Zygote

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Melatonin protects oogenesis from hypobaric hypoxia-induced fertility damage in mice

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Research Article

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Summary

Environmental hypoxia adversely affects reproductive health in humans and animals at high altitudes. Therefore, how to alleviate the follicle development disorder caused by hypoxia exposure and to improve the competence of fertility in plateau non-habituated female animals are important problems to be solved urgently. In this study, a hypobaric hypoxic chamber was used for 4 weeks to simulate hypoxic conditions in female mice, and the effects of hypoxia on follicle development, proliferation and apoptosis of granulosa cells, reactive oxygen species (ROS) levels in MII oocyte and 2-cell rate were evaluated. At the same time, the alleviating effect of melatonin on hypoxic exposure-induced oogenesis damage was evaluated by feeding appropriate amounts of melatonin daily under hypoxia for 4 weeks. The results showed that hypoxia exposure significantly increased the proportion of antral follicles in the ovary, the number of proliferation and apoptosis granulosa cells in the follicle, and the level of ROS in MII oocytes, eventually led to the decline of oocyte quality. However, these defects were alleviated when melatonin was fed under hypoxia conditions. Together, these findings suggest that hypoxia exposure impaired follicular development and reduced oocyte quality, and that melatonin supplementation alleviated the fertility reduction induced by hypoxia exposure.

Introduction

Follicle development accompanied by changes in granulosa cell function and oocyte maturation underlies the physiology of female fertility. Mammalian follicle development exhibits cyclical changes and consists of three main stages: follicle recruitment and priming, selection and dominance, and maturation and ovulation (Edson *et al*., 2009). During follicle development, the developmental capacity of oocytes is closely related to the function of granulosa cells (Boucret *et al*., 2015; Shen *et al*., 2021). In the initial stage of follicle development, a group of primordial follicles in the ovary is recruited and starts to grow at the same time as the degeneration of the corpus luteum. With the help of a series of cytokines and gonadotropins, the granulosa cells proliferate rapidly; this promotes follicle growth and, eventually, the follicular cavity appears. As the follicle develops, granulosa cells produce large amounts of oestrogen and transport ATP and small molecule metabolites to the oocyte for its development and maturation (Monniaux, 2016). Therefore, the follicle provides an ideal microenvironment for oocyte development and maturation, such as the hypoxic environment within the follicular cavity, which is necessary for egg maturation and ovulation (Redding *et al*., 2008; Tam *et al*., 2010; Lim *et al*., 2021).

About 140 million people live in areas higher than 2500 m, and about 40 million people travel to areas above 2500 m yearly (Moore *et al*., 1998). The hypoxic stress caused by high altitude has been proven harmful to non-adapted humans (Moore *et al*., 2001). Notably, the effect of hypoxia on female fertility has been described by various authors who have indicated that females exposed to hypoxia exposure experienced deficiency of pre-ovulatory follicle development, birth weight reduction, embryo loss, and intrauterine growth restriction (Parraguez *et al*., 2005, 2006, 2014; Hartinger *et al*., 2006). Only a few studies have shown that follicular growth is restricted when female follicles are exposed to hypoxic conditions *in vitro* (Connolly *et al*., 2017; Ma *et al*., 2019). Although hypoxic environmental stress has been shown to be detrimental to female fertility, the mechanism and how to release this effect still needs further investigation. A previous study demonstrated that hypobaric hypoxia would cause oxidative damage, which could be alleviated by antioxidants (Farias *et al*., 2010). Numerous studies have shown that the antioxidant activity of melatonin secreted by the pineal gland plays a positive role in regulating the reproductive process of female animals.

Melatonin plays a vital role in regulating circadian rhythms, pubertal development, and seasonal adaptation in animals (Reiter *et al*., 2014; Han *et al*., 2017; Jiang *et al*., 2021b; Cipolla-Neto *et al*., 2022). In recent years, its critical role in animal reproductive activity has been widely

demonstrated. Melatonin regulates gonadal hormone levels through the hypothalamic–pituitary–gonadal axis or targets the gonads to regulate germ cell development (Berlinguer *et al.*, 2009; Cipolla-Neto *et al.*, 2022). In female reproduction, melatonin could relieve oxidative stress (Zou *et al.*, 2020), delay ovarian ageing (Tong *et al.*, 2017), reduce granulosa cell apoptosis (Jiang *et al.*, 2021b), and promote egg maturation and embryonic development (Liu *et al.*, 2019b). Melatonin can remove or inhibit the production of ROS, reducing the damage of hypoxic stress to animals (Poeggeler *et al.*, 1994; Amaral and Cipolla-Neto, 2018; Gonzalez-Candia *et al.*, 2019). Melatonin can also improve fetal pulmonary circulation and cardiac function by increasing the activity of antioxidant enzymes and resisting the fetal pulmonary hypertension syndrome caused by perinatal hypoxia in high-altitude animals (Reiter *et al.*, 2018; Gonzaléz-Candia *et al.*, 2021). All these suggest that melatonin has a promising application against hypoxic stress.

In this study, we used hypoxic stress mice as an animal model to study the mechanism of hypoxic stress affecting female reproduction. The melatonin-hypoxia group was used to assess the role of melatonin in alleviating hypoxic reproductive stress in females. Alleviating the effect of melanin on the reproductive damage of hypoxic stress mice provides a theoretical reference for further research and improvement of the effect of hypoxic stress on the reproductive activity of female animals.

Materials and methods

Animals

CD-1 mice were purchased from the Charles River Laboratory Animal Centre (Beijing, China) and housed in a controlled environment with food and water *ad libitum*. Female CD-1 mice, 4–6 weeks of age, were divided randomly into the following treatments:

- Control: The control group were maintained in normal conditions.
- Hypoxia: The hypoxia group was housed in a hypobaric hypoxic chamber and treated with vehicles for 4 weeks.
- Hypoxia+MT: Melatonin with hypoxia group housed under the same hypoxia conditions, and that received melatonin powder (Sigma, M5250) dissolved in corn oil at a one-time oral dose of 30 mg/kg body weight daily for 4 weeks.

All animals used in the experiment were conducted under the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Welfare and Ethics Committee at West Anhui University.

Hypoxia treatment evaluation

Mouse heart tissue was removed immediately after 4 weeks of hypoxic treatment and rinsed with normal saline, and then moisture was adsorbed onto a filter paper. The weights of the right ventricle (RV) and left ventricle plus ventricular septum (LV+S) were recorded to calculate RV/[LV+S] (Chen *et al.*, 2019).

Ovary histological analysis

The histological analysis of ovaries followed a previous study (Xu *et al.*, 2020). Briefly, mouse ovarian tissues were fixed in 4% PFA for 2 h and then dehydrated using a series of graded ethanol (30%, 50%, 70%, 95%, and 100%). The tissues were embedded in paraffin (Leica, German) and cut into 5-μm sections (Leica RM2235, German) with serial section. After drying at 42°C, the sections were rehydrated and stained with haematoxylin and eosin (H&E). Images were acquired using a microscope (Nikon ECLIPSE E200, Japan) with a charge-coupled device (CCD; MshOt MS60, China). The classification of follicles was based on the shape of the granulosa cell and the number of layers surrounding the oocyte as described by Hadek (1965). Finally, each ovary was cut sequentially into 10 sections, and the proportions of preantral, antral, mature follicles, and corpus luteum were counted in each section.

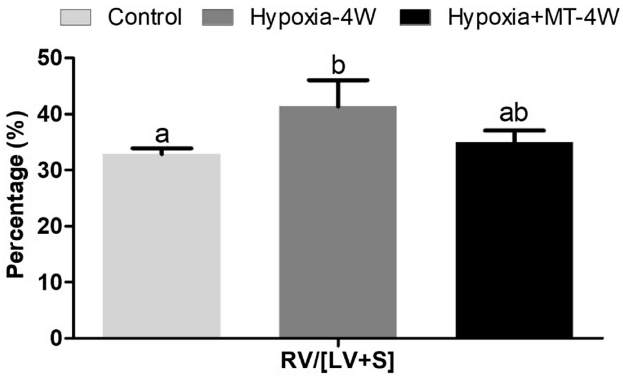

Figure 1. Effect of acute hypobaric hypoxia and melatonin on right ventricular hypertrophy index in female mice. Mice were submitted to acute hypobaric hypoxia with or without treatment of melatonin. Control: Mice were treated with normal environment. Experiment: 4 weeks treatment. At least four mice were counted per group. Significance between different groups is represented by different letters ($P < 0.05$).

Granulosa cell proliferation assay

For immunohistochemistry, ovary sections were rehydrated and endogenous peroxidase activity was blocked in 3% H_2O_2 for 10 min at room temperature (RT). Rehydrated tissues were washed three times with phosphate-buffered saline (PBS: NaH_2PO_4, Na_2HPO_4, NaCl; pH 7.4). Sections were blocked in 10% goat serum for 1 h and incubated with a primary antibody against proliferating cell nuclear antigen (PCNA; 1:200, Ruiying Technology, RLM3031) in antibody dilution buffer overnight at 4°C. Sections were washed three times with PBS and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:200, Proteintech, SA00013–6). Sections were washed three times with PBS and visualized using 3,3-diaminobenzidine (DAB; ab64238) for 3 min. Sections were counterstained with haematoxylin and observed under a microscope (Nikon, E200, Japan). Image Pro Plus software was used to calculate the percentage of PCNA-positive cells in total granulosa cells in preantral and antral follicles.

TUNEL assay

Mouse ovarian tissue sections were rehydrated in an ethanol gradient and then stained using a TUNEL apoptosis detection kit (Beyotime Biotechnology, C1090) to detect apoptosis. Briefly, the tissue sections were treated with proteinase K (20 μg/ml) without DNase at 37°C for 15 min. Sections were washed three times with PBS and incubated with TUNEL detection solution for 1.5 h at RT. Sections were washed three times with PBS and stained with

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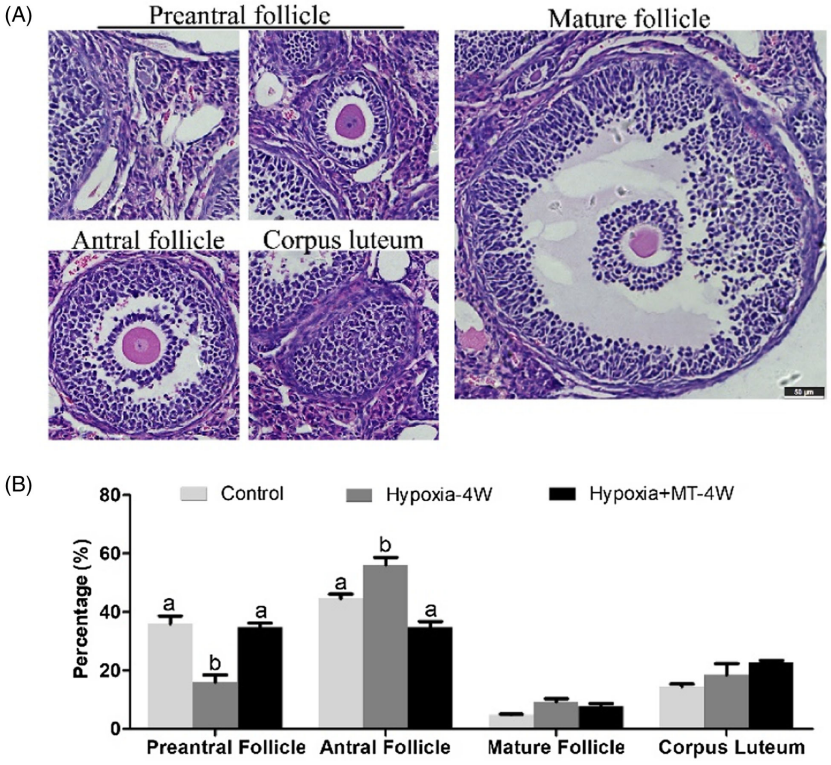

Figure 2. Quantification of preantral, antral, mature follicles and corpora lutea in mouse ovaries from control, Hypoxia, and Hypoxia+MT animals. (A) Classification of different developmental stage follicles in mouse ovaries. (B) The proportion of different developmental stage follicles per ovarian cross-section from control, Hypoxia, and Hypoxia+MT female mice. At least 10 ovary sections were counted per mouse. Significance between different groups is represented by different letters ($P < 0.05$). Black scale bar = 50 μm.

H33342 (1 μg/ml) for 1 min. After washing, samples were mounted with the anti-fluorescence quencher, and digital images were captured on a Leica DMR fluorescence microscope (Mannheim, Germany). The number of apoptotic granulosa cells was counted in the antral follicle using Image Pro Plus software.

In vitro *fertilization and embryo culture*

The *in vitro* fertilization (IVF) procedure was performed as described previously (Chen *et al.*, 2019). Briefly, matured (MII) oocytes from the oviduct ampulla were inseminated with capacitated sperm and incubated with HTF medium at 37°C in 5% CO_2 in air. After 4 h post-fertilization, oocytes were washed and cultured in KSOM medium at 37°C in 5% CO_2 in air. Proportions of 2-cell embryos were assessed at 24 h post-fertilization.

Measurement of intracellular ROS level

To measure intracellular ROS levels, matured (MII) oocytes were incubated in M2 solution containing 20 μM, 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA; Life Technologies, Invitrogen TM, C6827) for 30 min (37°C, 100% humidity, and 5% CO_2 concentration), and then washed three times in M2 solution containing 3 g/l BSA for 5 min each. Finally, oocytes were placed under a fluorescence microscope and measured at 460 nm excitation with a filter, and fluorescence images were recorded as TIFF files. After deducting the background value, fluorescence intensities were quantified using ImageJ software (Version 1.48; National Institutes of Health, Bethesda, MD, USA; Zou *et al.*, 2020).

Statistical analysis

All quantitative data are presented as the mean ± standard error of the mean (SEM) for at least three biological replicates. Data were analyzed using one-way analysis of variance (ANOVA) followed by a Duncan multiple comparison test using SPSS 21.0 software (SPSS Inc.). Next, 10 sections were used to count the proportions of corpora luteum and different types of follicles; at least 10 follicles were used for cell apoptosis detection. Differences between means were considered significant at a P-value < 0.05.

Results

Hypoxia exposure impairs the normal follicle development of mice

To study the effect of hypoxic stress on reproductive fertility, female mice were exposed to hypoxia for 4 weeks to simulate hypoxia exposure to explore changes in fertility. The ratio of RV/[LV+S] was used to assess the success of the mouse model. As shown in Figure 1, RV/[LV+S] was higher in the hypoxia exposure group (Hypoxia) compared with the control group (P <0.05). This result indicated that the hypoxic stress mouse model was successfully established.

As a basis for female fertility, we first examined the follicle development of oogenesis. After hypoxia exposure, the normal oogenesis process was impaired with the increased preantral (primordial, primary, and secondary follicles) and antral follicle proportion in the ovary (Figure 2). While there was little change in corpora luteum and mature follicle proportion, which indicated that normal folliculogenesis was impaired by hypoxia exposure.

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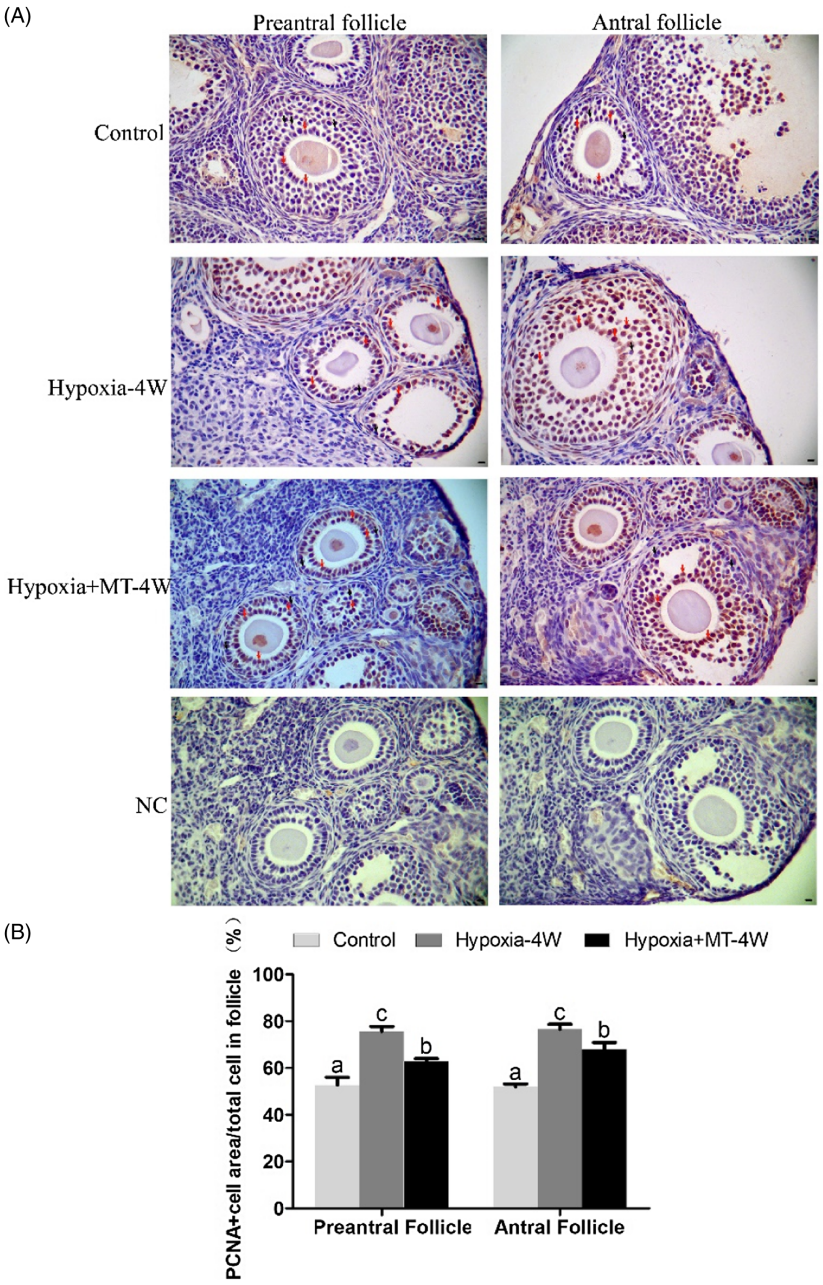

Figure 3. Proliferation activity of granulosa cells in follicles from control, Hypoxia, and Hypoxia+MT female mice. (A) Representative images of immunohistochemical staining of the proliferation cell marker PCNA. (B) Proliferation activity differences of granulosa cells in growing follicles (preantral and antral follicles) from the control, Hypoxia and Hypoxia+MT female mice. At least 10 follicles were counted per mouse. Significance between different groups is represented by different letters ($P < 0.05$). The red arrows indicate high proliferative granulosa cells. The black arrows indicate low proliferative granulosa cells. Black scale bars = 10 μm.

Melatonin reverses the disorder of follicle development caused by hypoxia exposure

as melatonin is often used to relieve oxidative stress, we wanted to examine whether melatonin could relieve the disorder in female follicle development caused by hypoxic stress. The increased RV/[LV+S] caused by hypoxia exposure could be reversed by melatonin feeding ($P > 0.05$). There was no significant difference between the control and Hypoxia+MT groups (Figure 2). This result supported the cardioprotective effects of an oral administration of melatonin in female mice that suffer from hypoxia exposure.

Then, we further explored if melatonin feeding could rescue the impaired folliculogenesis induced by hypoxia exposure. The follicle counting results showed that the preantral and antral follicle proportion in the Hypoxia+MT group was significantly lower than that in the Hypoxia group but had no difference from the control group. The results suggested that melatonin can improve follicular dysplasia caused by hypoxic stress.

Melatonin relieves the hyper-proliferation of granulosa cells induced by hypoxia exposure

Follicles are composed of oocytes and granulosa cells, and the development of follicles is mainly mediated by the proliferation and renewal of granulosa cells (Monniaux, 2016). Next, we analyzed the proliferation activity of granulosa cells under hypoxic stress. We chose PCNA as a proliferation cell marker in this assay. The result showed that PCNA-positive granulosa cells were significantly higher in the Hypoxia group than the other groups ($P < 0.05$; Figure 3B). These results demonstrated that hypoxic stress induced hyper-proliferation of granulosa cells, which would trigger the disorder of folliculogenesis.

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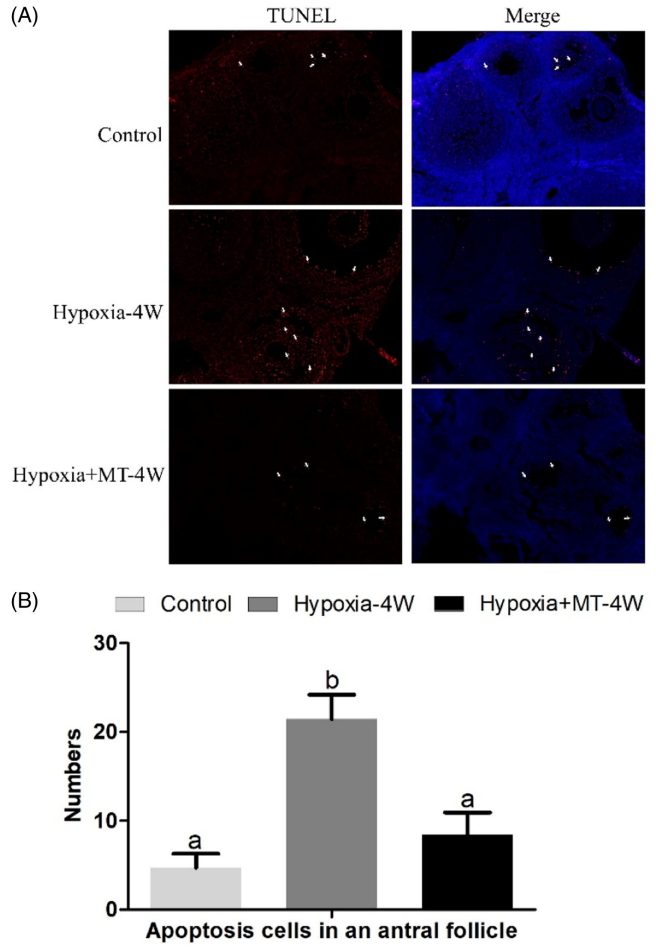

Figure 4. Apoptosis rate of granulosa cells in antral follicles from control, Hypoxia, and Hypoxia+MT female mice. (A) Representative images of fluorescence staining of DNA fragment marker TUNEL. (B) Apoptosis rate differences of granulosa cells in antral follicles from control, Hypoxia, and Hypoxia+MT female mice. At least 10 follicles were counted per group. Significance between different groups is represented by different letters ($P < 0.05$). The white arrows indicate apoptotic granulosa cells in the follicle.

Furthermore, we also analyzed whether the addition of melatonin affected the hyper-proliferation of granulosa cells induced by hypoxic exposure. As shown in Figure 3B, compared with the Hypoxia group, PCNA-positive granulosa cells in the Hypoxia+MT group were significantly reduced ($P < 0.05$). These results indicated that hypoxic exposure induced the proliferation of granule cells in mouse follicles. At the same time, melatonin partly overcame the excessive proliferation of granulosa cells caused by hypoxia exposure during follicle development.

Melatonin reduces apoptosis of granulosa cells in antral follicles of hypoxic mice

The above result showed that, although hypoxia induced an increasing proportion of antral follicles on the ovary, it did not change the rate of mature follicles. To further analyze the reasons for this phenomenon, we analyzed the apoptosis of granulosa cells in antral follicles. We examined the apoptosis of granulosa cells in follicles from control, Hypoxia, and Hypoxia+MT groups using TUNEL-staining targeting DNA fragments. The apoptosis rate of granulosa cells in antral follicles in the Hypoxia group was significantly increased than that in other groups ($P < 0.05$; Figure 4B). However, there was no obvious difference between the Hypoxia+MT group and the control group in the apoptosis rate of granulosa cells ($P > 0.05$; Figure 4B). These results suggested that hypoxic stress-induced follicular developmental disorders may be caused by the apoptosis of granulosa cells. Melatonin could rescue the apoptosis of granulosa cells during follicle development caused by hypoxia exposure.

Melatonin rescues the hypoxic stress-induced decline in early embryonic development

To further confirm the effect of hypoxia exposure on early embryonic development, we carried out superovulation and *in vitro* culture experiments. After hCG injection, the number of MII oocytes obtained from these three groups showed no difference, which indicated that hypoxic stress did not affect ovulation (Figure 5A). During the subsequent embryo development, the cleavage rate in Hypoxia was significantly lower compared with the control group ($P < 0.001$), whereas melatonin feeding could rescue the decreased embryo development ability induced by hypoxia exposure (Figure 5B). These data indicated that hypoxia exposure was detrimental to early embryo development. At the same time, melatonin improved the developmental competence of oocytes caused by hypoxia exposure.

Melatonin attenuates oxidative stress in oocytes induced by hypoxic exposure

Because the developmental competence of the MII oocytes was severely impaired by hypoxia exposure, ROS levels in the oocytes were measured to evaluate intracellular oxidative stress in the control, Hypoxia, and Hypoxia+MT groups. The ROS level in oocytes from the Hypoxia group was significantly higher than that of the corresponding control group ($P < 0.05$; Figure 6A,B). However, when melatonin was fed, intracellular ROS levels (MII oocytes) were decreased, and showed no difference when compared with the control group ($P > 0.05$). Therefore, melatonin acts against ROS accumulation in MII oocytes from the oxidative stress caused by hypoxia exposure.

Discussion

Our study describes the effects of melatonin administration on follicle development and oocyte quality in a mice model of hypoxia stress. In this study, we provided strong evidence that acute hypoxic stress caused the accumulation of growing follicles in the ovaries of mice, increased proliferation and apoptosis of granulosa cells in the follicle, and induced ROS levels to increase in oocytes, which ultimately led to the reduction of oocyte developmental potential. However, feeding antioxidant melatonin effectively inhibited abnormal follicle development and excessive proliferation and apoptosis of granulosa cells, and reduced the ROS level in eggs, therefore alleviating the fertility damage caused by hypoxia stress.

The abnormal granulosa cell cycle under an hypoxic environment resulted in the accumulation of growing follicles in the ovary. Previous studies have shown that a basal hypoxic microenvironment can limit the growth of antral follicles and reduce the diameter of the follicle *in vitro* (Connolly *et al.*, 2017; Ma *et al.*, 2019). In this work, the proportion of antral follicles increased significantly in the Hypoxia group, but there was no significant difference in corpora lutea and mature follicle

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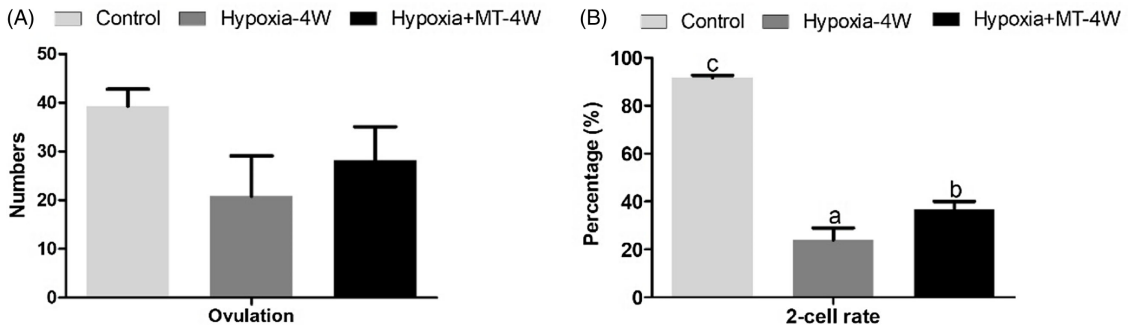

Figure 5. Effect of acute hypobaric hypoxia with or without melatonin on ovulation and early embryo development. (A) The three experimental groups had no differences on the number of ovulation. (B) The difference of 2-cell rates from control, Hypoxia, and Hypoxia+MT groups. At least 60 oocytes were used per group. Bars with different letters indicate significant difference ($P < 0.001$).

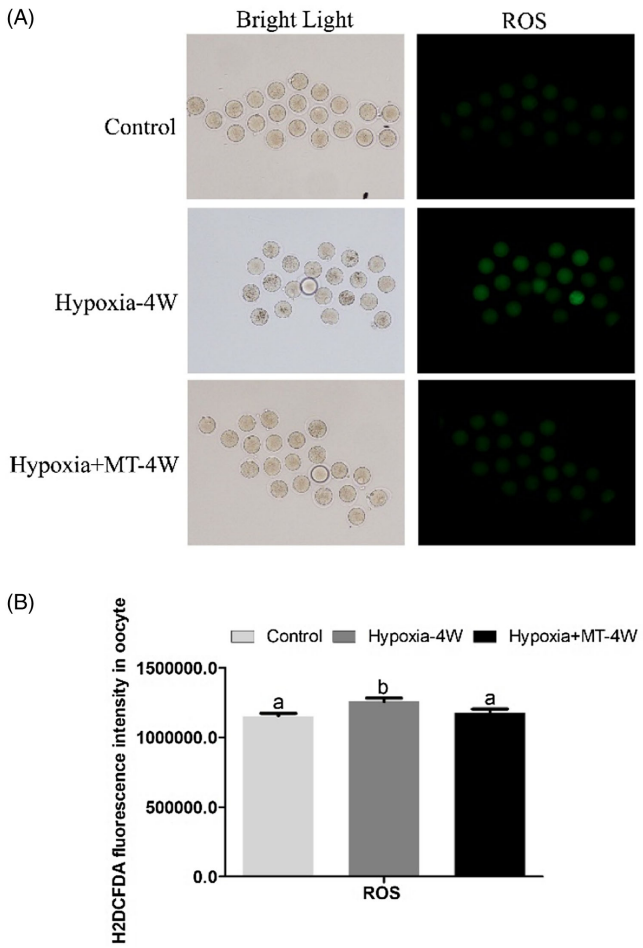

Figure 6. Effect of acute hypobaric hypoxia with or without melatonin on ROS levels in MII oocytes. (A) Representative images of fluorescence staining of ROS marker H_2DCFDA in MII oocytes from control, Hypoxia, and Hypoxia+MT female mice. (B) The three experimental groups had differences in ROS levels (H_2DCFDA fluorescence). At least 20 oocytes were counted per group. Significance between different groups is represented by different letters ($P < 0.05$).

proportion. The findings of these analyses uncovered that hypoxia causes antral follicle atresia. The poor GC number and function led to decreased production of steroid hormones and may trigger follicular atresia (Shen *et al.*, 2012; Zhang *et al.*, 2022). Previous studies have reported that oxidative stress caused by ovarian hypoxia is the main cause of ovarian ageing and GC dysfunction (Shen *et al.*, 2018; Jiang *et al.*, 2021a). Oxidative stress also alters the expression of more than 70 downstream genes, which leads to the overproduction of ROS and initiation of apoptotic cell death in ovarian follicles and granulosa cells (Lim and Luderer, 2011). In this study, the proliferation and apoptosis of granulosa cells were increased in the antral follicles, and there was no difference in the number of ovulations under hypoxia exposure. Therefore, oxidative stress injury of granulosa cells induced by hypoxia is the main cause of antral follicular accumulation in the ovary.

Hypobaric hypoxia causes ROS, a significant increase in mature oocytes, and results in the developmental failure of early embryos. Previously, it has been demonstrated that hypoxia modifies the activity of the cytochrome chain responsible for mitochondrial oxidative phosphorylation, resulting in a decrease in ATP synthesis and increased ROS (Turrens, 2003), at the same time as a decrease in the activity of the cellular antioxidant system, which may lead to oxidative injury and apoptosis (Adam-Vizi, 2005; Ramanathan *et al.*, 2005; Coimbra-Costa *et al.*, 2017). Under normal conditions, the cell produces a ROS level that acts beneficially for tissue regeneration, intracellular redox regulation, and embryogenesis. However, an excess of ROS can oxidize cellular molecules, such as lipids, carbohydrates, amino acids, and nucleic acids, modifying their functions and compromising cellular viability by producing lipid peroxidation, mitochondrial damage, and apoptosis. This impairs oocyte nuclear and cytoplasmic maturation, and subsequent embryo development and quality (Ambrogi *et al.*, 2017). In the present study, we showed a high ROS level in MII oocytes along with the decreasing embryo cleavage rate in the Hypoxia group. We proposed that hypoxia may induce excessive ROS accumulation in mature oocytes, resulting in oocyte oxidative injury and poor developmental ability.

Several studies have demonstrated the beneficial effects of melatonin against oxidative stress-stimulated granulosa cell damage. Melatonin treatment can markedly prevent the apoptosis of porcine granulosa cells during follicular atresia via its membrane receptors and its free-radical scavenging activity *in vitro* (He *et al.*, 2016). It was proposed that melatonin alleviates hypoxia-induced apoptosis of granulosa cells by reducing ROS production and activating the MTNR1B–PKA–Caspase8/9 pathway in porcine (Tao *et al.*, 2021). In addition, several studies have demonstrated that melatonin is against hypoxia-induced proliferation of cancer cells for preventing tumour progression (Chuffa *et al.*, 2015; Bastani *et al.*, 2021; Estaras *et al.*, 2022). In our study, melatonin relieved hypoxia-induced proliferation and the apoptosis of granulosa cells, which in turn led to a significant reduction in

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the proportion of antral follicles compared with the Hypoxia group. Findings from these data suggested that melatonin effectively modulated granulosa cell cycle and restored follicular development under hypoxia environment.

Melatonin alleviated the limit of follicle growth and improved oocyte quality under hypoxia exposure in female animals. Previous studies have shown that acute hypoxic stress caused follicle development, arrest, LH secretion deficiency, and luteal area reduction in sheep, and antioxidants partially alleviated the reproductive damage caused by hypoxic stress (Parraguez *et al.*, 2013, 2014). In this study, we showed that the process of follicle development and ROS level in the oocyte returned to normal while feeding melatonin in hypoxia-exposure female mice. Melatonin has a positive regulatory effect on the secretion of oestrogen and reducing of ROS in the ovary or oocytes. On the one hand, melatonin combined with melatonin receptors on the membrane of granulosa cells to activate the activity of aromatase CYP19A1 and to promote oestrogen synthesis and antral follicle maturation (Liu *et al.*, 2019a; Cheng *et al.*, 2020). On the other hand, melatonin is known as an antioxidant or free-radical scavenger by reducing the excessive production of ROS, thereby protecting against cardiac dysfunction under hypoxia (Poeggeler *et al.*, 1994; Gonzaléz-Candia *et al.*, 2021). Previous studies have suggested that adding melatonin (MT) for *in vitro* maturation improves the developmental competence of oocytes by scavenging reactive oxygen species (ROS) through melatonin receptor 2 in the cumulus–oocyte complex (Kim *et al.*, 2020; Zhu *et al.*, 2021). Melatonin acts on the Nrf2 signalling pathway through its receptors to induce the expression of downstream antioxidant proteins, thereby reducing the accumulation of ROS and enhancing the *in vitro* development potential of oocytes under stress (Guo *et al.*, 2021). A recent study confirmed that the expression of the melatonin receptor 2 (*MT2*) gene was identified in granulosa cells and oocytes (Jin *et al.*, 2022). Based on these studies, we suggested that melatonin may directly act on the oocyte itself or indirectly through granulosa cells. Furthermore, melatonin improved the adaptation of follicular cell response to the hypoxic environment and against oxidative damage induced by hypoxia (Tanabe *et al.*, 2015; Liu *et al.*, 2019a).

In summary, the present study showed that melatonin plays an important role in alleviating female fertility damage caused by acute hypoxic stress in mice, such as adjusting follicle development, maintaining the balance of proliferation and apoptosis in granulosa cells, decreasing ROS accumulation, and improving egg quality. Therefore, our findings support melatonin as a plausible treatment for reproductive diseases that coexist with hypoxia and oxidative stress. Future studies are required to understand the protection mechanism of melatonin against reproductive damage caused by hypoxic stress in female animals.

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Competing interests. The authors declare no conflicts of interest.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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