby promoting downstream defense gene expression.

The final part of this study aimed to investigate whether MT1 is an important upstream molecule of the Nrf2/ARE pathway in whole transplanted ovaries. Previous research reported that activation of the melatonin MT1 receptor by the agonist ramitinib attenuated oxidative stress and inflammation through the Nrf2 signaling pathway[35]. Similar findings were observed in the current study, with melatonin effectively activating MT1. The fluorescence signal of Nrf2 was significantly attenuated in the presence of the non-selective inhibitor luzindole, whereas the selective receptor inhibitor 4P-PDOT had no significant effect on the Nrf2 fluorescence signal. Additionally, western blot showed a significantly reduced Nrf2 protein expression with melatonin MT1 blockade but was almost unaffected by MT2 selective inhibitors. These findings suggested that melatonin can exert a protective effect through MT1 regulation of the Nrf2/ARE pathway.

Nevertheless, the limitations of the current study should be acknowledged. First, rat ovaries with blood vessels were used in the experiments, and the surgical protocol differed from the actual clinical application due to the volume limitation; uniform perfusion of cryoprotectant is more easily achieved in smaller organs. Future studies may be performed using closer-to-human ovaries, such as sheep ovaries, to yield results that may be more reproducible in humans. Second, melatonin indirectly promoted Nrf2 expression, but whether melatonin exerts a direct regulatory effect on Nrf2 and its response components remains unknown. This potential effect may be further explored in future studies.

5. Conclusion

In conclusion, the present study demonstrated for the first time that melatonin can protect against oxidative stress and inflammatory damage caused by ischemia-reperfusion in rat cryopreserved whole ovaries, maintaining oocyte quality and quantity as well as endocrine function of the ovary. This study also explored the signal transduction pathway and confirmed that melatonin activates the MT1/NEF2/ARE signaling pathway in exerting its protective effects. These results suggested that melatonin is a promising protective agent in whole ovarian transplantation.

Declarations

Declaration of interests

The authors declare that they have no known competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Ethics declarations

The study was approved by the Ethics Review Committee of the Obstetrics and Gynecology Hospital of Fudan University approved Grant No.202209027Z .

Consent for publication

Not applicable.

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Table 1

Table 1 is available in the Supplementary Files section.

Supplementary Information

Supplementary Information is not available with this version

Figures

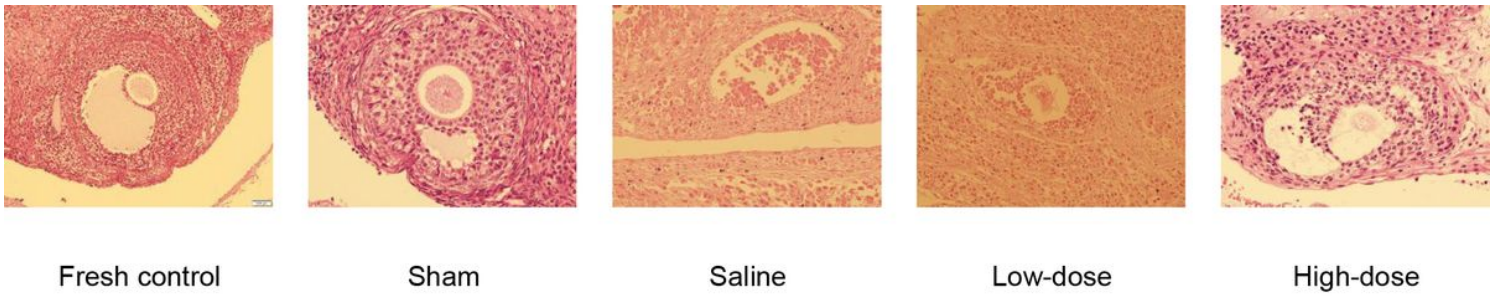

Figure 1

Morphological changes of follicles in each group (HE×400 times)

The morphology of the ovaries was better preserved with higher melatonin doses, and the ovarian tissues were collected for HE staining after 4 consecutive days of melatonin injection postoperation. Compared with the control group, the follicles in the saline group showed disorganized granulosa cells, which were sparse in number and detached from the basement membrane, and the oocyte nuclei disappeared. In the low-dose melatonin group, the follicle morphology was more complete, but the granulosa cells were still disorganized and detached from the basement membrane in some areas; normal oocyte nuclei were visible. In the high-dose melatonin group, the follicle morphology was closest to that of the control group, with normal follicle morphology and granulosa cell number. In the high-dose melatonin group, the follicle morphology was the most similar to that of the control group, with normal follicle morphology, number of granulosa cells, and cell arrangement.

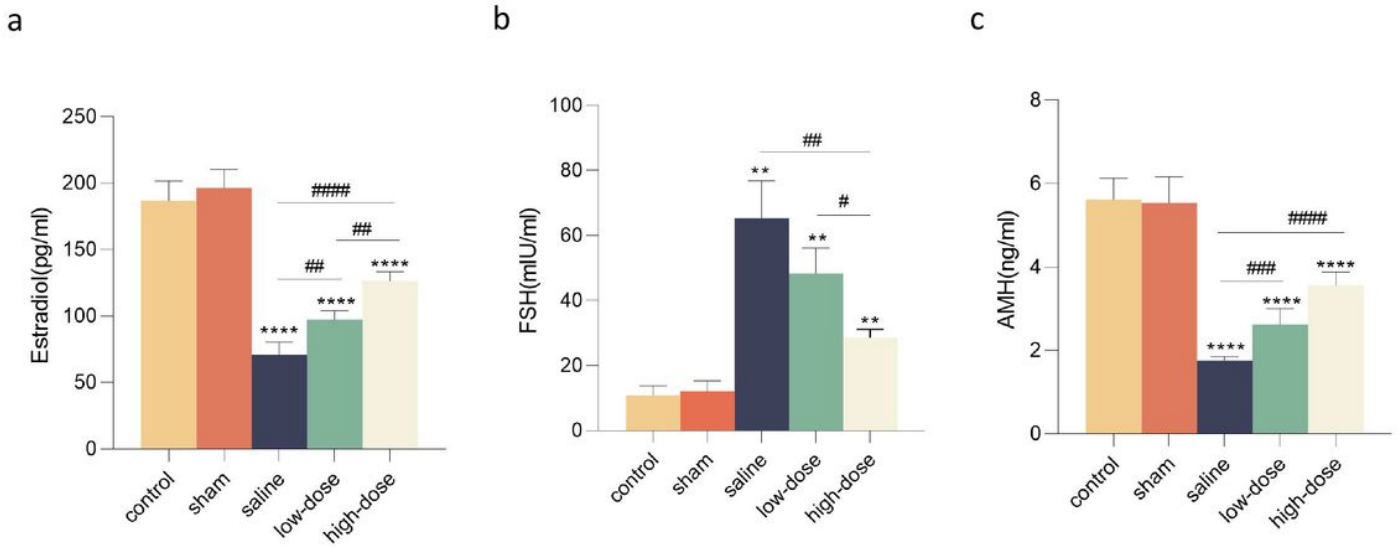

Figure 2

Effect of melatonin on serum endocrine hormones in rats on the fourth day after whole ovarian cryotransplantation.

(a). Serum estradiol levels were significantly lower in all three transplantation groups compared to the control and sham groups, and significantly lower in the saline group than in the high-dose and low-dose groups. Serum estradiol levels were significantly higher in the high-dose group than in the low-dose group.

(b). Serum FSH levels were significantly higher in all three transplantation groups than in the control and sham groups, significantly higher in the saline group than in the high-dose and low-dose groups, and significantly lower in the high-dose group than in the low-dose group.

(c). Serum AMH levels were significantly lower in all three transplantation groups than in the control and sham groups, but were significantly higher in the high-dose and low-dose groups compared to the saline group. However, no significant difference was found between the high-dose and low-dose groups. (Compared with the sham group, **P<0.01, ****P<0.001; comparison between the three transplantation groups, # P<0.05, ## P<0.01, ## P<0.005, #### P<0.001)

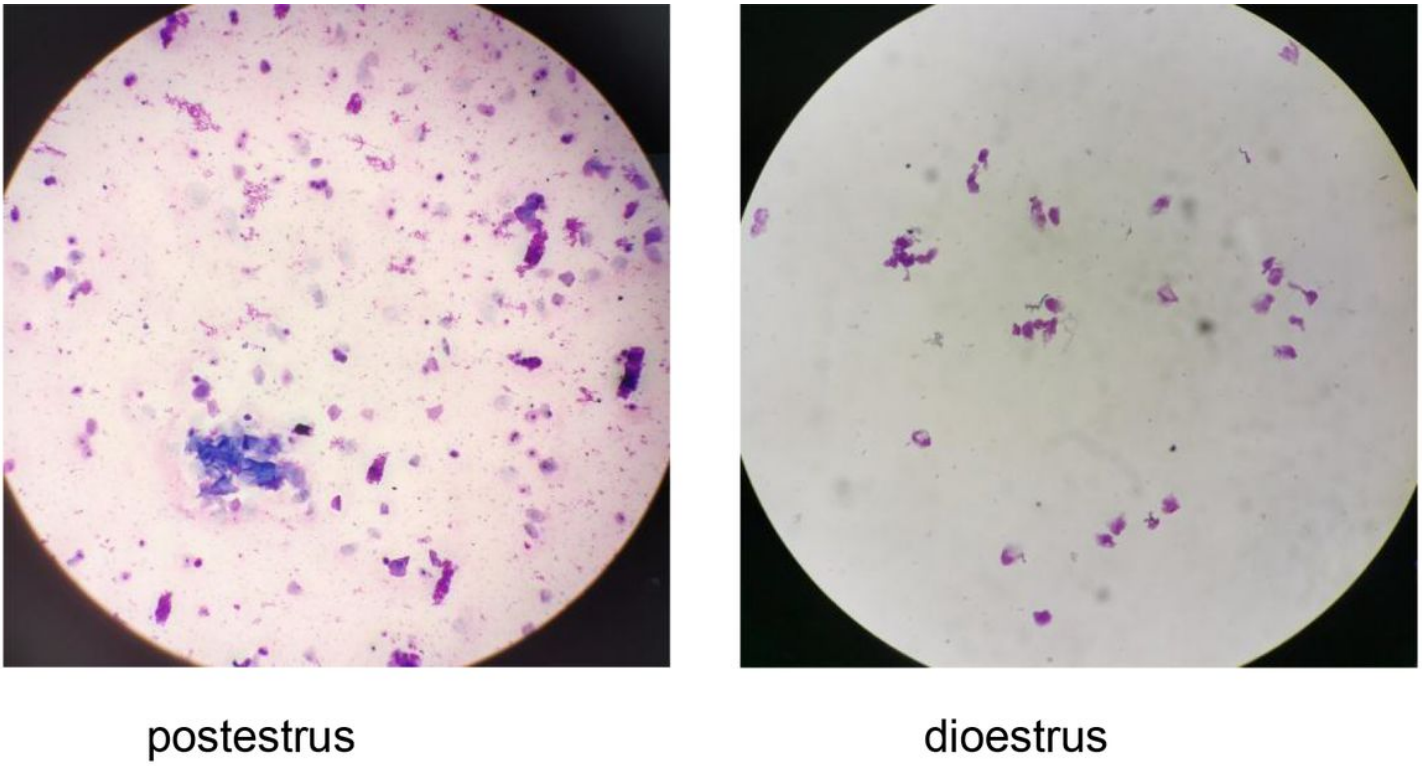

Figure 3

The vaginal exfoliated cells of the recipient rats displayed a postestrus or dioestrus morphology within one week after transplantation.

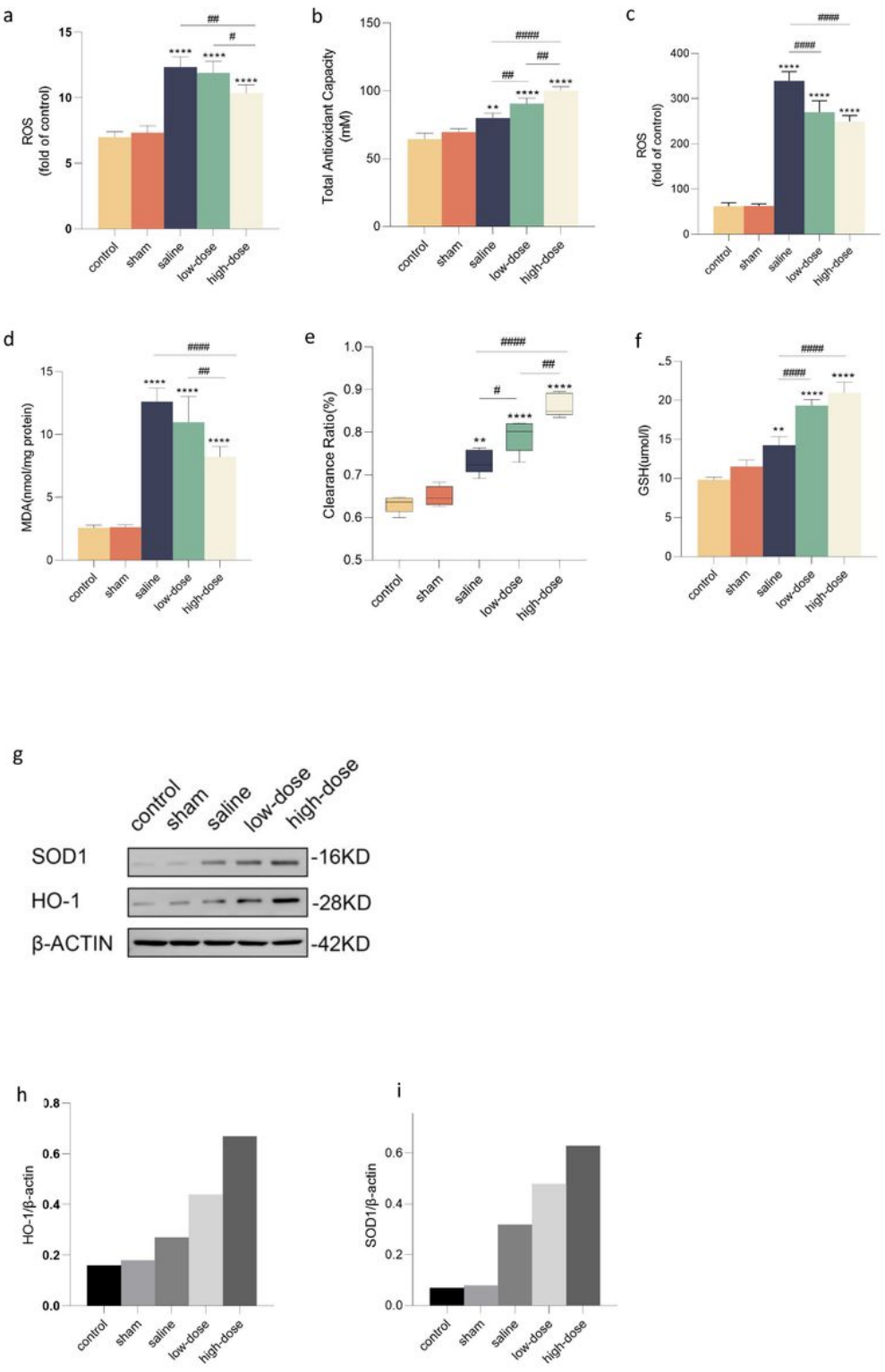

Figure 4

Effect of melatonin on the antioxidant function of ovaries of whole frozen transplanted rats on the fourth postoperative day.

(a). Serum ROS levels were significantly higher in the three transplanted groups than in the control and sham groups, significantly higher in the saline group than in the high-dose melatonin group, and

significantly higher in the low-dose group than in the high-dose group.

(b). Serum TAC levels were significantly higher in the three transplanted groups than in the control and sham groups, significantly lower in the saline group than in the two melatonin groups, and significantly lower in the low-dose group than in the high-dose group.

(c-f). The tissue ROS, MDA, hydroxyl radical scavenging rate, and GSH levels of the rats in the three transplanted groups were significantly higher than those in the control and sham groups, with ROS in the saline group significantly higher than those in the high-dose and low-dose groups. The MDA levels in the saline and low-dose groups were significantly higher than those in the high-dose group, and hydroxyl radical scavenging rate and GSH in the saline group were significantly lower than those in the high-dose and low-dose groups.

(g). Western blot results of the antioxidant enzymes SOD and HO-1 in the melatonin group were stronger than that in the control, sham, and saline groups, and expression was stronger in the high-dose group than the low-dose group.

(h-i). Western blot quantification results. (Compared with the sham group, **P<0.01, ****P<0.001; comparison between the three groups of transplantation, # P<0.05, ## P<0.01, #### P<0.001)

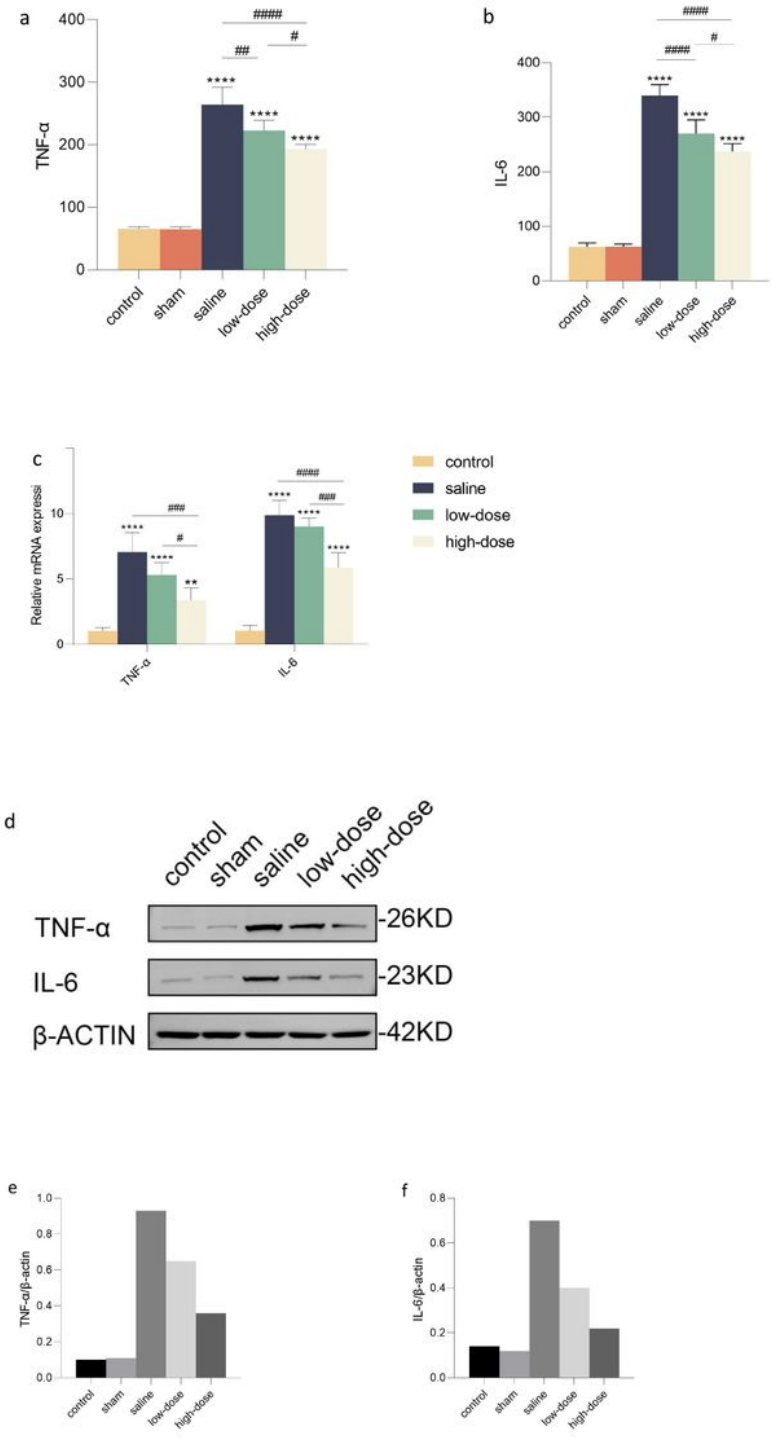

Figure 5

Effect of melatonin on inflammatory factors in the ovaries of whole frozen transplanted rats on the fourth postoperative day.

(a, b). Serum IL-6 and TNF-α levels were significantly higher in the three transplanted groups compared to the control and sham groups. They were significantly higher in the saline group than in both melatonin

groups, and significantly higher in the low-dose group than in the high-dose group.

(c). The levels of IL-6 and TNF-α in ovarian tissues of the three transplanted groups were significantly higher than that in the control group, and significantly higher in the saline and low-dose groups than in the high-dose melatonin group.

(d). A higher protein expression of IL-6 and TNF-α was observed in the physiological saline group than that in all other groups. The expression of inflammatory factors gradually decreased with increasing melatonin dose.

(e,f). Western blot quantification results. (Compared with the control group, **P<0.01, ****P<0.001; comparison between the three transplantation groups, # P<0.05, ## P<0.01, #### P<0.001)

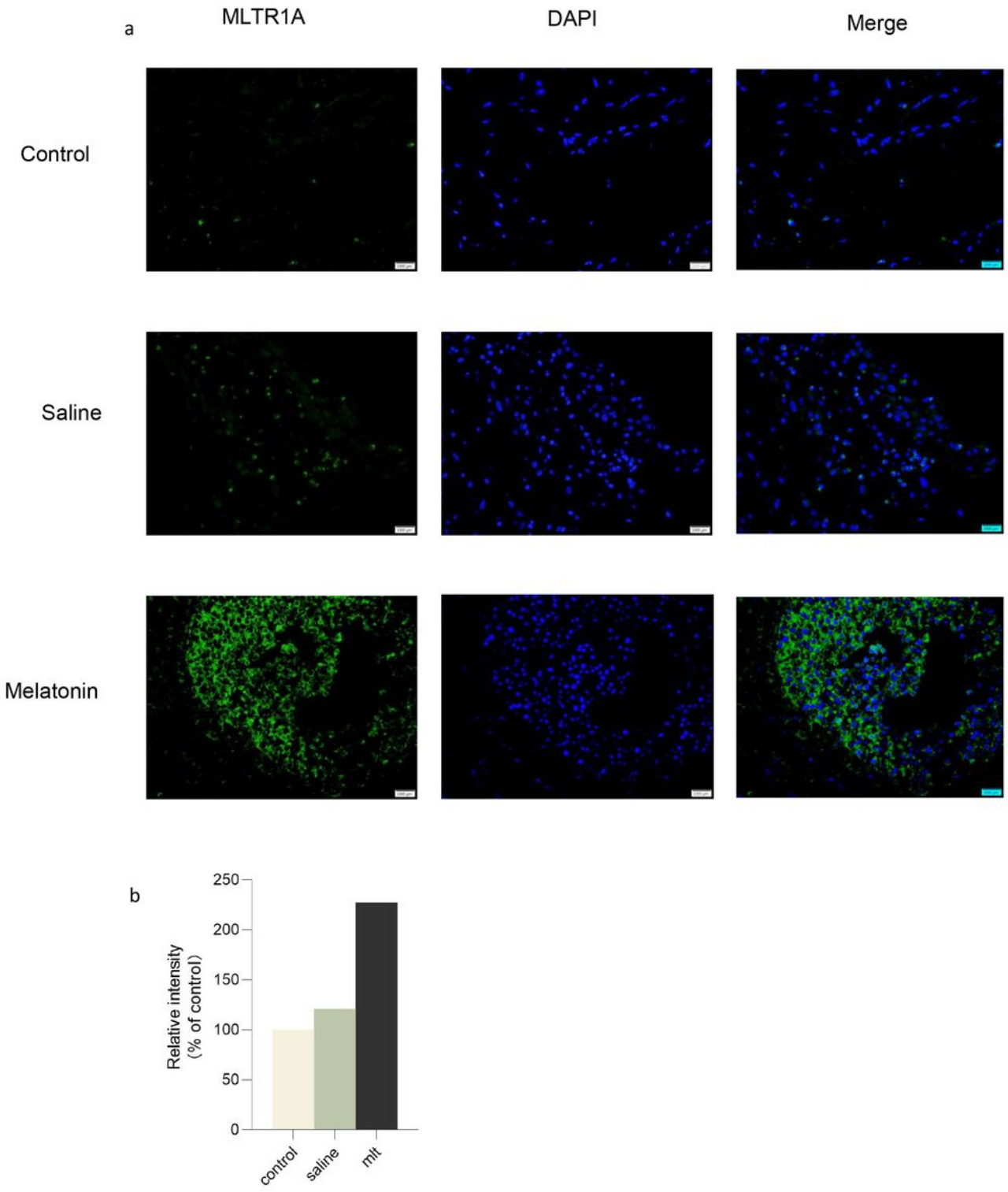

Figure 6

MT1 receptor expression in whole frozen transplanted ovaries.

(a). A weak melatonin receptor MT1 fluorescence signal was observed in the control group, with a small amount of green fluorescence. On the fourth postoperative day, enhanced MT1 staining of the tissue was seen in the saline group. The MT1 immunofluorescence staining signal of ovarian tissue was

significantly stronger than that in the control and saline groups on the 4th day of continuous melatonin use after transplantation.

(b). Quantitative analysis of immunofluorescence in each group.

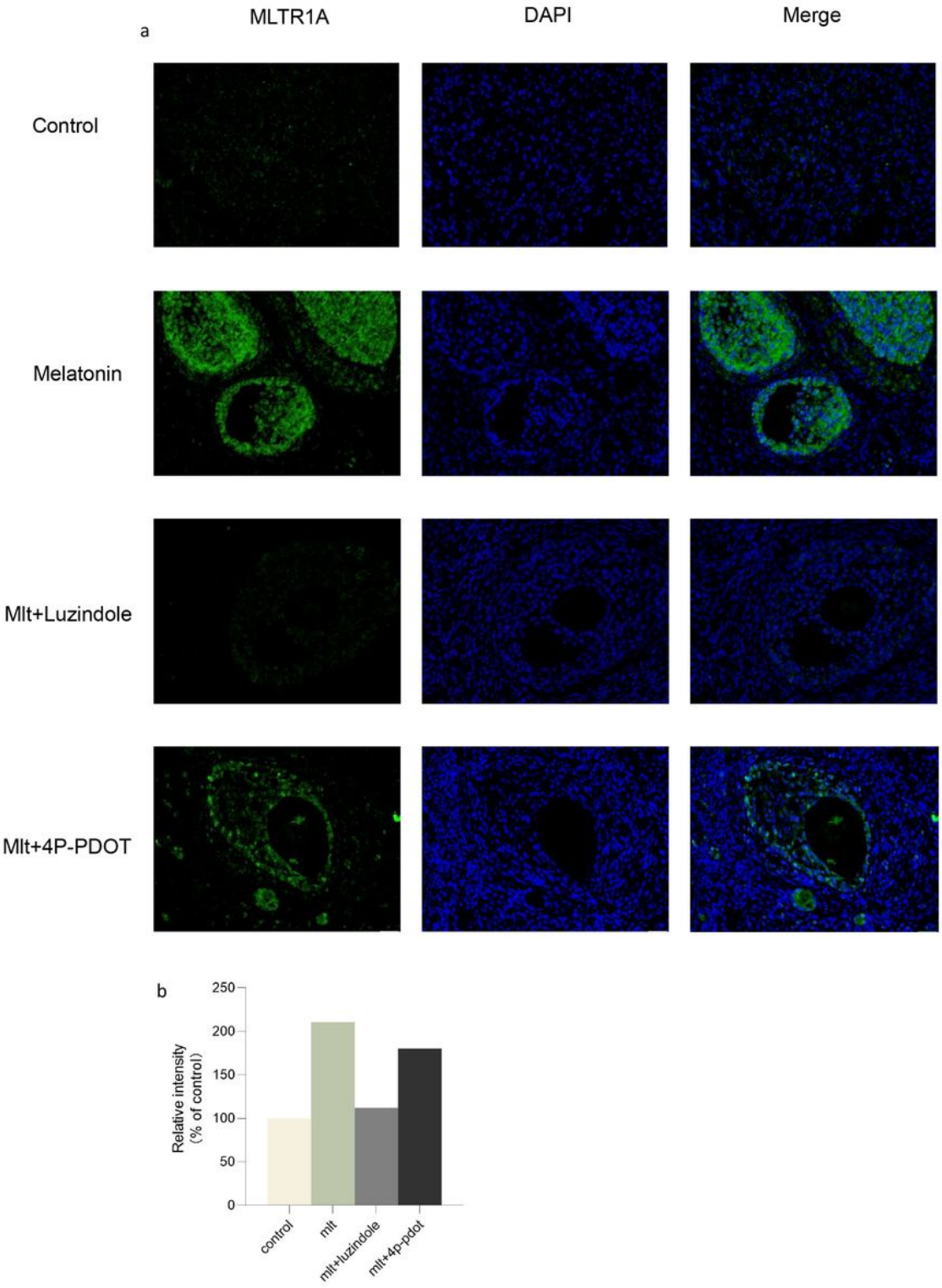

Figure 7

MT1 receptors were inhibited by the non-selective inhibitor luzindole, with little effect from the MT2-specific inhibitor 4P-PDOT.

(a). The melatonin receptor MT1 fluorescence signal was weak in the control group, displaying a weak green fluorescence. On day 4 of continuous melatonin administration after transplantation, strong immunofluorescence staining of MT1 was seen in ovarian tissues. The fluorescence intensity was significantly weaker in the luzindole group than in the melatonin and 4P-PDOT groups, while the fluorescence signal was almost unaffected in the 4P-PDOT group. (b). Quantitative analysis of immunofluorescence in each group.

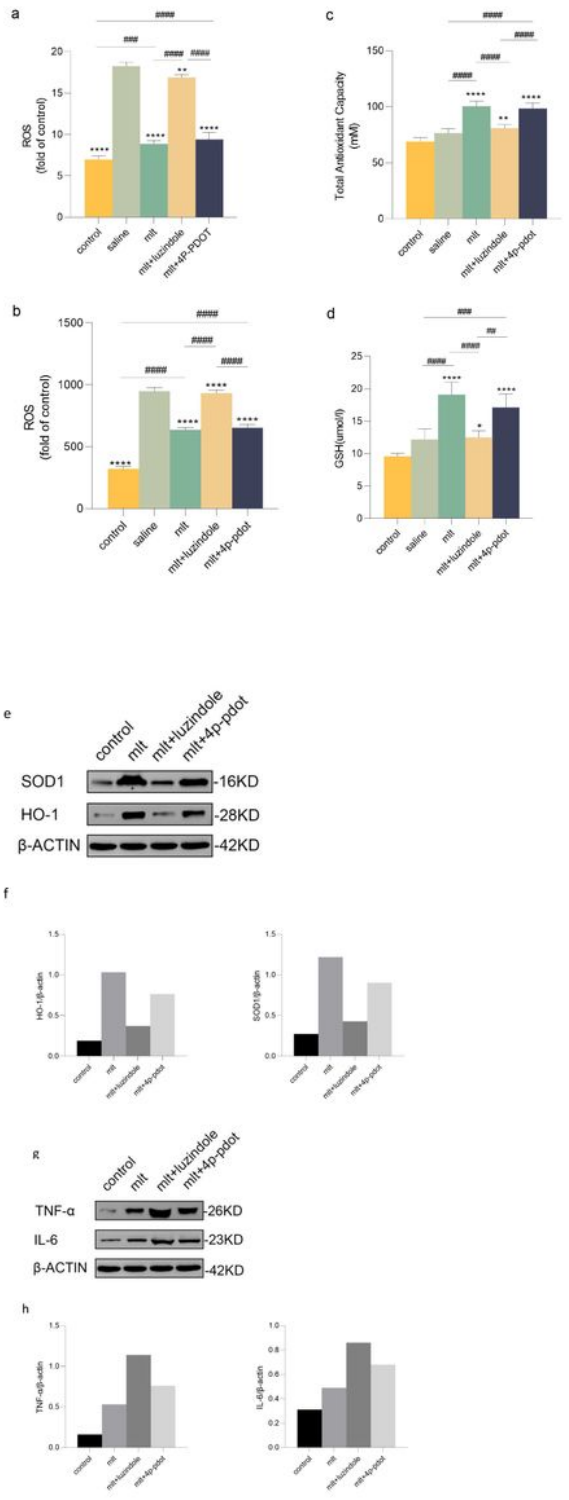

Figure 8

Melatonin receptor inhibitors can affect the levels of oxidative stress and inflammatory factors in cryopreserved and transplanted whole ovaries.

(a, b) The serum and tissue ROS levels in the melatonin group were significantly lower than in the saline group but significantly higher compared to the control group.The serum and tissue ROS levels in the

luzindole group were significantly higher than in the 4P-PDOT and melatonin groups, with no significant differences between the saline and 4P-PDOT groups.

(c, d). The serum TAC and tissue GSH levels in the melatonin group were significantly higher than those in the control, saline, and luzindole groups, and significantly higher in the 4P-PDOT group than in the luzindole group.

(e, f) Melatonin promoted the expression of downstream antioxidant enzymes SOD1 and HO-1. The expression in the luzindole group was significantly lower than that in the melatonin group, but still mildly higher than that in the control group; the expression of antioxidant enzyme proteins in the 4P-PDOT group was close to that in the melatonin group.

(g, h). Melatonin inhibited the expression of downstream inflammatory factors IL-6 and TNF-α, with the expression in the luzindole group being significantly higher than in the melatonin and fresh control groups. The expression of inflammatory factors in the 4P-PDOT group was close to that in the melatonin group. (Compared with saline group, **P<0.01, ****P<0.001; comparison between groups, ## P<0.01, ### P<0.005, #### P<0.001)

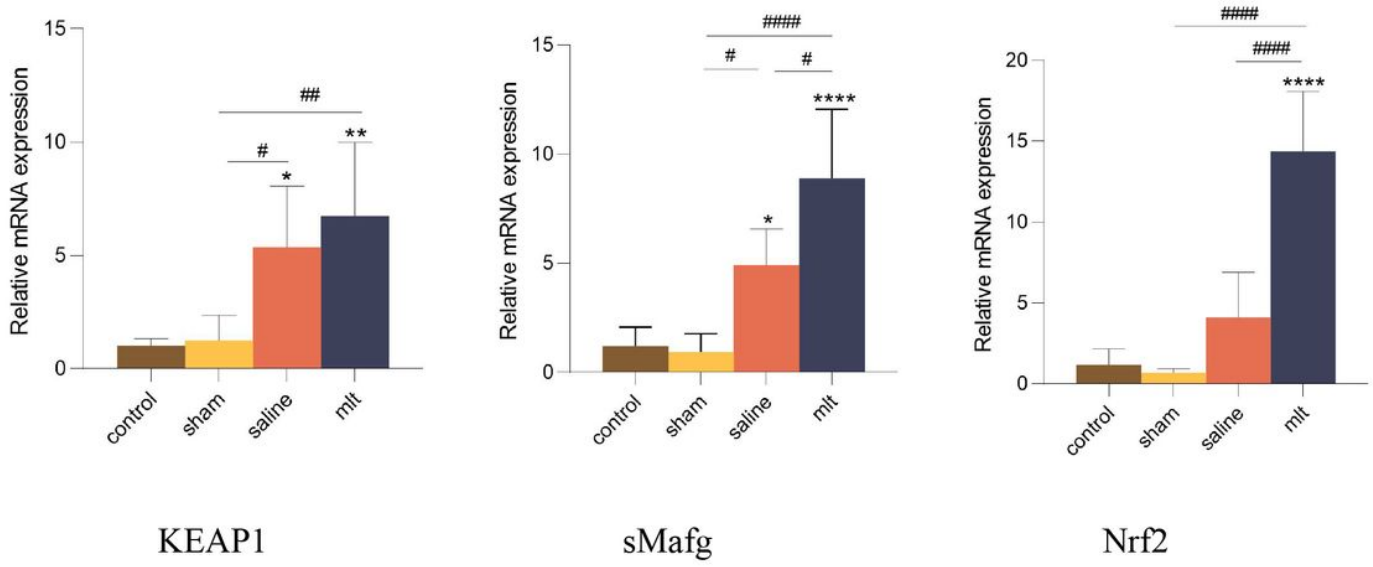

Figure 9

Melatonin regulates KEAP1, sMafg, and Nrf2 mRNA expression in cryopreserved and transplanted whole ovaries.

Continuous melatonin administration over the first four postoperative days resulted in a significant increase in sMafg and Nrf2 mRNA expression in ovarian tissues compared to the control, sham and saline groups. In addition, KEAP1 mRNA expression was significantly higher than in the control and sham groups.

(Compared with the saline group, *P<0.05, **P<0.01, ****P<0.001; compared among the surgical groups, #P<0.05, ## P<0.01, #### P<0.001)

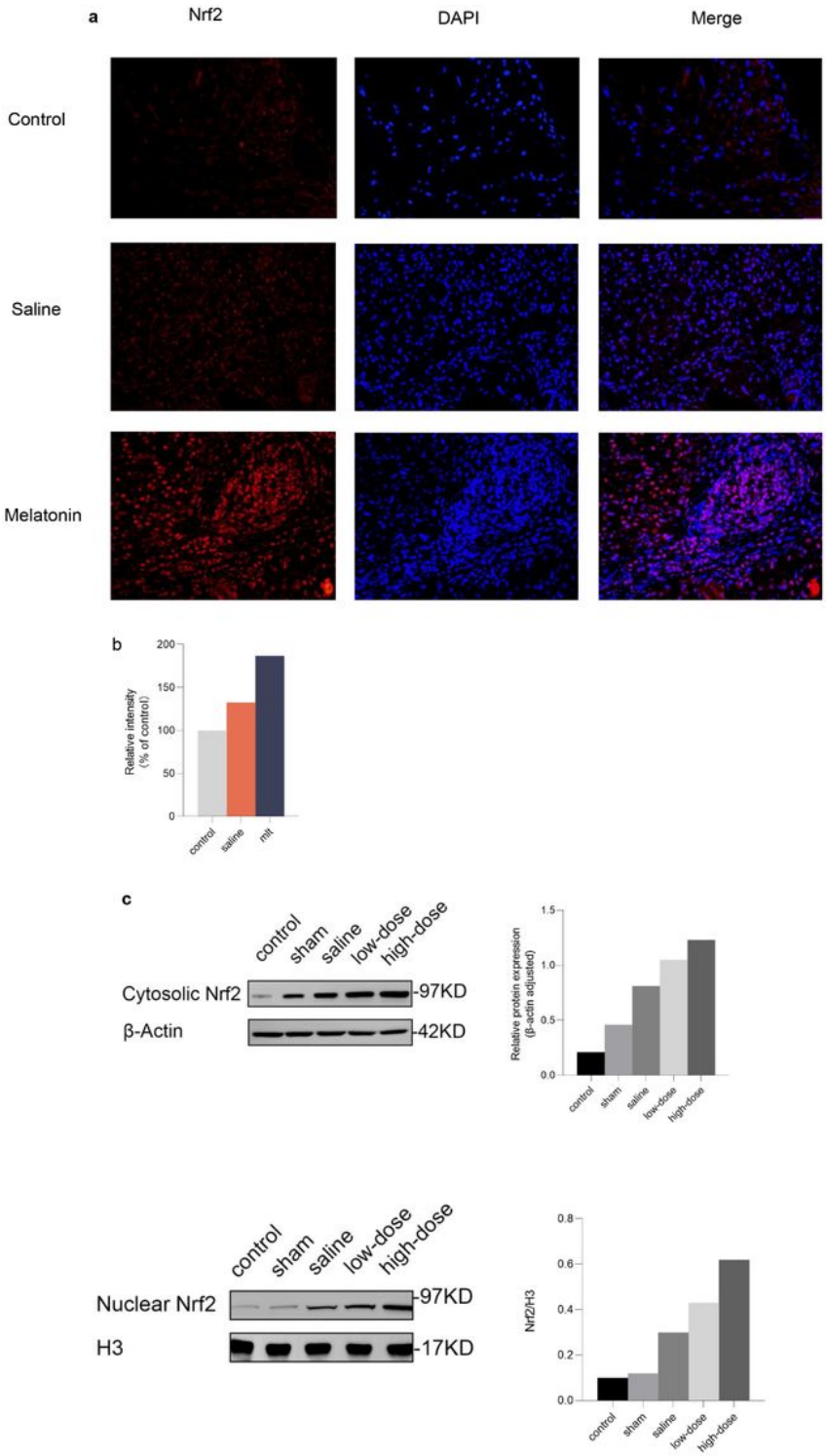

Figure 10

Melatonin enhancement of Nrf2 expression and nuclear translocation in cryopreserved and transplanted whole ovaries.

(a). A weak Nrf2 fluorescence signal was observed in the control group, as evidenced by the weak red fluorescence. On day 4 after ovarian transplantation in the saline group, tissue Nrf2 staining was slightly stronger than in the control group, and overlapping red fluorescence and blue DAPI immunofluorescence were seen in the nucleus. On the 4th day of continuous postoperative melatonin administration, the Nrf2 immunofluorescence staining of ovarian tissues was significantly stronger than that in the control and saline groups, and the red fluorescence overlapped with the blue DAPI in the nucleus.

(b). Quantitative analysis of immunofluorescence in each group.

(c). With increasing melatonin dose, the cytoplasmic and nuclear Nrf2 protein levels were significantly higher than those in the control, sham, and saline groups.

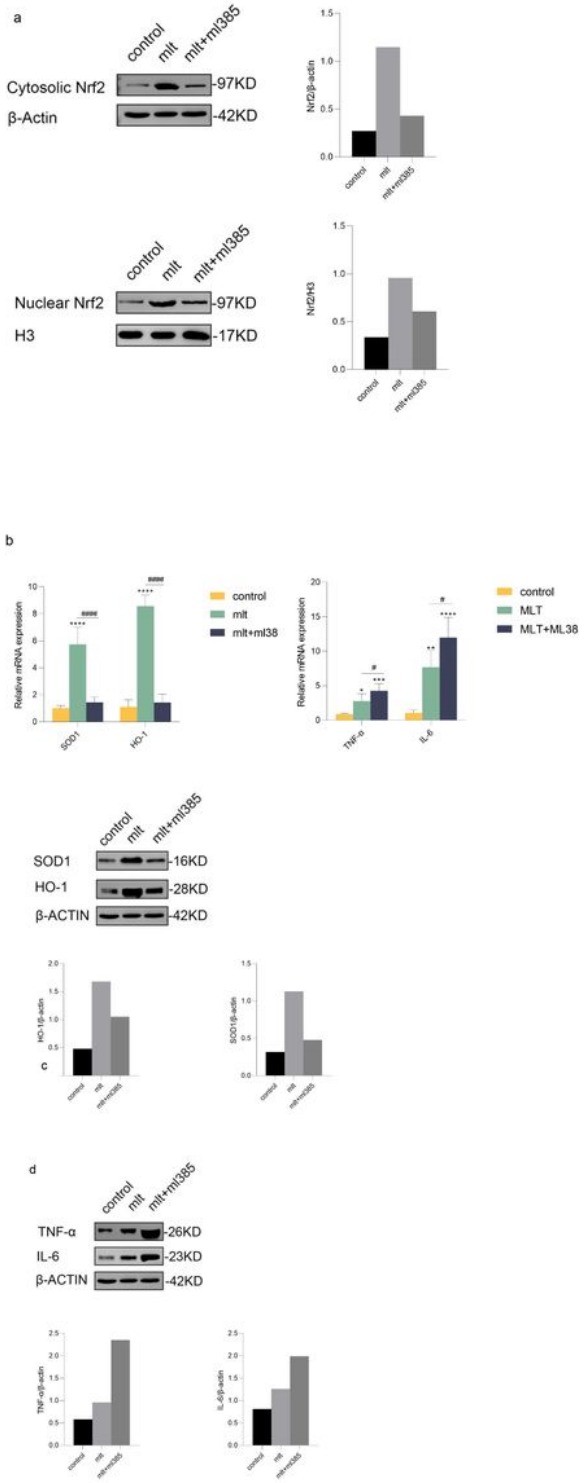

Figure 11

The Nrf2-specific inhibitor ML385 reverses melatonin-promoted cytoplasmic cytosolic Nrf2, downstream antioxidant enzyme expression, and inhibition of inflammatory factors.

(a). Cytoplasmic cytosolic Nrf2 protein levels were significantly higher in the melatonin group than in the control group, and Nrf2 protein levels in the ML385 group were significantly lower than in the melatonin

group but higher than that in the control group.

(b-d). Downstream SOD1 and HO-1 mRNA and protein expression were significantly lower in the ML385 group than in the melatonin group, and inflammatory factors IL-6 and TNF-α mRNA and protein expression were significantly higher than in the melatonin group.

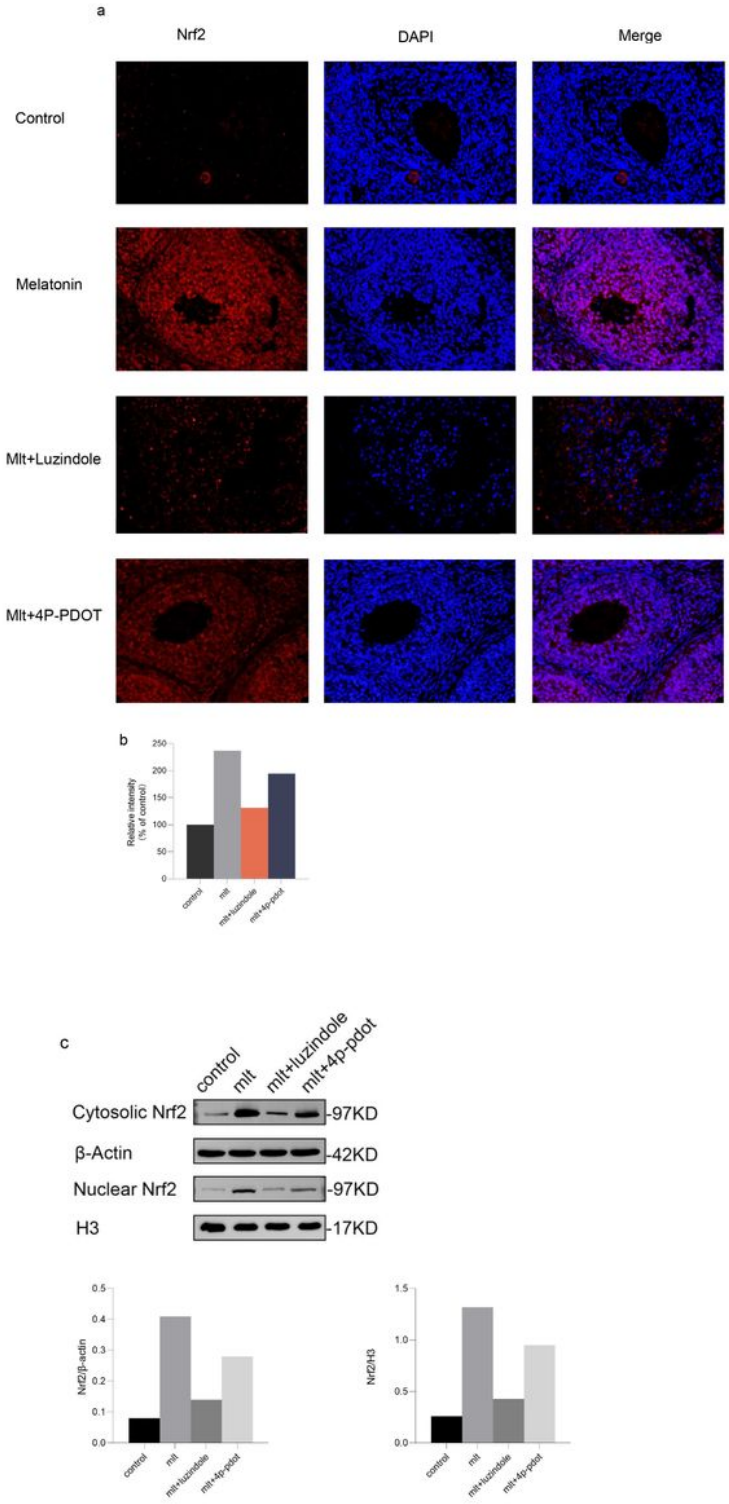

Figure 12

Melatonin affects Nrf2 expression and nuclear translocation via the MT1 receptor.

(a). Nrf2 fluorescence signal was weak in the control group. On the 4th day of continuous melatonin administration after transplantation, ovarian tissues showed strong Nrf2 immunofluorescence, which overlapped with the blue DAPI fluorescence in the nucleus. The Nrf2 signal in the luzindole group was significantly weaker and slightly higher than that in the control group. In contrast, the Nrf2 red fluorescence signal in the 4P-PDOT group was almost unaffected. The immunofluorescence of each group was quantified. (c). The cytoplasmic cytosolic Nrf2 protein expression was significantly stronger in the melatonin group than in the control group, significantly weaker in the luzindole group than in the melatonin and 4P-PDOT groups, and slightly higher than in the control group.

Supplementary Files

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- tables.pdf