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Melatonin relieves heat-induced spermatocyte apoptosis in mouse testes by inhibition of ATF6 and PERK signaling pathways

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ABSTRACT

Normal spermatogenic processes require the scrotal temperature to be lower than that of the body as excessive heat affects spermatogenesis in the testes, reduces sperm quality and quantity, and even causes infertility. Endoplasmic reticulum stress (ERS) is a crucial factor in many pathologies. Although several studies have linked ERS to heat stress, researchers have not yet determined which ERS signaling pathways contribute to heat-induced testicular damage. Melatonin activates antioxidant enzymes, scavenges free radicals, and protects the testes from inflammation; however, few studies have reported on the influence of melatonin on heat-induced testicular damage. Using a murine model of testicular hyperthermia, we observed that heat stress causes both ERS and apoptosis in the testes, especially in the spermatocytes. These observations were confirmed using the mouse spermatocyte cell line GC2, where the *Atf6* and *Perk* signaling pathways were activated during heat stress. Knockout of the above genes effectively reduced spermatocyte damage caused by heat stress. Pretreatment with melatonin alleviated heat-induced apoptosis by inhibiting the *Atf6* and *Perk* signaling pathways. This mitigation was dependent on the melatonin receptors. *In vivo* experiments verified that melatonin treatment relieved heat-induced testicular damage. In conclusion, our results demonstrated that ATF6 and PERK are important mediators for heat-induced apoptosis, which can be prevented by melatonin treatment. Thus, our study highlights melatonin as a potential therapeutic agent in mammals for subfertility/infertility induced by testicular hyperthermia.

Keywords: Heat stress; ATF6; PERK; Melatonin; Spermatocyte

INTRODUCTION

Infertility affects 10%–15% of all reproductive-aged couples and is of major clinical, social, and economic concern (Lotti & Maggi, 2018). Male infertility mainly manifests by changes in sperm density, quantity, quality, or volume (Kumar & Singh, 2015). Scrotal temperature is currently recognized as an important factor of male infertility by the World Health Organization, with a strong link found between increased testicular temperature and decreased fertility (Jung &

Schuppe, 2007). Various factors, such as occupation, living habit, and clinical disease, can raise the temperature of the testes. Prolonged occupational heat exposure, such as for chefs and welders, can increase scrotal temperature, resulting in a decrease in semen quality (Thonneau et al., 1997). Long-term use of laptop computers and extended periods of sitting (e.g., professional drivers) can also increase scrotal temperature, leading to decreased fertility (Garolla et al., 2013).

Spermatogenesis requires the scrotal temperature to be lower than that of the body (Danno et al., 2000). Studies have shown that men with higher scrotal temperature show subfertility/infertility, and sperm quality worsens as the temperature increases (Mieusset et al., 1987). Spermatocytes and mature sperm cells are sensitive to changes in temperature. The cells most vulnerable to heat damage in humans and rats are zygotene and pachytene spermatocytes and early round spermatozoa (Cooke & Saunders, 2002). Heat stress damages spermatogenic cells through apoptosis, autophagy, DNA damage, and excessive production of reactive oxygen species. Increased testicular temperature also interferes with the function and morphology of Sertoli cells and disrupts the tight connection between cells and blood-testis barrier (Zhang et al., 2020).

The endoplasmic reticulum (ER) regulates cell function by controlling calcium ion homeostasis, lipid synthesis, and protein folding. When protein synthesis and transportation are impaired or Ca^{2+} uptake and release are dysfunctional, many unfolded or misfolded proteins accumulate in the ER cavity, causing ER stress (ERS) (Santofimia-Castaño et al., 2016). To resist the effects of ERS, cells initiate a protective unfolded protein response (UPR) to restore ER protein folding homeostasis (Hetz & Papa, 2018). ERS can be induced by three independent signaling pathways, including activated transcription factor 6 (ATF6), inositol-requiring enzyme (IRE1), and protein kinase R-like endoplasmic reticulum kinase (PERK) (Davenport et al., 2008). BiP/GRP78 is an important molecular chaperone in the ER lumen and plays an important role in the synthesis of related proteins in the cascade signaling pathway (Ibrahim et al., 2019).

The relationship between heat stress and ERS varies according to the intensity of heat stimulation and the type of cells involved. Heat stress usually accompanies other stress responses, as seen in bovine granulosa cells where *Nrf2*-mediated oxidative stress and ERS are triggered after heat stimulation (Alemu et al., 2018). Heat stress also has different effects on ERS signaling pathways. PERK reduces cell damage and maintains cell homeostasis by activating eIF2α phosphorylation (Khoutorsky et al., 2016). Furthermore, heat stress can cause the down-regulation of IRE1α, which effectively activates autophagy and then the UPR negative feedback system by regulating the IRE1α-XBP-1 axis (Homma & Fujii, 2016).

Melatonin (N-acetyl-5-methoxytryptamine) is a neuroendocrine hormone with strong antioxidant activities secreted by the pineal gland (Tan et al., 2007) and synthesized in the testes (Frungieri et al., 2017). This hormone exhibits a distinct circadian synthesis profile, with high levels at night and low levels during the day,

synchronized with the environmental light/dark cycle. Melatonin activates antioxidant enzymes, scavenges free radicals (Amaral & Cipolla-Neto, 2018), and protects the testes from inflammation (Frungieri et al., 2017). It has both fat-soluble and water-soluble characteristics and can directly pass through cell membranes (Claustrat & Leston, 2015). Melatonin can also regulate the homeostasis of apoptosis and autophagy. Phosphorylated BECLIN1 can bind to BCL2, increase free BAX in cells, and cause mitochondrial-dependent apoptosis (Pan et al., 2018). Melatonin improves spermatogonial stem cell apoptosis caused by reactive oxygen species and P53 by promoting the expression of MnSOD and SIRT1 (Li et al., 2018). Melatonin can alleviate reproductive damage caused by heat stress by reducing apoptosis and oxidative stress and strengthening the tight junctions between Sertoli cells (Zhang et al., 2020). The function of melatonin depends on melatonin receptors 1 and 2 (MT1 and MT2), which are G protein-coupled membrane receptors that can heterodimerize with the G protein receptor. Interactions between MT1 and G protein receptors can influence cellular functions (Samanta, 2020).

In the current study, we explored the relationship between ERS and heat-induced testicular damage and investigated the role of ATF6/PERK in spermatocyte apoptosis induced by heat stress. We also tested whether melatonin application protects against heat-induced testicular damage via activation of melatonin receptors and inhibition of ERS (Supplementary Figure S1).

MATERIALS AND METHODS

Ethics approval and consent to participate
All animal experiments were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of the People's Republic of China, Policy No. 2 006 398) and were approved by the Animal Care and Use Center of Northwest A&F University, Shaanxi, China (approval No. 201902A299).

Animals
Experiments were performed on male ICR mice (30–35 g, 9 weeks old) obtained from the Laboratory Animal Center of the Fourth Military Medical University, China. The mice were maintained at 20±2 °C and 50%–70% humidity under a 12 h:12 h light:dark cycle. All experimental animals and procedures were approved by the Institutional Animal Care and Use Committee of Northwest A&F University.

Induction of testicular heat stress
Healthy male ICR mice (Dashuo, China) were exposed to a single heat stress treatment at 42 °C. Specifically, the mice were anesthetized, and the lower part of the body, including the hind legs and scrotum, were submerged in a 42 °C thermostatic water bath for 20 min. Following treatment, the mice were placed in an animal room and provided with normal water and mouse chow for recovery over 6–24 h. Finally, the treated mice were euthanized, and testicular samples were collected. The mice were divided into four groups (control and heat-treated groups). For heat treatment, 15 mice were divided into three groups (6, 12, and 24 h) for one

independent experiment, with the treated mice analyzed at each time point. Three independent experiments were performed.

To explore whether melatonin pretreatment can protect mice from the effects of heat-induced testicular damage, the mice were injected with 20 mg/kg melatonin for 7 consecutive days before heat treatment. In the heat treatment group, 10 male mice were divided into two groups (con and pre-mel) in one independent experiment; in the no heat stress group, 10 male mice were also divided into two groups (con and pre-mel). The testes were extracted after 12 h of recovery. Three independent experiments were conducted.

Histological analysis

For histological analysis, testes were fixed in 4% paraformaldehyde (PFA) solution and testicular sample sections were embedded in paraffin and stained with hematoxylin and eosin (H&E). The integrity and structural modifications of tubule sections were evaluated semi-quantitatively, Briefly, a score between 0 and 5 was given for (i) nuclear and (ii) epithelial alterations, with 0 representing the complete absence of alteration and 5 representing the most severe alterations (Dumont et al., 2015; Milazzo et al., 2008).

Cells

The mouse spermatocyte cell line (GC-2) and human embryonic kidney epithelial cell line (293T) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) with 2 mmol/L L-glutamine (Invitrogen, Thermo Fisher Scientific, USA), 80 U/mL streptomycin, 100 U/mL penicillin (Invitrogen, Thermo Fisher Scientific, USA), and 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, USA). The cells were cultured at 37 °C in a humidified 5% CO_2 incubator and passaged with a 1:5 subculture ratio. Trypsin-EDTA solution (2–3 mL) was added to the flask and cells were observed under an inverted microscope until the cell layer was dispersed. The cells were dissociated by 0.25% trypsin-EDTA (Invitrogen, Thermo Fisher Scientific, USA) and inoculated to the culture dish, with the medium exchanged every 24 h.

Thermal stress induction in cells

Cells ($5×10^3$) were seeded in each well of a 48-well plate. After the cells adhered to the wall for 24 h, they were incubated at 42 °C for 2 h. The cells were then cultured normally for 4 h.

Reverse transcription

We used a FastKing RT Kit (Tiangen Biotech, China) for reverse transcription. Firstly, we used 2 µL 5×gDNA buffer and 1 µg RNA, with water to 10 µL, at 42 °C for 3 min. Secondly, we used 2 µL 10×King RT buffer, 1 µL FastKing RT enzyme mix, and 2 µL FQ-RT primer mix, with water to 10 µL, at 42 °C for 15 min and 95 °C for 3 min.

Quantitative real-time polymerase chain reaction (qRT-PCR)

We used 10 µL 2×SuperReal Color PreMix (Tiangen Biotech, China) for qRT-PCR, 0.2 µL reverse primer, 0.2 µL forward primer, 1 µL cDNA, and 8.6 µL distilled water. The qPCR instrument was purchased from BIO-RAD (CFX Connect Optics Module, Serial NO. 788BR08727, Singapore). The

reaction steps were: 95 °C for 15 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s, then 72 °C for 10 min, and 4 °C for permanent preservation. All datasets are expressed as fold-change relative to the control, i.e., delta Ct using *Gapdh* or *β-actin* as the housekeeping genes. The primers used for qRT-PCR are shown in Supplementary Table S1. Each primer was localized to the extron.

Immunofluorescence staining

The preparation of the cell and testis samples is described above. Immunofluorescence staining was performed as reported recently (Xu et al., 2020). All antibody details are given in Supplementary Table S2.

Western blot (WB) analysis

Protein was extracted from testicular tissue and cells using RIPA lysis buffer (Beyotime, China) with phosphatase inhibitor and phenylmethane sulfonyl fluoride (PMSF) (Beyotime, China). Details on WB analysis are described in our previous article (Yang et al., 2021). The antibodies used are shown in Supplementary Table S2. Semi-quantification of WB was performed via Image J.

TUNEL assay

Cell and testis sample treatment is described above. Cells were fixed with 4% PFA for 30 min and washed three times with phosphate-buffered saline (PBS). We added 0.3% Triton X-100 (Beyotime, China) to penetrate the cell membrane for 10 min, then washed the cells twice using PBS. The samples were added with 50 µL TUNEL assay solution (Beyotime, China) and incubated at 37 °C for 60 min, while protected from light. The cells were incubated with 1 µg/mL Hoechst 33 342 dye solution at room temperature for 5 min in the dark, then washed with PBS. The samples with apoptotic cells were visualized by fluorescence microscopy.

Testis sections were subjected to the following reagents in sequence: xylene (1) for 10 min, xylene (2) for 10 min, absolute ethanol for 3 min, 90% alcohol for 3 min, 70% alcohol for 3 min, and distilled water for 2 min. We added 20 µg/mL DNase-free proteinase K to the sections, which were then incubated at 25 °C for 20 min and washed three times with PBS. The slides were incubated with terminal deoxynucleotidyl transferase (TdT) enzyme for 1 h at 37 °C, followed by counterstaining with Hoechst 33 342 for 5 min. Finally, the sections were observed by fluorescence microscopy.

Flow cytometry

An Annexin V-FITC/PI apoptosis kit (Multisciences, China) was used to assess cell apoptosis distribution. Cells ($1×10^5$) were seeded in a 6-well plate and preprocessed with melatonin for 24 h. According to the description above, cells were induced by heat stress. The cells were then collected in 1.5 mL tubes and resuspended in 500 µL cold 1×binding buffer. To each tube was added 5 µL Annexin V-FITC and 10 µL PI, followed by incubation for 5 min at room temperature in the dark. Cells were analyzed on an EPICS ALTRA flow cytometer (Beckman Altra, USA). Annexin V-FITC (Ex= 488 nm; Em=530 nm) was detected using the FITC detection channel and propidium iodide (PI) (Ex=535 nm; Em=615 nm) was detected using the PI detection channel. The cells were

single stained, with the negative control group set to 0.1%–0.3%. Data were analyzed using Expo32 v1.2.

Knockdown of PERK and ATF6 in GC2 cells

Primer sequences for down-regulating PERK and ATF6 short hairpin RNAs (siRNAs) were designed using the Contech siRNA designer as follows:

siRNAs-PERK: GATCCGCAGGTCCTTGGTAATCATCTCAA GAGGATGATTACCAAGGACCTGCTTTTTTg
siRNAs-ATF6: AATTCAAAAAAAAGAGCAGATCGAAGTCAT ACTCTTGATATGACTTCGATCTGCTCTTg

The siRNAs (Sangon, China) were inserted into the CD513B-U6 vector to obtain sh-PERK-CD513B-U6 and sh-ATF6-CD513B-U6 eukaryotic expression plasmids. The inserts were verified and sequenced by Sangon Biotech (China). The inserts were verified using PCR and double restriction enzyme digestion with EcoR I and BamH I. The 293-T cells were used for lentiviral packaging. In total, 1×10^5 GC2 cells were cultured in a 6-well plate before infection with the lentivirus for 12 h. The infected cells were screened by puromycin and detected by qRT-PCR.

Statistical analysis

All data are presented as mean±standard deviation (*SD*) and were analyzed using unpaired two-tailed Student's *t*-tests. *P*-values of <0.05 and <0.01 were considered statistically significant and highly statistically significant, respectively.

RESULTS

Heat stress causes testicular damage in male mice

To identify the effects of hyperthermia, mouse testes were exposed to heat by immersing the scrotum into a 42 °C thermostatic water bath for 20 min. The mice were examined at 6, 12, and 24 h after heat treatment. Based on H&E staining, heat-stress exposure caused obvious testicular damage at 12 h (Figure 1A; Supplementary Figure S2A). Although there was no significant change in testicular size, the testicular weight/body weight ratio decreased at 12 h after heat treatment (Figure 1B, C). Based on TUNEL assay, apoptosis was found in the testicular tissue at 12 h after heat treatment (Figure 1D; Supplementary Figure S2B). The mRNA expression levels of *Caspase3*, *Caspase9*, *Caspase12*, and *Hsp 70* indicated that heat stress and apoptosis both occurred in the male mouse testes (Figure 1E). Hyperpyrexia enhanced the expression of HSP70 and cleaved CASPASE3 at the protein level (Figure 1F–G). Statistical analysis verified the above results. Hence, our results confirmed that heat stress can cause testicular apoptosis and damage.

Atf6 and Perk signaling pathways are enhanced by heat stress in mouse testes

To explain why heat stress induced testicular damage and apoptosis, we extracted mRNA from the heat-treated mouse testes and performed qRT-PCR for markers of ERS (*Grp78*) and key factors of the three signaling pathways (ATF6, IRE1, PERK). Results showed that the expression levels of ATF6, GRP78, and PERK were significantly higher in heat-treated mice (Figure 2A, B). Importantly, based on TUNEL and BOULE co-immunostaining, most apoptosis-positive cells

were located in the spermatocytes (Figure 2C). Immunofluorescence staining revealed that the heat-exposed testes mainly activated the ATF6 and p-PERK proteins rather than p-IRE1 (Figure 2D–F; Supplementary Figure S2C–E). Hence, we speculated that hyperthermia may promote activation of the *Atf6* and *Perk* signaling pathways, with spermatocytes being the most severely affected cells.

Heat stress induces spermatocyte apoptosis and ERS

We used the mouse spermatocyte cell line GC2 to address whether hyperthermia affected spermatocyte proliferation *in vitro*. qRT-PCR showed that the expression levels of *Hsp70* and *Hsp27*, members of the heat shock protein family expressed in large quantities when stimulated by heat, were up-regulated at each time point (0, 2, 4, and 6 h) after 42 °C exposure (Figure 3A). At 4 h after exposure to 42 °C, the mRNA expression levels of *Grp78*, *Chop*, and *Caspase3* and the ERS signaling pathways were significantly increased (Figure 3B, C). These results implied that heat stress promoted spermatocyte apoptosis and ERS, but the changes to different ERS signaling pathways were not consistent. Western blotting and statistical analysis showed that the expression levels of p-PERK and ATF6 increased significantly at different time points, while the phosphorylation level of IRE showed no significant or consistent change. The ER apoptotic protein CASPASE12 was also slightly up-regulated (Figure 3D, E). To verify the above results, immunofluorescence and TUNEL staining were used on GC2 cells after 2 h of heat treatment and 4 h of recovery. The number of TUNEL-positive cells increased significantly after heat stress (Figure 3F; Supplementary Figure S2F). The activation of HSP27 and HSP70 indicated that exposure to 42 °C caused heat stress in spermatocytes, while the high expression of GRP78 in the cytoplasm and localization of CHOP in the nucleus indicated the presence of ERS (Figure 3G, H; Supplementary Figure S2G). Moreover, the marked changes in the expression levels of p-PERK and ATF6 in comparison with IRE1 demonstrated that the three different ERS signaling pathways play different roles in heat-induced spermatocyte damage (Figure 3I; Supplementary Figure S2G). Thus, our results indicated that p-PERK and ATF6 play dominant roles in spermatocyte damage induced by high temperature.

Knockdown of ATF6 and PERK relieves heat-induced damage

To address whether ATF6 and PERK induced GC2 cell apoptosis, two cell lines were established, i.e., cells with ATF6 knocked down (si-ATF6) and cells with PERK knocked down (si-PERK). qRT-PCR showed an ~80% decrease in the expression levels of *Atf6* and *Perk* in cells following ATF6 and PERK knockdown (Figure 4A; Supplementary Figure S3A). As an internal control, western blotting and statistical analysis showed that the expression levels of ATF6 and p-PERK were reduced in si-RNA (Figure 4B, C). Both cell lines were exposed to 43 °C for 2 h individually, then incubated at 37 °C for 4 h. When the expression levels of ATF6 and PERK decreased in the GC2 cells, the expression levels of apoptotic genes *Caspase12*, *Caspase3*, and *Chop* also subsequently decreased (Figure 4D–F; Supplementary Figure S3B). Taken together, these results indicate that hyperthermia can induce
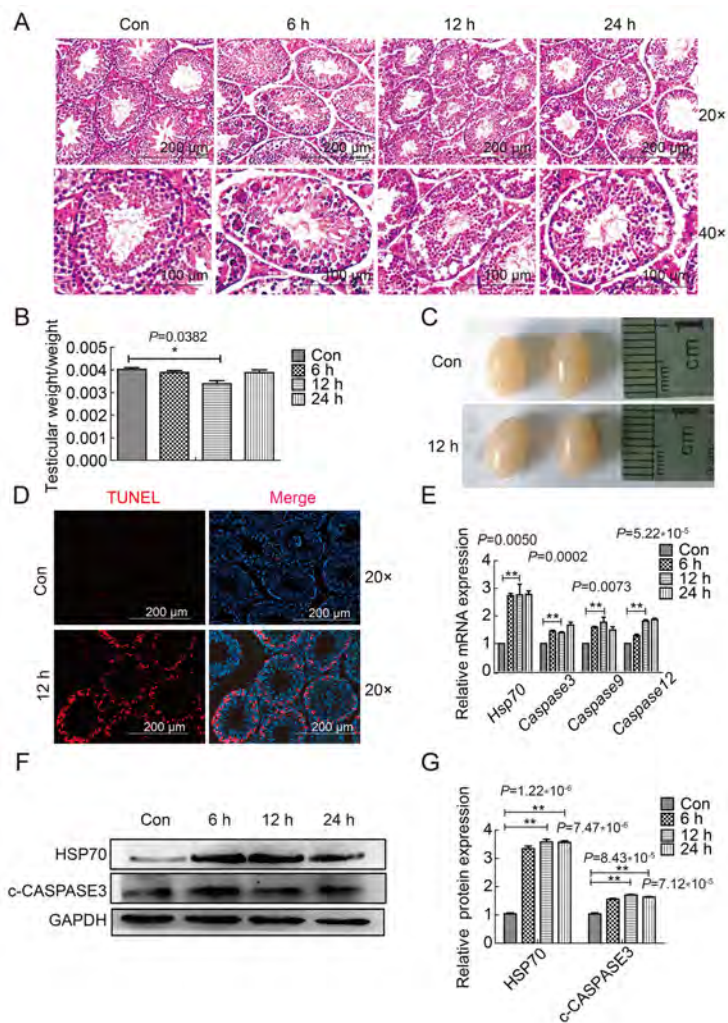
Figure 1 Heat stress causes testicular damage in male mice

A: Hematoxylin and eosin staining showing testicular damage at 6, 12, and 24 h after heat treatment. B: Testicular weight/body weight ratios of all mouse groups presented as a bar graph at 6, 12, and 24 h after heat treatment. C: Morphology of testes after 12 h of recovery. D: TUNEL staining. Red fluorescence indicates TUNEL positive. Hoechst 33342 (blue)-stained cell nuclei. E: qRT-PCR of *Hsp70*, *Caspase3*, *Caspase9*, and *Caspase12* expression. F: Western blotting of HSP70 and c-CASPASE3 expression at 6, 12, and 24 h after heat treatment. Con: Control. Data are mean±*SD* (*n*=3); statistical significance is expressed as: *: P<0.05; **: P<0.01.

spermatocyte apoptosis and ERS, while inhibition of ATF6 and PERK can significantly alleviate cell damage.

Melatonin reduces spermatocyte damage caused by heat stress via ATF6 and PERK

Melatonin is reported to regulate ERS, autophagy, and apoptosis in different cell types, such as tumor cells, tumor microenvironment (TME) cells, and liver cells (Fernández et al., 2015; Mortezaee et al., 2019). Therefore, we further explored the effects of melatonin on relieving spermatocyte damage induced by heat stress through ERS signaling pathways. Flow cytometry was used to determine the occurrence of cell apoptosis in melatonin and heat-treated spermatocytes. We found that the level of apoptosis with 10^{-6} mmol/L melatonin (6.4%) pretreatment was significantly lower than that in the heat-stress group (16.6%) 4 h after exposure (Figure 5A). The rate of apoptosis in the 10^{-6}

mmol/L melatonin-treated cells was lower than that in the heat-stress group, as determined by PI staining (Figure 5B, C). We detected the expression levels of markers of ERS and apoptosis in the testes of the four heat-treated groups at 4 h after heat treatment. Results showed that *Caspase3* and *Caspase12*, which are marker genes for apoptosis, decreased at the mRNA level following melatonin pretreatment (Figure 5E; Supplementary Figure S3C). The mRNA expression levels of *Perk* and *Atf6* were also inhibited by 10^{-7} melatonin pretreatment (Figure 5D; Supplementary Figure S3C). Western blot analysis showed that the changes in the protein levels of ATF6, p-PERK, c-CASPASE3, and CASPASE12 were consistent with the mRNA levels (Figure 5F–H). Our results suggest that melatonin inhibits the *Atf6* and *Perk* signaling pathways, and thus plays a protective role against heat-induced damage in spermatocytes.
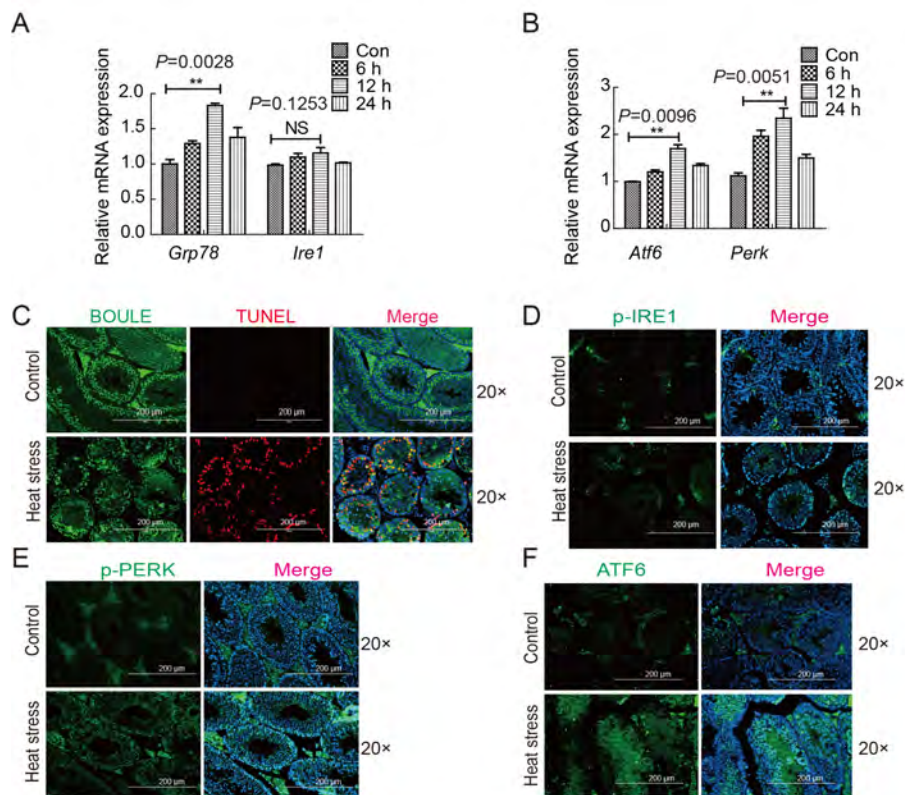
Figure 2 *Atf6* and *Perk* signaling pathways are enhanced by heat stress in testes

A, B: mRNA expression of ERS-related genes (*Grp78*, *Ire1*, *Perk* and *Atf6*) in testes at 6, 12, and 24 h after heat treatment. C: Immunofluorescence and TUNEL double staining showing spermatocyte apoptosis after 12 h of recovery from heat stress. D, E, F: Representative immunofluorescent images of pIRE1 (D), pPERK (E), and ATF6 (F) All images were merged from pIRE1, pPERK, or ATF6 (green) and Hoechst 33342-stained nuclei (blue). Scale bar: 200 µm. Con: Control. Data are mean±*SD* (*n*=3); statistical significance is expressed as: ** : *P*<0.01. NS: Not significant.

MT1/MT2 receptors are essential for melatonin to relieve heat stress in spermatocytes

Melatonin activates two G protein-coupled receptors, MT1 and MT2, to exert beneficial actions in a variety of physiological processes (Liu et al., 2016). To evaluate the importance of melatonin receptors in inhibiting ERS and apoptosis, we performed western blotting to detect the expression levels of MT1 and MT2 in spermatocytes pretreated with melatonin. Results confirmed that MT1 and MT2 were activated in the melatonin-pretreated spermatocytes (Figure 6A, B). Immunofluorescence staining also showed that the expression of MT1 and MT2 increased significantly upon the addition of melatonin (Figure 6C; Supplementary Figure S2H). Furthermore, when luzindole was used as an inhibitor against MT1 and MT2 expression, suppression was observed (see Figure 3), and the protective effects of melatonin on spermatocyte damage caused by heat stress were eliminated (Figure 6D, E). These data suggest that the effects of melatonin on spermatocytes are mediated by the MT1 and MT2 receptors.

Melatonin alleviates apoptosis of testicular spermatocytes caused by heat stress in vivo

To verify the potential of melatonin as a clinical drug to alleviate heat stress, we conducted a complementary experiment in male mice. Before 42 °C heat treatment to the testes, mice were exposed to consecutive injections of melatonin (20 mg/kg bw/day) for 7 days, with testicular tissue sections subsequently examined by H&E staining. In the group without melatonin pretreatment, clumps of cells appeared in the lumen and the tubules showed vacuolization after heat treatment. In contrast, in the melatonin pretreatment group, numerous tubules were seen with normal seminiferous epithelium and vacuolization was rarely observed (Figure 7A). TUNEL staining was used to further examine the anti-apoptotic effects of melatonin on the testes in heat-stressed mice. The rate of apoptosis in testes treated with melatonin was significantly lower than that in the heat-stress treatment group (Figure 7B; Supplementary Figure S2I). To evaluate the effects of melatonin on inhibiting ERS, we used western blotting to detect the expression levels of PERK and ATF6 in testes pretreated with melatonin. Results confirmed that PERK and ATF6 were activated in the melatonin-pretreated group (Figure 7C). Immunofluorescence staining showed that the expression of ATF6 was significantly reduced in the melatonin-pretreated group (Figure 7D). Cumulatively, our data indicate that melatonin can protect the testes from damage caused by heat stress.

DISCUSSION

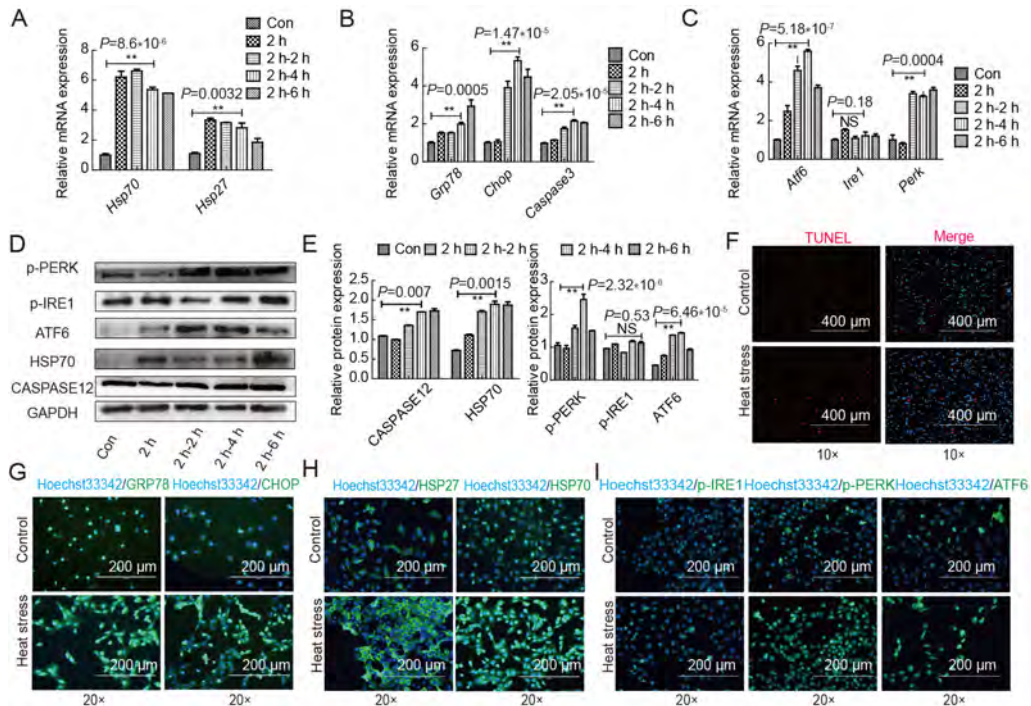As global temperatures rise, heat stress has become an

Figure 3 Heat stress induces spermatocyte apoptosis and endoplasmic reticulum stress

A: mRNA expression of heat shock genes (*Hsp27* and *Hsp70*) in spermatocytes (GC2 cell line) at 0, 2, 4, and 6 h after heat treatment for 2 h. B: mRNA expression of apoptosis genes (*Caspase3* and *Chop*) and Grp78 in spermatocytes at 0, 2, 4, and 6 h after heat treatment for 2 h. C: mRNA expression of ERS-related genes (*Ire1*, *Perk* and *Atf6*) in spermatocytes at 0, 2, 4, and 6 h after heat treatment for 2 h. D: Western blotting of ERS-related proteins (p-IRE1, p-PERK, and ATF6), apoptosis protein (CASPASE12), and heat shock protein (HSP70) in spermatocytes at 0, 2, 4, and 6 h after heat treatment for 2 h. E: Image J analysis of (D). F: TUNEL staining for apoptosis at 4 h after heat treatment for 2 h. Cells showing red fluorescence are positive. Hoechst 33342 (blue)-stained cell nuclei. G: Immunofluorescent images showing GRP78 and CHOP in spermatocytes at 4 h after heat treatment for 2 h. H: Immunofluorescent images showing heat shock proteins (HSP27 and HSP70) in spermatocytes at 4 h after heat treatment for 2 h. I: Immunofluorescent images showing ERS-related proteins (p-IRE1, p-PERK, and ATF6) in spermatocytes at 4 h after heat treatment for 2 h. All images were merged from pIRE1, pPERK, or ATF6 (green) and Hoechst 33342-stained nuclei (blue). Scale bar: 200 μm. Con: Control, hs: Heat stress. Data are mean±*SD* (*n*=3); statistical significance is expressed as: **: *P*<0.01, NS: Not significant.
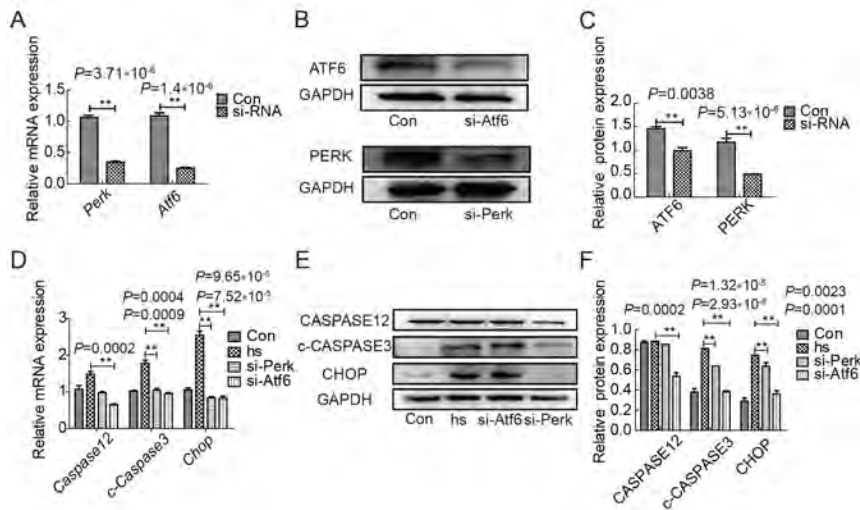

Figure 4 Knockdown of ATF6 and PERK relieves heat-induced damage

A: qRT-PCR of Perk and Atf6 mRNA expression in si-Perk and si-Ptf6 cell lines. B: Western blotting of PERK and ATF6 expression in si-Perk and si-Atf6 cell lines. C: Image J analysis of (B). D: qRT-PCR of apoptosis-related genes (*Caspase12*, *Caspase3*, and *Chop*) in si-Perk and si-ATF6 cell lines at 4 h after heat treatment for 2 h. E: Western blotting of apoptosis-related protein (CASPASE12, CASPASE3, CHOP) expression in si-Perk and si-ATF6 cell lines at 4 h after heat treatment for 2 h. F: Image J analysis of (E). Con: Control, hs: Heat stress. Data are mean±*SD* (*n*=3); statistical significance is expressed as: **: *P*<0.01.
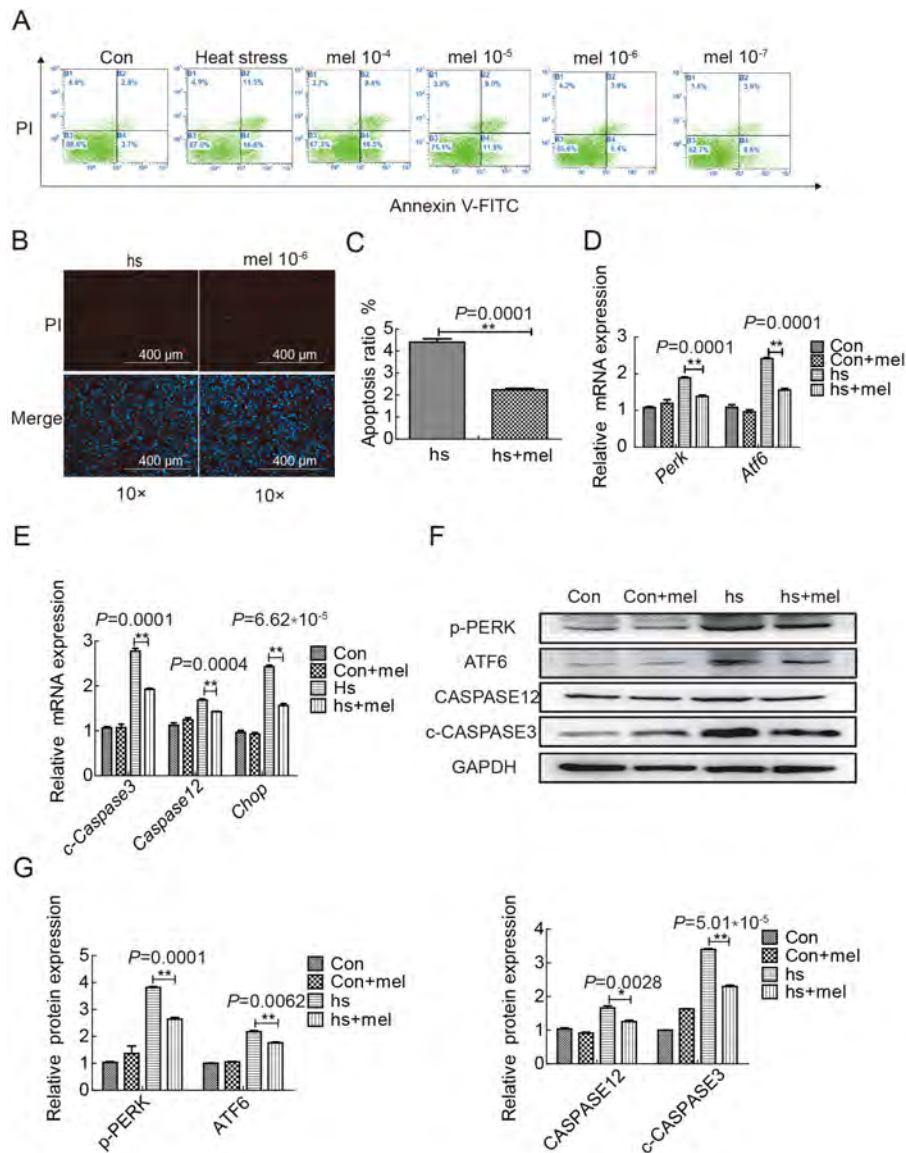
Figure 5 Melatonin relieves spermatocyte damage caused by heat stress through ATF6 and PERK

A: Flow cytometry was used to determine cell apoptosis in different melatonin treatment groups (i.e., con, 0, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} mmol/L) at 4 h after heat treatment. B: PI staining was used to detect apoptosis in 10^{-6} mmol/L melatonin-treated spermatocytes at 4 h after heat treatment. C: Statistical analysis of (B). D: qRT-PCR of ERS-related genes (*Perk* and *Atf6*) in 10^{-6} mmol/L melatonin-treated spermatocytes at 4 h after heat treatment. E: qRT-PCR of apoptosis-related genes (*Caspase3*, *Caspase12*, and *Chop*) in different groups, i.e., con, con+mel (10^{-6} mmol/L melatonin-treated spermatocytes), hs (4 h after heat treatment for 2 h), and hs+mel (10^{-6} mmol/L melatonin-treated spermatocytes at 4 h after heat treatment). F: Western blotting of apoptosis-related proteins (CASPASE12 and c-CASPASE3) and ERS-related proteins (p-PERK and ATF6) in con, con+mel 10^{-6} μmol/L melatonin-treated spermatocytes), hs (4 h after heat treatment for 2 h), and hs+mel (10^{-6} mmol/L melatonin-treated spermatocytes at 4 h after heat treatment) groups. G, H: Image J analysis of (F). Con: Control, hs: Heat stress, mel: melatonin. Data are mean±*SD* (*n*=3); statistical significance is expressed as: *: *P*<0.05; **: *P*<0.01.

important factor affecting the reproductive performance of mammals and increases the risk of subfertility/infertility in humans (Leyk et al., 2019). Thus, research on methods that effectively retain fertility during heat stress is crucial. In the current study, we explored the use of melatonin as a potential target for remedying heat-induced testicular damage and spermatocyte apoptosis, and elucidated the underlying protective mechanism of melatonin, which acts via modulation of the ATF and p-PERK signaling pathways.

During spermatocyte meiosis and sperm cell formation, thick spermatocytes and round spermatids are particularly sensitive to high temperature (Yadav et al., 2018). This was evident from our experiments, which showed an increase in the expression of HSP70 after scrotal heat treatment for 20 min at 42 °C. The testes also showed a series of injuries, such as testicular atrophy and tubule vacuolation. Spermatocyte
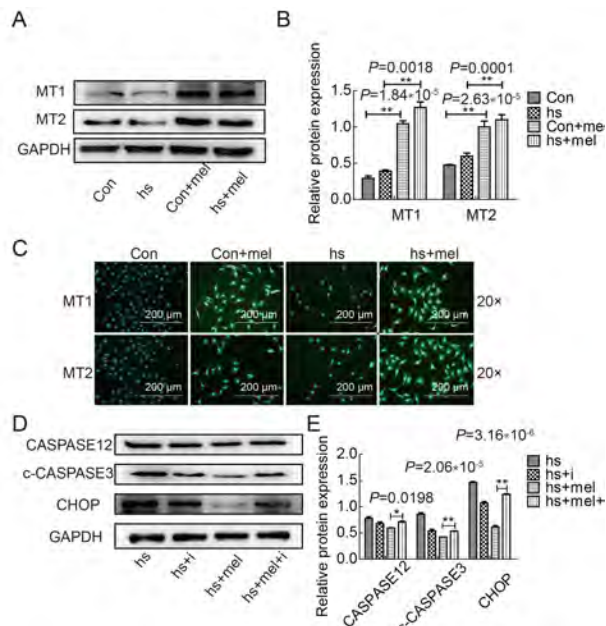
Figure 6 MT1/MT2 receptors are essential for melatonin to relieve heat stress in spermatocytes

A: Western blotting of melatonin receptor protein expression (MT1 and MT2) in con, hs (4 h after heat treatment for 2 h), con+mel (10^{-6} mmol/L melatonin-treated spermatocytes), and hs+mel (10^{-6} mmol/L melatonin-treated spermatocytes at 4 h after heat treatment) groups. B: Image J analysis of (A). C: Immunofluorescent images showing melatonin receptor protein (MT1 and MT2) in con, hs, con+mel, and hs+mel groups. D: Western blotting of apoptosis-related proteins (CASPASE12, c-CASPASE3, and CHOP) in hs (4 h after heat treatment for 2 h), hs+i (luzindole), hs+mel (10^{-6} mmol/L melatonin-treated spermatocytes at 4 h after heat treatment), and hs+mel+i (luzindole). E: ImageJ analysis of (D). Con: Control, hs: Heat stress, mel: melatonin. Data are mean±*SD* (*n*=3); statistical significance is expressed as: *: *P*<0.05; **: *P*<0.01.

Figure 7 Melatonin alleviates apoptosis of testicular spermatocytes caused by heat stress *in vivo*

A: Hematoxylin and eosin staining of testicular damage in con, mel, hs, and hs+mel groups. B: TUNEL staining (red fluorescence represents TUNEL positive staining), Hoechst 33342 (blue)-stained cell nuclei. C: Western blotting of ERS-related proteins (ATF6 and p-PERK) in con+mel, con, hs, and hs+mel groups. D: Immunofluorescent images showing ATF6 in con+mel, con, hs, and hs+mel groups. Con: Control, hs: Heat stress, mel: melatonin. Data are mean±*SD* (*n*=3); statistical significance is expressed as: *: *P*<0.05; **: *P*<0.01.

apoptosis was induced in the testes shortly after heat treatment. Studies have shown that heat stress induces ERS in the spermatocytes of mice and activates the UPR, leading to apoptosis (Kim et al., 2013). Chemicals such as BIS phenol-α and diethylstilbestrol disrupt ER homeostasis by inducing IRE1 phosphorylation and CHOP expression in rats, which has a toxic effect on testicular spermatogenesis (Jiang et al., 2016). Different ERS pathways play different roles in response to heat stress. P-EIF2 can reduce the pathological thermal sense of the dorsal root ganglia and reduce cell damage and maintain cell homeostasis (Khoutorsky et al., 2016). In the present study, we demonstrated the relationship between the ERS signaling pathways and spermatocyte apoptosis in mouse testes exposed to heat stress. We also evaluated the response of ERS-related genes and found that the *Atf6* and *Perk* signaling pathways play important roles in thermal stress in mouse testes. In contrast, IRE1 was not found to play an obvious role in thermal stimulus recovery. Previous studies have shown that heat stress in HeLa cells can lead to down-regulation of IRE1, activation of autophagy, and activation of the UPR negative feedback system through
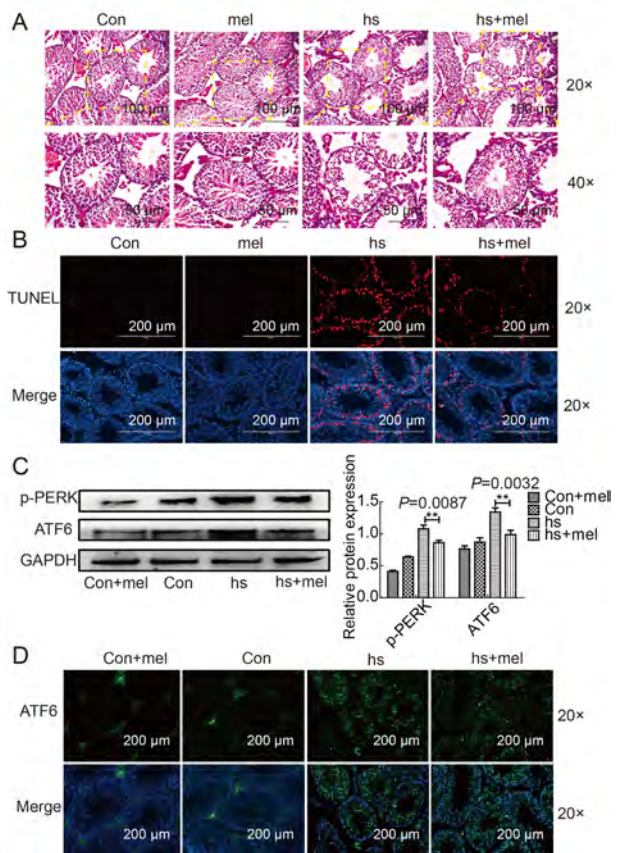
the regulation of the IRE1-XBP1 axis (Homma & Fujii, 2016). Therefore, the role of IRE1 as a special target in heat stress needs to be further explored. The activation of *Chop* is known to induce ERS and apoptosis (Kim et al., 2020). Our results further substantiated that heat stress can activate ERS-associated apoptotic genes, such as *Caspase12* and *Chop*. Thus, heat stress appears to influence spermatocyte homeostasis, potentially causing male subfertility and inferior sperm quality.

Melatonin is a hormone synthesized and secreted by the pineal gland and the testes. As a powerful antioxidant, melatonin plays a direct physiological role in the regulation of sperm function and an important protective role in testicular development and testosterone synthesis. As a local regulator, melatonin directly affects the synthesis and secretion of androgens by mesenchymal cells and regulates the production of steroid hormones by supporting cell proliferation and energy metabolism, thus affecting spermatogenesis (Yu

et al., 2018). Melatonin can also regulate apoptosis and autophagy homeostasis. Under this state, phosphorylated BECLIN1 binds to BCL2, damages the BCL2 and BAX complex, increases free BAX in cells, and causes mitochondria-dependent apoptosis (Pan et al., 2018). Melatonin can reduce reproductive damage caused by heat stress and improve the tight connections of supporting cells by reducing apoptosis and oxidative stress (Zhang et al., 2020). Our results are consistent with previous reports indicating that melatonin can effectively alleviate different ERS signaling pathways and reduce spermatocyte apoptosis (Cui et al., 2017). The effects of melatonin are dose dependent, with excessively high concentrations not only failing to relieve apoptosis caused by heat stress, but also aggravating cell death; in contrast, excessively low concentrations show no significant effects (unpublished data). In the present work, a melatonin concentration of 10^{-7} was found to be highly effective for spermatocyte apoptosis remission, as detected by flow cytometry. Melatonin inhibited the *Atf6* and *Perk* signaling pathways, down-regulated the expression of *Chop* and *Caspase12*, and reduced the apoptosis of spermatocytes caused by high temperature. In mammals, melatonin activity is often mediated through its interactions with the G protein-coupled MT1 and MT2 receptors (Dubocovich et al., 2010). In the spermatocyte cell line GC2, the role of melatonin was dependent on both MT1 and MT2. This was confirmed using specific melatonin inhibitors to inhibit the two receptors, which eliminated the protective effects of melatonin on apoptosis and subsequently on ERS. *In vivo* studies were also carried out to test the clinical potential of melatonin using continuous intraperitoneal injections of melatonin. Results confirmed our hypothesis that melatonin could prevent testicular injury caused by thermal stimulation and reduce testicular cell apoptosis.

In conclusion, melatonin showed considerable potential as an agent to reduce spermatocyte apoptosis and testicular damage caused by hyperthermia. By inhibiting the *Atf6* and *Perk* signaling pathways, we found that the three different ERS signaling pathways play different roles in heat stress (Figure 8). This study offers valuable support for the (pre-) clinical practice of melatonin treatment, which could help protect the testes from damage caused by heat stimulation and thus improve male reproductive health.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

D.Z.Q., J.L.H. and S.P. conceived and designed the study. D.Z.Q., H.C., D.H.Y., W.L.H., C.H., J.S. and B.L.L. performed the experiments. D.Z.Q. performed statistical analyses. D.Z.Q., H.C., and S.P. wrote and edited the manuscript. All authors read and approved the final version of the manuscript.
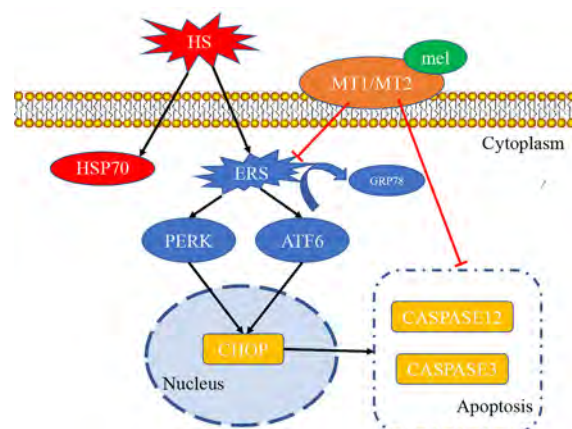

Figure 8 Illustration of melatonin relieving heat-induced spermatocyte apoptosis in mouse testes

In this study, we concluded that heat stress causes both ERS and apoptosis in testes, especially in spermatocytes, and p-PERK and ATF6 play dominant roles in spermatocyte damage induced by high temperature. Furthermore, pretreatment with melatonin alleviates heat-induced apoptosis by inhibiting ATF6 and PERK signaling pathways. This mitigation depends on melatonin receptors (MT1/MT2).

REFERENCES

Alemu TW, Pandey HO, Wondim DS, Gebremedhn S, Neuhof C, Tholen E, et al. 2018. Oxidative and endoplasmic reticulum stress defense mechanisms of bovine granulosa cells exposed to heat stress. *Theriogenology*, **110**: 130−141.

Claustrat B, Leston J. 2015. Melatonin: physiological effects in humans. *Neurochirurgie*, **61**(2–3): 77−84.

Cooke HJ, Saunders PTK. 2002. Mouse models of male infertility. *Nature Reviews Genetics*, **3**(10): 790−801.

Cui Y, Ren L, Li B, Fang J, Zhai Y, He X, et al. 2017. Melatonin relieves busulfan-induced spermatogonial stem cell apoptosis of mouse testis by inhibiting endoplasmic reticulum stress. *Cellular Physiology and Biochemistry*, **44**(6): 2407−2421.

Danno S, Itoh K, Matsuda T, Fujita J. 2000. Decreased expression of mouse Rbm3, a cold-shock protein, in Sertoli cells of cryptorchid testis. *The American Journal of Pathology*, **156**(5): 1685−1692.

Davenport EL, Morgan GJ, Davies FE. 2008. Untangling the unfolded protein response. *Cell Cycle*, **7**(7): 865−869.

do Amaral FG, Cipolla-Neto J. 2018. A brief review about melatonin, a pineal hormone. *Archives of Endocrinology and Metabolism*, **62**(4): 472−479.

Dubocovich ML, Delagrange P, Krause DN, Sugden D, Cardinali DP, Olcese J. 2010. International Union of Basic and Clinical Pharmacology. LXXV. Nomenclature, classification, and pharmacology of G protein-coupled melatonin receptors. *Pharmacological Reviews*, **62**(3): 343−380.

Dumont L, Arkoun B, Jumeau F, Milazzo JP, Bironneau A, Liot D, et al. 2015. Assessment of the optimal vitrification protocol for pre-pubertal mice testes leading to successful in vitro production of flagellated spermatozoa. *Andrology*, **3**(3): 611−625.

Fernández A, Ordóñez R, Reiter RJ, González-Gallego J, Mauriz JL. 2015. Melatonin and endoplasmic reticulum stress: relation to autophagy and apoptosis. *Journal of Pineal Research*, **59**(3): 292−307.

Frungieri MB, Calandra RS, Rossi SP. 2017. Local actions of melatonin in

somatic cells of the testis. *International Journal of Molecular Sciences*, **18**(6): 1170.

Garolla A, Torino M, Sartini B, Cosci I, Patassini C, Carraro U, et al. 2013. Seminal and molecular evidence that sauna exposure affects human spermatogenesis. *Human Reproduction*, **28**(4): 877−885.

Hetz C, Papa FR. 2018. The unfolded protein response and cell fate control. *Molecular Cell*, **69**(2): 169−181.

Homma T, Fujii J. 2016. Heat stress promotes the down-regulation of IRE1α in cells: an atypical modulation of the UPR pathway. *Experimental cell Research*, **349**(1): 128−138.

Ibrahim IM, Abdelmalek DH, Elfiky AA. 2019. GRP78: a cell's response to stress. *Life Sciences*, **226**: 156−163.

Jiang X, Chen HQ, Cui ZH, Yin L, Zhang WL, Liu WB, et al. 2016. Low-dose and combined effects of oral exposure to bisphenol A and diethylstilbestrol on the male reproductive system in adult Sprague-Dawley rats. *Environmental Toxicology and Pharmacology*, **43**: 94−102.

Jung A, Schuppe HC. 2007. Influence of genital heat stress on semen quality in humans. *Andrologia*, **39**(6): 203−215.

Khoutorsky A, Sorge RE, Prager-Khoutorsky M, Pawlowski SA, Longo G, Jafarnejad SM, et al. 2016. eIF2α phosphorylation controls thermal nociception. *Proceedings of the National Academy of Sciences of the United States of America*, **113**(42): 11949−11954.

Kim JH, Park SJ, Kim TS, Park HJ, Park J, Kim BK, et al. 2013. Testicular hyperthermia induces Unfolded Protein Response signaling activation in spermatocyte. *Biochemical and Biophysical Research Communications*, **434**(4): 861−866.

Kim SY, Hwang S, Choi MK, Park S, Nam KY, Kim I. 2020. Molecular mechanisms underlying the effects of the small molecule AMC-04 on apoptosis: roles of the activating transcription factor 4-C/EBP homologous protein-death receptor 5 pathway. *Chemico-Biological Interactions*, **332**: 109277.

Kumar N, Singh AK. 2015. Trends of male factor infertility, an important cause of infertility: a review of literature. *Journal of Human Reproductive Sciences*, **8**(4): 191−196.

Leyk D, Hoitz J, Becker C, Glitz KJ, Nestler K, Piekarski C. 2019. Health risks and interventions in exertional heat stress. *Deutsches Arzteblatt International*, **116**(31–32): 537−544.

Li B, He X, Zhuang MR, Niu BW, Wu CY, Mu HL, et al. 2018. Melatonin ameliorates busulfan-induced spermatogonial stem cell oxidative apoptosis in mouse testes. *Antioxidants & Redox Signaling*, **28**(5): 385−400.

Liu JB, Clough SJ, Hutchinson AJ, Adamah-Biassi EB, Popovska-Gorevski M, Dubocovich ML. 2016. MT$_1$ and MT$_2$ melatonin receptors: a therapeutic perspective. *Annual Review of Pharmacology and Toxicology*, **56**: 361−383.

Lotti F, Maggi M. 2018. Sexual dysfunction and male infertility. *Nature Reviews Urology*, **15**(5): 287−307.

Mieusset R, Bujan L, Mondinat C, Mansat A, Pontonnier F, Grandjean H. 1987. Association of scrotal hyperthermia with impaired spermatogenesis in infertile men. *Fertility and Sterility*, **48**(6): 1006−1011.

Milazzo JP, Vaudreuil L, Cauliez B, Gruel E, Massé L, Mousset-Siméon N, et al. 2008. Comparison of conditions for cryopreservation of testicular tissue from immature mice. *Human Reproduction*, **23**(1): 17−28.

Mortezaee K, Najafi M, Farhood B, Ahmadi A, Potes Y, Shabeeb D, et al. 2019. Modulation of apoptosis by melatonin for improving cancer treatment efficiency: an updated review. *Life Science*, **228**: 228−241.

Pan P, Zhang HM, Su LX, Wang XT, Liu DW. 2018. Melatonin balance the autophagy and apoptosis by regulating UCP2 in the LPS-induced cardiomyopathy. *Molecules*, **23**(3): 675.

Samanta S. 2020. Melatonin: an endogenous miraculous indolamine, fights against cancer progression. *Journal of Cancer Research and Clinical Oncology*, **146**(8): 1893−1922.

Santofimia-Castaño P, Izquierdo-Alvarez A, de la Casa-Resino I, Martinez-Ruiz A, Perez-Lopez M, Portilla JC, et al. 2016. Ebselen alters cellular oxidative status and induces endoplasmic reticulum stress in rat hippocampal astrocytes. *Toxicology*, **357–358**: 74−84.

Tan DX, Manchester LC, Terron MP, Flores LJ, Reiter RJ. 2007. One molecule, many derivatives: a never-ending interaction of melatonin with reactive oxygen and nitrogen species?. *Journal of Pineal Research*, **42**(1): 28−42.

Thonneau P, Ducot B, Bujan L, Mieusset R, Spira A. 1997. Effect of male occupational heat exposure on time to pregnancy. *International Journal of Andrology*, **20**(5): 274−278.

Xu SS, Qin DZ, Yang H, He C, Liu WQ, Tian N, et al. 2020. SerpinB1 promotes the proliferation of porcine pancreatic stem cells through the STAT3 signaling pathway. *The Journal of Steroid Biochemistry and Molecular Biology*, **198**: 105537.

Yadav SK, Pandey A, Kumar L, Devi A, Kushwaha B, Vishvkarma R, et al. 2018. The thermo-sensitive gene expression signatures of spermatogenesis. *Reproductive Biology and Endocrinology*, **16**(1): 56.

Yang DH, Wei YD, Lu QZ, Qin DZ, Zhang MF, Du XM, et al. 2021. Melatonin alleviates LPS-induced endoplasmic reticulum stress and inflammation in spermatogonial stem cells. *Journal of Cellular Physiology*, **236**(5): 3536−3551.

Yu K, Deng SL, Sun TC, Li YY, Liu YX. 2018. Melatonin regulates the synthesis of steroid hormones on male reproduction: a review. *Molecules*, **23**(2): 447.

Zhang PF, Zheng Y, Lv YH, Li FY, Su LH, Qin YW, et al. 2020. Melatonin protects the mouse testis against heat-induced damage. *Molecular Human Reproduction*, **26**(2): 65−79.