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Original Research

Melatonin Alleviates BPA-Induced Testicular Apoptosis and Endoplasmic Reticulum Stress

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Abstract

Background: The impact of melatonin on bisphenol A (BPA)-induced testicular apoptosis and endoplasmic reticulum (ER) stress was explored. **Methods**: The mice received BPA (50 mg/kg) by gavage for 30 days while being injected with 20 mg/kg melatonin. Protein expressions were detected with western blotting. The Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) assay measured testicular cell apoptosis. Testosterone was quantified by employing enzyme-linked immunosorbent assay (ELISA). **Results**: Melatonin promoted the development of seminiferous tubules, restored the orderly arrangement of the germ cells, and increased epithelial layers in the seminiferous tubules in BPA-treated mice. Moreover, in BPA-treated mouse testicular cells, melatonin markedly upregulated melatonin receptor 1A (MTNR1A) and melatonin Receptor 2 (MTNR2) expressions while downregulating ER molecular chaperones glucose-regulated protein 78 (GRP78) and glucose-regulated protein 94 (GRP94). Furthermore, it decreased p-PERK, p-IRE1, and ATF6α, as well as the apoptotic proteins cysteine-containing aspartate-specific proteases-12 (caspase-12) and cleaved cysteine-containing aspartate-specific proteases-3 (cleaved caspase-3), causing the suppression of testicular cell apoptosis. Additionally, melatonin increased the levels of cytochrome P450 17α-hydroxylase/20-lyase (CYP17A1), 17β-hydroxysteroid dehydrogenase 3 (17β-HSD3), and 3β-hydroxysteroid dehydrogenase 4 (3β-HSD4), in the ER, and elevated testosterone levels in testicular tissue. **Conclusions**: Melatonin can significantly alleviate testicular apoptosis and ER stress induced by BPA, which is because of the upregulation of melatonin receptor expression in testicular cells, inhibition of ER stress-related pathways, and enhancement of testosterone synthesis.

Keywords: melatonin; bisphenol A; endoplasmic reticulum stress; apoptosis; testosterone

1. Introduction

Bisphenols are widely produced organic compounds worldwide [1]. They are applied in the production of plastic bottles, food packaging, inner coatings of beverage cans, thermal paper, and medical devices, among other products. These products can release monomers (such as bisphenol A (BPA)/S/C/F) under conditions such as heating, acidity, or alkalinity [1]. BPA monomers can enter the human body through the digestive system, vertical transmission between mother and fetus, respiratory system, and contact with the skin and eyes. BPA disrupts male reproductive function [2,3]. BPA exposure can increase sperm DNA damage and decrease semen quality [4]. Additionally, BPA damages spermatogenesis and Sertoli cells in male rats [5,6]. Consequently, the exposure to BPA has garnered considerable attention from both the public and the scientific community.

BPA can trigger apoptosis and endoplasmic reticulum (ER) stress of neuronal, hepatic, and testicular cells [7–9]. BPA exerts a concentration-dependent effect on the upregulation of ER stress pathway proteins in mouse testicular tissue. Furthermore, BPA induces the upregulation of cleaved-caspase-3 in testicular cells, leading to apoptosis [10]. Sulforaphane can target the ER stress signaling pathway in liver cells, thus alleviating lipid droplet accumulation and key enzyme levels responsible for fat synthesis induced by BPA [11]. Nano-selenium has been found to alleviate BPA-induced intestinal damage by inhibiting the expression of ER stress signaling proteins such as protein kinase R-like ER kinase (PERK), inositol-requiring protein 1-α (IRE1), and activating transcription factor 6 (ATF6) [12]. However, there is a lack of research on drugs specifically targeting ER stress in testicular cells to alleviate BPA-induced testicular cell damage.

Melatonin [13] can regulate the function of the reproductive system. In the female reproductive system, it can enhance the total antioxidant capacity of the ovary and delay ovarian aging by increasing the superoxide dismutase level [14]. It enhances the developmental ability of embryos after cryopreservation and vitrified oocytes by capturing free radicals and improving a series of events in oxidative stress [15,16]. Furthermore, it can effectively alleviate the developmental damage to ovarian oocytes by some insecticides, such as acetamiprid [17], rotenone [18], and imidacloprid [19]. Additionally, melatonin also regulates the male reproductive system by enhancing testosterone synthesis and secretion, promoting testicular growth and development, and improving semen quality [20–22]. Melatonin exhibits potent antioxidant properties, which al-

leviate damage to testicular tissue caused by anticancer drugs [23,24], radiation therapy [25,26], bisphenols [27–32], herbicides [33], and heavy metals [34,35]. Melatonin, when combined with bisphenol, enhances sperm viability, density, and testosterone content [27,29]. Both vitamin E and melatonin effectively mitigate reproductive damage caused by bisphenols [27,36]. Melatonin is considered a more suitable agent for combating the toxicity of bisphenols [1]. Melatonin can alleviate ER stress in various tissues and cells [27–29]. It can relieve ER stress and apoptosis in chondrocytes caused by osteoarthritis, by inhibiting the IRE1-α-X-box-binding protein 1-C/EBP homologous protein (IRE1α-XBP1S-C/EBP homologous protein (CHOP)) signaling pathway [27]. Melatonin can also reduce ER stress triggered by obesity or diabetes in kidney cells by down-regulating glucose-regulated protein 78 (GRP78), IRE1, and ATF6 [28]. In testicular tissue, melatonin can suppress apoptosis and attenuate ER stress in testicular cells caused by heavy metal cadmium by suppressing ER stress proteins phosphorylated eukaryotic initiation factor 2α (p-EIF2α), CHOP, GRP78, and X-box-binding protein 1 (XBP-1) [29]. Nevertheless, it remains unclear whether melatonin can inhibit apoptosis and alleviate ER stress-induced injury in testicular cells resulting from BPA exposure.

Here, the impact of melatonin on testicular apoptosis and ER stress caused by BPA was investigated. Sexually mature male mice were simultaneously intervened with both BPA and melatonin. The testicular tissue development, the expressions of ER chaperone proteins and ER stress-related signaling pathway proteins, and testicular cell apoptosis were evaluated. Our findings may provide a novel therapeutic approach to alleviate ER stress in testicular cells and attenuate testicular injury.

2. Materials and Methods

2.1 Study Animals

Male sexually mature Kunming mice (weighing 35 g $\pm$ 2 g; n = 30; Changchun Yisi Experimental Animal Technology, Changchun, Jilin, China) were utilized in this study. The Ethics Committee of Jilin Medical University approved the experimental procedures (No. 2023-LW013). The study is reported following ARRIVE guidelines.

2.2 Preparation of BPA and Melatonin

The 20 mg/mL of BPA (239658, Sigma-Aldrich, St. Louis, MO, USA) was prepared by dissolving in olive oil. The 8 mg/mL of melatonin (M5250, Sigma-Aldrich) (0.24 g) was prepared by dissolving in 3 mL of anhydrous ethanol and subsequently 27 mL of normal saline to achieve a concentration of 8 mg/mL. The melatonin solution contained 10% ethanol, which has been recognized as non-toxic [37].

2.3 Grouping and Treatment of Animals

Thirty male mice were assigned randomly to the control, BPA, and BPA+melatonin groups. The BPA (50 mg/kg, a dose considered as the lowest-observed-adverse-effect level [38]), was administered to the BPA and BPA+melatonin groups daily by gavage. The control mice were administered an equivalent volume of olive oil daily. Meanwhile, the BPA+melatonin group was given 20 mg/kg melatonin (i.p.) every other day [39,40]. The every-other-day administration approach can attenuate the stress damage induced by daily handling and injections in mice. Meanwhile, normal saline (equivalent volume) was given to the control and BPA groups. The mice in all three groups were treated with the respective interventions for 30 days, after which they were euthanized by cervical dislocation to obtain their testes. The right testis of each mouse was fixed, while the left testis was used for western blotting and enzyme-linked immunosorbent assay (ELISA).

2.4 Hematoxylin-Eosin (HE) Staining

Following 24 of fixation in Bouin's fixative solution (BL-GO16, Nanjing Sumberger Biotechnology Co., Ltd., Nanjing, Jiangsu, China), the testis tissue was subjected to gradient ethanol dehydration, xylene treatment for 15 min, and paraffin embedment. Then, the tissue sections were subjected to treatment with xylene and graded ethanol. Followingly, staining with hematoxylin and eosin was performed. Finally, after dehydration and xylene treatment, the sections were examined using the Olympus BX53 microscope (Tokyo, Japan).

2.5 Western Blotting Analysis

Mouse testicular tissues were incubated with RIPA lysis buffer and 1% PMSF (P0013B and P1006, Beyotime, Beijing, China) to extract proteins. After protein concentration determination, electrophoresis, and transfer, we conducted incubation with primary antibodies against rabbit melatonin receptor 1A (MTNR1A) (ab87639, Abcam, Cambridge, UK), melatonin Receptor 2 (MTNR2) (ab155678, Abcam), GRP78 (ab21685, Abcam), GRP94 (ab238126, Abcam), p-PERK (3179, CST, Boston, MA, USA), p-EIF2α (3398, CST), p-IRE1 (ab124945, Abcam), XBP-1 (ab220783, Abcam), ATF6α (ab37149, Abcam), cleaved cysteine-containing aspartate-specific proteases-3 (cleaved caspase-3) (ab2302, Abcam), cysteine-containing aspartate-specific proteases-12 (caspase-12) (35965, CST), cytochrome P450 17α-hydroxylase/20-lyase (CYP17A1) (ab125022, Abcam), 17β-hydroxysteroid dehydrogenase 3 (17β-HSD3) (bs-3905R, Bioss, Beijing, China), 3β-hydroxysteroid dehydrogenase 4 (3β-HSD4) (sc515120, Santa Cruz, Dallas, TX, USA), and β-actin (ab8226, Abcam). After that, a goat anti-rabbit HRP-labeled secondary antibody (31210, Thermo Scientific Pierce, Waltham, MA, USA) was employed for incubation, followed by enhanced chemiluminescent detection. ImageJ software V1.8.0 (http

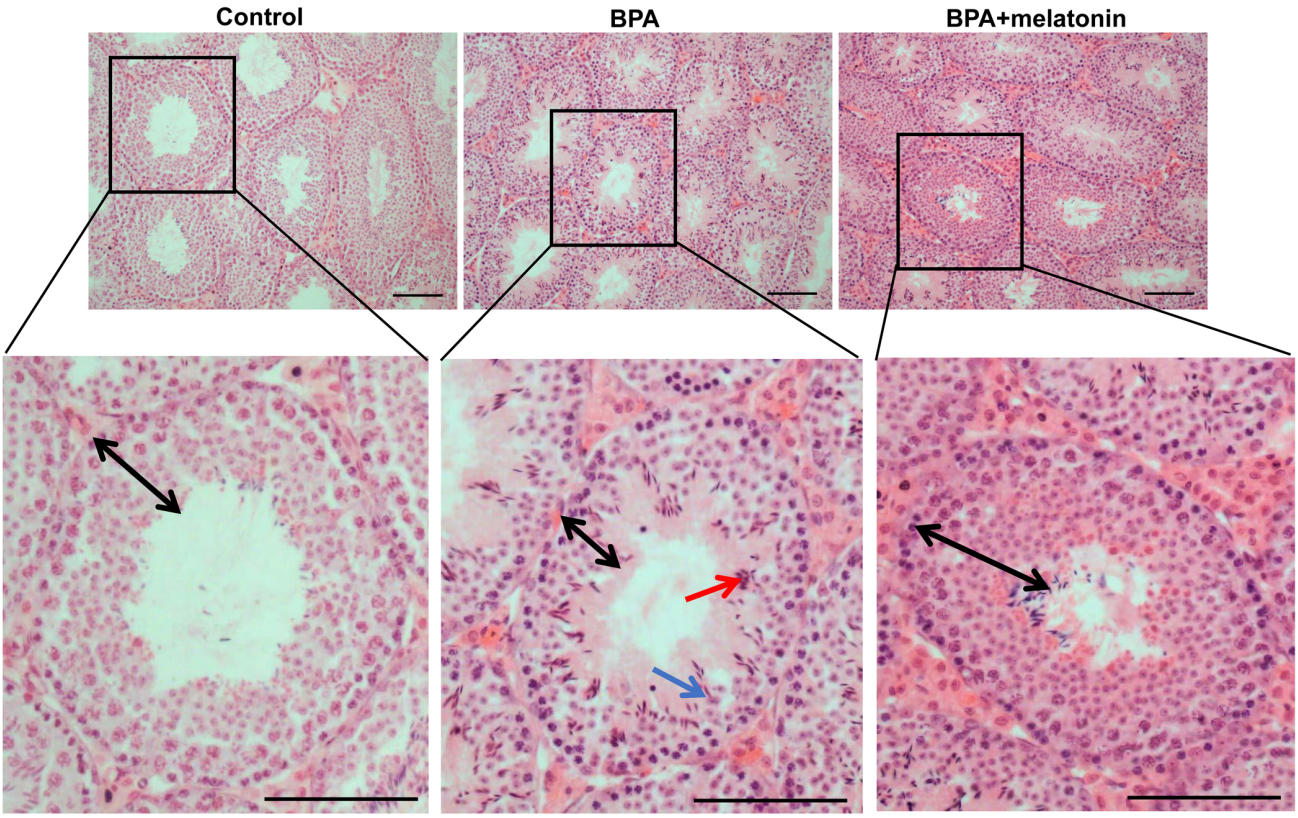

Fig. 1. HE staining of mouse testicular tissue. HE staining results of the control, BPA, and BPA+melatonin groups are presented (Scale bar = 100 µm). The thickness of the spermatogenic epithelium is indicated by the double-headed arrow. The shedding of germinal epithelium is represented by the red arrow. The disordered arrangement of germ cells is indicated by the blue arrow. HE, Hematoxylin-Eosin; BPA, bisphenol A.

s://imagej.net/ij/) analyzed protein levels. The blots were cut before hybridization with antibodies. As a result, images of sufficient length were unavailable.

2.6 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling L (TUNE) Assay

Testicular cell apoptosis was evaluated with the Terminal Deoxynucleotidyl Transferase dUTP Nick end Labeling (TUNEL) cell apoptosis detection kit (MK1024, Wuhan Boster Biotechnology Co., Ltd., Wuhan, Hubei, China). The specific procedure was as follows: the paraffin sections of testicular tissue were de-parafinized, followed by gradient ethanol treatment. After proteinase treatment for 10 min at 37 °C, the TUNEL reaction was performed through treating the sections with terminal deoxynucleotidyl transferase and digoxigenin-labeled dUTP for 2 h at 37 °C, which labeled the 3′-OH end of the fragmented DNA. Following blocking, the sections were treated with the biotinylated digoxigenin antibody diluted in Streptavidin-Biotin Complex (SABC) for 0.5 h at 37 °C. The sections were further incubated with SABC-FITC for 0.5 h at 37 °C. The nuclei were stained with DAPI (C1005, Beyotime). The stained tissue sections were mounted using an anti-quenching mounting medium. Intestinal tissue served as the positive control. The stained tissues were observed under an Olympus BX53 microscope, with TUNEL-positive cells emitting green fluorescence. For each group, testicular tissues from three mice were used, and three sections per mouse were stained. Five randomly selected regions were used to quantify apoptotic cells.

2.7 ELISA

After homogenizing the mouse testicular tissue and centrifugation, we quantified the testosterone in the supernatant with a testosterone detection kit (YJ001948, Shanghai Yuanju Biotechnology Center, Shanghai, China). The OD450 was determined utilizing a microplate reader. The testosterone content was quantified using the standard curve.

2.8 Statistical Methods

SPSS 26.0 (http://www.spss.com.hk/software/statistics) evaluated the data, which are described by mean ± standard deviation. We conducted multi-comparison with one-way ANOVA and the LSD test. The $p < 0.05$ indicates significant differences.

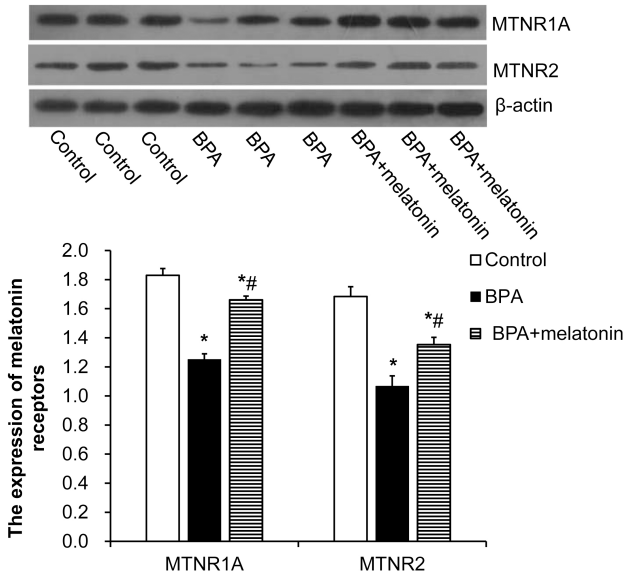

Fig. 2. Expression of MTNR1A and MTNR2 in mouse testicular tissue. MTNR1A and MTNR2 expression in mouse testicular tissue was assessed with western blotting. The relative expression levels of MTNR1A and MTNR2 were compared after grayscale analysis. $^{\#}p < 0.05$, *vs.* the BPA group; $^{*}p < 0.05$, *vs.* the control; N = 3. MTNR1A, melatonin receptor 1A; MTNR2, melatonin Receptor 2.

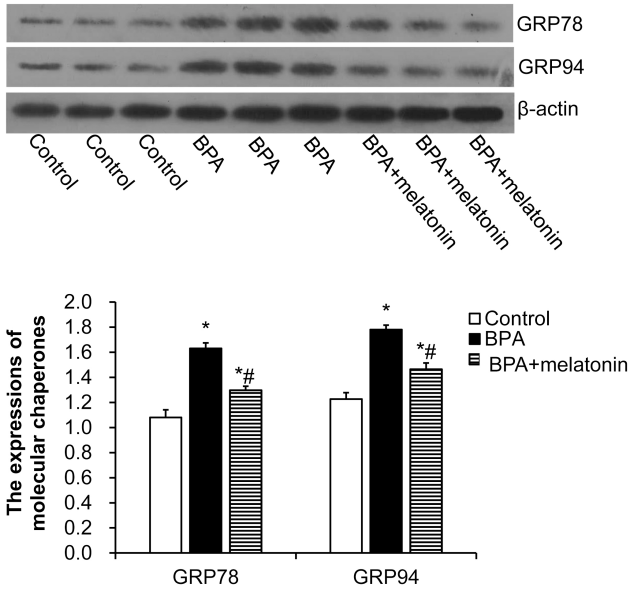

Fig. 3. Expression of molecular chaperones GRP78 and GRP94 in mouse testicular tissue cells. GRP78 and GRP94 expressions in mouse testicular tissue were measured with western blotting. Their relative expression levels were compared after grayscale analysis (N = 3). $^{\#}p < 0.05$, *vs.* the BPA group; $^{*}p < 0.05$, *vs.* the control. GRP78, glucose-regulated protein 78; GRP94, glucose-regulated protein 94.

3. Results

3.1 Melatonin Alleviates the Damage to Mouse Testicular Tissues Caused by BPA

The testes were fixed and stained with HE. In the BPA group, the epithelium of the seminiferous tubules in mouse testicular tissues became thinner. There was a reduction in germ cell number, disordered arrangement, and shedding of the germinal epithelium into the lumen (Fig. 1). In the control and BPA+melatonin groups, the layers of germinal epithelium were increased, with an orderly arrangement of germ cells, and no shedding of germinal epithelium was observed (Fig. 1). Therefore, melatonin mitigated the damage to mouse testicular tissues induced by BPA.

3.2 Melatonin Increases the Levels of Melatonin Receptors in the Testicular Tissues of Mice after BPA Exposure

We assessed the expression of melatonin receptors MTNR1A and MTNR2 by using western blotting (Fig. 2). Both MTNR1A and MTNR2 expressions in the BPA and BPA+melatonin groups were significantly lower than the control ($p < 0.05$). However, their expression in the BPA+melatonin group elevated significantly in comparison to that in the BPA group ($p < 0.05$). Hence, the melatonin receptor expressions in the testicular tissues of BPA-treated mice were up-regulated by melatonin.

3.3 Melatonin Suppresses ER Molecular Chaperones in BPA-Treated Mouse Testicular Tissues

Compared to the control, the BPA and the BPA+melatonin groups exhibited significantly elevated GRP78 and GRP94 in the testicular tissues, which are ER molecular chaperones (Fig. 3) ($p < 0.05$), as demonstrated by western blotting. Furthermore, their expression in the BPA+melatonin group was significantly lower than that in the BPA group ($p < 0.05$). Therefore, melatonin down-regulates GRP78 and GRP94 in mouse testicular tissues exposed to BPA.

3.4 Melatonin Reduces the Expression of ER Stress Pathway Proteins in the Testicular Tissues of Mice after BPA Exposure

The induction of ER stress principally comprises IRE1, ATF6, and PERK signaling pathways [41]. The protein expression levels of these pathways were assessed through Western blot analysis (Fig. 4A,B). Both the BPA and BPA+melatonin groups had significantly elevated levels of p-PERK, XBP-1, p-IRE1, p-EIF2α, and ATF6α than the control ($p < 0.05$). Conversely, their levels in the BPA+melatonin group were reduced than in the BPA group ($p < 0.05$). This data suggests that melatonin can down-regulate ER stress pathway proteins in the testicular tissues of mice after BPA exposure.

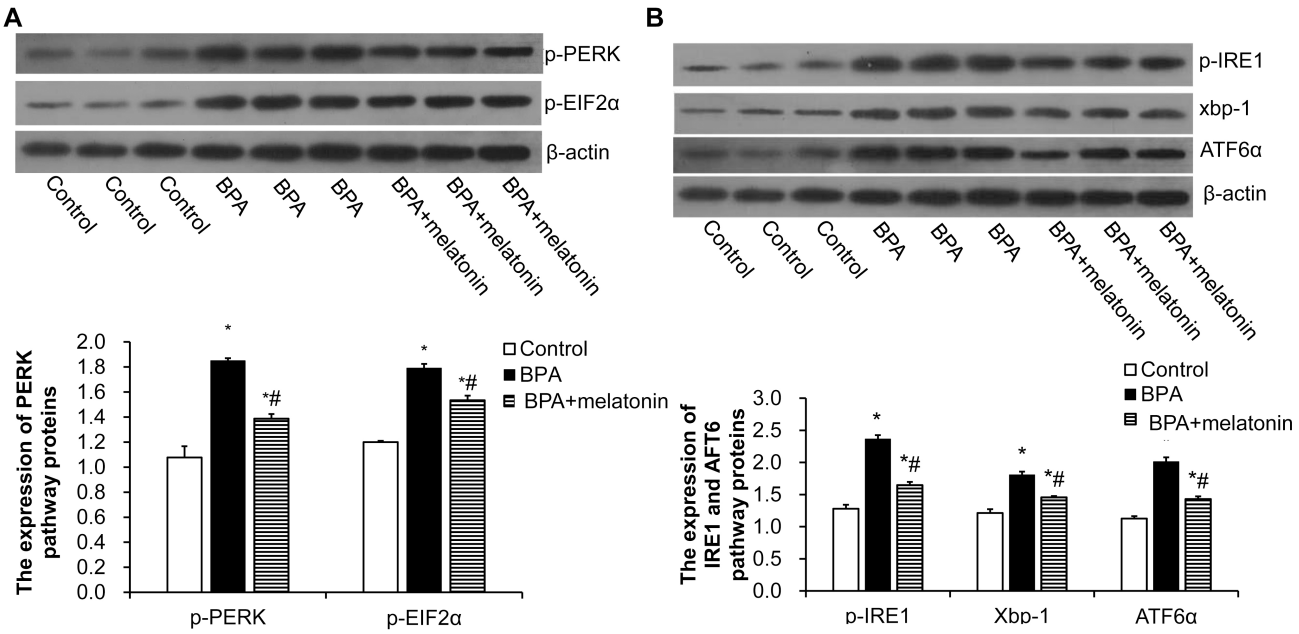

Fig. 4. Expression of PERK, IRE1, and ATF6α signaling pathway proteins under endoplasmic reticulum stress in mouse testicular tissue. (A) Western blot analysis of p-PERK and p-EIF2α in the PERK signaling pathway. The relative p-PERK and p-EIF2α levels were compared after grayscale value analysis (N = 3). (B) Western blot analysis of IRE1 and ATF6α in mouse testicular tissue. The blots were cut before hybridization with antibodies. As a result, images of sufficient length were unavailable. Through grayscale analysis, the relative p-IRE1, XBP-1, and ATF6α levels in each group were compared. $^{\#}p < 0.05$, *vs.* the BPA group; $^{*}p < 0.05$, *vs.* the control; N = 3. p-IRE1, phosphorylated inositol-requiring protein 1-α; ATF6, activating transcription factor 6; p-PERK, phosphorylated protein kinase R-like ER kinase; p-EIF2α, phosphorylated eukaryotic initiation factor 2α; XBP-1, X-box-binding protein 1.

3.5 Melatonin Reduces Apoptosis in Mouse Testicular Tissues Treated with BPA

Western blotting revealed that both the BPA and BPA+melatonin groups exhibited significantly elevated expression of p-Caspase-3 and Caspase-12 than the control (Fig. 5) ($p < 0.05$). Conversely, the BPA+melatonin group had significantly reduced p-caspase-3 and caspase-12 than the BPA group (Fig. 5) ($p < 0.05$).

TUNEL assay was employed to detect apoptosis in mouse testicular cells. Cells emitting green fluorescence indicate the presence of DNA fragmentation. Apoptosis primarily occurred in spermatogonia and interstitial cells of mouse testicular cells (Fig. 6A). The BPA and BPA+melatonin groups had significantly more apoptotic testicular cells than the control ($p < 0.05$) (Fig. 6B). However, the BPA+melatonin group had significantly fewer apoptotic testicular cells than the BPA group ($p < 0.05$) (Fig. 6B).

Collectively, apoptosis in mouse testicular tissues after treatment with BPA is attenuated by melatonin.

3.6 Melatonin Elevates ER-Related Steroidogenic Enzymes and Testosterone in the Testicular Tissues of Mice after BPA Exposure

The expression of the ER-related steroidogenic enzymes CYP17A1, 17β-HSD3, and 3β-HSD4 in testicular tissues was determined using western blotting, while ELISA assessed the testosterone levels. Both the BPA and BPA+melatonin groups had significantly reduced CYP17A1, 17β-HSD3, and 3β-HSD4 than the control, as demonstrated in Fig. 7 ($p < 0.05$). Conversely, the expressions of these enzymes in the BPA+melatonin group were significantly ($p < 0.05$) elevated than those in the BPA group. The BPA group had a significantly ($p < 0.05$) declined testosterone level in the testicular tissue than the control, whereas the BPA+melatonin group exhibited a significantly elevated ($p < 0.05$) testosterone level than the BPA group (Fig. 8). The testosterone level did not significantly alter between the BPA+melatonin and the control groups ($p > 0.05$). Thus, melatonin could up-regulate the levels of ER-related steroidogenic enzymes (CYP17A1, 17β-HSD3, and 3β-HSD4) and testosterone secretion in the testicular tissues of mice after BPA exposure.

4. Discussion

In recent decades, both humans and animals have been increasingly exposed to BPA, bisphenol S, bisphenol C, bisphenol F, bisphenol AF, and similar compounds [42,43]. BPA and its analogs, due to their heat resistance, high fracture resistance, and electrical resistance, are utilized in the production of polycarbonate plastics and epoxy resins as cross-linking chemicals. Currently, extensive experimental, epidemiological, and clinical research is focus-

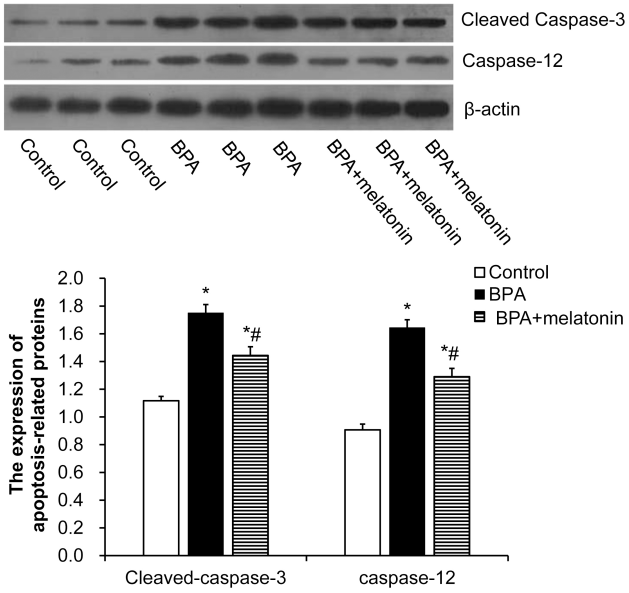

Fig. 5. Expression of apoptotic proteins related to endoplasmic reticulum stress in mouse testicular tissue. Western blotting detected cleaved caspase-3 and caspase-12 in mouse testicular tissue. Their relative expression levels were compared after grayscale analysis. $^{\#}p < 0.05$, *vs.* the BPA group; $^{*}p < 0.05$, *vs.* the control; N = 3. Cleaved caspase-3, cleaved cysteine-containing aspartate-specific proteases-3; caspase-12, cysteine-containing aspartate-specific proteases-12.

ing on the safety of bisphenol compounds, especially BPA, which exhibits estrogen-like activity and serves as an endocrine disruptor [44–46]. People are gradually substituting BPA with analogs such as bisphenol AF in products [47]. BPA can accumulate in the bodies of males or male animals, leading to testicular tissue damage, affecting the development of seminiferous tubules, as well as the synthesis and secretion of testosterone [42,48]. BPA can also cause ER stress in tissue cells, leading to cellular apoptosis, which is also an important pathway for BPA-induced testicular cell damage [49,50]. Importantly, it has been found that antioxidants can reverse reproductive damage caused by environmental pollutants and protect the reproductive system [51]. Melatonin, with powerful antioxidant capabilities, can suppress the expression of ATF6, IRE1, and PERK proteins, which are related to the signal pathway of ER stress, thereby alleviating heat stress-induced cell apoptosis [23,52]. Our study explored the function of melatonin against BPA-caused ER stress in mouse testicular cells. The results demonstrated suppressed the PERK, IRE1, and ATF6 signal pathways of ER stress, reduced testicular cell apoptosis, increased testosterone levels, and protected against the BPA-caused damages.

The unfolded protein response (UPR) is activated to rectify the issue of protein folding defects. Despite this, its prolonged and excessive activation overwhelms its protective effects, resulting in impaired ER function/homeostasis, cellular damage, and apoptosis [41]. ER stress upregulates GRP78 and GRP94, which are glycoproteins residing in the ER. Their activation regulates apoptosis triggered by toxic injury [41]. Furthermore, ER stress triggers the activation of three primary signaling pathways within the ER membrane: PERK, IRE1, and ATF6, which then initiate downstream gene transcription to alleviate ER stress [41]. ER stress can arise from exposure to various factors, including drugs [53], environmental pollutants [54], and toxic substances [55]. BPA exposure can cause ER damage in mouse oocytes, hepatocytes, and endometrial stromal cells, leading to elevated levels of CHOP, p-EIF2α, p-PERK, GRP78, p-IRE1, and ATF6 [11,56,57]. BPA-caused ER stress is dependent on its concentration [11,56,57]. Our research revealed that BPA not only stimulated GRP78 and GRP94 expressions in testicular tissues but also up-regulated the PERK, IRE1, and ATF6 ER stress signaling, subsequently causing significant ER stress.

Melatonin, an important antioxidant, alleviates ER stress in spermatogonial stem cells and testicular stromal cells by binding to the MTNR1A and MTNR2, thereby down-regulating p-PERK, Xbp-1, p-IRE1, and GRP78 [58,59]. Furthermore, melatonin can reduce the expression of p-EIF2α protein through the melatonin receptor pathway, thereby alleviating cadmium-induced ER stress in testicular cells [60]. Additionally, melatonin mitigates ER stress-induced damage in testicular tissue caused by tunicamycin [61], thereby reducing apoptosis in spermatogonial stem cells and preserving testicular spermatogenic function. It is currently unknown whether melatonin can alleviate ER stress in testicular cells caused by BPA through receptor pathways. In this study, we simultaneously exposed mouse testicular tissues to melatonin and BPA and found that melatonin significantly elevated MTNR1A and MTNR2 levels, reduced GRP78 and GRP94 levels, and inhibited the PERK, IRE1, and ATF6 signaling pathways involved in ER stress, thus alleviating ER stress in testicular cells. Therefore, melatonin, as an endogenous hormone, could alleviate the ER stress in testicular cells induced by BPA through receptor-mediated pathways. It is highly likely to become a novel therapeutic drug for improving male fertility.

Cell apoptosis acts as a protective mechanism to maintain organism homeostasis [62]. Caspase-12 regulates ER stress-induced cell apoptosis. Excessive ER stress can activate caspase-12, while ER stress-independent apoptosis does not involve this step [63]. The expression of caspase-12 can distinguish whether cell apoptosis is triggered by mitochondrial stress or ER stress. ER damage triggers the activation of procaspase-12, leading to its cleavage, conversion, and activation into caspase-12. Activated caspase-9 is subsequently cleaved into procaspase-3 and other effector caspases, ultimately leading to cell apoptosis [64]. Caspase-3 and caspase-12 are two critical proteins involved in ER stress-induced cell apoptosis. BPA upregulates caspase-3 and caspase-12 proteins, causing apoptosis

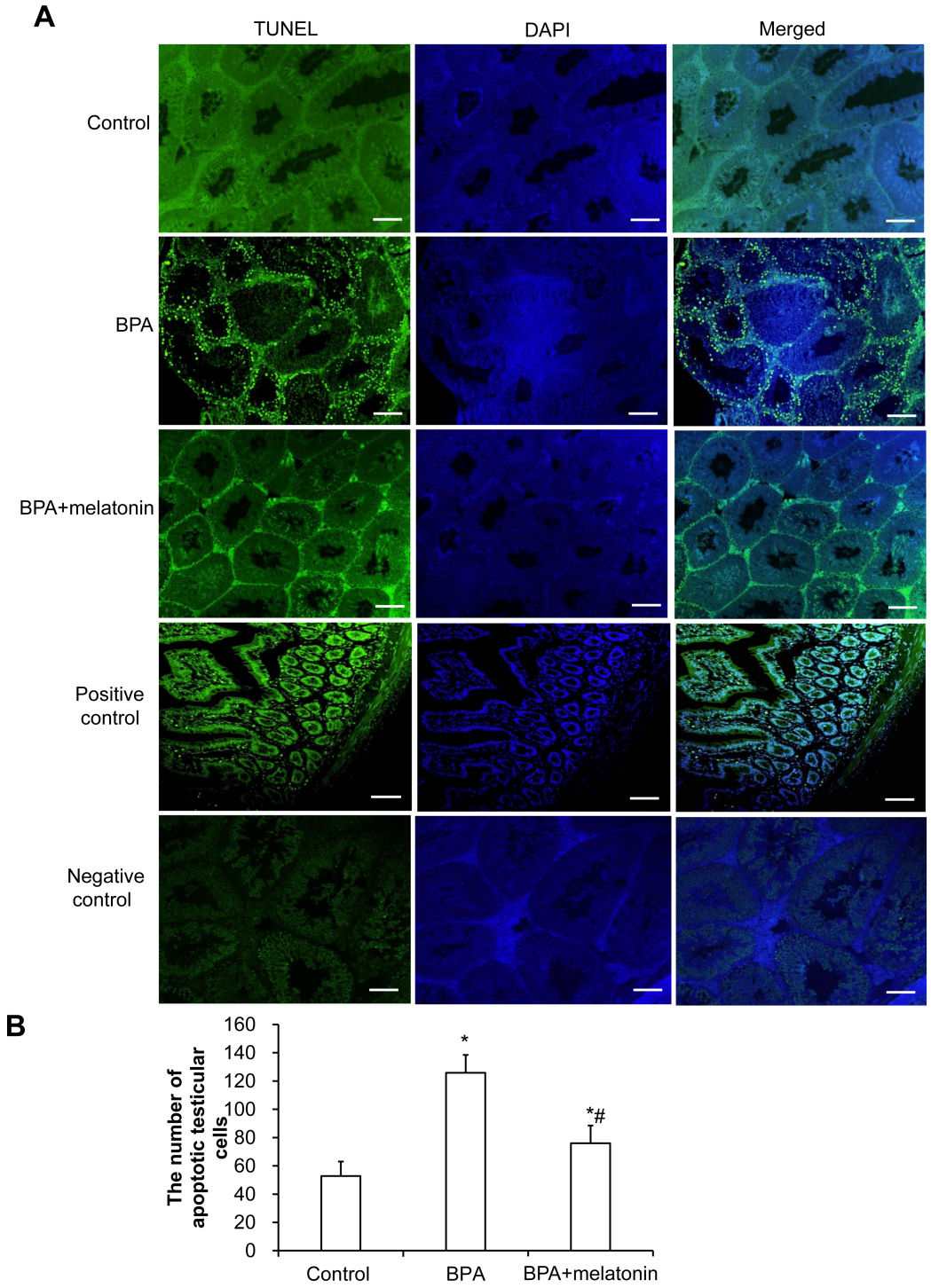

Fig. 6. Detection of apoptosis in mouse testicular tissue. (A) Apoptosis in mouse testicular tissue was detected using TUNEL. Green fluorescence indicates apoptotic cells (Scale = 100 μm). (B) The apoptotic testicular cell number was statistically analyzed. $^{\#}p < 0.05$, *vs.* the BPA group; $^{*}p < 0.05$, *vs.* the control; N = 3. TUNEL, Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling.

in mouse brain cells and non-parenchymal liver cells [9,65]. Melatonin suppresses cleaved caspase-3 in spermatogonial stem cells, thus preventing spermatogonial stem cell apoptosis [66]. Consistently, we demonstrated that melatonin suppressed BPA-induced cleaved caspase-3, caspase-12, and cell apoptosis, and protected testicular tissues.

Interstitial cell apoptosis is the predominant form of testicular cell apoptosis induced by BPA [67]. Testicular interstitial cells are critical for testosterone synthesis and release [68]. Prolonged exposure to BPA leads to reduced testosterone synthesis and secretion [67]. The ER housing key enzymes like CYP17A1, 3β-HSD4, and 17β-HSD3

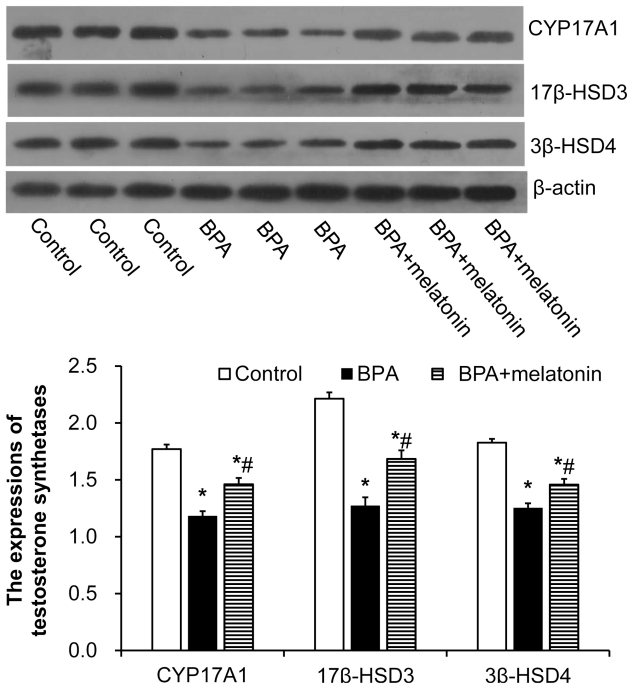

Fig. 7. Expression of endoplasmic reticulum-associated steroidogenic enzymes in mouse testicular tissue. The expressions of CYP17A1, 17β-HSD3, and 3β-HSD4 in mouse testicular tissue were examined utilizing western blotting. Relative levels of CYP17A1, 17β-HSD3, and 3β-HSD4 were compared after grayscale analysis (N = 3). $^{\#}p < 0.05$, *vs.* the BPA group; $^{*}p < 0.05$, *vs.* the control. CYP17A1, cytochrome P450 17α-hydroxylase/20-lyase; 17β-HSD3, 17β-hydroxysteroid dehydrogenase 3; 3β-HSD4, 3β-hydroxysteroid dehydrogenase 4.

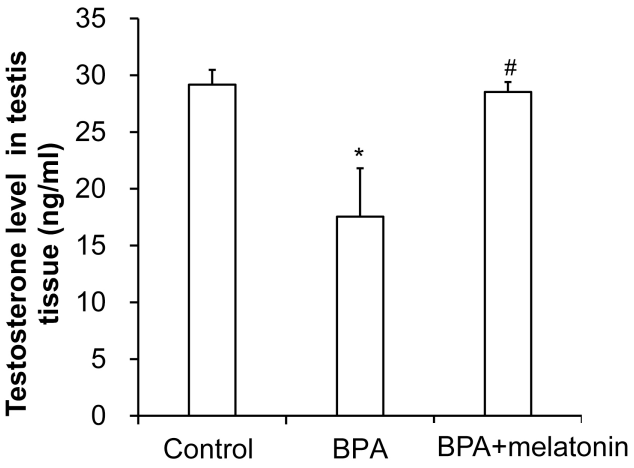

Fig. 8. Testosterone content in mouse testicular tissue. The content of testosterone in mouse testicular tissue was measured using enzyme-linked immunosorbent assay (ELISA). $^{\#}p < 0.05$, *vs.* the BPA group; $^{*}p < 0.05$, *vs.* the control; N = 5.

is crucial for testosterone synthesis [69]. BPA suppresses the activity of CYP17A1, 3β-HSD, and 17β-HSD3 in both rat and human testicular cells, leading to decreased testosterone synthesis and secretion [70]. Here, we found that under the action of melatonin, the expression of CYP17A1, 3β-HSD4, and 17β-HSD3 in testicular tissues, which were decreased due to BPA, were significantly elevated, and the levels of testosterone in testicular tissues were significantly increased. We suppose that melatonin may promote the development of seminiferous tubules, arrange germ cells in an orderly manner, increase the number of layers of the germinal epithelium, and protect testicular spermatogenic function.

5. Conclusions

As a potent antioxidant, melatonin enhances the expression of MTNR1A and MTNR2 on the membrane of testicular cells, inhibits ER stress and apoptosis caused by BPA, improves the expression of testosterone synthases on the ER, promotes testosterone synthesis and secretion, and protects the normal structure of testicular seminiferous tubules. Melatonin shows promise as a drug for preventing and treating oxidative stress-induced injury in testicular tissue in clinical practice. However, elucidating the mechanism by which melatonin inhibits ER stress in testicular cells is challenging and requires further investigation in subsequent cell experiments.

Abbreviations

BPA, Bisphenol A; ER, endoplasmic reticulum; MTNR1A, melatonin receptor 1A; MTNR2, melatonin Receptor 2; GRP78, glucose-regulated protein 78; p-EIF2α, phosphorylated eukaryotic initiation factor 2α; p-IRE1, phosphorylated inositol-requiring protein 1-α; p-PERK, phosphorylated protein kinase R-like ER kinase; XBP-1, X-box-binding protein 1; ATF6α, activating transcription factor 6; cleaved caspase-3, cleaved cysteine-containing aspartate-specific proteases-3; CYP17A1, cytochrome P450 17α-hydroxylase/20-lyase; CHOP, C/EBP homologous protein; caspase-12, cysteine-containing aspartate-specific proteases-12; 17β-HSD3, 17β-hydroxysteroid dehydrogenase 3; GRP94, glucose-regulated protein 94; 3β-HSD4, 3β-hydroxysteroid dehydrogenase 4; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

GW and XP designed the study. QQ, LF, JL, and DX collected the data and performed the statistical analysis. QQ and XP analyzed and interpreted the data. QQ and LF collected the funds. QQ was a major contributor

to manuscript writing. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

The experimental procedures involving the mice were approved by the Ethics Committee of Jilin Medical University (No. 2023-LW013). The study is reported following ARRIVE guidelines.

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Conflict of Interest

The authors declare no conflict of interest.

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