Original Paper

Melatonin Relieves Busulfan-Induced Spermatogonial Stem Cell Apoptosis of Mouse Testis by Inhibiting Endoplasmic Reticulum Stress

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Key Words

Melatonin • Busulfan • Spermatogonial stem cells • Apoptosis • Endoplasmic reticulum stress

Abstract

Background/Aims: Busulfan is commonly used for cancer chemotherapy. Although it has the advantage of increasing the survival rate of patients, it can cause male infertility via damaging the testes and reducing sperm counts. Therefore, the underlying mechanism should be explored, and new agents should be developed to protect the male reproductive system from busulfan-induced damage. Endoplasmic reticulum stress (ERS) is considered a key contributor to numerous pathologies. Despite several studies linking ERS to toxicants, studies have yet to determine whether ERS is a contributing factor to busulfan-induced testicular damage. Melatonin is a well-known broad-spectrum antioxidant, anti-inflammatory and antitumour agent, but the effects of melatonin on busulfan-induced ERS in mouse testes damage are less documented. *Methods:* The effects of melatonin were measured by immunofluorescence staining, Western blot, qRT-PCR analysis and flow cytometry assay. The underlying mechanism was investigated by measuring ERS. *Results:* We found that ERS was strongly activated in mouse testes (*in vivo*) and the C18-4 cell line (*in vitro*) after busulfan administration. ERS-related apoptosis proteins such as caspase-12, CHOP and caspase-3 were activated, and the expression of apoptotic proteins such as P53 and PUMA were upregulated. Furthermore, we investigated whether melatonin reduced the extent of damage to mouse testes and improved the survival rates of busulfan-treated mice. When exploring the underlying mechanisms, we found melatonin could counteract ERS by decreasing the expression levels of the ERS markers GRP78, ATF6, pIRE1 and XBP1 in mouse testes and mouse SSCs (C18-4 cells). Moreover, it blocked the activation of ERS-related apoptosis proteins caspase-12, CHOP and caspase-3 and suppressed P53 and PUMA expression stimulated by busulfan both *in vivo* and *in vitro*. *Conclusion:* Our results demonstrate that ERS is an important mediator for busulfan-induced apoptosis. The attenuation of ERS by melatonin can prevent busulfan-treated SSCs apoptosis

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and protect busulfan-treated testes from damage. Thus, this study suggests that melatonin may alleviate the side effects of busulfan for male patients during clinical treatment.

Introduction

Advances in chemotherapy for cancer treatment in young patients have resulted in increased survival and cure rates [1]. However, fertility is often impaired by alkylating agents, such as busulfan, which is a commonly used chemotherapeutic drug [2-4]. Therefore, the quality of life and the preservation of fertility have emerged as significant issues [5]. New methods should be developed to prevent testicular damage induced by alkylating agents [6, 7].

The endoplasmic reticulum (ER) is an intracellular organelle that controls protein folding, intracellular calcium homeostasis, and lipid synthesis [8]. Environmental stresses and increased protein synthesis often lead to the accumulation of unfolded proteins in the ER, and this phenomenon is called ER stress (ERS). The ERS pathway consists of three different branches: activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1), and protein kinase RNA-like ER kinase (PERK) [9].

ERS performs a dualistic function. It can restore ER homeostasis, but if ERS persists chronically at high levels, cellular functions can be negatively affected [10]. Chronic ERS is considered a key contributor to numerous human diseases, such as diabetes and neurodegeneration [11, 12]. Hence, targeting the components of ERS has been employed as a therapeutic strategy to control these ERS-associated pathologies [13, 14]. The IRE1 signalling pathway protects against ERS and controls the development and survival of immune cells [15]. ATF6 activates the expression of a repertoire of antioxidant genes and serves an important role as a link between the ERS and oxidative stress gene programmes [16]. Tuberous sclerosis complex-1 ablation induces prominent endoplasmic ERS responses by activating the PERK signalling pathways [17]. Moreover, ERS can also be induced by alkylating agents in renal epithelial cells [18].

Melatonin is secreted by the pineal gland, which is regulated by dark-light and seasonal cycles in mammals [19]. It acts as an antioxidant by reducing oxidative stress and increasing the activity of antioxidant enzymes in the body [20]. Melatonin protects melanocytes from DNA damage and oxidative stress through activation of NRF2 and its target enzymes [21]. It is well reported that melatonin can ameliorate ischaemic brain injury, preserve the functional integrity of the blood-brain barrier, and induce neurogenesis and cell proliferation through a receptor-dependent mechanism [22].

Melatonin is also involved in ER homeostasis [23]. It enhances the expression of the ERS proteins ATF6 and CHOP, improves survival rates, and successfully attenuates liver injury [24]. Furthermore, melatonin treatment alleviates ERS during bleomycin-induced pulmonary fibrosis [25]. Melatonin affects the regulation of testicular development and male reproduction by stimulating the secretion of gonadotropins and testosterone [26]. It also prevents cisplatin-induced primordial follicle loss [27]. In addition, the research recently published by our group has reported that melatonin has beneficial effects on oxidative apoptosis of mouse SSCs induced by busulfan [28]. Therefore, we aim to assess whether ERS is involved in busulfan-induced SSC apoptosis and testicular damage. The present study will provide a theoretical basis for alleviating the side effects caused by busulfan in clinical cancer treatment.

In this study, the beneficial effects of melatonin on busulfan-induced testicular damage and mouse spermatogonial stem cell (SSC) apoptosis were demonstrated. Our results revealed that the presence of melatonin directly provided resistance to busulfan-induced apoptosis and correlated with ERS. *In vitro* and *in vivo* evidence demonstrated that the administration of melatonin could ameliorate testicular damage caused by busulfan. Therefore, melatonin might be useful as a potential drug to counteract the side effects of busulfan because of its advantages, including high efficiency, low toxicity and endogenous effects.

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Materials and Methods

Animal experiments

Experiments were performed on healthy eight-week-old ICR male mice weighing between 25 and 30 g. The mice were obtained from the animal centre of the Fourth Military Medical University and were housed in wire cages at 25°C in a 12 h/12 h light/dark cycle at 70% humidity and fed ad libitum. The mice were maintained and the experiments were completed in accordance with the Guide for the Care and Use of Laboratory Animals at Northwest A&F University.

In this experiment, busulfan and 4PBA were diluted in DMSO, and melatonin was dissolved in 1% ethanol (in normal saline). Eighty age-matched mice were randomly separated into four groups: the CON group received vehicle injection (DMSO), the BU group received a single busulfan treatment (30 mg/kg), the BU+MEL group received melatonin (10 mg/kg/day) after busulfan treatment for 4 weeks, and the BU+4PBA group received 4PBA (100 mg/kg/day) after busulfan treatment for 4 weeks.

Cell experimental protocol

C18-4 cells, which are type A spermatogonial stem cells, were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F12 (DMEM/F12, Gibco, Grand Island, NY) supplemented with 10% foetal bovine serum (FBS, Gibco), 2 mM of L-glutamine (Invitrogen, Carlsbad, CA), and 100 unit/ml of penicillin and streptomycin (Invitrogen). The cells were subcultured every 3 days and maintained at 37°C in an incubator humidified with 5% CO_2. The cells were dissociated by treating with 0.25% trypsin-EDTA (Invitrogen) and reseeded into multi-well plates for subsequent analysis. The medium was changed every 2 d. In this procedure, busulfan was dissolved in DMSO to 10^{-4} μM. Melatonin was dissolved in 1% ethanol in physiological saline to 10^{-7} M. 4PBA was dissolved in DMSO to 1 mM. Cells were cultivated with busulfan (BU), busulfan and melatonin (BU+MEL), or busulfan and 4PBA (BU+4PBA) for 24 h prior to the experiments.

Histology analysis

For histological analyses, testes were dissected from CON, BU, BU+MEL and BU+4PBA mice. After fixation in 4％ PFA solution, the samples were embedded in paraffin blocks. Sections were cut and then stained with haematoxylin and eosin (HE).

For sperm morphological analyses, cauda epididymal sperm from CON, BU, BU+MEL or BU+4PBA mice were spread onto slides with PBS and air-dried. Sperm smears were stained with Giemsa.

Immunofluorescence staining

Immunofluorescence staining was performed as described in our previous studies [29]. The details of the antibodies used are listed in Table 1.

Western blot

The testes were collected, and protein extracts were obtained after surgery. The proteins were quantified using a BCA protein assay kit (Beyotime, China). The proteins were separated through SDS–PAGE and subsequently transferred to Immobilon NC membranes (Millipore, Boston, MA, USA). The membranes were blocked with 6% non-fat milk in TBST at room temperature, incubated with primary antibodies (the details are listed in Table 1) overnight at 4°C and washed thrice with TBST for 15 min each. The membranes were incubated at 37°C for 1 h with appropriate secondary antibodies, including goat anti-rabbit IgG-HRP (1:3000, Boster) and rabbit anti-mouse

Table 1. The primary antibody information

Antibody	Catalog No.	Corporate Brand	Dilution	Immusourcer
PLZF	12-9320-82	eBioscience	1:200	Mouse IgG
VASA	ab13840	Abcam	1:200	Rabbit IgG
ATF6	D262665-0010	Sangon Biotech	1:100	Rabbit IgG
XBP1	E1916	Santa cruz	1:100	Rabbit IgG
pIRE1	bs-4308R	Biosynthesis Biotechnology	1:200	Rabbit IgG
CHOP	bs-20669R	Biosynthesis Biotechnology	1:200	Rabbit IgG
PUMA	BA1802	Boster biological technology	1:200	Rabbit IgG
P53	bs-0033R	Biosynthesis Biotechnology	1:200	Rabbit IgG
Caspase3	bs-0081R	Biosynthesis Biotechnology	1:200	Rabbit IgG
Caspase12	bs-1105R	Biosynthesis Biotechnology	1:200	Rabbit IgG
MT1	bs-0027R	Biosynthesis Biotechnology	1:500	Rabbit IgG
MT2	bs-0963R	Biosynthesis Biotechnology	1:500	Rabbit IgG
GRP78	BA4293	Boster biological technology	1:500	Rabbit IgG
Gapdh	KM9002T	Monoclonal	1:1000	Rabbit IgG

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IgG-HRP (1:2000, Boster) and then washed with TBST thrice for 15 min each time. Protein bands were detected using a Bio-Rad imaging system (Bio-Rad, Hercules, CA, USA) and quantified using Quantity One (West Berkeley, CA, USA).

Table 2. The qRT-PCR primer sequences and the length of the amplified products

Primer	Sense Primer sequence 5'→3'	Antisense Primer sequence 5'→3'	Tm°C	Length of products (bp)
GRP78	AGAAACTCCGGCGTGAGGTAGA	TTCCTGGACAGGCTTCATGGTAG	59	176
XBP1	AAGAACACGCTTGGGAATGG	CTGCACCTGCTGCGGAC	58	113
CHOP	AGCTGGAAGCCTGGTATGAGGA	AGCTAGGGACGCAGGGTCAA	58	134
Caspase3	ATGGAGAACAACAAAACCTCAGT	TTGCTCCCATGTATGGTCTTTAC	58	74
Caspase12	CTGGCTCTCATCATCTGCAACAA	CGGCCAGCAAACTGCATTAAC	58	173
Gapdh	TGGCCTTCCGTGTTCCTAC	GAGTTGCTGTTGAAGTCGCA	58	178

qRT-PCR analysis

Total RNA was extracted from testes using the Trizol reagent (Qiagen, Beijing, China) according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA using a Reverse Transcriptase Reagent kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. qRT-PCR analysis was conducted in triplicate on a CFX96 Real-time PCR system (Bio-Rad Ltd, Berkeley, CA, USA) according to the directions of the BioEasy SYBRGreen I RT-qPCR kit (Bioer Co. Ltd., Hangzhou, China). The qRT-PCR was performed as described previously [28]. *Gapdh* expression was used as the house-keeping control. The comparative CT method was used to measure the relative gene expression. The primers are listed in Table 2.

Flow cytometry assay

Cell cycle distribution was assessed by propidium iodide (PI) staining. Adherent cells were dissociated with 0.25% trypsinization. The samples were washed with PBS, stained with 50 μg/ml of PI and 50 μg/ml of RNase A at 37°C for 20 min and analysed through flow cytometry (Altra; Beckman Co.).

Cell apoptosis was evaluated through annexin V-FITC and PI staining. The cells for the apoptosis test were resuspended gently in 500 μl of PBS. Then, 5 μl of annexin V-FITC and 5 μl of PI were added to the medium and mixed gently. The cells were incubated at room temperature for 10 min in the dark and subjected to FACS analysis.

The intracellular Ca^{2+} level was measured by using the Fluo-3 AM (Beyotime, China). Cells (5×10^5) were pretreated with BU, BU and MEL or 4PBA for 24 h, loaded with Fluo-3 AM for 1 h and then subjected to flow cytometric analysis.

Data analysis

Statistical analyses were performed using one-way ANOVA with the Newman–Keuls multiple range test. Student's *t* test was performed when only two pairs of data were compared. Data were represented as the mean ± SD, and statistical significance was expressed as follows: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. All data are representative of at least three different experiments and were analysed with GraphPad Prism (La Jolla, CA, USA).

Results

Melatonin alleviated busulfan-induced testicular damage in mice

To examine whether melatonin treatment influences the regeneration of spermatogenesis in the male mouse genital tract impaired by busulfan, we histopathologically analysed the testes and epididymis of different treatment groups. The results showed that the testicular mass of the busulfan-treated mice was reduced compared to control testes (48 ± 13% versus control group, P < 0.05, Fig. 1A). 4PBA works as a chemical chaperone by inhibiting ERS [30]. To determine whether the protective mechanism of melatonin is through suppression of ERS, we introduced 4PBA and found that the testicular weight was significantly increased in the melatonin and 4PBA treatment groups compared to busulfan-treated testes (62 ± 15% and 68 ± 22% versus the busulfan-treated group, respectively, P < 0.05, Fig. 1B). Approximately
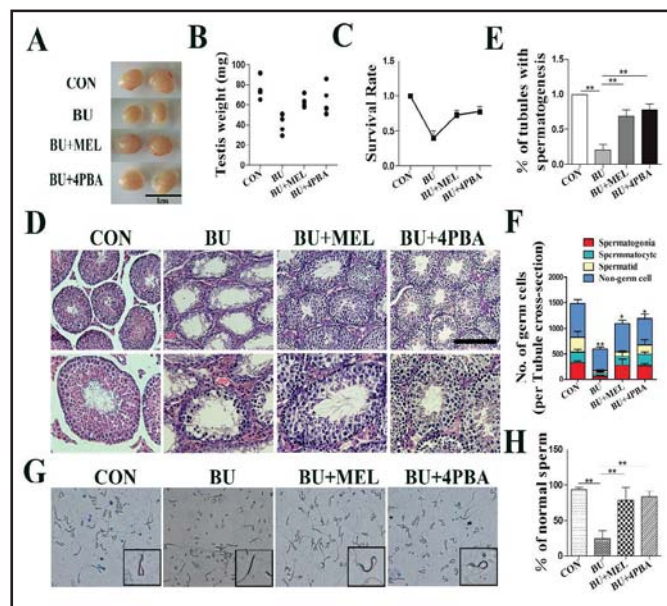
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50% of the mice died roughly 30 days after they were treated with busulfan, but the death rate was only 17% and 22% in the melatonin- and 4PBA-treated mice, respectively (Fig. 1C).

No histopathological abnormalities were observed in the control testes, and almost all seminiferous tubules were undergoing complete spermatogenesis. The busulfan-treated testes consisted of thin seminiferous epithelia, large lumens, and testicular vein congestion. Most seminiferous tubules were devoid of germ cells, and numerous degenerating germ cells could be observed in the seminiferous tubules of the busulfan-treated testes. Melatonin significantly enhanced the spermatogenic recovery after busulfan treatment was administered (Fig. 1D). The proportion of tubules with spermatogenesis was declined in the busulfan treatment group compared to control testes (P < 0.05). However, the percentage of seminiferous tubule with spermatogenesis in testes of the melatonin- and 4PBA-treated group were increased than that of the busulfan-treated group

Fig. 1. Effect of melatonin on busulfan-induced testes injury. (A) Morphology of the testes from 4-week-old mice after treatment with busulfan (BU), busulfan in combination with melatonin (BU+MEL), busulfan in combination with 4PBA (BU+4PBA) and control (CON). (B) The weight of all groups of mice is presented as a bar graph. (C) Survival rate of the different groups. (D) Representative images of HE-stained testicular cross sections from the four treatment groups. Scale bars = 200 μm. (E) Percentage of seminiferous tubules with capacity of spermatogenesis was calculated across multiple HE cross-sections (relative to the total number of tubules). (F) Germ cell number was determined in the testes of each group. (G) Sperm morphology. (H) Relative ratio of normal sperm per epididymis was counted. Data are represented as the mean ± SD (n = 3), and statistical significance is expressed as follows: * P<0.05 and ** P<0.01.

(48 ± 28% and 57 ± 7% versus busulfan-treated group, respectively, P < 0.05, Fig. 1E). The quantitative data indicated that a significant number of germ cells lost in busulfan treatment group. Moreover, the total number of spermatogonial cells was drastically reduced in the busulfan treatment group compared to control group (74 ± 21%, P < 0.05, Fig. 1F). Exposure of busulfan decreased the number of normal sperm per epididymis compared to control mice (73 ± 13%, P < 0.05). The count of normal sperm was increased 2.2 and 2.4-fold in the melatonin- and 4PBA-rescued groups relative to the busulfan-treated group (P < 0.05, Figs. 1G–H). These results suggest that melatonin significantly prevented busulfan-induced testicular damage and spermatogonium loss.

Melatonin inhibited the expression of ERS-related genes in testes

To analyse the protective mechanism of melatonin for the testes against toxicity caused by busulfan, we used 4PBA and investigated the protein expression of the ERS indicators ATF6 and PERK in the testes. Consistent with histopathological findings, our results demonstrated that the intensity of the immunofluorescent signal for ATF6 and pIRE1 was markedly increased in the majority of busulfan-treated mice compared with the control mice (P < 0.05). The administration of melatonin or 4PBA significantly decreased the expression levels of ATF6 and pIRE1 (P < 0.05, Fig. 2A–D).

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Fig. 2. Detection of ERS-related molecules in mouse testes. The expression of ERS-related molecules in mouse testes after treatment with CON, BU, BU+MEL and BU+4PBA. (A, C) Representative immunofluorescent images show ATF6 expression (A) and pIRE1 (C). All images were merged from ATF6 or pIRE1 (green), PLZF (red) and Hoechst33342-stained nuclei (blue). Experiments were performed in triplicate. Boxed regions of interest are enlarged and shown on the bottom line. Scale bar, 100 μm. (B, D) Quantitative analysis of the immunofluorescent signal for ATF6 (B) and pIRE1 (D). (E) The mRNA expression of ERS-related genes (GRP78, IRE1, XBP1 and ATF6) in testes via qRT-PCR analysis. (F) The protein expression of GRP78, IRE1, XBP1 and ATF6 in mouse testes detected by Western blot. (G) ImageJ analysis showing the mean density of the results of (F). Data are represented as the mean ± SD (n = 3). * P<0.05, ** P<0.01, and *** P<0.001.
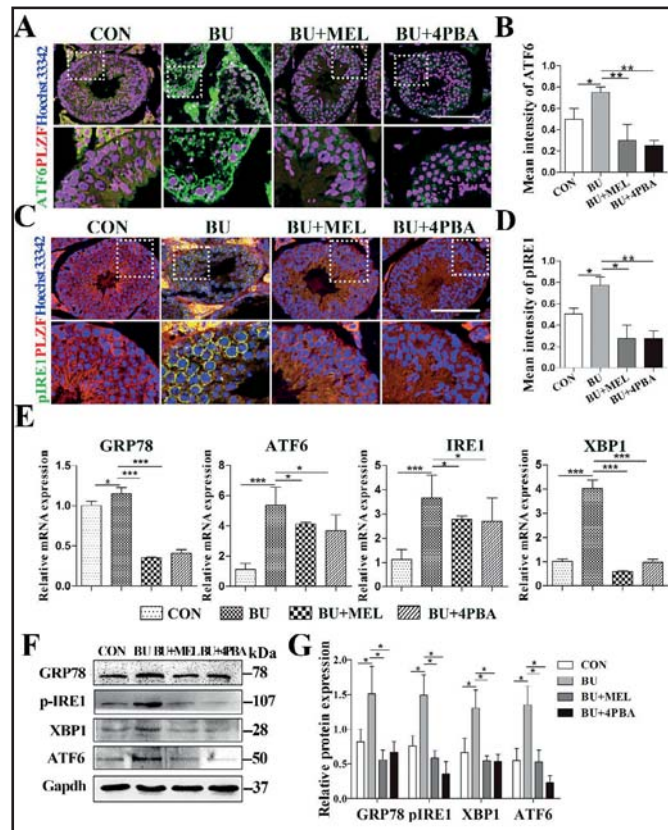
Fig. 3. Expression of ERS-related apoptotic molecules in teste. The expression of ERS-related molecules in mouse testes after treatment with CON, BU, BU+MEL and BU+4PBA. Experiments were performed in triplicate. Boxed regions of interest are enlarged and shown on the bottom line. Scale bar, 200 μm. (A, B) Representative immunofluorescence images (A) and their fluorescence intensity analysis (B) indicating CHOP expression. (C, D) Representative immunofluorescence images (C) and their fluorescence density analysis (D) indicating PUMA expression. (E) The testicular mRNA expression levels of CHOP, PUMA, caspase-12 and caspase-3 were examined by qRT-PCR. (F) Western blot detection of apoptotic proteins CHOP, caspase-12, PUMA and caspase-3. (G) ImageJ analysis showing the grey value of (F). All data are presented as the mean ± SD (n = 3), and statistical significance is shown as follows: *P<0.05, **P<0.01, and ***P<0.001.

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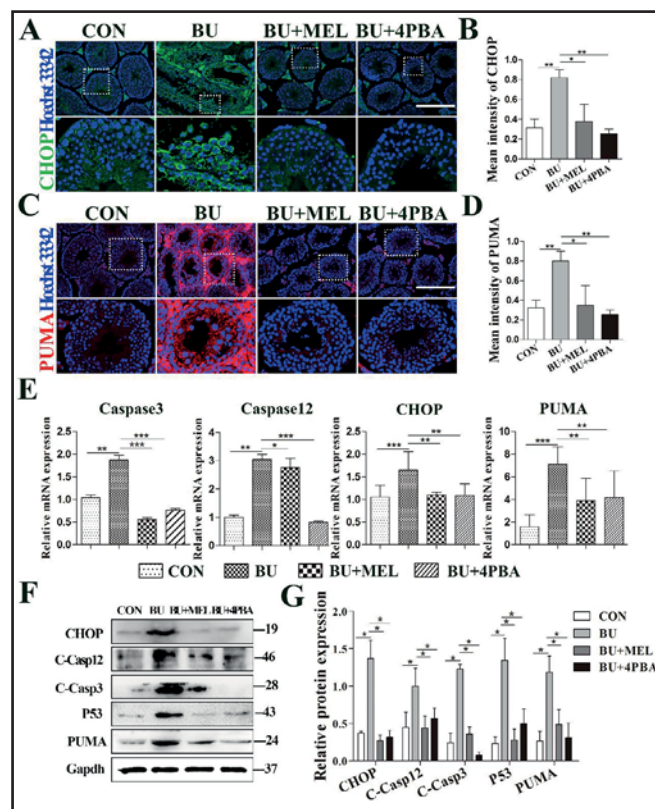
We also assessed the expression levels of ERS-related genes in the testes through qRT-PCR. Compared with that in the control group, the mRNA expression of GRP78, Atf6, pIRE1, and Xbp1 in the busulfan-treated group was significantly increased (0.14-, 2.26-, 2.99-, and 3.79-fold increases compared with the control group, respectively, $P < 0.05$). However, this increasing trend was significantly reversed when the treatment was combined with melatonin or 4PBA ($P < 0.05$, Fig. 2E).
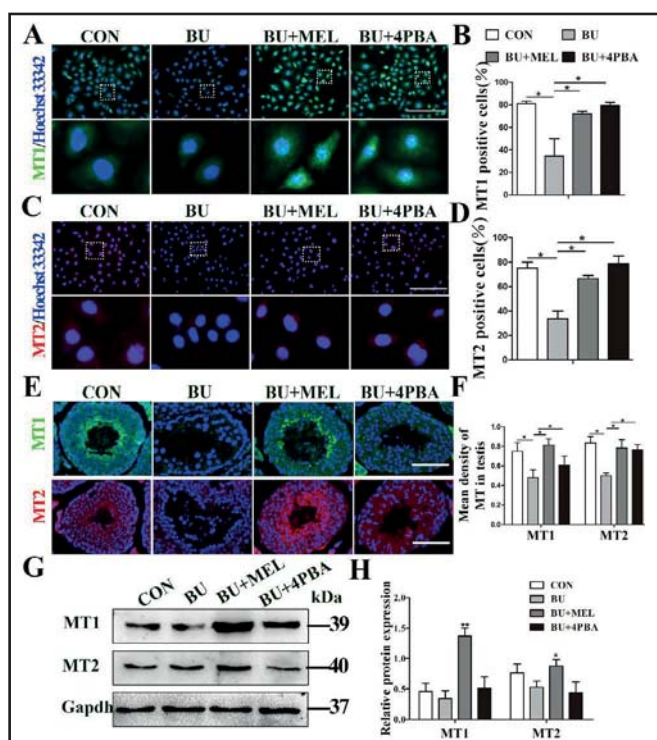
Western blotting was applied to examine the effect of ERS activation. In the control group, a low and basal activation level of the ERS-related proteins was detected. After busulfan was added, the expression of GRP78, pIRE1, XBP1, and ATF6 was clearly stimulated. In the presence of melatonin or 4PBA, the activation effect was reversed ($P < 0.05$, Fig. 2F–G). Therefore, melatonin protected the testes from ERS damage.

Melatonin suppressed the expression of ERS-related apoptotic genes in testes

To evaluate the protective mechanism of melatonin, we investigated the protein expression of the ERS-related apoptotic genes CHOP and PUMA through an immunofluorescence assay. CHOP had a high level of expression after busulfan treatment but low expression in the melatonin- and 4PBA-treated groups ($P < 0.05$, Fig. 3A–B). Compared to control mice, intense fluorescent signals of PUMA were found in the busulfan-treated group. Conversely, weak signals were observed in the melatonin- and 4PBA-treated groups ($P < 0.05$, Fig. 3C–D). The mRNA expression levels of CHOP, PUMA, caspase-3 and caspase-12 were examined through qRT-PCR analysis, and similar to the immunofluorescence analysis, the mRNA expression level of ERS-related apoptotic genes was increased relative to the control group after busulfan was administered, whereas the increase was significantly compromised by melatonin or 4PBA ($P < 0.05$, Fig. 3E).

Western blotting results further revealed that co-administration of busulfan with melatonin or 4PBA significantly downregulated the expression of the ERS-mediated apoptosis

Fig. 4. Expression of melatonin receptors MT1 and MT2. The expression of MT1 and MT2 in mouse testes or C18-4 after treatment with CON, BU, BU+MEL and BU+4PBA. (A) Immunofluorescent assay for MT1 in C18-4 cells. Scale bar, 200 μm. (B) Quantitation analysis of MT1-positive cells in A. (C) Immunofluorescent assay of MT2 in C18-4 cells. Boxed regions of interest are enlarged and shown on the bottom line. Scale bar, 200 μm. (D) Quantitation analysis of MT1-positive cells in C. (E) Immunofluorescent assay of MT1 and MT2 in mouse testes with different treatments. Scale bar, 100 μm. (F) Quantitation of mean immunofluorescent density signals for MT1 and MT2 in E. (G) Western blot analysis of MT1 and MT2 in C18-4 cells. (H) ImageJ analysis showing the grey value of (G). All experiments were performed in triplicate. Data are presented as the mean ± SD (n = 3). Statistical significance is expressed as follows: * P<0.05, ** P<0.01, and *** P<0.001.

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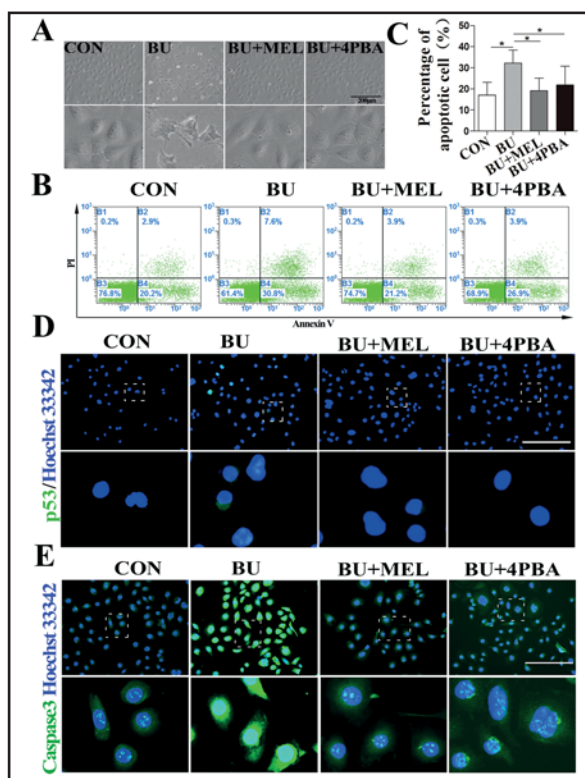Cui et al.: Melatonin Relieves Busulfan-Induced Testis Damage

protein CHOP, cleaved caspase-12, PUMA, and cleaved caspase-3, all of which were upregulated by busulfan (P < 0.05, Fig. 3F–G). Therefore, ERS might be activated by busulfan and act to further induce testicular cell apoptosis and spermatogonium loss. The combined treatment with melatonin abolished the adverse effects of busulfan on the testes.

Melatonin increased MT1 and MT2 expression levels

To evaluate the importance of melatonin receptors (MT) in facilitating the therapeutic functions of melatonin, we performed an immunofluorescence analysis of melatonin receptors in the testes and mouse SSCs. The untreated cells showed normal signals of MT1 and MT2. However, only weak signals were found in busulfan-treated cells. In contrast, the fluorescent signals of MT1 and MT2 were highly enriched in melatonin- and 4PBA-treated cells (Fig. 4A–C). The quantitative results of the immunofluorescence assay showed that the positive signals were weaker in the busulfan-treated group than in the control cells. The fluorescent signals of MT1 and MT2 were enhanced when melatonin was administered compared with the busulfan-treated group. Moreover, 4PBA treatment increased the signals of MT1 and MT2 (P < 0.05, Fig. 4B–D). Similar to cell immunofluorescence analysis, testicular fluorescence

Fig. 5. Effect of melatonin on the apoptosis of C18-4 cells. (A) The morphological changes of C18-4 cells after different treatments. The images are representative of at least three independent experiments. (B) The apoptosis of C18-4 cells was examined by flow cytometry analysis. (C) Percentages of the apoptotic cells that were analysed in B. (D) Immunofluorescent assay of P53. (E) Immunofluorescent assay of cleaved caspase 3. The images are representative of at least three independent experiments. Boxed regions of interest are enlarged and shown on the bottom line. Scale bar, 200 μm. Mean ± SD was calculated from at least three determinations. * P<0.05.

analysis revealed that intense MT1 and MT2 fluorescent signals were observed in melatonin- or 4PBA-treated testes; however, weak signals were found in the seminiferous tubules of the testes treated with busulfan (Fig. 4E). The quantitation results confirmed that the MT1 and MT2 expression levels were increased in the melatonin-treated group compared with the busulfan-treated group. 4PBA administration increased MT1 and MT2 expression compared with that of the busulfan group (P < 0.05, Fig. 4F). Western blotting further confirmed that administration of melatonin significantly upregulated the expression of MT1 and MT2 (P < 0.05, Fig. 4G–H). These data suggested that the protective role of melatonin against mouse testicular damage was mediated by MT1 and MT2.

Melatonin inhibited mouse SSC apoptosis

The mouse C18-4 cell line, which is a type A SSC, was examined to verify the direct effects of melatonin and busulfan on spermatogonial cells. To confirm the involvement of ERS, we co-treated the cells with 4PBA. Co-treatment with melatonin was also administered to assess the role of melatonin in busulfan-induced apoptosis. After treatment with busulfan, the morphological characteristics of the C18-4 cells were shrunken, and their surface was

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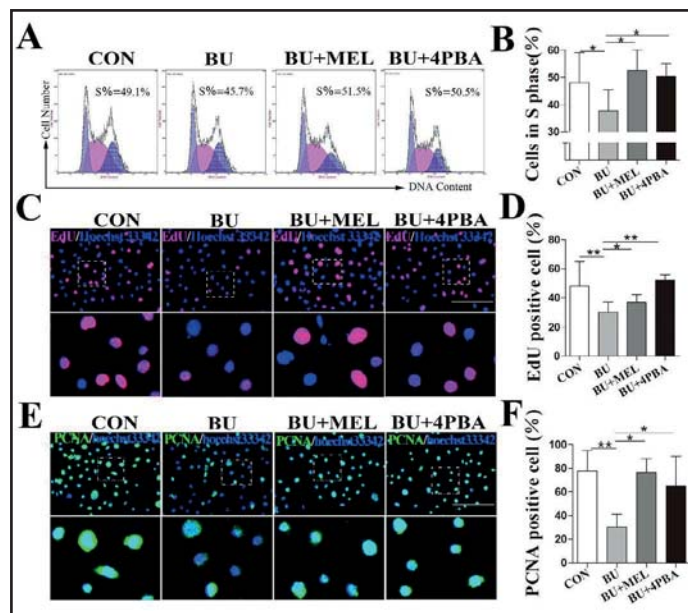Cui et al.: Melatonin Relieves Busulfan-Induced Testis Damage

rough in comparison to the control cells, as revealed by light microscopy. Conversely, the co-administration of melatonin or 4PBA significantly reversed these morphological changes (Fig. 5A). We counted the number of annexin V FITC-positive cells through flow cytometry analysis. Compared to the control group, busulfan treatment increased the percentage of apoptotic cells; however, the administration of melatonin or 4PBA decreased the percentage of apoptotic cells compared with the busulfan-treated cells (P < 0.05, Fig. 5B–C). To confirm these results, we analysed the apoptotic proteins P53 and cleaved caspase-3 through immunofluorescence assay. Intense fluorescent signals indicating the presence of apoptosis were observed in the busulfan-treated cells. Conversely, only slight fluorescence was detected in the melatonin- and 4PBA-treated cells (Fig. 5D–E). These experiments suggested that melatonin treatment could sufficiently enhance the resistance of mouse SSCs to busulfan-induced apoptosis.

Fig. 6. Effect of melatonin on the proliferation of C18-4 cells. (A) The cell cycle proportions of C18-4 cells detected by flow cytometry. (B) The percentage of C18-4 cells in S phase. (C) Representative images of the EdU assay in C18-4 cells in different treatment groups. Scale bar, 200 μm. (D) EdU-positive rate of C18-4 cells. (E) Immunofluorescent assay of proliferative protein PCNA. Scale bar, 200 μm. (F) Percentage of PCNA positive cells in different treatment groups were counted. All experiments were repeated at least three times. Boxed regions of interest are enlarged and shown on the bottom line. Scale bar, 200 μm. Data are presented as the mean ± SD (n = 3), and statistical significance is expressed as follows: * P < 0.05 and ** P < 0.01.

Melatonin promoted mouse SSC proliferation

Cell cycle distribution was measured to explore the effects of melatonin and busulfan on the proliferation of mouse SSCs. Our results indicated that the percentage of busulfan-treated cells in the S phase was reduced compared with the control group. In contrast, the percentage of S-phase cells increased in the melatonin- and 4PBA-treated cells (P < 0.05, Fig. 6A–B). Cell proliferation was observed by using the EdU kit. The cell proliferation rate was reduced in the busulfan treatment group compared with that of the control group (18.5 ± 9%, P < 0.05). Conversely, melatonin promoted the proliferation activity of mouse SSCs. Compared with the busulfan-treated group, the proliferation of the SSCs was increased in the 4PBA-treated group (P < 0.05, Fig. 6C–D). To confirm these results, we analysed the proliferative protein PCNA through immunofluorescence assay and found that the number of positive cells was reduced in the busulfan-treated group relative to the control cells (P < 0.05). In contrast, the percentage of PCNA-positive cells in the melatonin- and 4PBA-treated group increased (P < 0.05, Fig. 6E–F). These data suggest that melatonin may promote SSC survival and drive their proliferation to promote regeneration after busulfan treatment.

Melatonin inhibited ERS activation in mouse SSCs

C18-4 cells were examined through immunofluorescence assays to clarify whether spermatogenic cells treated with busulfan *in vitro* induced ERS and to determine the effect
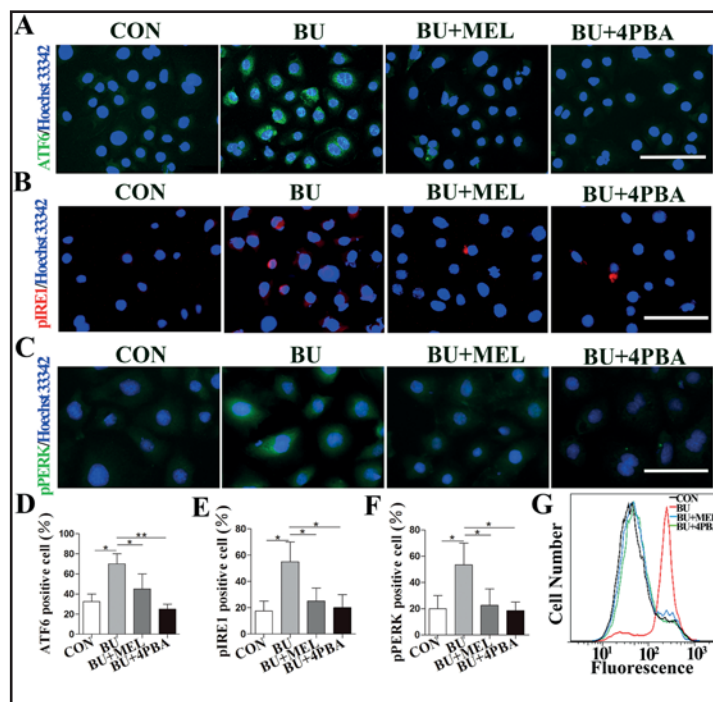
of melatonin on the expression of ERS-related molecules. The ERS indicator ATF6 had a high level of expression in the busulfan group but low expression in melatonin- and 4PBA-treated cells (P < 0.05, Fig. 7A, D). Cell imaging revealed that the pIRE1 signals were enhanced in the busulfan group relative to the control group. In comparison, this strong signal was suppressed by either melatonin or 4PBA (P < 0.05, Fig. 7B, E). Intense fluorescent signals of pPERK indicated that higher ERS levels occurred in the busulfan-treated cells than in the control group. Conversely, low levels of fluorescence showed that the ERS level was reduced in melatonin- and 4PBA-treated cells compared with the busulfan treatment group (P < 0.05, Fig. 7C, F). We also measured intracellular Ca^{2+} levels through Fluo-3AM staining because ER is a major calcium-storage organelle, and its calcium content is essential for proper protein folding and transport. The disruption of calcium homeostasis can disturb ER functions and induce ERS [31]. Our results indicated that the intracellular Ca^{2+} concentration increased in C18-4 cells treated with busulfan. Conversely, the co-treatment of busulfan with melatonin or 4PBA failed to increase intracellular Ca^{2+} levels (Fig. 7G). Therefore, busulfan enhanced the activation of ERS in C18-4 cells, but co-treatment with melatonin resulted in improved ER homeostasis.

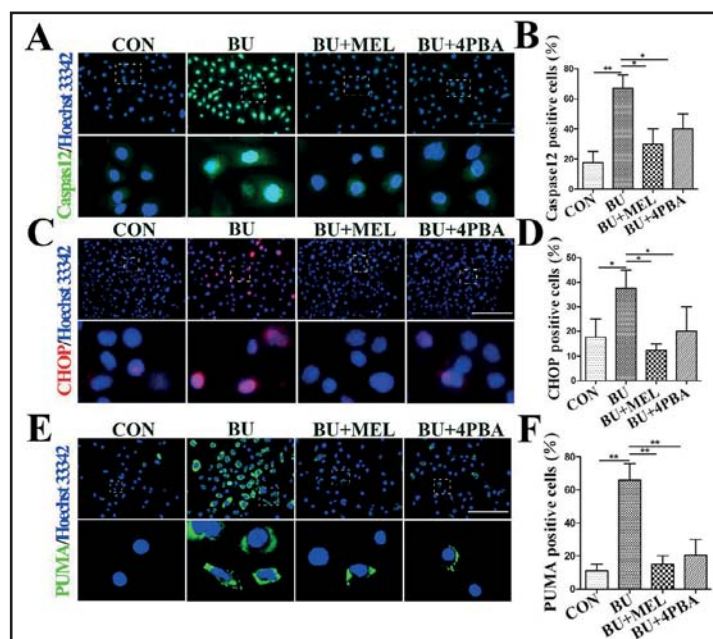*Melatonin counteracted ERS-related apoptosis in mouse SSCs*

To obtain insights into the mechanism by which busulfan induces apoptosis and to clarify whether melatonin prevents apoptosis caused by ERS in mouse SSCs, we performed immunofluorescence analysis. The results demonstrated that the signals from the ERS-related apoptotic marker cleaved caspase-12 were enhanced in the busulfan-treated cells compared with the control cells. In contrast, a relatively weak fluorescence signal was detected in the cells treated with busulfan combined with melatonin or 4PBA (P < 0.05, Fig. 8A–B). The quantitative results of the immunofluorescence assay of whole cells revealed that the CHOP expression level was increased in the busulfan-treated cells relative to the control cells. Conversely, the signal was suppressed when the cells were co-treated with melatonin or 4PBA (P < 0.05, Fig. 8C–D).

We also investigated PUMA expression, which is reported to be a proapoptotic gene induced by ERS [32]. Our results showed that melatonin suppressed PUMA expression

Fig. 7. Expression of ERS-related proteins in C18-4 cells. The C18-4 cells were treated with CON, BU, BU+MEL and BU+4PBA. The expression of ERS-related proteins and the intracellular Ca^{2+} level in C18-4 was detected. (A–C) Immunofluorescence images of ERS indicators ATF6 (A), pIRE1 (B) and pPERK (C) in C18-4 cells. Scale bar, 100 μm. (D-F) Immunofluorescence images of pIRE1 in C18-4 cells. Scale bar, 100 μm. (D) Statistical analysis of the positive rate of ATF6 (D), pIRE1 (E) and pPERK (F). (G) Analysis of the intracellular Ca^{2+} level was measured by flow cytometry. Experiments were performed in triplicate. All data are presented as the mean ± SD (n = 3). Statistical significance is expressed as follows: * P<0.05 and ** P<0.01.

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Fig. 8. Expression of ERS-related apoptosis markers in C18-4 cells. (A, B) Representative immunofluorescence images (A) and positive rate analysis (B) of caspase-12 in C18-4 cells. (C, D) Representative immunofluorescence images (C) and positive rate analysis (D) of CHOP in C18-4 cells. (E, F) Representative immunofluorescence images (E) and positive rate analysis (F) of PUMA in C18-4 cells. Boxed regions of interest are enlarged and shown on the bottom line. Scale bar, 200 μm. Data are presented as the mean ± SD of three separate determinations. Statistical significance was indicated as follows: * P < 0.05 and ** P < 0.01.

stimulated by busulfan (P < 0.05, Fig. 8E–F). From these results, we conclude that mouse SSCs exposed to busulfan undergo ERS-related apoptosis, but in contrast, cells co-administered melatonin or 4PBA conferred higher resistance than busulfan-treated cells.

Discussion

Chemotherapeutic drugs, such as busulfan, are often used to treat cancer and increase the survival rates of patients. However, these chemotherapeutics may induce temporary or permanent reproductive toxicity in male patients. Children and adolescents with testicular cancer or leukaemia are at high risk of developing permanent sterility because of chemotherapeutic application. Therefore, strategies that effectively preserve fertility during the course of cancer treatment should be developed [33, 34]. In this study, we provide evidence that melatonin is sufficient to counteract busulfan-induced testicular damage and SSC apoptosis. Moreover, we found that the protective effect of melatonin is dependent on ERS alleviation.

The ER is an important organelle involved in protein folding, calcium homeostasis maintenance, and lipid biosynthesis [35]. ERS occurs when unfolded proteins accumulate and the folding capacity of ER chaperones exceeds the capacity of the ER lumen to facilitate their disposal. It is a major contributor to various diseases, such as cancer, diabetes and Alzheimer's disease [36, 37]. As such, targeting the components of the ERS signal pathway has been widely explored as a therapeutic strategy [38].

Despite a large number of studies linking ERS to toxicants, currently, studies have yet to determine whether ERS is a contributing factor to busulfan-induced testicular damage and apoptosis [39, 40]. In this study, we demonstrated the relationship between testicular damage and ERS pathways in response to busulfan. We evaluated ERS-related genes and found an increased expression of GRP78, ATF6, pIRE1, and XBP1 in busulfan-treated mouse testes. This finding suggested that ERS could be responsible for busulfan-induced testicular damage. A recent report showed that activation of ATF4 and CHOP could enhance protein synthesis and subsequently induce ERS and apoptosis [41]. Our results further demonstrated that busulfan treatment increased the expression of ERS-related apoptotic proteins, such as CHOP and cleaved caspase-12. We also found an increase in P53 and PUMA protein levels in response to busulfan treatment, which could be further confirmed by evaluating levels

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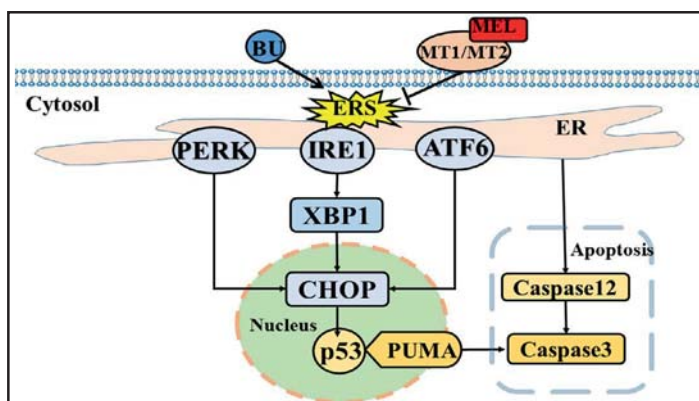Cui et al.: Melatonin Relieves Busulfan-Induced Testis Damage

of apoptosis because P53 acts as a transcription factor that regulates the expression of apoptosis-related genes [42], and PUMA is a potent pro-apoptotic protein because of its ability to bind and neutralize all pro-survival BCL2 members [43]. Therefore, ERS regulation could involve apoptotic actions in testicular damage. Our experiments on mouse SSCs were consistent with that on the mouse testes. These findings (both *in vitro* and *in vivo*) suggest that inhibition of ERS might be a target of novel therapeutics for male infertility.

Melatonin and its metabolites are well-known broad-spectrum antioxidants and free radical scavengers that regulate various molecular pathways, such as inflammation, proliferation, apoptosis, and metastasis, during different pathological conditions [44-46]. It has been documented that melatonin modulates the signalling of neuronal cell death [47] and bladder cancer cells induced by ERS [48]. Melatonin significantly reverses tunicamycin-induced ERS in human hepatocellular carcinoma cells and improves cytotoxic responses to doxorubicin by increasing the expression of CHOP [49]. A couple of studies revealed that melatonin alleviates Cd-induced cellular stress and germ cell apoptosis in testes [50] and prevents bisphenol A-induced apoptosis by controlling BCL2 expression and ameliorating oxidative stress in the testes and sperm [51]. Furthermore, melatonin had a positive influence on the efficiency of male germ cell differentiation [52], and it can promote goat SSC proliferation by stimulating GDNF production in Sertoli cells [53].

Similarly, our data showed that melatonin contributed to the proliferation of mouse SSCs, but the proliferation was suppressed when they were exposed to busulfan. Indeed, our recent study has shown that melatonin markedly reduces the extent of testicular injury by inhibiting oxidative stress [28]. However, whether melatonin could relieve busulfan-induced ERS in testes and SSCs was previously unclear. Here, we showed that melatonin significantly alleviated busulfan-induced testicular ERS by suppressing the expression levels of the key ERS genes pIRE1 and ATF6 and inhibiting the ERS-related apoptotic genes CHOP and caspase-12. Moreover, the integrity of the testes was significantly preserved by melatonin treatment. Previous studies reported that the effects of melatonin were mediated by the specific receptors MT1 and MT2 [54]. Melatonin pretreatment inhibited the activating role of tunicamycin on ERS through melatonin receptor-mediated IRE-1 signalling in skeletal muscle cells [55]. Similarly, our study suggests that the expression of MT1 and MT2 was increased in exogenous melatonin-treated mouse testes and SSCs, and these findings imply that the protective role of melatonin against mouse testicular damage could be exerted through MT1 and MT2.

4PBA is known to be a major repressor of ERS by improving the folding capacity of misfolded proteins [30]. It is capable of counteracting the toxic effect of proteinuria through suppressing ATF4 and attenuating ERS in chronic kidney disease [56]. To elucidate the mechanism behind the inhibition of ERS by melatonin, 4PBA was utilized. We found that the expression of ERS-related genes and apoptosis genes decreased to normal levels when 4PBA was co-administered with busulfan. Similar to 4BPA, melatonin is able to protect against cerebral IR injury through inhibiting ERS via PERK and IRE1 signals [57]. Downregulation of

Fig. 9. Schema diagram illustrating the mechanism of how melatonin blocked busulfan-induced spermatogonial stem cell apoptosis in mouse testes.

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ATF-6 by melatonin contributes to ERS-induced apoptosis of human hepatoma cells [58]. In the current study, we found that melatonin could abrogate busulfan-induced ERS in mouse SSCs by inhibiting the ERS-related genes IRE1 and PERK, which further depressed pro-apoptotic transcript CHOP activation, then inhibited P53 expression and resulted in the reduction of PUMA, and consequently decreased the accumulation of cellular caspase, thereby preventing cell apoptosis (Fig. 9). Thus, we conclude that melatonin treatment significantly protected mouse SSCs from busulfan-induced apoptosis and protected testes from damage in busulfan-treated mice. This study suggests that melatonin could provide potential therapeutic advantages by protecting the testes from busulfan-induced injury.

Taken together, our study demonstrated that the activation of ERS signalling contributed to busulfan-induced SSC apoptosis and that melatonin treatment significantly protected mouse SSCs from busulfan-induced apoptosis. This study provides basic information that helps us to understand the recovery of spermatogenesis in men after cancer treatment and provides guidance about the possible effects that new agents might have on the testes of male patients with cancer.

Acknowledgements

Disclosure Statement

The authors declare no conflicts of interest.

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