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# Effects of Melatonin on Spermatozoa Activity and Associated Mechanisms in Heat-Stressed Rats

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**Abstract:** In animals, heat stress (HS) disrupts spermatogenesis, reducing sperm quality and, in severe cases, potentially inducing the loss of male reproductive function. Melatonin confers significant resistance to oxidative stress and apoptosis; however, its specific effects on rat spermatocytes and the mechanism underlying its anti-HS effects remain inadequately explored. Therefore, this study aimed to analyze the effects of melatonin at different concentrations on sperm cell activity in heat-stressed rats. Modeling heat stress injury, sperm viability and density assay, sperm plasma membrane integrity analysis, and oxidative stress assay of testicular tissue were conducted. The results revealed that HS caused sperm cell injury. However, the intraperitoneal injection of melatonin effectively improved spermatozoa quality, and a dose of 1 mM significantly alleviated the HS-induced damage. Moreover, HS increased the levels of oxidative and endoplasmic reticulum (ES) stress in rat testicular tissues, inducing germ cell apoptosis and pathological changes. Similarly, melatonin treatment improved sperm cell viability and density, inhibited germ cell apoptosis, and reduced oxidative and ES stress levels. Overall, melatonin effectively reduced the adverse effects of HS on rat sperm cells, and an intraperitoneal injection of 1 mM (0.6966 mg) melatonin facilitated the normal production of spermatozoa. Notably, its mechanism may involve reduced ES and oxidative stress levels in testicular tissues, increased expression of the anti-apoptotic protein Bcl-2, and inhibition of germ cell apoptosis.

**Keywords:** Melatonin; Rat; Spermatocyte; Spermatogenesis; Antioxidant

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## 1. Introduction

Spermatozoa are specialized germ cells that develop from spermatogonia and are characterized by several quality indicators, including motility, density, plasma membrane integrity [1], acrosome integrity, and DNA damage, all of which are associated with the female fertilization rate. Heat stress (HS) increases ROS production in the testis, disrupting sperm membrane integrity and inducing DNA damage, which results in decreased fertilization rates [2]. ROS levels can also increase during the *in vitro* storage of human semen [3]. Furthermore, the freeze-thaw process affects sperm quality [4] and reduces their motility [5,6].

Melatonin has a wide range of applications in the human body and is produced in many organs [7]. It facilitates the reduction of ROS levels [8], improves sleep quality [9], and exerts other effects, such as regulating the nervous system [10] and increasing oxygen radical scavenging capacity [11]. Additionally, melatonin has demonstrated efficacy as a potential antiviral drug [12].

In recent years, melatonin has been studied more extensively for applications in food preservation [13], cell culture [14], oxidative stress regulation, and as a potential treatment for Alzheimer's [15] and Parkinson's diseases [16]. In the 1980s, a study [17] demonstrated the regulatory effects of melatonin on reproduction, wherein subcutaneous injection of 100 μg melatonin in rats produced a series of pathological changes in the reproductive system. Furthermore, *in vitro* culture of melatonin-stimulated undifferentiated spermatogonial stem cells significantly increased proliferation efficiency [18] and increased testosterone secretion from interstitial cells, indicating more efficient meiosis and spermatocyte differentiation [19]. Melatonin has also been shown to enhance chemotherapy-induced cancer cytotoxicity by increasing apoptosis [20]. Additionally, melatonin receptor blockade has been reported to significantly promote human chorionic gonadotropin-induced apoptosis in mice [21].

Spermatogenesis is a complex process that involves several specialized physiological changes from single cells to mature sperm cells [22]. For effective spermatogenesis, the external temperature must be lower than the body temperature [23]. However, sperm cells are affected and multiple proteins are dysregulated when the external temperature is higher than the scrotal temperature [24]. Pathological changes in testicular tissues also occur in response to HS, with the primary consequence being the apoptosis of germ cells. Excessive germ cell apoptosis can lead to cryptorchidism and infertility [25]. Germ cells are particularly susceptible to HS owing to their high mitotic activity, with the most vulnerable cells in humans and rats being spermatogonia and early-round spermatocytes [26]. HS in the testes can cause many harmful events [27]. ROS has been shown to be significantly associated with oxidative stress [28]. Chronic exposure and increased ROS concentrations impede various cellular functions [29], leading to detrimental effects on organisms [30]. One study observed that nearly half of male infertility patients have significantly elevated ROS levels in semen samples [31].

Melatonin exhibits strong antioxidant properties [32] and provides protective and regulatory effects against oxidative stress; however, its *in vivo* action on rat sperm cells and the associated regulatory mechanisms remain elusive. Therefore, this study aimed to determine the effects of different concentrations of melatonin on HS-induced sperm cell damage.

## 2. Materials and methods

### 2.1. Experimental material

Sperm samples were obtained from rats, and sperm dilutions were prepared in a laboratory. Phosphate-buffered saline was obtained from Hyclone (Logan, UT, USA). Fluorescein diacetate (FDA), propidium iodide, melatonin, hematoxylin, eosin, and glycine were obtained from Sigma-Aldrich (Burlington, MA, USA). Neutral gum, anhydrous ethanol, xylene, sodium chloride, potassium chloride, potassium dihydrogen phosphate, xylene, and 1-bromo-3-chloropropane were obtained from China National Pharmaceutical Chemical Corporation (Beijing, China). Protein extraction reagent and bicinchoninic acid (BCA) protein quantification kit were obtained from Beyotime Biotech (Jiangsu, China). Furthermore, 4',6-diamidino-2-phenylindole (DAPI), GRP78, and ATF-6 were obtained from Abcam (Cambridge, UK). Rainbow 180 Broad Spectrum Protein Marker (11-180KD), wet transfer buffer, and stripping buffer were obtained from Tendril (Boulder, CO, USA). Bovine serum albumin, ammonium persulfate, N,N,N',N'-tetramethylethylenediamine, Ponceau S staining solution, and bromphenol blue were obtained from AMRESCO (Solon, OH, USA). P-elF2α CST, BCL-2, Bax, GAPDH, and CHOP were obtained from ImmunoWay (Plano, TX, USA). Total RNA Rapid Extraction Kit and

Rapid De-genomic Reverse Transcription Premix were obtained from Hocker Biologicals (Hangzhou, China).

## 2.2. Experimental methods

### 2.2.1. Animal origin and handling

This study was approved by the Ethics Committee of the Zhejiang Academy of Agricultural Sciences (approval number: 2024ZAASLA110). Eight-week-old healthy male SPF-grade CD® (SD) IGS rats were used in this study. Sexually mature rats were housed individually in a temperature-controlled room (20–25°C) with a 12 h/12 h light/dark cycle and good indoor ventilation. The rats had *ad libitum* access to drinking water and feed. The experimental period commenced after a 1-week acclimation period. Subsequently, the rats were randomly divided into six groups: control group A (administered 3 mL saline) and HS groups B (model group; HS + 3 mL saline), L (HS + 3 mL of 10 mM melatonin), M (HS + 3 mL of 0.01 mM melatonin), H (HS + 3 mL of 0.1 mM melatonin), and E (HS + 3 mL of 1 mM melatonin). The HS + melatonin groups were injected with the same volume of the corresponding concentration of melatonin daily for 10 days, after which HS modeling was conducted.

For HS exposure, the rats were anesthetized using isoflurane airflow configured with a gas anesthesia device. Subsequently, the testicular section of the rats was immersed in a water bath at 42°C for 30 min. The rats did not experience any pain and did not require any pain management.

### 2.2.2. Sperm viability and density assays

The tail of the epididymis of rats was placed in saline at 37°C and clipped. Subsequently, sperm suspension was added to pre-warmed slides for sperm viability assays and observed under an optical microscope (Nikon ECLIPSE E100; Nikon Instruments, Tokyo, Japan). The percentages of A-grade sperm, B-grade sperm, forward sperm (PR), and sperm motility were calculated. A sample aliquot of 10 μL was added to a hemocyte-counting plate to count the sperm and determine the density ($10^6$/mL).

### 2.2.3. Sperm plasma membrane integrity assay

A 0.5-mL aliquot of freshly collected sperm suspension was removed from the cryopreservation tubes and centrifuged at 25°C for 2 min at 1,000 rpm. The supernatant was removed using a Thermo Micro17R centrifuge (Massachusetts, USA). The sperm density was adjusted to $5 \times 10^6$/mL and stained with FDA staining solution at 37°C, shielded from light exposure. Subsequently, a propidium iodide staining solution was added, and the coated film was counted and observed using a fluorescence microscope (Nikon Ts2-FC; Nikon Instruments, Tokyo, Japan).

### 2.2.4. Mitochondrial membrane potential assays

Spermatozoa were centrifuged at 25°C for 2 min at 1,000 rpm, and the supernatant was removed using a Thermo Micro17R centrifuge (Massachusetts, USA). The sperm concentration was adjusted to $1 \times 10^6$. Subsequently, 20 μL of rat spermatozoa cells were aspirated, and 1 mL of JC-1 fluorescent probe solution was added. The samples were incubated for 20 minutes at 37°C in a cell culture incubator. After centrifugation and discarding the supernatant, the JC-1 staining solution was added and centrifuged again. The previous step was repeated. Finally, 200 μL of buffer was aspirated and analyzed using a flow cytometer (Agilent, NovoCyte, California, USA).

### 2.2.5. Hematoxylin and eosin staining

Paraffin sections of rat testis tissue were dewaxed with water, and the sections were stained with hematoxylin for 3–5 min, washed, differentiated, and washed again. Subsequently, the sections were stained with gradient alcohol of 85% and 95%, followed by staining with eosin for 4 min, then analyzed under a light microscope (Nikon ECLIPSE E100; Nikon Instruments, Tokyo, Japan).

### 2.2.6. TUNEL staining

Paraffin sections of rat testis tissue were deparaffinized with water for proteinase K repair. The sections were shaken dry, and a membrane-breaking working solution was added to cover the tissue samples. The samples were incubated for 20 min at 25°C. The nuclei of the cells were re-stained with DAPI. The samples were sealed, and images were captured using a fluorescence microscope (Nikon Ts2-FC; Nikon Instruments).

### 2.2.7. Oxidative stress assay of testicular tissues

Malondialdehyde (MDA) levels in rat testicular tissues were determined by conducting colorimetric analysis using the thiobarbituric acid assay. Glutathione peroxidase (GSH-Px) activity in rat testis tissues was determined using a colorimetric assay, whereas superoxide dismutase (SOD) and catalase (CAT) levels were measured using a microassay. The kits were provided by Abbkine Biotechnology Co. (Wuhan, China).

### 2.2.8. Western blotting

Testicular tissue samples (0.1 g) were weighed and placed in an Eppendorf (EP) tube. The tissue samples were injected with lysis buffer containing radioimmunoprecipitation assay solution and phosphorylated protease inhibitor, ground, lysed at 0°C, centrifuged to remove the supernatant, and quantified using the BCA assay kit. Protein samples were added to the comb wells of the gel using the protein marker. Subsequently, electrophoresis was performed, and the membrane was washed several times with Tris-buffered saline with Tween (TBST) washing solution and incubated at 25°C for 4 h for closure. Polyvinylidene fluoride (PVDF) membranes were incubated overnight at 4°C with the primary antibody solution, removed, and washed several times with TBST. Subsequently, the membranes were washed with TBST after a 2-h incubation period at 25°C with the secondary antibody. Protein banding experiments were performed using an ultrasensitive ECL development kit. The PVDF membranes were transferred to a protein gel imaging system, and a working solution was added dropwise onto the PVDF membranes to generate target protein bands.

### 2.2.9. qPCR

Rat testicular tissues were ground into a fine powder. The powder was collected in EP tubes, lysed, and allowed to stand. RNA was extracted from the testicular tissue using a Total RNA Rapid Extraction Kit, and the RNA template was placed in a new PCR tube. All-in-one first-strand synthesis Master Mix and dsDNase were added to the tube, mixed thoroughly, and let it sit for 2 min on a PCR instrument and at 65°C for 2 min to remove genomic DNA contamination. The sample was warmed at 55°C for 15 min and incubated at 85°C for 5 min to terminate the reaction. The PCR amplification conditions: pre-denaturation at 94°C for 3 min; cycling (40 times) at 94°C for 10 s and 60°C for 30 s; melting curve 60°C–95°C, increasing at a rate of 0.5°C/10 s. The primer sequences are listed in **Table 1**.

**Table 1.** Target sequences

| | Forward | Reverse |
|---|---|---|
| CHOP | GTCACAAGCACCTCCCAAAG | TCTTCCTCTTCTTCCTCCTGAG |
| ATF-6 | TCTTCTCCTCGGTCCACAGA | ACCAGTGACAGGCTTCTCTTC |
| Bax | TGGAGCTGCAGAGGATGATT | CAGGGCCTTGAGCACCACTT |
| Bcl-2 | GCTGAGGCAGAAGGGTTATG | GCCCCCTTGAAAAAGTTCAT |
| Caspase-3 | TGCGGTATTGAGACAGACAGT | GCGGTAGAGTAAGCATACAGGA |
| GAPDH | GAAACCCATCACCATCTTCCAG | GCCAGTAGACTCCACGACATA |

## 2.3. Statistical analysis

Data were analyzed using SPSS software, and differences between multiple groups were analyzed using one-way analysis of variance (ANOVA) following a normal distribution and meeting the chi-squared test criteria. Further two-by-two comparisons between groups were made using Tukey's test. *P*-values < 0.05 were considered statistically significant.

# 3. Results

## 3.1. Effects of melatonin on sperm cell viability and density

The sperm survival rate in the model group was significantly lower than that in the control group A ($P < 0.01$), and the sperm survival rate in groups M and L was significantly higher than that in the model group B ($P < 0.01$) (**Table 2** and **Figure 1**). There was a significant difference in sperm motility between the heat stress group and all melatonin groups. In addition, sperm cell motility was significantly higher in group E than in the other groups.

**Table 2.** Changes in sperm survival rate in each group ($n = 3$)

| Group | Sperm survival rate |
|---|---|
| Control group (A) | 0.65 ± 0.03 |
| Model group (B) | 0.21 ± 0.04 |
| 10 mM melatonin group (L) | 0.37 ± 0.01 |
| 0.01 mM melatonin group (M) | 0.48 ± 0.01 |
| 0.1 mM melatonin group (H) | 0.51 ± 0.02 |
| 1 mM melatonin group (E) | 0.53 ± 0.02 |

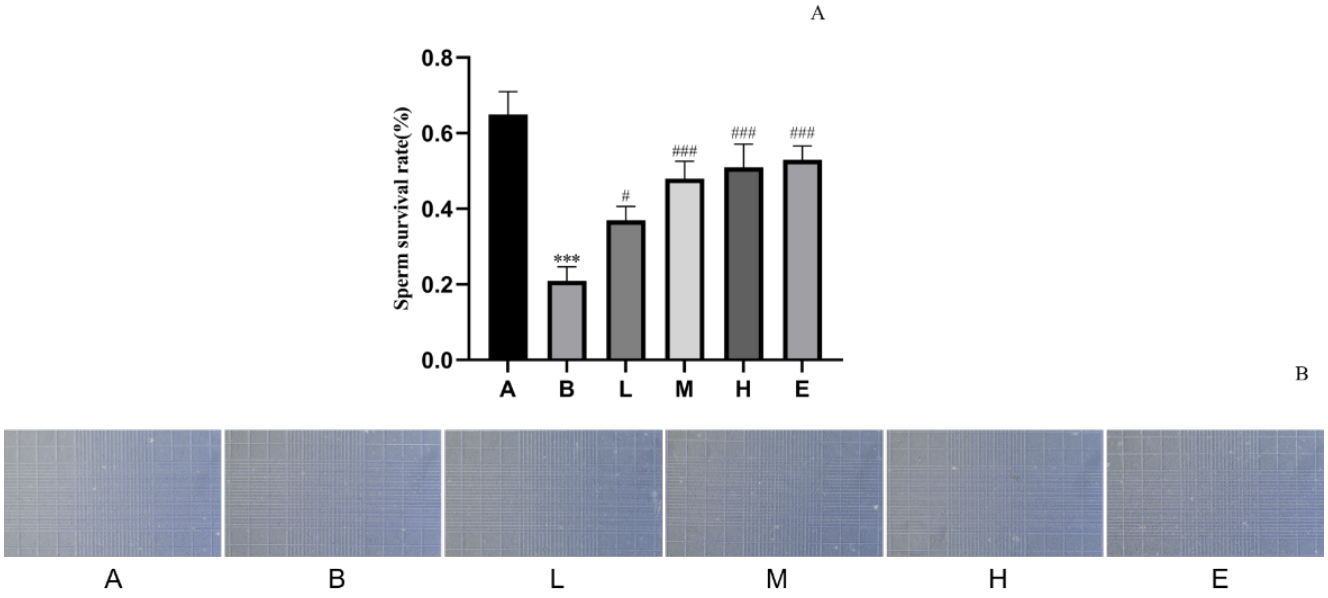


**Figure 1.** Sperm **(a)** survival and **(b)** density changes in each group. ***$P < 0.001$ compared with the blank control group A; #$P < 0.05$, ###$P < 0.001$ compared with the heat stress + intraperitoneal injection of 3 mL saline group B. Magnification 100×

## 3.2. Effects of different melatonin doses on mitochondrial membrane potential (MMP) of sperm cells

Spermatocyte MMP was significantly higher in the control group A than in the heat stress-treated group B ($P < 0.01$) (**Table 3** and **Figure 2**). Compared with that in group B, the cellular MMP of spermatozoa in melatonin groups L, M, H, and E increased significantly ($P < 0.01$). Significant differences in their intra-mitochondrial depolarization were also found compared to the heat stress group (**Figure 2a**; $P < 0.01$). The change in MMP in group E was smaller and lower in amplitude than that of group B.

**Table 3.** Rates of change in sperm mitochondrial membrane potential in each rat group ($n = 3$)

| Group | A | B | L | M | H | E |
|---|---|---|---|---|---|---|
| MMP (%) | 6.1 | 38.3 | 27.6 | 26.2 | 19.5 | 15.1 |

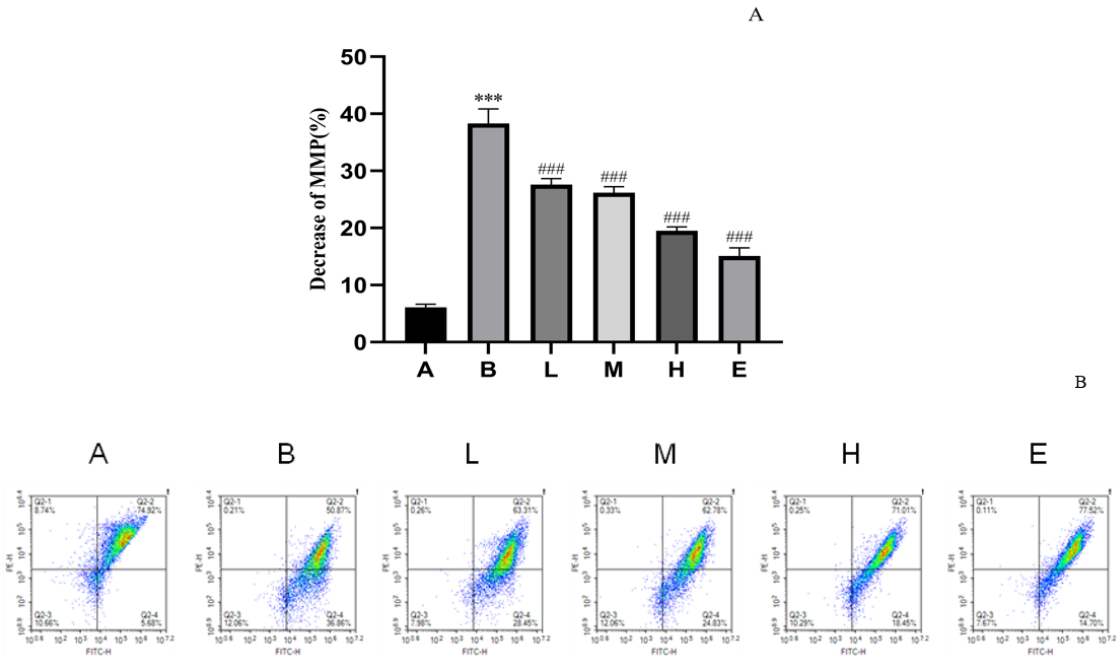


**Figure 2.** (a) MMP analytics and (b) flow cytometric analysis of spermatozoa in each group. ***P < 0.001 compared to the blank control group A; ###P < 0.001 compared to the heat stress + intraperitoneal injection of 3 mL saline group B.

## 3.3. Effects of different melatonin doses on the plasma membrane integrity of sperm cells

Sperm plasma membrane integrity was significantly lower ($P < 0.01$) in group B than in group A. However, sperm plasma membrane integrity was significantly higher ($P < 0.01$) in groups L, M, H, and E than in group B (**Table 4** and **Figure 3**). No dead sperm cells due to plasma membrane disruption were observed at a melatonin dose of 1 mM.

**Table 4.** Sperm plasma membrane integrity in each rat group ($n = 3$)

| Group | A | B | L | M | H | E |
|---|---|---|---|---|---|---|
| Percentage of plasma membrane integrity (%) | 92.33 | 7.67 | 20.33 | 39.17 | 62.00 | 84.17 |

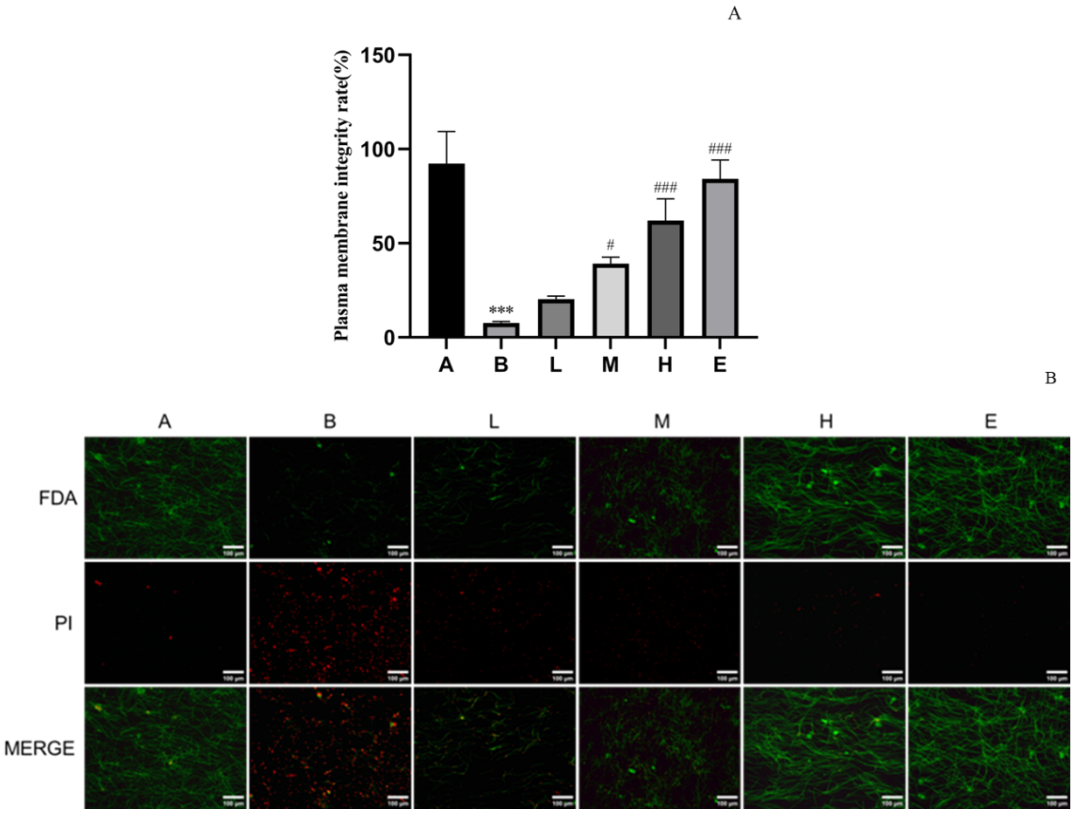


**Figure 3.** (a) Plasma membrane integrity and (b) fluorescent staining analysis of changes in spermatozoa in the control, model, and treatment groups. ***P < 0.001 compared with the blank control group A; #P < 0.05, ###P < 0.001 compared to the heat stress + intraperitoneal injection of 3 mL saline group B.

## 3.4. H&E staining

Hematoxylin and eosin (H&E) analysis (**Figure 4**) revealed that group B exhibited clear, blank defects in the seminiferous tubules of testicular tissues, and the blank areas of the H&E sections of rats in each treatment group were notably less than those of group B. The experimental data proved that the absence of germ cells in the rats was progressively ameliorated by pretreatment with melatonin. Group E had the fewest missing germ cells and was similar in appearance to that of the control group A. This finding indicates that melatonin pretreatment reduced HS-induced germ cell damage.

## 3.5. TUNEL sectioning

TUNEL analysis (**Figure 5**) revealed that the amount of green fluorescence produced by the cells in the seminiferous tubules owing to the exposure of nuclei was significantly higher in group B than in the control group. However, the amount of green fluorescence emitted by the nuclei in the seminiferous tubules (caused by the unnatural death of cells) was alleviated by intraperitoneal injection of melatonin, Apoptosis of germ cells in testicular tissue was significantly reduced in group E compared to group B, The number of germ cell apoptosis was significantly lower in group E than in remission.

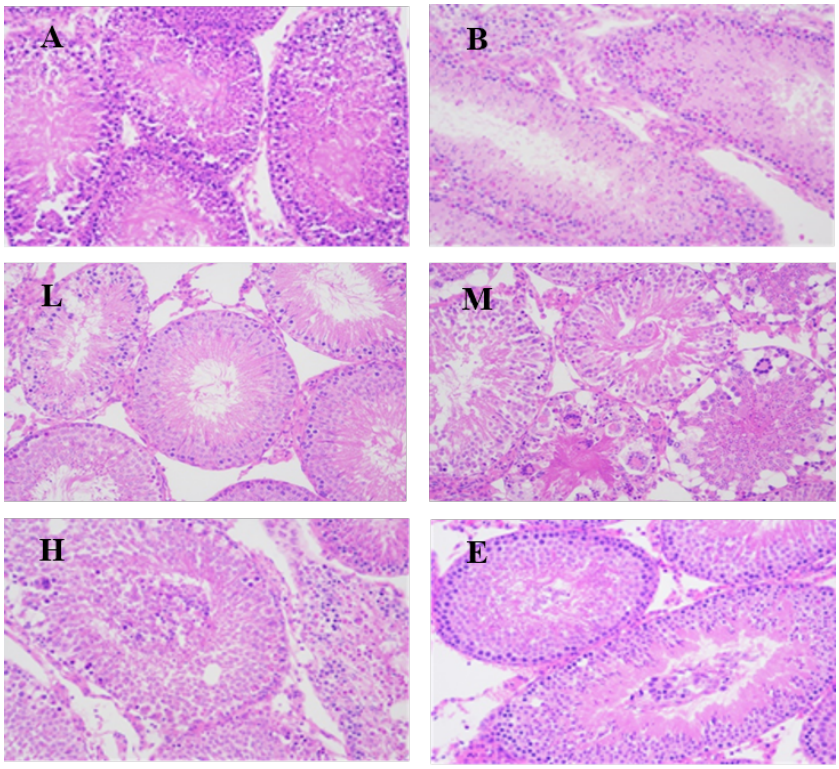


**Figure 4.** Hematoxylin and eosin sections of rat testicular tissues of different groups.

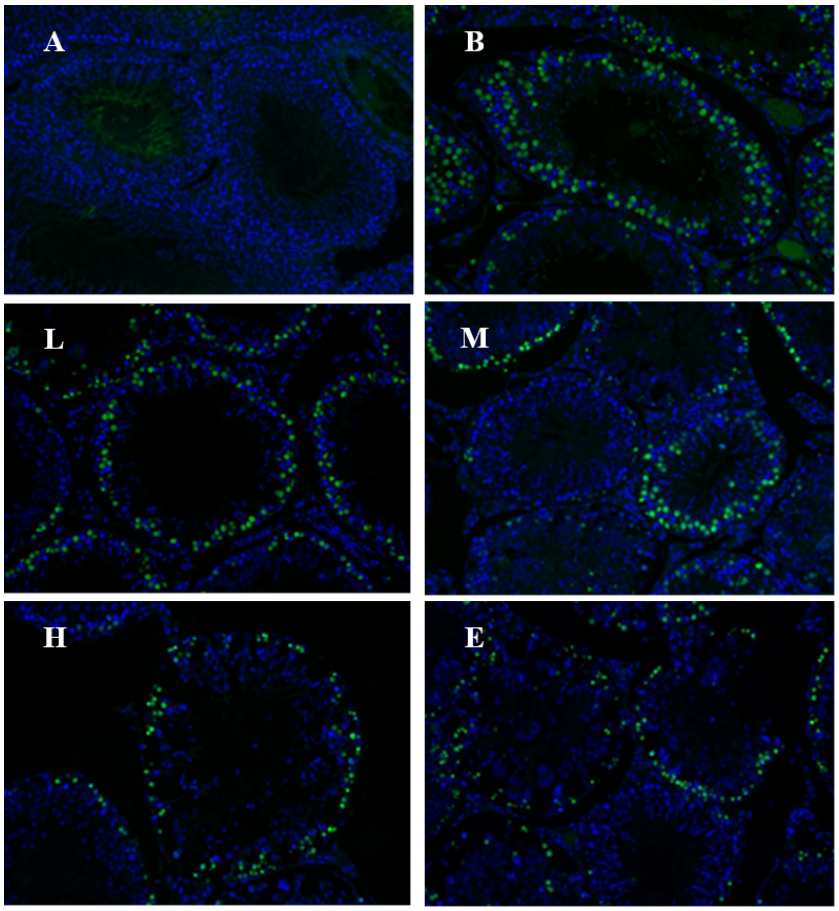


**Figure 5.** TUNEL sections of rat testicular tissues of different groups

## 3.6. Oxidative stress in rat testicular tissues

MDA and CAT levels in rat testicular tissues were significantly elevated after HS treatment compared to those in the control group (**Figure 6a** and **c**; $P < 0.001$). Compared with those of group B, MDA and CAT levels in rat testicular tissues were decreased in other melatonin groups, and group E is less different from the normal group (**Figure 6a** and **c**; $P < 0.001$). The GSH-Px ratio was significantly lower in group B than in the control group (**Figure 6b**; $P < 0.001$), with a significant difference in group E (**Figure 6b**; $P < 0.01$). Regarding the SOD inhibition ratio, group B showed a significant decrease, compared with the control group (**Figure 6d**; $P < 0.01$).

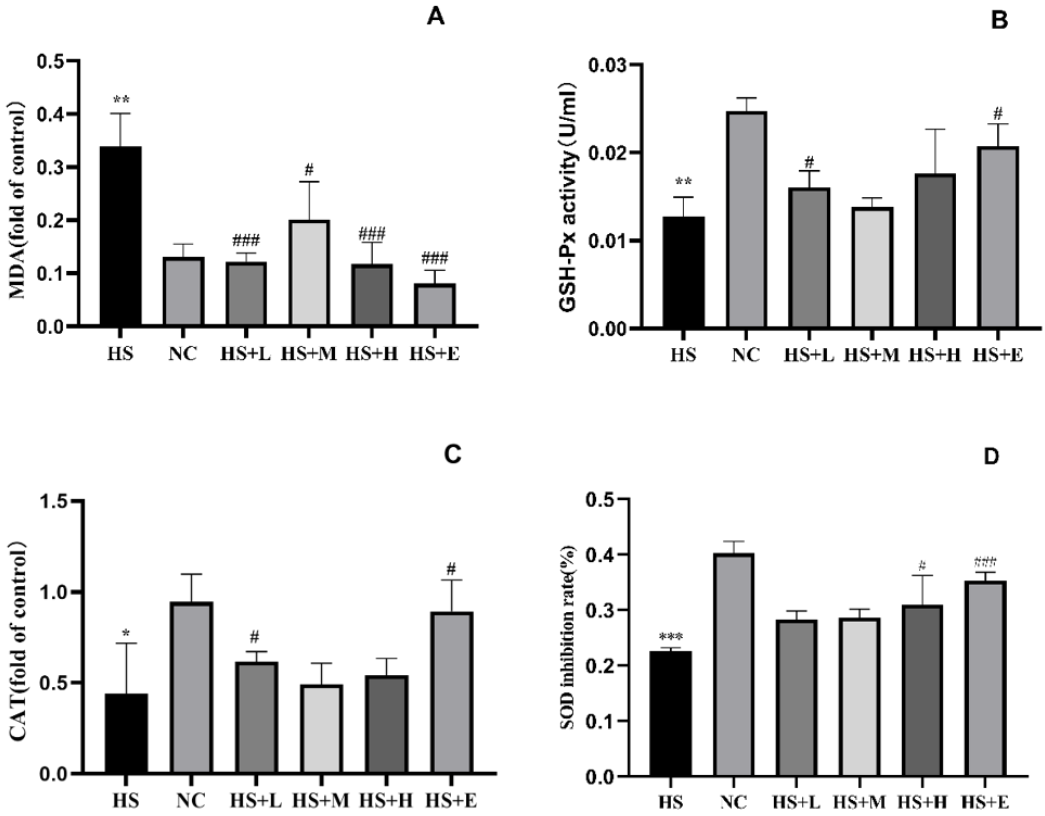


**Figure 6.** Oxidative stress levels in testicular tissues of different groups. Abbreviation: HS, heat stress; NC, normal control

## 3.7. Endoplasmic reticulum stress in rat testicular tissues

The expression and mRNA levels of proteins in the endoplasmic reticulum (ER) stress pathway were measured. The results show that GRP78 protein levels increased significantly in group B compared to the control group (**Figure 7a**; $P < 0.001$). However, melatonin reduced the HS-induced upregulation of GRP78 levels, with smaller differences in protein expression levels in group E compared to the control group A (**Figure 7a**; $P < 0.05$). CHOP, an important transcriptional protein, was also detected in the testicular tissues, exhibiting higher expression levels in group B (**Figure 7b** and **f**; $P < 0.01$). However, melatonin treatment significantly reduced the HS-induced upregulation of CHOP expression, with protein expression levels in group E differing significantly from those of the other treatment groups (**Figure 7b** and **f**; $P < 0.001$ and $P < 0.01$). ATF-6 was highly expressed in HS-exposed rat testicular tissues but was significantly reduced after melatonin treatment, with the highest decrease observed in group E (**Figure 7e** and **g**; $P < 0.001$). Phosphorylation levels increased after HS treatment but improved following melatonin treatment (**Figure 7d**; $P < 0.001$). These results indicate that melatonin significantly improved ER stress and reduced oxidative stress in the testicular tissues at a dose of 1 mM.

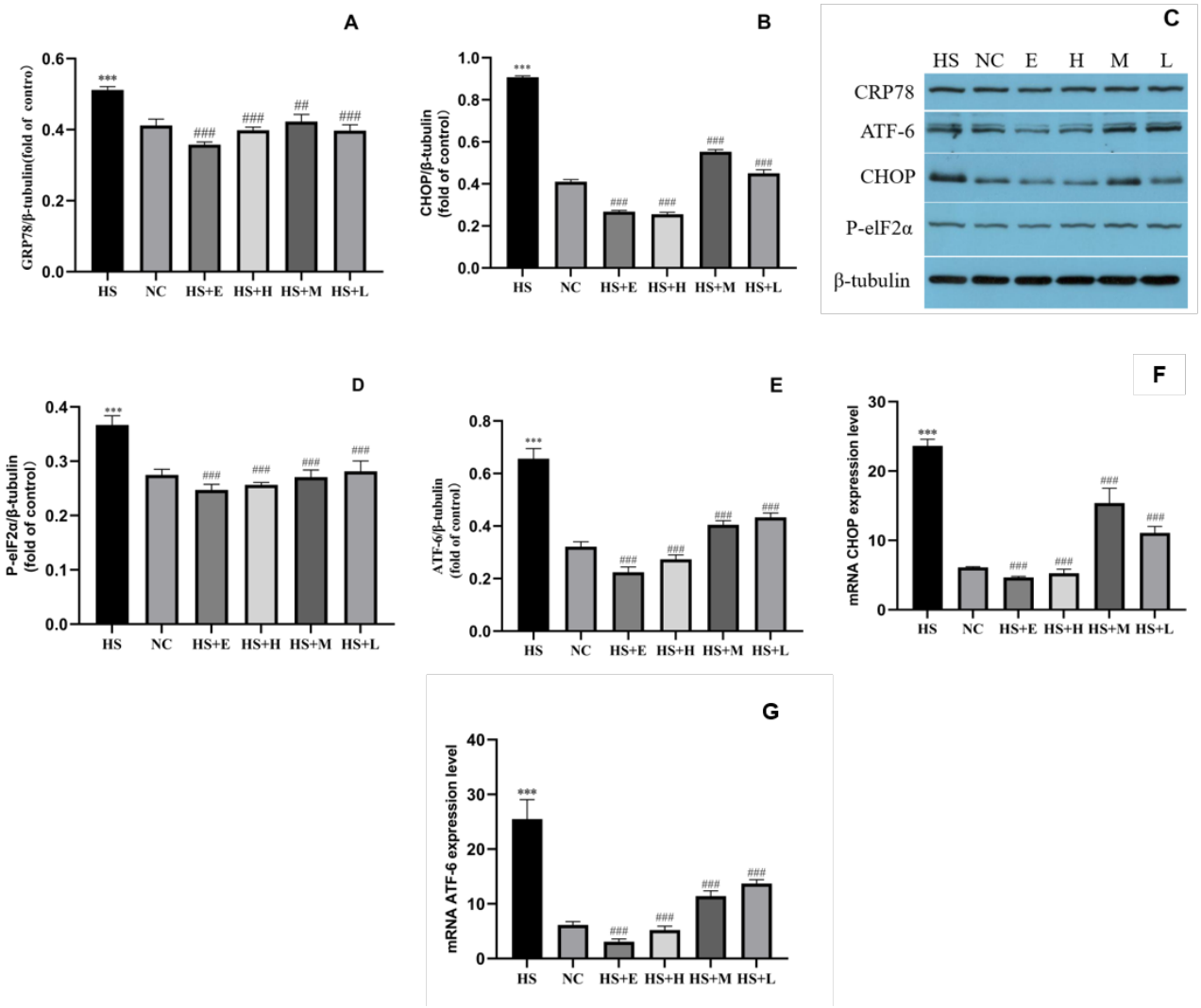


**Figure 7.** Melatonin reduced endoplasmic reticulum stress in rat testicular tissues.

## 3.8. Status of apoptotic pathways in rat testicular tissues

The expression of the apoptotic proteins Casp3 and Bax and the anti-apoptotic protein Bcl-2 was measured. The results showed that HS increased the expression of the apoptotic pathway proteins in testicular tissues. Cytochrome Casp3, an important protein in the ER stress pathway, exhibited significantly increased mRNA expression levels (**Figure 8c**; $P < 0.001$). Bcl-2 has been demonstrated to combine with the pro-apoptotic protein Bax for cell dimerization upon ER stress. Notably, the protein and mRNA levels in testicular tissues showed an increasing trend in the Bcl-2:Bax ratio with melatonin treatment compared with that in group B (**Figure 8a** and **d**; $P < 0.001$). The HS-induced reduction of the Bcl-2:Bax ratio in testicular tissues was most significantly ameliorated in group E (**Figure 8a** and **d**; $P < 0.001$), demonstrating that a dose of 1 mM melatonin was effective in reducing testicular cell apoptosis.

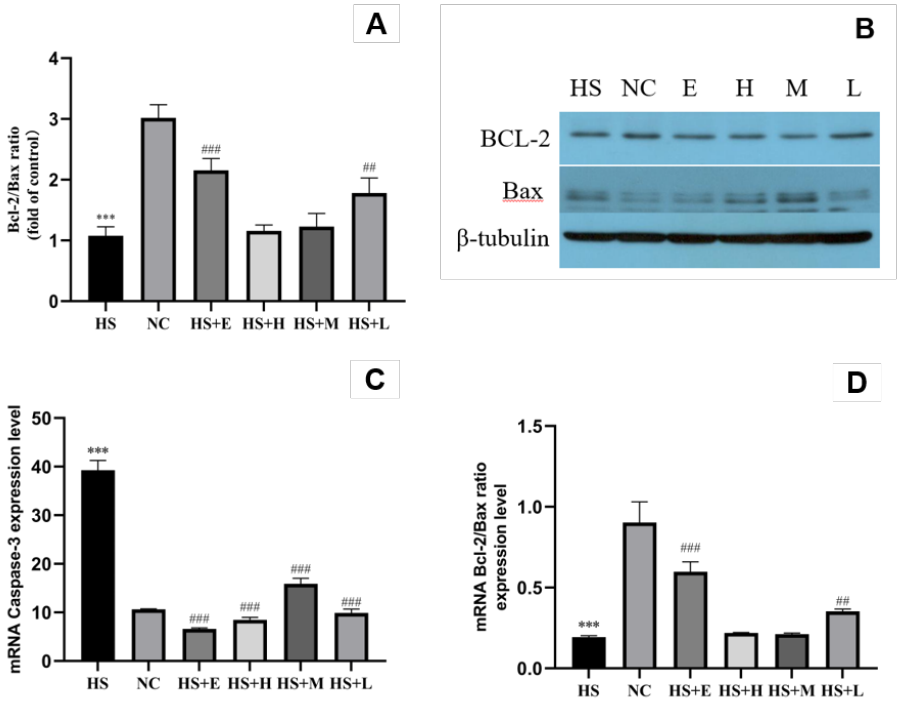


**Figure 8.** Melatonin reduced apoptosis in rat testicular tissues

## 4. Discussion

Several studies have demonstrated that scrotal heat therapy or heat stress (HS)-induced oxidative stress alters spermatogenesis [33], with oxidative damage emerging as a primary cause of male infertility. HS affects sperm quality, and even short periods of heat stress can pose a threat to sperm quality [34]. Elevated testicular temperature can disrupt the regulation and homeostasis of organisms [35], induce oxidative stress in germ cells [36], and damage sperm DNA [37]. In addition to HS, multiple methods exist that affect sperm quality. A study demonstrated that high temperature and psychological stress equally affect sperm quality in rats [38]. Moreover, sperm quality is similarly affected upon exposure to different types of radiation [39]; for example, sperm quality is reduced by radiofrequency electromagnetic field exposure [40]. Indicators of stag sperm cells under HS have also been reported to improve with the addition of botanical supplements to herbivore diets [41]. Furthermore, studies have demonstrated the efficacy of dietary antioxidants in combating the effects of HS [42], such as dietary onion powder [43] and betaine [44], which have been reported to improve reproductive indicators. Melatonin, a hormone with strong antioxidant properties, has been used to treat osteoarthritis [45], slow down aging [46], and manage anxiety [47,48].

The present study revealed that sperm activity in rats was affected by HS, consistent with previous reports [49]. Zhao *et al*. demonstrated that HS significantly reduces sperm quality, which was improved by melatonin [50]. Some scholars have found that melatonin attenuated HS-induced gamete apoptosis in mice primarily by inhibiting the ATF-6 and PERK signaling pathways [51], consistent with the regulatory mechanisms that have been experimentally validated using rats. Some studies have demonstrated that melatonin can be used in human mesenchymal stem cell-based therapies to treat oxidative stress injury in patients [52]. In an *in vitro* preservation experiment, melatonin positively affected frozen ram spermatozoa [53]. However, none of these studies proposed an effective dosing concentration of melatonin. Herein, it was observed that a 1 mM melatonin

dose could safeguard the process of spermatogenesis, which is consistent with the findings of previous *in vitro* experimental investigations of the effects of melatonin on spermatogenesis; this investigation may provide a basis for the use of melatonin in future *in vitro* experiments.

This study has some limitations. First, the safety pharmacology of melatonin was not considered, necessitating further pharmacokinetics and pharmacodynamics experiments. Furthermore, regarding the melatonin regulation mechanism, the trend of protein expression of the PERK, ATF-6, and IREI pathways was inconsistent. Given that melatonin may regulate the three pathways differently, these mechanisms require more in-depth investigations.

In summary, melatonin offers significant potential as a therapeutic agent for infertility, abnormal spermatogenesis, soft spermatogenesis, and cryptorchidism. Furthermore, it could be used for the optimization of sperm-related assisted reproduction technology as a sperm cell dilution preservation agent and cryoprotectant. However, the mechanism of melatonin production as an endogenous hormone requires further exploration.

## 5. Conclusion

In this study, HS affected the oxidative and ER stress levels of rat testicular tissues, inducing sperm cell damage, germ cell apoptosis, and pathological changes. However, melatonin alleviated the HS-induced damage, reduced the level of oxidative stress, repaired the ER stress state, improved sperm quality, and reduced germ cell apoptosis. The degree of apoptosis inhibition varied with different doses of melatonin. However, our results demonstrated that a melatonin dose of 1 mM exhibited the greatest effect in alleviating oxidative and ER stress relative to the other melatonin doses and effectively reduced apoptosis and protected spermatogenesis.

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## Disclosure statement

The authors declare no conflict of interest.

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