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# Melatonin alleviates cadmium-induced cellular stress and germ cell apoptosis in testes

Abstract: Increasing evidence demonstrates that melatonin has an anti-apoptotic effect in somatic cells. However, whether melatonin can protect against germ cell apoptosis remains obscure. Cadmium (Cd) is a testicular toxicant and induces germ cell apoptosis. In this study, we investigated the effects of melatonin on Cd-evoked germ cell apoptosis in testes. Male ICR mice were intraperitoneally (i.p.) injected with melatonin (5 mg/kg) every 8 hr, beginning at 8 hr before $CdCl_2$ (2.0 mg/kg, i.p.). As expected, acute Cd exposure resulted in germ cell apoptosis in testes, as determined by terminal dUTP nick-end labeling (TUNEL) staining. Melatonin significantly alleviated Cd-induced testicular germ cell apoptosis. An additional experiment showed that spliced form of XBP-1, the target of the IRE-1 pathway, was significantly increased in testes of mice injected with $CdCl_2$. GRP78, an endoplasmic reticulum (ER) chaperone, and CHOP, a downstream target of the PERK pathway, were upregulated in testes of Cd-treated mice. In addition, acute Cd exposure significantly increased testicular eIF2$\alpha$ and JNK phosphorylation, indicating that the unfolded protein response (UPR) pathway was activated by $CdCl_2$. Interestingly, melatonin almost completely inhibited Cd-induced ER stress and the UPR in testes. In addition, melatonin obviously attenuated Cd-induced heme oxygenase (HO)-1 expression and protein nitration in testes. Taken together, these results suggest that melatonin alleviates Cd-induced cellular stress and germ cell apoptosis in testes. Melatonin may be useful as pharmacological agents to protect against Cd-induced testicular toxicity.

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Key words: cadmium, endoplasmic reticulum stress, germ cell apoptosis, heat stress, melatonin, testis

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## Introduction

Cadmium (Cd) is a major occupational and environmental toxicant. Cd is frequently used in electroplating, pigments, paints, welding, and Ni–Cd batteries. Workers in these occupations are exposed to Cd at significantly higher levels than the general public [1]. The general population is exposed to Cd via drinking water, food, and cigarette smoking [2]. As a well-known endocrine-disrupting chemical, Cd is not only a regulator of hypothalamus and pituitary hormone secretion [3, 4] but also disrupts testicular testosterone production [5, 6]. Increasing evidence demonstrated that environmental exposure to Cd is associated with male infertility and the poor semen quality in humans [7]. According to several earlier studies, there was a significant inverse correlation between blood Cd level and semen quality [8, 9]. In addition, oxidative DNA damage in human spermatozoa is associated with Cd concentration in seminal plasma [8]. Even a low level of Cd accumulation in semen might contribute to male infertility by reducing sperm quality [10]. Cd is a testicular toxicant in rodent animals [11]. Several studies showed that Cd induces germ cell apoptosis in testes [12, 13].

Several studies showed that Cd-induced male reproductive damage was associated with oxidative stress in testes [14, 15]. Recent studies found that several antioxidants protected against Cd-induced testicular oxidative stress and male reproductive damage [16–18]. Melatonin is the major secretory product of the pineal gland. As a potent antioxidant, melatonin and its metabolites directly scavenge a variety of free radicals [19–23]. In addition, melatonin reduces free radical levels via stimulating the activities of antioxidative enzymes [24–27]. According to an earlier study, melatonin alleviated Cd-induced expression of inducible nitric oxide synthase and lipid peroxidation in rat hypothalamus and anterior pituitary [28]. A recent study found that melatonin counteracted Cd-evoked oxidative stress in liver [29]. Interestingly, melatonin prevents the estrogenic effects of subchronic administration of cadmium on mammary glands and uterus [30]. On the other hand, melatonin has an anti-apoptotic effect in somatic cells [31, 32]. However, whether melatonin can protect against germ cell apoptosis remains obscure.

In this study, we investigated the effects of melatonin on Cd-induced testicular germ cell apoptosis in mice. We demonstrate for the first time that melatonin protects mice from Cd-induced germ cell apoptosis in testes. In addition, melatonin alleviated Cd-induced testicular oxidative stress and endoplasmic reticulum (ER) stress.

## Materials and methods

### Chemicals and reagents

$CdCl_2$ and melatonin were from Sigma Chemical Co. (St. Louis, MO, USA). HO-1, 3-nitrotyrosine (3-NT) and phosphor-JNK antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). GRP78 and phosphor-eIF2$\alpha$ antibodies were from Cell Signaling Technology (Beverley, MA, USA). $\beta$-actin antibody was from Boster Bio-Technology Co. Ltd (Wuhan, China). Chemiluminescence (ECL) detection kit was from Pierce Biotechnology (Rockford, IL, USA). TRI reagent was from Molecular Research Center, Inc. (Cincinnati, OH, USA). RNase-free DNase was from Promega Corporation (Madison, WI, USA). All other reagents were purchased from Sigma Chemical Co. if not stated.

### Animals and treatments

Adult male CD-1 mice (8 wks old, 28–32 g) were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories, Inc (Wilmington, MA, USA). The animals were allowed free access to food and water at all times and were maintained on a 12-hr light/dark cycle in a controlled temperature (20–25°C) and humidity (50 ± 5%) environment. To investigate the effects of melatonin on Cd-induced testicular ER stress and germ cell apoptosis in testes, all mice except controls were i.p. injected with $CdCl_2$ (2.0 mg/kg). Some mice were i.p. injected with melatonin (5 mg/kg) every 8 hr, beginning at 8 hr before Cd treatment. The control mice received an equal volume of normal saline or equal doses of melatonin. Testes were collected at 24 hr after Cd treatment. The testes were divided into two parts: left one was kept at −80°C for subsequent Western Blot and RT-PCR. The other part of the testes was immersed in modified Davidson's fluid (mDF) for 12–24 hr for testicular histology and apoptosis analysis. All procedures on animals followed the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

### Terminal dUTP nick-end labeling (TUNEL) staining

For the detection of apoptosis, paraffin-embedded sections were stained with the TUNEL technique using an in situ apoptosis detection kit (Promega) according to the manufacturer's protocols. To assess apoptosis in testicular cells, 200 different seminiferous tubules were observed in predetermined different fields in each section at a magnification of ×400. A histogram of the number of TUNEL-positive germ cells per seminiferous tubule and the percentages of the number of seminiferous tubules containing TUNEL-positive germ cells were analyzed.

### Immunoblotting

Immunoblotting was performed using testicular lysates. In brief, protein extracts from each sample were added to a gel loading buffer (100 mM Tris, pH 6.8, 20% glycerol, 200 mM DTT, 4% SDS, 0.03% bromophenol blue) and boiled for 5 min. Proteins (50 $\mu$g/sample) in loading buffer were subjected to electrophoresis in 10–15% SDS-polyacrylamide gel for 3 hr. The gel was transferred electrophoretically onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA, USA) and blocked in 5% nonfat powdered milk in Dulbecco's PBS (DPBS) overnight at 4°C. The membranes were incubated for 2 hr with the following antibodies: HO-1, 3-nitrotyrosine (3-NT), GRP78, phosphor-eIF2$\alpha$, and p-JNK. $\beta$-actin was used as a loading control for total proteins. After washes in DPBS containing 0.05% Tween-20 four times for 10 min each, the membranes were incubated with goat anti-rabbit or goat anti-mouse IgG antibody for 2 hr. The membranes were then washed four times in DPBS containing 0.05% Tween-20 for 10 min each, followed by signal development using an enhanced chemiluminescence (ECL) detection kit from Pierce Biotechnology.

### Testicular histology

Two cross sections from each testis were embedded in parafilm using standard procedures performed by Pathological Lab at Anhui Medical University. Paraffin-embedded tissues were serially sectioned. At least two nonserial sections were stained with hematoxylin and eosin (H&E) using standard procedures for morphological analyses.

### Isolation of total RNA and RT-PCR

Fifty milligrams of testis was collected from each mouse. Total cellular RNA was extracted using TRIzol reagent according to the manufacturer's instructions. RNase-free DNase was used to remove genomic DNA. The integrity and concentration of RNA was determined by measuring absorbance at 260 nm followed by electrophoresis on agarose gels. For the synthesis of cDNA, 1.0 $\mu$g of total RNA from each sample was resuspended in a 20-$\mu$L final volume of reaction buffer, which contained 25 mM Tris–HCl, pH 8.3, 37.5 mM KCl, 10 mM dithiothreitol, 1.5 mM $MgCl_2$, 10 mM of each dNTP, and 0.5 mg oligo$(dT)_{15}$ primer. After the reaction mixture reached 42°C, 20 units of RT was added to each tube, and the sample was incubated for 60 min at 42°C. Reverse transcription was stopped by denaturing the enzyme at 95°C. The final PCR mixture contained 2.5 $\mu$L of cDNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 200 $\mu$M dNTP mixture, 1 U of Taq DNA polymerase, 1 $\mu$M sense and antisense primers, and 50 $\mu$L of sterile water. The reaction mixture was covered with mineral oil. Following primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Company (Shanghai, China), according to sequence designs previously described by others. GAPDH, 5′-GAG GGG CCA TCC ACA GTC TTC-3′ and 5′-CAT CAC CAT CTT CCA GGA GCG-3′; XBP-1, 5′-ACA AGC TTG GGA ATG GAC AC-3′ and 5′-CCA TGG GAA GAT GTT CTG GG-3′. The amplified PCR products were subjected to electrophoresis at 75 V through 1.5% agarose gels (Sigma) for 45 min. The pBR322 DNA digested with Alu I was used for molecular markers (MBI Fermentas, Vilnius,

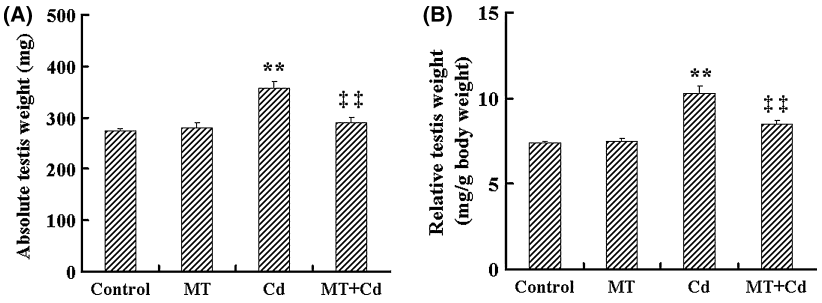


*Fig. 1.* Effects of melatonin and cadmium (Cd) on testis weight. Male mice were injected with $CdCl_2$ (2.0 mg/kg). Some mice were administered with melatonin as Materials and methods. Testes were collected at 24 hr after Cd treatment. Testes were weighed. (A) Absolute testis weight; (B) relative testis weight. All data were expressed as means ± S.E.M. (n = 12). $^{**}P < 0.01$ as compared with controls. ‡‡$P < 0.01$ as compared with Cd group.

LT, USA). Agarose gels were stained with 0.5 mg/mL ethidium bromide (Sigma) TBE buffer.

### Statistical analysis

All quantified data were expressed as means ± S.E.M. ANOVA and the Student–Newman–Keuls post hoc test were used to determine differences among different groups.

## Results

An obvious testicular edema was observed at 24 hr after mice were treated with $CdCl_2$. Consistent with testicular edema, absolute testis weight was significantly increased in Cd-treated mice. Interestingly, melatonin almost completely inhibited Cd-induced testicular edema. In addition, melatonin significantly alleviated Cd-induced elevation of the absolute testis weight (Fig. 1A) and the relative testis weight (Fig. 1B). The effects of melatonin on Cd-induced testicular pathohistological damage are presented in Fig. 2. As expected, a slight necrosis of seminiferous tubules and a moderate hemorrhage in the interstitium were observed in testes of Cd-treated mice. Interestingly, melatonin obviously alleviated Cd-induced pathohistological damage in testes.

The effects of melatonin on Cd-induced testicular germ cell apoptosis were analyzed. As shown in Fig. 3A, most of TUNEL+ cells were observed mainly in germ cells of seminiferous tubules. Further analysis showed that melatonin significantly reduced the frequency of tubules with more than six TUNEL+ cells (Fig. 3B). In addition, melatonin significantly reduced the number of tubules with TUNEL+ cells (Fig. 3C) and the number of TUNEL+ cells per tubule (Fig. 3D).

The effects of melatonin on Cd-induced testicular ER stress were then analyzed. As shown in Fig. 4A, the expression of testicular GRP78, an ER chaperone and ATF6 target, was significantly upregulated in testes of Cd-treated mice. Melatonin significantly attenuated Cd-induced upregulation of testicular GRP78. Next, the level of phosphorylated eIF2α (p-eIF2α), a downstream target of the PERK pathway, was measured in testes of mice injected with $CdCl_2$. As expected, the level of p-eIF2α was significantly increased in testes of mice administered with $CdCl_2$.

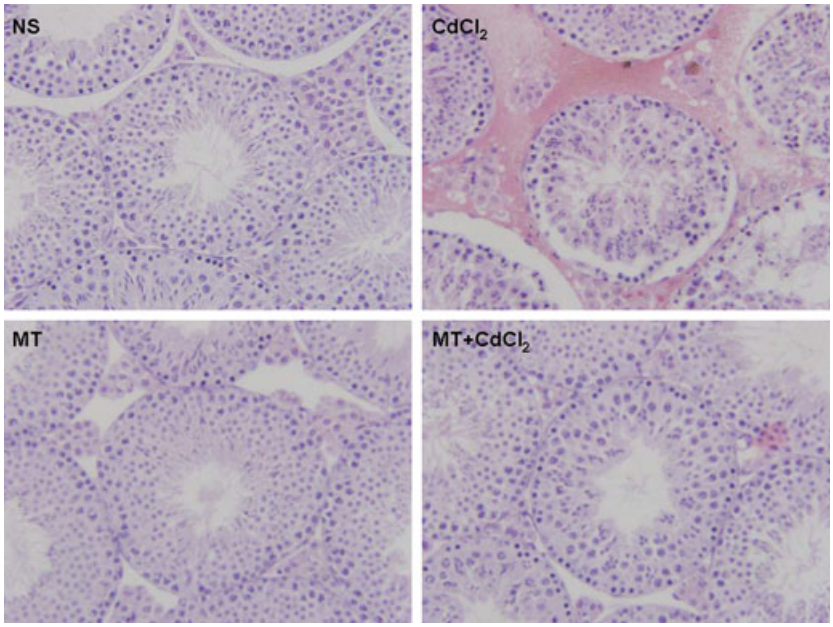


*Fig. 2.* Effects of melatonin on cadmium (Cd)-induced pathohistological damage in testes. Male mice were injected with $CdCl_2$ (2.0 mg/kg). Some mice were administered with melatonin as Materials and methods. Testes were collected at 24 hr after Cd treatment. Testicular cross sections were stained with H&E. Original magnification: 400×.

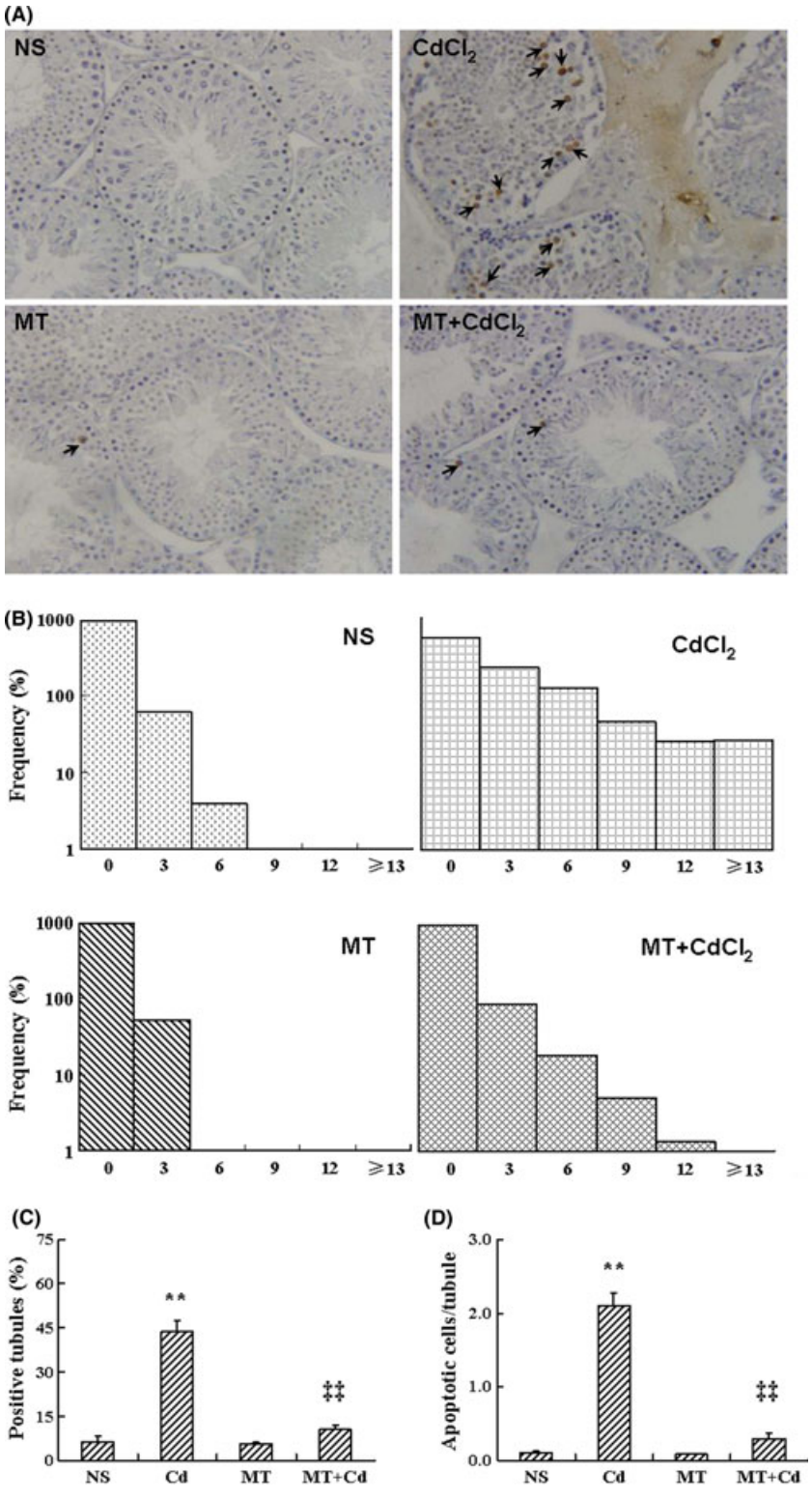


*Fig. 3.* Effects of melatonin on cadmium (Cd)-induced testicular germ cell apoptosis. (A) Male mice were injected with $CdCl_2$ (2.0 mg/kg). Some mice were administered with melatonin as Materials and methods. Testes were collected at 24 hr after Cd treatment. Germ cell apoptosis was detected by TUNEL staining. Arrow showed apoptotic germ cells in seminiferous tubules. (B) A histogram of the number of TUNEL-positive germ cells per seminiferous tubule. (C) Percentages of the number of seminiferous tubules containing TUNEL-positive germ cells. (D) The number of TUNEL + germ cells per seminiferous tubule. All data were expressed as means ± S.E.M. (n = 12). $^{**}P < 0.01$ as compared with controls. $‡‡P < 0.01$ as compared with Cd group.

Melatonin significantly attenuated Cd-induced testicular eIF2α phosphorylation (Fig. 4B). The expression of CHOP, a downstream target of the PERK and ATF6 pathways, was analyzed. As shown in Fig. 4D, the level of testicular CHOP was significantly increased in Cd-treated mice. Melatonin significantly alleviated Cd-induced upregulation of CHOP in testes. Finally, the effects of melatonin on X box-binding protein (sXBP)-1 were analyzed. As expected, the level of sliced XBP-1 (sXBP-1) was significantly increased in testes of Cd-exposed mice, indicating that inositol-requiring ER-to-nucleus signal kinase (IRE) 1α signaling was activated by Cd. Melatonin obviously alleviated Cd-induced elevation of sXBP-1 in testes (Fig. 5). Interestingly, the level of phosphorylated JNK (p-JNK),

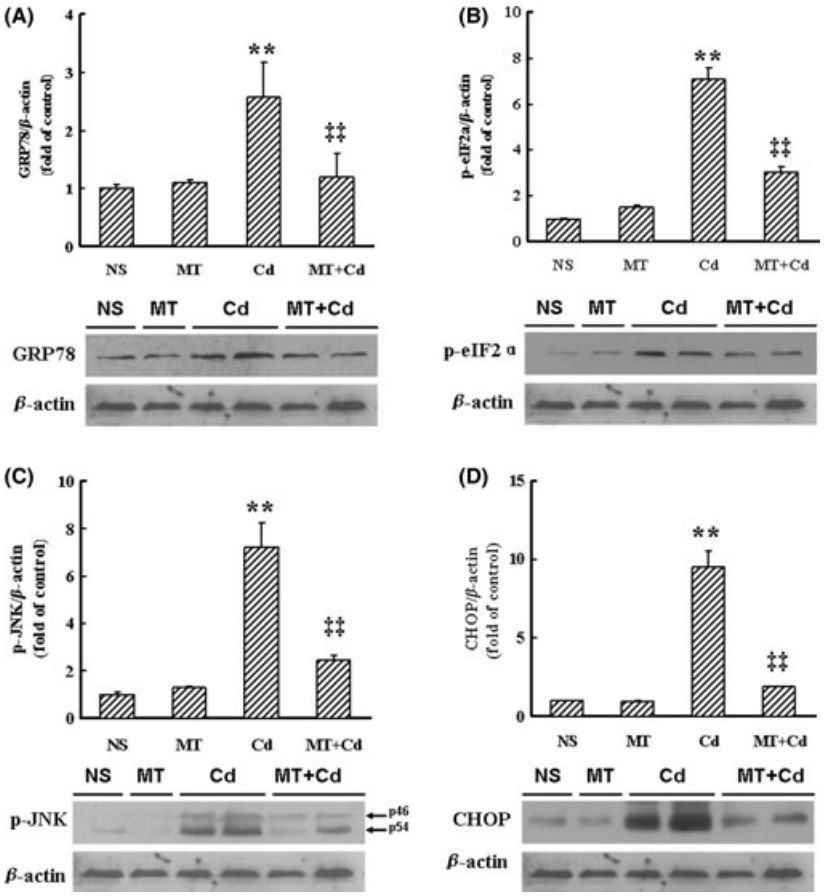


*Fig. 4.* Effects of melatonin on cadmium (Cd)-induced ER stress in testes. Male mice were injected with $CdCl_2$ (2.0 mg/kg). Some mice were administered with melatonin as Materials and methods. Testes were collected at 24 hr after Cd treatment. Testicular GRP78, p-eIF-2α, CHOP, and p-JNK were measured using Western blot. (A) GRP78; (B) p-eIF-2α; (C) CHOP; and (D) p-JNK. All data were expressed as means ± S.E.M. (n = 3–6). $^{**}P < 0.01$ as compared with controls. ‡‡$P < 0.01$ as compared with Cd group.

a downstream target of the IRE1 pathway, was also significantly increased in testes of Cd-exposed mice. Melatonin significantly attenuated Cd-induced testicular JNK phosphorylation (Fig. 4C).

The effects of melatonin on Cd-induced upregulation of testicular HO-1 were analyzed. As shown in Fig. 6, acute Cd exposure significantly upregulated the expression of HO-1 in testes. Interestingly, melatonin significantly alleviated Cd-induced upregulation of testicular HO-1 expression. 3-NT is a specific marker for protein nitration. The effects of melatonin on Cd-induced testicular protein nitration are presented in Fig. 7. As expected, testicular 3-NT intensity was significantly enhanced in Cd-treated mice. Melatonin significantly attenuated Cd-induced protein nitration in testes.

## Discussion

Cd is associated with a poor sperm quality in human being [7, 33]. In addition, Cd induces testicular germ cell apoptosis in rodent animals [12, 13] and in humans [34, 35]. In the present study, we showed that a slight necrosis of seminiferous tubules and a moderate hemorrhage in the interstitium in testes were observed in Cd-treated mice. Importantly, the number of apoptotic germ cells per tubule was significantly increased in Cd-treated mice. In addition, acute Cd exposure obviously elevated the percentage of tubules with apoptotic germ cells. Increasing evidence demonstrates that melatonin has an anti-apoptotic effect [36–38]. In vivo, melatonin alleviated ischemia-reperfusion-induced apoptosis in neural cells through repression of the mitochondrial permeability transition pore [39, 40]. In our laboratory, we found that melatonin attenuated lipopolysaccharide-induced apoptotic liver damage in D-galactosamine-sensitized mice [41]. According to a recent study, melatonin protected kidney grafts from ischemia/reperfusion injury through the repression of the apoptosis after experimental kidney transplantation [42]. In the present study, we found that melatonin alleviated Cd-induced pathohistological damage. Importantly, the frequency of tubules with more than six apoptotic germ cells was significantly reduced when mice were administered with melatonin. In addition, melatonin significantly reduced the percentage of tubules with apoptotic germ cells and the number of apoptotic germ cells per tubule. These results are in agreement with an earlier study by our laboratory [43], in which melatonin attenuated 2-bromopropane-induced testicular germ cell apoptosis in rats. These data demonstrate that melatonin could protect against germ cell apoptosis in testes.

The ER is an important organelle required for cell survival and normal cellular function. In the ER, nascent proteins are folded with the assistance of ER chaperones. The ER is sensitive to alterations in cellular homeostasis. When unfolded and misfolded proteins are retained in the ER lumen, ER stress occurs and the unfolded protein response (UPR) is activated [44–46]. Numerous studies have demonstrated that ER stress and the UPR are

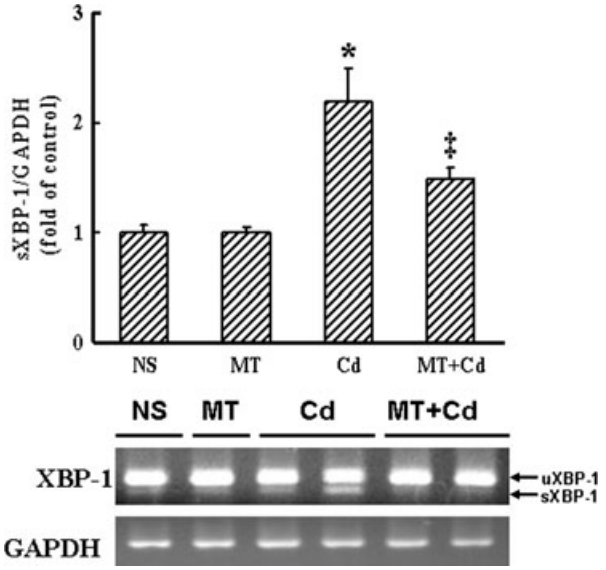


*Fig. 5*. Effects of melatonin and cadmium (Cd) on the level of unspliced and spliced forms of XBP-1 in testes. Male mice were injected with $CdCl_2$ (2.0 mg/kg). Some mice were administered with melatonin as Materials and methods. Testes were collected at 24 hr after Cd treatment. Unspliced and spliced forms of XBP-1 were measured using RT-PCR. All data were expressed as means ± S.E.M. (n = 3–6). $^*P < 0.05$ as compared with controls. ‡$P < 0.05$ as compared with Cd group.

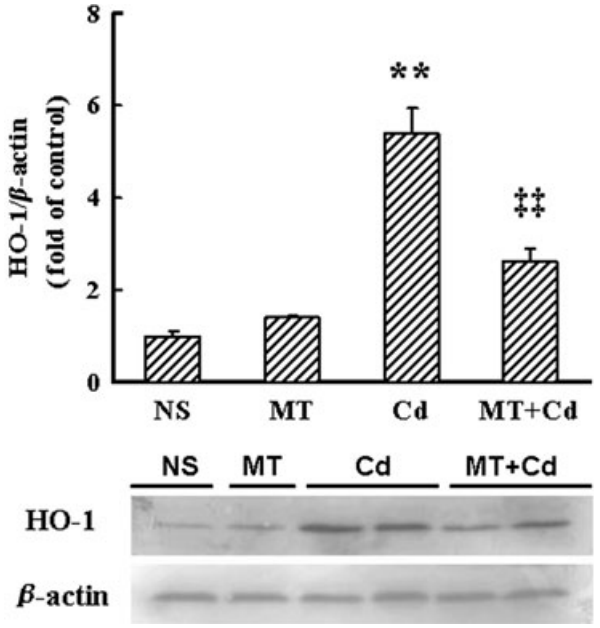


*Fig. 6*. Effects of melatonin on cadmium (Cd)-induced upregulation of testicular HO-1. Male mice were injected with $CdCl_2$ (2.0 mg/kg). Some mice were administered with melatonin as Materials and methods. Testes were collected at 24 hr after Cd treatment. The expression of HO-1 in testes was detected using Western blot. HO-1 was normalized to β-actin level in the same samples. The densitometry unit of the control was assigned as 1. All data were expressed as means ± S.E.M. (n = 3–6). $^{**}P < 0.01$ as compared with controls. ‡‡$P < 0.01$ as compared with Cd group.

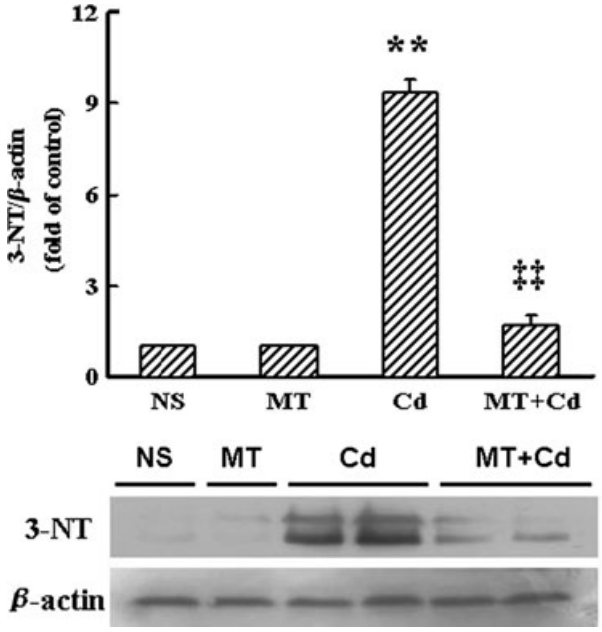


*Fig. 7*. Effects of melatonin on cadmium (Cd)-induced protein nitration in testes. Male mice were injected with $CdCl_2$ (2.0 mg/kg). Some mice were administered with melatonin as Materials and methods. Testes were collected at 24 hr after Cd treatment. 3-NT was measured using Western blot. 3-NT was normalized to β-actin level in the same samples. The densitometry unit of the control was assigned as 1. All data were expressed as means ± S.E.M. (n = 3–6). $^{**}P < 0.01$ as compared with controls. ‡‡$P < 0.01$ as compared with Cd group.

involved in the process of the apoptosis in somatic cells [47–49]. According to a recent study, nonylphenol induces apoptosis in rat testicular Sertoli cells via endoplasmic reticulum stress [50]. In the present study, we showed that acute Cd exposure caused ER stress and activated the UPR in testes. First, GRP78, an important ER molecular chaperone, was upregulated in testes of Cd-treated mice. Second, the level of phosphorylated eIF2α, a downstream target of the PERK pathway, was increased in testes, indicating that PERK pathway was activated by Cd. Third, the level of the sliced XBP-1 mRNA in testes was increased, indicating that IRE1 pathway was activated by Cd. In addition, the expression of CHOP, a downstream target of the PERK and ATF6 pathways, was upregulated in testes of Cd-treated mice. Finally, acute Cd exposure significantly increased the level of JNK phosphorylation in testes. Melatonin is an inhibitor of ER stress. According to our recent study, melatonin obviously attenuated lipopolysaccharide-induced placental ER stress in mice [51]. In addition, melatonin prevented arsenite-induced apoptosis via the suppression of ER signaling and mitochondrial pathway [52, 53]. In the present study, we showed that melatonin significantly attenuated Cd-induced upregulation of testicular GRP78. Moreover, the level of testicular sXBP-1 was significantly decreased when mice were administered with melatonin. In addition, melatonin inhibited Cd-evoked eIF2α and JNK phosphorylation in testes. Importantly, melatonin obviously repressed Cd-induced upregulation of CHOP in testes. These results suggest that melatonin could alleviate Cd-induced ER stress in testes.

Melatonin-mediated protection against Cd-induced germ cell apoptosis might be associated with its alleviation of ER stress in testes.

Heme oxygenase (HO) catalyzes the rate-limiting step in the degradation of heme to yield equimolar amounts of biliverdin, carbon monoxide, and iron. Three isoforms of the HO protein, HO-1, HO-2 and HO-3, have been identified. HO-2, a 36-kDa protein, is constitutively expressed at high levels in testes, mainly in spermatogonia, as well as spermatocytes, spermatids, and residual bodies of the seminiferous epithelium, whereas HO-1, a stress-inducible enzyme, is expressed at a low level in testes, mainly in Sertoli and Leydig cells [54]. It has been demonstrated that testicular HO-1 expression is upregulated in response to stress [55]. In the present study, we showed that acute Cd exposure significantly upregulated the expression of HO-1 in testes. There is increasing evidence that heme oxygenases play important roles in the cellular defense against oxidative stress [56]. Melatonin is a potent regulator of HO-1. A recent study showed that melatonin alleviated cardiopulmonary bypass–induced renal damage through the induction of HO-1 [57]. Another study found that melatonin upregulated the expression of pancreatic HO-1 in cerulein-induced pancreatitis [58]. According to a recent report, the capacity of melatonin to modulate Nrf2 pathway was associated with increased HO-1 expression in streptozotocin-induced diabetic neuropathy [59]. Moreover, melatonin synergistically increases resveratrol-induced HO-1 expression through the inhibition of ubiquitin-dependent proteasome pathway [60]. On the other hand, the induction of HO-1 is associated with oxidative stress [61]. Indeed, the present study found that testicular 3-NT intensity, a marker of protein nitration and oxidative stress, was significantly enhanced in Cd-treated mice. Interestingly, melatonin almost completely inhibited Cd-induced testicular protein nitration and thus downregulated the expression of HO-1 in testes. These results are in agreement with others, in which melatonin reduced the level of hypothalamic HO-1 in Cd-treated rats [28] and downregulated the expression of hepatic HO-1 in old castrated female rats [62]. An earlier study demonstrates that Cd-evoked HO-1 upregulation in Leydig cells contributes to germ cell apoptosis in testes [12]. Taken together, these results suggest that melatonin could effectually alleviate testicular oxidative stress, attenuate testicular HO-1 upregulation, and thus protect against Cd-induced germ cell apoptosis in testes.

In summary, the present study indicates that acute Cd exposure resulted in oxidative stress and ER stress in testes, which might contribute, at least partially, to Cd-induced testicular germ cell apoptosis. Melatonin attenuates Cd-evoked testicular oxidative stress and ER stress. Importantly, melatonin alleviates Cd-induced germ cell apoptosis in testes. Thus, melatonin may be useful as pharmacological agents to protect against Cd-induced testicular damage.

## Acknowledgements

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