Melatonin protects the mouse testis against heat-induced damage

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Abstract

Spermatogenesis, an intricate process occurring in the testis, is responsible for ongoing production of spermatozoa and thus the cornerstone of lifelong male fertility. In the testis, spermatogenesis occurs optimally at a temperature 2-4 °C lower than that of the core body. Increased scrotal temperature generates testicular heat stress and later causes testicular atrophy and spermatogenic arrest, resulting in a lower sperm yield and therefore impaired male fertility. Melatonin (N-acetyl-5-methoxytryptamine), a small neuro-hormone synthesized and secreted by the pineal gland and the testis, is widely known as a potent free-radical scavenger; it has been reported that melatonin protects the testis against inflammation and reactive oxygen species generation thereby playing anti-inflammatory, -oxidative and -apoptotic roles in the testis. Nevertheless, the role of melatonin in the testicular response to heat stress has not been studied. Here, by employing a mouse model of testicular hyperthermia, we systematically investigated the testicular response to heat stress as well as the occurrence of autophagy, apoptosis and oxidative stress in the testis. Importantly, we found that pre-treatment with melatonin attenuated heat-induced apoptosis and oxidative stress in the testis. Also, post-treatment with melatonin promoted recovery of the testes from heat-induced damage, probably by maintaining the integrity of the Sertoli cell tight-junction. Thus, we for the first time provide the proof-of-concept that melatonin can protect the testis against heat-induced damage, supporting the potential future use of melatonin as a therapeutic drug in men for sub/infertility incurred by various testicular hyperthermia factors.
Keywords

Melatonin; testis; spermatogenesis; apoptosis; autophagy; heat stress
Introduction

Infertility, defined as a failure to conceive after 1 year of unprotected coitum (Zegers-Hochschild et al. , 2009), is of major clinical, social and economic concerns in this day and age. Infertility affects 10-15% of couples of reproductive age, and in about half of the cases a male factor is often involved, characterized by a decline in sperm density, count, quality or seminal volume (Kumar and Singh, 2015, Sharlip et al. , 2002). Factors undermining male fertility include chemo/radio-therapy, electromagnetic fields, microbial and parasitic infection, cryptorchidism, varicocele, testicular inflammation and hyperthermia (Kumar and Singh, 2015), etc. Of these, testicular hyperthermia, often caused by high scrotal temperature, is deleterious to spermatogenesis and a contributor to the lowered sperm quality and even male infertility (Durairajanayagam et al., 2015).

In the testis, spermatogenesis occurs optimally at a temperature 2-4 °C lower than that of the core body, and an increase of 1 °C can be accompanied by a 14% decline in spermatogenesis (Wang et al. , 1997). Increased scrotal temperature generates testicular heat stress and later causes testicular atrophy and spermatogenic arrest (Munkelwitz and Gilbert, 1998), resulting in a lower sperm yield and therefore impaired male fertility. Previous studies have demonstrated that male germ cells are generally susceptible to heat stress, with pachytene and diplotene spermatocytes as well as early round spermatids being most vulnerable (Lue et al. , 1999). The responses of male germ cells to testicular hyperthermia include germ cell apoptosis (Absalan et al. , 2010, Yin et al. , 1997) and autophagy (Eisenberg-Lerner et al., 2009, Zhang et al. , 2012), as well as generation of reactive oxygen species (ROS) (Ahotupa
and Huhtaniemi, 1992, Ikeda et al., 1999, Ishikawa et al., 2005, Li et al., 2006, Lue et al., 2003, Paul et al., 2009a). In addition, the sperm DNA integrity is damaged, as shown by defective synapsis and DNA strand breaks (Paul et al., 2008, Paul et al., 2009b). Testicular somatic cells, such as Sertoli cells and Leydig cells, are also influenced by heat stress, with the compromised ability to support germ cell development (Aktas and Kanter, 2009, Cai et al., 2011, Chen et al., 2008). Despite the involvement of all these factors, the various responses can occur in parallel, through diverse pathways, and there seems to be some degree of crosstalk among them.

Melatonin (N-acetyl-5-methoxytryptamine) is a small neuro-hormone mainly synthesized and secreted by the pineal gland (Ji et al., 2012b, Reiter et al., 2017, Schaefer and Hardeland, 2009). Also synthesized by the testis (Frungieri et al., 2017), melatonin is able to promote testicular development by modulating testosterone secretion, FSH action, as well as proliferation and differentiation of testicular somatic cells and germ cells (Baburski et al., 2015, Frungieri et al., 2017, Heindel et al., 1984). Melatonin is widely known as a potent free-radical scavenger; it has been reported that melatonin protects the testis against inflammation and ROS generation thereby playing anti-inflammatory, -oxidative and -apoptotic roles in the testis (Frungieri et al., 2017). By using animal models, increasing evidence has demonstrated that melatonin can relieve testicular damage resulting from, for example hyperlipidemia (Ji et al., 2012a), gonadal torsion (Kanter, 2010), varicocele (Semercioz et al., 2003b), irradiation (Take et al., 2009), vitrification (Hemadi et al., 2014) or busulfan treatment (Cui et al., 2017, Li et al., 2018). Melatonin may also play direct roles in male germ cell development (Frungieri et al., 2017), as it can
enhance the efficiency of spermatogonial stem cell (SSC) transplantation (Navid et al., 2017b) and facilitate SSC differentiation into haploid germ cells in vitro (Deng et al., 2016). Nevertheless, the impact of melatonin on the response to testicular hyperthermia, if any, has not been investigated.

Here, by employing a testicular hyperthermia mouse model, we systematically investigated the testicular response to heat stress, the occurrence of autophagy, apoptosis and oxidative stress in the testis and, more importantly, the utility of melatonin pre/post-treatment as a preventive measure for the heat-induced testicular insults. The underlying mechanisms were explored as well. Overall, the principal aim of the present study was to identify the role of melatonin in the response to testicular hyperthermia and as a result we, for the first time, provide the proof-of-concept that melatonin can indeed protect the testis against heat stress.
Materials and Methods

Animals

C57BL/6J mice were obtained from the laboratory animal center of Fourth Military Medical University, China. The mice were kept at 20 ± 2°C and 50-70% humidity under a 12h light-dark cycle. All experimental animals and procedures used in this study were approved by the Institutional Animal Care and Use Committee of Northwest A&F University.

Induction of testicular hyperthermia

Male C57BL/6J mice aged 10-12 weeks, with the body weight (b.w.) 22-25g and core body temperature 32 °C were subjected to single heat stress treatment at 39°C or 42°C. Technically, the mice were sedated, and the lower parts of the body (hind legs, tail and scrotum) were submerged in a thermostatically controlled water bath for 20min. Following the treatment, these mice were dried, returned to the animal room, and housed with food and water provided ad libitum. The treated mice were then allowed to recover for 6h-35d before sacrifice. In the 39°C or 42°C treatment group, 25 mice were used in one independent experiment, with five mice analyzed at each time point (6h, 12h, 24h, 48h and 7d) after treatment. Three independent experiments were performed, and 165 mice were used in total.

To investigate the protective effects of pre-treatment with melatonin against heat-induced testicular damage, the mice were injected i.p. with 20mg/kg b.w. melatonin (M5250, Sigma-Aldrich, St. Louis, MO, USA) (Lv et al., 2018) at 2h before 42°C treatment. Accordingly, to explore whether post-treatment of melatonin promotes the recovery of testes from heat-induced damage, the mice were treated with
melatonin (20mg/kg b.w./day) immediately after the 42 °C treatment by i.p. injection for 14 consecutive days. In the 42 °C only or 42 °C plus melatonin pre/post-treatment group, 30 mice were used in one independent experiment, with five mice analyzed at each time point (6h, 12h, 7d, 14d, 21d and 35d) after treatment. Selection of the time points is primarily based on previous reports (Li et al., 2013, Setchell et al., 2001) in which mouse testicular histology was typically analyzed every 7 days up to the day 35, going through a full mouse spermatogenic cycle. In this case, another 225 mice were used for three independent experiments.

For subsequent experiments, the treated mice were euthanased, followed by testis removal. For all individuals, one testis was weighed and fixed overnight in Bouin’s solution for histological and immunohistochemical analyses, and the other testis was snap-frozen in liquid nitrogen and stored at -80°C for protein extraction.

**Histological analysis of testes**

Histological analysis after hematoxylin and eosin (H&E) staining was performed as previously reported (Zheng et al., 2019).

**Immunofluorescence**

Testis sections were prepared and treated as previously described (Zheng et al., 2018). Subsequently, the sections were incubated with primary antibodies (Table I) at 4 °C overnight. Next day, the sections were washed with PBS and incubated with donkey anti-rabbit/mouse secondary antibodies (Alexa Fluor 488/594, 1:400,
Invitrogen, L.A., USA) at 37 °C for 1h, followed by nuclear staining with 4, 6-diamidino-2-phenylindole (DAPI, 1:1,000; Bioworld Technology Inc., Bloomington, USA). For negative controls, the corresponding isotype IgGs were used in place of the primary antibodies. Digital images were captured with a Nikon Eclipse 80i fluorescence microscope camera (Tokyo, Japan).

Transmission electron microscopy

The sample preparation and ultrastructural analysis by transmission electron microscopy were performed as recently reported (Zheng et al., 2019). In brief, testis tissue was perfused with 2.5% glutaraldehyde, followed by washing with PBS, fixation in 1% OsO4 and embedding in Araldite. The ultra-thin sections were stained with uranyl acetate and lead citrate, and visualized under a transmission electron microscope (JEM.1010, JEOL, Tokyo, Japan).

Western blot analysis

Total proteins were extracted and the western blot assay was performed as previously reported (Zheng et al., 2019). The blots were incubated with primary antibodies (Table I) overnight at 4 °C followed by washing with Tris-buffered saline Tween-20 (TBST). Then, membranes were incubated with horse-radish peroxidase-conjugated rabbit anti-goat IgG, goat anti-rabbit IgG or goat anti-mouse IgG (1:2,000, CWBIO) for 2h at room temperature followed by washing with TBST. Proteins were detected using Pierce ECL Western Blotting Substrate (Thermo, LA, USA), visualized with ChemiDox XRS (Bio-Rad, CA, USA) and digital images were
captured. The protein band density was analyzed by Image-Pro Plus (Media Cybernetics, MD, USA).

**TUNEL assay**

To detect the presence of apoptotic cells in the heat-treated testes, testis sections were deparaffinized and rehydrated, and the TUNEL assay was carried out following the manufacturer’s protocol (Apoptosis Detection kit, Shanghai, Vazyme). Briefly, the slides were digested with proteinase-K at a concentration of 20 μg/mL for 20min and washed with PBS at room temperature. The slides were then incubated with equilibration buffer for 30min and with terminal deoxynucleotidyl transferase (TdT) enzyme for 1h at 37°C in a humidified chamber, followed by counterstaining with DAPI for 10min. The slides with apoptotic cells were finally visualized by fluorescence microscopy. For quantification of TUNEL+ cells, at least 500 cells from five mice were analyzed in each group. To quantify the percentage of tubules with TUNEL+ cells and the average number of TUNEL+ cells per tubule, at least 100 seminiferous tubules from five mice were analyzed in each group.

**Malondialdehyde assay**

Frozen testis tissue was homogenized in ice-cold RIPA solution. The homogenate was centrifuged at 5000 ×g for 15min at 4°C, and the malondialdehyde (MDA) level in the supernatant was analyzed using an MDA assay kit (Beyotime, Shanghai, China) according to the manufacturer’s instruction. This kit utilizes the thiobarbituric acid method to monitor MDA-reactive products spectrophotometrically. The absorbance of
the organic layer was measured at 532 nm. Data were expressed as micromoles of MDA per gram of testis protein.

Analysis of antioxidant status

Testicular samples were prepared as described above, and the parameters of oxidative stress, such as the activities of the enzymatic antioxidant total superoxide dismutase (T-SOD) and catalase (CAT), were analyzed subsequently. The T-SOD levels were determined by measuring absorbance at 560 nm. The CAT activity was measured as absorbance at 405 nm using an assay based on the consumption of H₂O₂. Both parameters for oxidative stress were analyzed using commercial kits (Beyotime, Shanghai, China) according to the manufacturers’ instructions.

Statistics

Statistical analyses were performed with Student’s t-test or one-way ANOVA followed by Duncan’s multiple range test using SPSS V22.0 statistical software (IBM, USA). Data were presented as the mean ± S.E.M. and differences were considered statistically significant at p<0.05.
Results

Heat treatment induces spermatogenic abnormality and oxidative stress in the mouse testis

To systematically probe the response of male germ cells to heat stress, we exposed mouse testes to heat by immersing the scrotum in a hot water bath at a distinct temperature (39 °C or 42 °C) for 20 min, and removed the testes at 6 h, 12 h, 24 h, 48 h and 7 d after heat treatment. H&E staining of the testis sections collected at different time points revealed that while the control mouse testes (core body temperature 32 °C) had normal histoarchitecture of seminiferous tubules and seminiferous epithelium, a short exposure of the testes to 42 °C did perturb spermatogenesis and spermatogenic subtypes. Specifically, the seminiferous epithelium became abnormal from 6 h post heat treatment, and karyopyknosis was discernable in some spermatocytes. At 12 h post treatment, multinucleated round spermatids could be observed, probably caused by the defects in intercellular bridges to support cell partition. At 24 h post treatment, most seminiferous epithelium exhibited signs of degeneration and vacuolization, and by 48 h, almost all spermatogenic cells except spermatogonia exfoliated and discharged into the tubule lumen. At 7 d post heat treatment, vacuolization along with the absence of germ cell layers dominated most tubules (Fig. 1A). In contrast, heat treatment of the testes at 39 °C had no apparent influence on germ cell morphology and testicular histology (Fig. 1B).

Previous studies have demonstrated that heat stress can act as a catalyst for oxidative stress in the testis (Ahotupa and Huhtaniemi, 1992, Ikeda et al., 1999,
To gain more insights into the testicular heat-induced oxidative stress, we analyzed several parameters at different time points post heat treatment. The 42 °C treatment significantly changed the levels of MDA and H$_2$O$_2$ (two markers for ROS), with an increase after heat treatment, culminating at 12h post treatment, then followed by a gradual decrease. By contrast, the levels of MDA and H$_2$O$_2$ remained stable in the 39 °C treatment group (Fig. 1C). The activity of two enzymatic antioxidants, CAT and SOD, showed a downward trend after 42 °C treatment, bottoming out at 12h post treatment, then followed by a steady increase. In the 39 °C treatment group, the activity of CAT marginally fluctuated, whereas that of SOD was decreased at 6h post treatment but later experienced a consistent increase (Fig. 1C). The overall data suggest that oxidative stress, indicated by the upregulation of ROS and downregulation of antioxidants, was induced in the 42 °C treatment group, which is likely a contributor to the observed spermatogenic abnormality.

Heat treatment induces autophagy, apoptosis and stress response in mouse testicular cells

To acquire more knowledge about the testicular response to heat stress and the underlying reasons for spermatogenic abnormality, we extracted proteins from the heat-treated mouse testes and performed western bolt analyses for markers of cellular stress, apoptosis and autophagy (Fig. 2). Interestingly, the expression patterns of these markers differed between the 39 °C and 42 °C treatment group. The 39 °C testicular treatment upregulated heat shock protein 2 (HSPA2), a constitutively expressed heat
shock protein (HSP), c-Jun N-terminal kinase (JNK), a stress-activated protein kinase, was transiently activated at 39 °C, characterized by the upregulation of phosphorylated-JNK (p-JNK) at 6h and 12h post heat treatment but returning to a normal level later. Notably, BECLIN 1 and LC3 II, which are perceived as markers for autophagy, were activated at 39 °C, while the cleaved-PARP1 (c-PARP1), an indicator of apoptosis, was not induced (Fig. 2), suggesting that the 39 °C treatment induced autophagy in the testis. In the 42 °C treatment group, HSPA2 was downregulated, whereas JNK (a cellular stress marker) was activated and sustained after heat treatment. p-BECLIN 1 was upregulated but experienced a downregulation from 12h post treatment, and a small activation of LC3 II was detected at 6h after 42 °C treatment (Fig. 2), unlike in the 39 °C treatment group. Nonetheless, c-PARP1, p53 and the pro-apoptotic BCL2-associated X (BAX) were all upregulated at 42 °C, along with the downregulation of BCL-2, an anti-apoptotic protein (Fig. 2), suggesting that apoptosis was predominantly induced in the mouse testis under the 42 °C heat stress condition.

To pinpoint the occurrence of autophagy in the 39 °C heat-treated mouse testis, we collected the testes at 24h after heat treatment, and visualized autophagosomes under a transmission electron microscope. More autophagosomes could be observed in the 39 °C treatment group (Fig. 3A, arrowheads and the structures labelled A). Further, the immunofluorescence result (Fig. 3B) showed that the 39 °C treatment generated strong LC3 I/II⁺ cells which were located at the basal compartment of seminiferous tubules. We quantified the LC3 I/II⁺ cells per tubule and analyzed their average fluorescence intensity by Image J, and observed an upward trend for both at 24h after the 39 °C treatment (Fig. 3B), corroborating that the 39 °C treatment induced
autophagy of mouse testicular cells. By contrast, more apoptotic bodies could be observed in the 42 °C treatment group (Fig. 3A, arrows), and the TUNEL assay revealed that increasing numbers of cells were undergoing apoptosis with time after the 42 °C treatment (Fig. 3C).

To study the testicular cell types undergoing apoptosis in response to 42 °C heat stress, we performed an immunofluorescence analysis for TUNEL and testicular cell markers. The co-staining results showed that 0.74 ± 0.25% of promyelocytic leukemia zinc finger-positive (PLZF+, undifferentiated spermatogonia), 1.42 ± 0.68% of KIT proto-oncogene-positive (c-KIT+ differentiating spermatogonia), 94.62 ± 3.22% of synaptonemal complex protein 3-positive (SCP3+, spermatocytes) and 7.64 ± 1.11% of SRY-box transcription factor 9-positive (SOX9+, Sertoli) cells were positive for the TUNEL staining at 24h after the 42 °C treatment (Fig. 4A, B). The western blot assay based on the whole testicular cell lysates further disclosed that the protein levels of PLZF, c-KIT and SOX9 showed an upward trend with time after the 42 °C treatment, in contrast to that of SCP3, the level of which was gradually declined (Fig. 4C), suggesting the progressive loss of SCP3+ cells in the testis. The overall data indicate that spermatocytes are the predominant cell type undergoing apoptosis in response to the 42 °C testicular treatment, in accordance with a previous report (Kim et al., 2012).

Intriguingly, by immunofluorescence, we observed that the 39 °C treatment led to an increase of heat shock protein family A member 2 (HSPA2) protein mainly in the cytoplasm at 24h later (Fig. 4D, the middle panel), while the HSPA2 localization vanished or shifted from the cytoplasm to the nucleus in the 42 °C treatment group.
Notably, almost all HSPA2\(^+\) cells with the altered localization were TUNEL-positive (Fig. 4D, the right panel). HSPA2 has been reported to play important roles in male meiosis and stress response (Bromfield et al., 2017, Dix et al., 1997, Eddy, 1999).

The switch of the HSPA2 localization to the nucleus might be related to the maintenance of chromatin stability in response to heat stress.

**Pre-treatment of melatonin attenuates heat-induced apoptosis and oxidative stress in the mouse testis**

To investigate the roles of melatonin in the response to testicular heat stress, the mice were treated with 20mg/kg b.w. melatonin at 2h before the 42 °C treatment. Selection of the dose for melatonin injection is primarily based on our previous study, where we found that melatonin at this dosage protected mouse SSCs against hexavalent chromium-induced apoptosis and epigenetic histone modification (Lv et al., 2018). Indeed, when pre-treated with melatonin, vacuolization of seminiferous tubules was clearly relieved, and there were more tubules with normal seminiferous epithelium and multiple layers of germ cells after heat treatment (Fig. 5A), suggesting that melatonin exerted a protective role against heat-induced testicular damage.

Consistently, the immunofluorescence analysis showed that the HSPA2 localization stabilized in the group pre-treated with melatonin 12h after the heat treatment (Fig. 5B, the upper panel), and the percentage of tubules with TUNEL\(^+\) cells, as well as the average number of TUNEL\(^+\) cells per tubule, were significantly reduced in relation to those in the 42 °C treatment-only group (Fig. 5B, the lower histograms).

To investigate the impact of melatonin on heat-induced oxidative stress in the...
testis, we measured the levels of MDA, H$_2$O$_2$, SOD and CAT in two groups of heat-treated testes, i.e. with or without the pre-treatment of melatonin. Following the 42 °C treatment, the levels of MDA and H$_2$O$_2$, markers for ROS, were significantly increased in the testis, while the activities of enzymatic antioxidants SOD and CAT were substantially decreased. Nevertheless, compared to the 42 °C treatment-only group, pre-treatment with melatonin did significantly reduce the generation of MDA and H$_2$O$_2$ but increase the activity of SOD and CAT (Fig. 5C), indicating that melatonin could relieve heat-induced oxidative stress in the mouse testis.

Pre-treatment of melatonin protects the testis against heat-induced damage by inhibiting the apoptotic JNK and p38 mitogen-activated protein kinase signals

Later, we interrogated the mechanism underlying the protective roles of melatonin against heat-induced testicular damage. We detected the expression of markers for cellular stress and apoptosis in two groups of heat-treated testes (with or without the pre-treatment of melatonin) at 12h after the heat treatment. Western blot results showed the induction of stress markers, such as JNK and p38, but not the extracellular regulated protein kinase (ERK) in the testis by the 42 °C treatment. However, pre-treatment with melatonin suppressed the activation of JNK and p38 mitogen-activated protein kinase (MAPK), demonstrated by the relatively lower levels of p-JNK and p-p38 in the melatonin pre-treatment group (Fig. 6A). The expression of c-PARP1, an apoptosis marker, as well as the pro-apoptotic BAX, was also inhibited by the pre-treatment of melatonin, and consistently HSPA2 and the anti-apoptotic BCL-2 were upregulated in the testis pre-treated with melatonin (Fig. 6B).
Taken together, we conclude that melatonin, by inhibiting the apoptosis-related JNK and p38 MAPK signaling pathways, protects the testis against heat-induced damage.

Post-treatment of melatonin promotes the recovery of testes from heat-induced damage

We presumed also that post-treatment of melatonin might promote the recovery of testes from heat-induced damage. To investigate this, after the 42 °C testicular treatment the mice were subjected to i.p. injections of melatonin for 14 consecutive days (20mg/kg b.w./day), and histological analyses of the testis sections were performed periodically after testicular heat treatment. In the group without melatonin post-treatment, most tubules showed vacuolization on d7 after heat treatment, and spermatogenesis slowly resumed from d14. By contrast, when post-treated with melatonin, numerous tubules with normal seminiferous epithelium and multiple layers of germ cells could already be observed on d7 (Fig. 7A), suggestive of the faster recovery of spermatogenesis, which was later validated by quantifying the percentage of abnormal tubules (Fig. 7B). The testicular index (testis weight/body weight) also went up in the melatonin post-treatment group at 7 days (Fig. 7C). Together, our data indicate that melatonin promotes the recovery of testes from heat-induced damage.

Post-treatment with melatonin maintains the integrity of the Sertoli cell tight-junction

Testicular heat treatment at 42 °C triggered a host of multinucleated round spermatids (Fig. 7A, at 12h, arrowheads). Nonetheless, this could not be typically
observed in the melatonin post-treatment group. A previous study reported that heat stress damaged Sertoli cells and compromised their ability to support round spermatids, resulting in the generation of multinucleated round spermatids (Li et al., 2016, Oliveira et al., 2007). In this sense, the preventative role of melatonin could be exerted by preserving Sertoli cells and, in particular, the tight-junctions between them. We thus performed immunofluorescence analysis for ZO-1 and β-catenin, two major components of the tight-junction, as well as androgen receptor (AR). In line with a previous study (Cai et al., 2011), ZO-1 and β-catenin showed a severely fractured staining pattern in seminiferous tubules at 12h after testicular heat treatment, while in the group with melatonin post-treatment, ZO-1 and β-catenin among Sertoli cells generally remained intact (Fig. 8, the upper and middle panels). As expected, post-treatment of melatonin also sustained the expression of AR in comparison with the heat treatment-only group (Fig. 8, the lower panel), since the loss of AR expression in Sertoli cells after heat treatment has been reported to be a primary contributor to the fractured tight-junction (Cai et al., 2011, Chen et al., 2008). Collectively, our results suggest that melatonin maintains the integrity of the Sertoli cell tight-junction, which may be crucial to the faster recovery of testes from heat-induced damage.
In this study, we presented results consistent with a previous article (Rockett et al., 2001), in that testicular heat treatment at 42 °C for 20 min triggered a series of damage symptoms in the testis such as testicular atrophy, perturbed seminiferous epithelium, tubule vacuolization, germ cell death and the fracture of the Sertoli cell tight-junction. The underlying causes could be apoptosis and oxidative stress, which were induced in the testis soon after heat treatment. By contrast, heat treatment of the testis at 39 °C had no evident impact on germ cell morphology and testicular histology, also in line with previous reports (Morgentaler et al., 1999, Rockett et al., 2001), suggesting a threshold of the testicular response to heat stress. Yet, we detected that the 39 °C treatment induced autophagy in the testis, which has so far not been reported. The 39 °C treatment also generated cellular stress in the testis, characterized by the upregulation of p-JNK, albeit to a lesser extent than the 42 °C treatment group. It is thus plausible that autophagy plays a role in relief of the slight cellular stress induced by the 39 °C treatment, thereby contributing to the maintenance of germ cell morphology and testicular histology. Nonetheless, researchers have summarized that a rise of 1 °C entails a 14% decline in spermatogenesis (Wang et al., 1997). Given this, albeit not fully surprising, our novel finding does provide further evidence that even low heat stress can influence the homeostasis of testicular cells, being a potential contributor to the inferior sperm quality and consequently male subfertility.

Our data revealed that the 42 °C heat treatment generated oxidative stress in the testis, indicated by the upregulation of ROS and downregulation of antioxidants, in accordance with previous studies (Kumagai et al., 2002, Li et al., 2006). In normal
testes, ROS are maintained at an acceptable level due to the presence of natural antioxidants, and when the balance between ROS and antioxidants is disturbed, oxidative stress will ensue, then followed by apoptosis (Ikeda et al., 1999, Kumagai et al., 2002). Yet, Peltola et al. (Peltola et al., 1995) reported that the decrease of antioxidants likely plays a less important role in the generation of oxidative stress than the increase of ROS, as the drop of antioxidants did not match the rise of DNA insults. Despite all these, it is clear that heat stress upsets the balance between ROS and antioxidants, being responsible for the induction of oxidative stress and apoptosis in the testis.

Intriguingly, we observed that the 42 °C treatment triggered loss or translocation of HSPA2 into nuclei, that correlates with cell apoptosis. Previous studies have suggested that HSPA2 plays important roles in stress response and male meiosis. HSPA2 in cytoplasm may protect the cells against stress and apoptosis, whereas its nuclear counterpart is implicated in synaptonemal complex formation, DNA repair and maintenance of DNA integrity (Bromfield et al., 2017, Dix et al., 1997, Eddy, 1999). By using a knock-out mouse model, Mori et al. (Mori et al., 1997) found that depletion of HSPA2 led to DNA fragmentation and apoptosis in pachytene spermatocytes. Here, we presume that HSPA2 translocation from cytoplasm to nuclei might be related to the maintenance of DNA integrity in the early stages of heat stress response, which remains to be investigated in future.

Melatonin, an indolamine secreted by the pineal gland and testes, is widely known to be involved in a variety of activities including cellular growth, energy metabolism and the intracellular redox status (Ji et al., 2012b, Reiter et al., 2017,
Previous studies have revealed that melatonin is able to protect the testis against inflammation and ROS generation (Guneli et al., 2008, Rocha et al., 2015, Semercioz et al., 2003a). In 1999, Badr et al. (Badr et al., 1999) reported that injection of melatonin to male mice before irradiation helped to preserve fertility. Since then, melatonin has been employed to relieve testicular damage resulting from adverse treatment. We thus presumed that melatonin might also make a difference in the testicular response to heat stress. As expected, the detrimental effects of heat stress on the testis were alleviated by melatonin pre-treatment, characterized by more intact seminiferous tubules with multiple layers of germ cells and the preserved testicular index. Our results further demonstrated that melatonin could effectively prevent ROS generation and the decline in the activity of antioxidant enzymes (SOD and CAT), suggesting that melatonin modulates the balance between ROS and antioxidants, thereby maintaining the intracellular redox status at a controllable level. In addition, a previous study suggested that the increased responsiveness of Sertoli cells to FSH induced by melatonin might help to prevent testicular insults (Heindel et al., 1984). Whether melatonin can also function in a similar way to ease heat-induced testicular damage warrants future exploration.

Previous studies reported the activation of diverse signaling pathways in the testis by heat stress (Durairajanayagam et al., 2015, Kim et al., 2013a). Kim et al. (Kim et al., 2013b) found that the anti-apoptotic phospho-JNK (p54) level in spermatocytes was minimally increased by one cycle of testicular hyperthermia but then gradually decreased after three cycles. Jia et al. (Jia et al., 2009) reported the induction of both ERK and p38 but not JNK signals by rat testicular heat stress. The inconsistency regarding JNK could be ascribed to differences in cell types or the temperature/time
dosages of heat treatment. Here we detected that the JNK and p38 signals were temperature dosage-dependent, with an intense and sustained activation of both signals by testicular heat treatment at 42 °C but not 39 °C. In fact, the JNK kinases, activated by cellular stressors and inflammatory mediators, can promote cell death or survival under particular circumstances. The early transient activation of JNK is often involved in stress tolerance and cell survival, while the intense and sustained activation typically associates with cell death (Luo et al., 2005, Shaulian and Karin, 2001, Wei et al., 2008). In the present study, the activated apoptosis-related JNK and p38 signals were impeded by pre-treatment of melatonin. Concomitantly, the pro-apoptotic c-PARP1 and BAX were downregulated, and the anti-apoptotic BCL-2 was upregulated, further demonstrating that melatonin prevents heat-induced apoptosis in the testis.

Moreover, we demonstrated that post-treatment of melatonin also promoted the recovery of testes from heat-induced damage, which is even more desirable in terms of human fertility. Melatonin post-treatment maintained testicular histology and enhanced the testicular index. The favorable role of melatonin may be exerted by preserving the Sertoli cell tight-junction, the destruction of which by testicular heat stress has been suggested as a cause of spermatogenic disturbance and thus male infertility (Cai et al., 2011, Chen et al., 2008). To be more precise, heat stress can lead to the severe fracture of the Sertoli cell tight-junction, depriving germ cells of the shield which is normally afforded by the tight-junction, eventually resulting in the perturbed spermatogenic process (Cai et al., 2011, Chen et al., 2008). Here we found that melatonin could maintain the integrity of the Sertoli cell tight-junction, probably by sustaining the expression of AR, as the loss of AR expression in Sertoli cells after
heat treatment has been proposed as a primary contributor to the fractured tight-junction (Chen et al., 2008). Consequently, germ cells are, for a large part, preserved and left in the normal seminiferous epithelium cycle, which is later reflected by the faster recovery of spermatogenesis. Besides, post-treatment of melatonin may have a direct influence on germ cells. Previous studies have reported that melatonin is able to promote SSC self-renewal (Navid et al., 2017a, Navid et al., 2017c) and early meiotic progression (Zhang et al., 2018) as well as development of haploid germ cells in vitro (Deng et al., 2016). Hence, the recovery of testes from heat-induced damage may be facilitated by synergistic roles of melatonin in the supportive Sertoli cells and in the germ cells.

In the female, a recent article reported that melatonin could delay postovulatory oocyte aging via SIRT1 (Yang et al., 2018). As shown in that study, aged postovulatory oocytes exhibited a series of phenotypes such as reduced expression of Siruin 1 (SIRT1) and manganese superoxide dismutase (MnSOD) at the protein level, an elevated ROS level, meiotic defects, mitochondrial dysfunction, autophagy and apoptosis. Melatonin supplementation in vitro induced SIRT1 expression, further upregulating MnSOD and downregulating the ROS level, eventually repressing oocyte aging and enhancing oocyte quality. It is thus concluded that melatonin acts as a SIRT1 regulator to prevent oocyte aging. In future, it would be appealing to investigate whether melatonin functions in a similar manner to protect the testis against heat stress.

To sum up, by employing a testicular hyperthermia mouse model, we systematically probed the testicular response to heat stress, the occurrence of
autophagy, apoptosis and oxidative stress in the testis and, more importantly, the utility of melatonin pre/post-treatment as a preventative measure for the heat-induced testicular insults. Since testicular hyperthermia perturbs spermatogenesis and leads to inferior sperm quality, gaining more insights into the response to testicular heat stress and the underlying molecular mechanisms could contribute to development of tailored therapies for heat-induced male subfertility. Furthermore, the proof-of-concept that melatonin can protect the testis against heat stress suggests the potential use of this indolamine as a therapeutic drug for sub/infertile men incurred by various testicular hyperthermia factors. In fact, melatonin has long been reported to correlate with sperm motility and progression, with significantly reduced levels in men suffering from oligozoospermia, asthenozoospermia or non-obstructive azoospermia compared to their fertile counterparts (Awad et al., 2006, Yie et al., 1991). In this sense, our study lends further support to the (pre-)clinical practice of melatonin treatment, not only for protective purposes, but also for improvement of male reproductive health in the long run.
Authors’ roles

P.Z., Y.Z. and Y.L. conceived the study. P.Z., Y.Z., F.L., L.S. and Y.Q. performed the experiments. P.Z., Y.Z., Y.L. and W.Z. analyzed the data. P.Z. and Y.Z. wrote the manuscript. All authors read and approved the final version.

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Conflict of interest

The authors have nothing to disclose.
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Table I Primary antibodies used in this study of mouse testes.

<table>
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<th>Antibody</th>
<th>Species Source</th>
<th>Supplier</th>
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<th>IHC</th>
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WB: Western blot; IHC: immunohistochemistry.
Figure 1 Heat treatment induces spermatogenic abnormality and oxidative stress in the mouse testis.

(A-B) Haematoxylin and eosin (H&E) staining of testis sections from mice subjected to 42 °C (A) or 39 °C (B) testicular heat treatment. Bar=100µm. (C) Analysis of the parameters for oxidative stress (malondialdehyde, MDA; hydrogen peroxide, H₂O₂; catalase, CAT; superoxide dismutase, SOD) in the heat-treated testis. Data are presented as the mean ± SEM of three independent experiments, with each experiment using 25 mice in the 42 °C or 39 °C treatment group and five mice analyzed at each time point. Control (CTR): the control mice with core body temperature 32 °C. Asterisks indicate statistical significance between the 42 °C and 39 °C treatment group at each time point. Statistical analyses were performed with Student’s t-test. *: p<0.05; **: p<0.01.
Figure 2 Western blot assay for autophagy and apoptosis-related proteins in the testes after testicular heat treatment at 39 °C or 42 °C. β-ACTIN is used as a loading control. Data are presented as the mean ± SEM of three independent experiments. Statistical analyses were performed with one-way ANOVA followed by Duncan’s multiple range test. In each experiment, five mice were analyzed at each time point. Different letters in the histogram mean significant differences between groups (p<0.05). CTR: the control mice with core body temperature 32 °C.
Figure 3 Heat treatment induces autophagy and apoptosis of mouse testicular cells.

(A) Upper panel: representative transmission electron micrographs of testis sections from treated mice at 24h after heat treatment. Arrowheads show the typical autophagosome, while arrows refer to apoptotic bodies. Lower panel: magnified transmission electron micrographs of testis sections from treated mice at 24h after the 39 °C treatment. A: autophagosomes; G: Golgi apparatus; L: lysosomes; M: mitochondria. Bar=2µm. (B) Upper panel: LC3 I/II immunostaining in the testis sections at 24h after the 39 °C treatment. NC: the isotype IgG in place of the primary antibody. Asterisks indicate the putative positive cells. Lower panel: the number of LC3 I/II+ cells per tubule (left) and their average fluorescence intensity in the testes at 24h after the 39 °C treatment. Data are presented as the mean ± SEM of three independent experiments, and in each experiment, at least 100 tubules from five mice were analyzed. Statistical analyses were performed with Student’s t-test. **: p<0.01. Bar=50µm. (C) TUNEL staining of testis sections from 42 °C testicular heat-treated mice. NC: the negative TUNEL control prepared by omitting the enzyme. CTR: the control mice with core body temperature 32 °C. Bar=100µm.
**Figure 4** Co-staining of TUNEL and testicular cell markers or heat shock protein family A member 2 in the mouse testis sections at 24h after heat treatment.

(A) Co-staining of TUNEL and promyelocytic leukemia zinc finger (PLZF), KIT proto-oncogene (c-Kit), synaptonemal complex protein 3 (SCP3) or SRY-box transcription factor 9 (SOX9) in the testis sections from 42 °C-treated mice. Bar=100µm. (B) Percentages of cells double-positive for both TUNEL and PLZF, c-Kit, SCP3 or SOX9 in the 42 °C-treated mouse testes. Data are presented as the mean ± SEM of three independent experiments, and in each experiment, at least 500 cells from five mice were analyzed. Statistical analyses were performed with one-way ANOVA followed by Duncan’s multiple range test. ***: p<0.001. (C) Western blot assay for testicular cell markers in the 42 °C-treated testes. β-ACTIN is used as a loading control. (D) Co-staining of heat shock protein family A member 2 (HSPA2) and TUNEL in the testis sections from heat-treated mice. CTR: the control mice with core body temperature 32 °C. Bar=50µm.
Figure 5 Pre-treatment of melatonin attenuates heat-induced apoptosis and oxidative stress in the mouse testis.

(A) H&E staining of testis sections from 42 °C testicular heat-treated mice with or without melatonin pre-treatment. Bar=100µm. (B) Upper panel: co-staining of HSPA2 and TUNEL in the testis sections at 12h after heat treatment. Bar=100µm. Lower panel: percentages of seminiferous tubules with TUNEL+ cells (left) and the average number of TUNEL+ cells per tubule (right) in the heat-treated testes at 12h after heat treatment. (C) Analysis of the parameters for oxidative stress (MDA, H2O2, SOD, CAT) in the heat-treated testes. Data are presented as the mean ± SEM of three independent experiments. In each experiment, five mice were analyzed at each time point. Statistical analyses were performed with one-way ANOVA followed by Duncan’s multiple range test. **: p<0.01; ***: p<0.001. CTR: the control mice with core body temperature 32 °C.
Figure 6 Western blot assay for stress (A) and apoptosis (B)-related markers in the testes of 42 °C-treated mice, with or without melatonin pre-treatment at 12h after heat treatment.

β-ACTIN is used as a loading control. Data are presented as the mean ± SEM of three independent experiments, with each experiment using five mice in one group. Statistical analyses were performed with one-way ANOVA followed by Duncan’s multiple range test. *: p<0.05; **: p<0.01. CTR: the control mice with core body temperature 32 °C.
Figure 7 Post-treatment with melatonin promotes the recovery of mouse testes from heat-induced damage.

(A) H&E staining of testis sections from 42 °C testicular heat-treated mice with or without melatonin post-treatment. Arrowheads point to the multinucleated round spermatids. Bar=100µm. (B) Percentages of abnormal seminiferous tubules in the heat-treated testes. (C) Testicular indexes (testis weight/body weight) of the heat-treated mice. Data are presented as the mean ± SEM of three independent experiments. In each experiment, five mice were analyzed at each time point. CTR: the control mice with core body temperature 32 °C. Asterisks indicate statistical significance between the 42 °C-only and 42 °C plus melatonin post-treatment group at each time point. Statistical analyses were performed with Student’s t-test. *: p<0.05; **: p<0.01.
**Figure 8** Staining of tight junction protein 1 ZO-1, β-catenin and androgen receptor in the testis sections from heat-treated mice at 12h after heat treatment.

AR: androgen receptor, NC: the isotype IgG in place of the primary antibody. CTR: the control mice with core body temperature 32 °C. Bar=100µm.