Ascorbic acid protects against cadmium-induced endoplasmic reticulum stress and germ cell apoptosis in testes

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ABSTRACT

Cadmium (Cd) is a testicular toxicant which induces endoplasmic reticulum (ER) stress and germ cell apoptosis in testes. This study investigated the effects of ascorbic acid on Cd-evoked ER stress and germ cell apoptosis in testes. Male mice were intraperitoneally injected with CdCl 2 (2.0 mg/kg). As expected, a single dose of Cd induced testicular germ cell apoptosis. Interestingly, Cd-triggered testicular germ cell apoptosis was almost completely inhibited in mice treated with ascorbic acid. Interestingly, ascorbic acid significantly attenuated Cd-induced upregulation of GRP78 in testes. In addition, ascorbic acid significantly attenuated Cd-triggered testicular IRE1α and eIF2α phosphorylation and XBP-1 activation, indicating that this antioxidant counteracts Cd-induced unfolded protein response (UPR) in testes. Finally, ascorbic acid significantly attenuated Cd-evoked upregulation of CHOP and JNK phosphorylation, two components in ER stress-mediated apoptotic pathway. In conclusion, ascorbic acid protects mice from Cd-triggered germ cell apoptosis via inhibiting ER stress and UPR in testes.

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1. Introduction

Cadmium (Cd) is one of major occupational and environmental toxicants. The general population is exposed to Cd via drinking water, food and cigarette smoking [1]. Cd is a reproductive toxicant in humans. Cd has been associated with male infertility and poor semen quality in humans [2]. Several epidemiological investigations demonstrated that there was a significant inverse correlation between blood Cd level and semen quality [3,4]. Our earlier report showed that Cd in seminal plasma could affect semen quality and oxidative DNA damage in human spermatozoa [5]. Even a low level of Cd accumulation in semen might contribute to male infertility by reducing sperm quality [6]. Cd is a testicular toxicant in rodents [7]. At high doses, a single dose of Cd causes interstitial edema, hemorrhage and necrosis, accompanied by damage to seminiferous tubules affecting sperm cells and their precursors [8,9]. Recently, several studies found that acute Cd exposure induced germ cell apoptosis in testes [10,11].

Endoplasmic reticulum (ER) is an important organelle required for cell survival. In the ER, nascent proteins are folded with the assistance of ER chaperones. The ER is sensitive to alterations in cellular homeostasis [12,13]. When ER function is disturbed, ER stress occurs. Under ER stress, the unfolded protein response (UPR) signaling is activated to recover or maintain ER function [14,15]. The UPR signaling is mediated by three transmembrane ER proteins: inositol requiring ER-to-nucleus signal kinase (IRE)1, activating transcription factor (ATF)6 and double-stranded RNA-activated kinase (PKR)-like ER kinase (PERK) [16,17]. If UPR fails to alleviate ER stress, the apoptosis pathway will be activated [18]. A recent report from our laboratory showed that acute Cd exposure caused ER stress and activation of the UPR signaling in testes [19]. Importantly, Cd-evoked ER stress and UPR signaling activation were mediated in germ cell apoptosis in testes [20]. Nevertheless, the mechanism of Cd-evoked ER stress and UPR signaling activation remained obscure.

Increasing evidence demonstrated that excess reactive oxygen species (ROS) production was associated with ER stress and the UPR [21]. Indeed, acute Cd exposure induced oxidative stress in testes [22]. Several recent studies showed that Cd caused lipid peroxidation in testes [23–26]. On the other hand, several studies showed that antioxidants alleviated ER stress and the activation of the UPR signaling [27]. Ascorbic acid is an antioxidant. An earlier report showed that ascorbic acid protected rat testes from Cd-induced oxidative stress [22]. In addition, ascorbic acid protected against Cd-induced impairment in spermatogenesis in rats [24]. In the present study, we investigated the effects of ascorbic acid on Cd-induced ER stress and UPR signaling in testis. We demonstrated for the first time that ascorbic acid protects mice from Cd-triggered germ cell apoptosis by inhibiting ER stress and UPR in testes.

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2. Materials and methods

2.1. Chemicals and reagents

Cadmium chloride (CdCl₂) and ascorbic acid were from Sigma Chemical Co. (St. Louis, MO). Antibodies for heme oxygenase (HO)-1 and phosphor–p38 enzyme were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for CCAAT/enhancer binding protein (C/EBP) and homologous protein (CHOP), glucose-regulated protein 78 (GRP78) and phosphor–eukaryotic translation initiation factor (eIF2) were from Cell Signaling Technology (Beverley, MA). β-Actin antibody was from Boster Bio-Technology Co. Ltd. (Wuhan, China). Chemiluminescence (ECL) detection kit and phosphor–moiost-requiring ER-to-nucleus signal kinase (IRE) 1α antibody were from Pierce Biotechnology (Rockford, IL). TRI reagent was from Molecular Research Center, Inc (Cincinnati, Ohio). RNase-free DNase was from Promega Corporation (Madison, WI). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) if not otherwise stated.

2.2. Animals and treatments

Adult male CD-1 mice (8 week-old, 28–32 g) were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laborato- ries, Inc. The animals were allowed free access to food and water at all times and were maintained on a 12-h light/dark cycle in a controlled temperature (20–25 °C) and humidity (50 ± 5%) environment. Our earlier study showed that the percentage of tubules with TUNEL+ germ cells and the number of apoptotic cells per tubule were highest at 24 h after Cd injection. In addition, most of apoptotic cells were observed in testes of mice treated with 2.0 mg/kg CdCl₂ [20]. In the present study, all mice except controls were intraperitoneally (i.p.) injected with 2.0 mg/kg of CdCl₂. In Cd + AA group, mice were i.p. injected with ascorbic acid (100 mg/kg) every 8 h, beginning at 8 h before Cd injection. The dose of ascorbic acid referred to others [28]. Testes were collected at 24 h after Cd injection. Testes were divided in two parts: left one was kept at −80 °C for Western blotting and RT-PCR. The other part of the testes was immersed in modified Davidson’s fluid (mDF) for 24 h for testicular histology and apoptosis analysis [29]. 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.

2.3. Testicular histology and terminal dUTP nick-end labeling (TUNEL) staining

Two cross sections from each testis were embedded in paraffin. 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. 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+ germ cells per seminiferous tubule and the percentages of the number of seminiferous tubules containing TUNEL+germ cells were analyzed.

2.4. 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 μg/sample) in loading buffer were subjected to electrophoresis in 10–15% SDS-polyacrylamide gel for 3 h. The gel was transferred electrophoretically onto a polyvinyldene fluoride membrane (Immobilon-P; Millipore Corp., Bedford, Massachusetts, USA) and blocked in 5% nonfat powdered milk in Dulbecco’s PBS (DPBS) overnight at 4 °C. The membranes were incubated for 2 h with the following antibodies: HO-1, GRP78, phosphor–eIF2α, phosphor–IRE1α and phosphor–JNK. β-Actin was used as a loading control for total proteins. After washes in DPBS containing 0.05% Tween-20 for 4 min each, the membranes were incubated with goat anti-rabbit or goat anti-mouse IgG antibody for 2 h. The membranes were then washed for 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 (Pierce Biotechnology, Rockford, IL).

2.5. Isolation of total RNA and RT-PCR

About 30 mg 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 electrophore- sis on agarose gels. For the synthesis of cDNA, 1.0 μg of total RNA from each sample was resuspended in a 20-μl final volume of reaction buffer, which contained 25 mM Tris–HCl (pH 7.5), 375 mM KCl, 10 mM dithiothreitol, 10 mM dNTP and 0.5 mg oligo(dT)₁₅ primer. After the reaction mixture reached 42 °C for 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 μl of cDNA, 1× PCR buffer, 1.5 mM MgCl₂, 200 μM dNTP mixture, 1.0 U of Taq DNA polymerase, 1 μM sense and antisense primers, and sterile water to 50 μl. 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 [30]. GAPDH, 5′-GAG GGG CCA TCA GCC TTC-3′ and 5′-CAT CAC CAT CTT CCA GGA GCG-3′; XBP-1, 5′-ACA AGC TTC GGA ATC AC-3′ and 5′-CCA TGG GAA GAT GTT CGG-3′. The amplified PCR products were subjected to electrophoresis at 75 V through 1.5% agarose gels (Sigma, St. Louis, MO) for 45 min. The pBluescript II DNA digested with Alu I was used for molecular markers (MBI Fermentas). Agarose gels were stained with 0.5 mg/ml ethidium bromide (Sigma, St. Louis, MO) TBE buffer.

2.6. Statistical analysis

Each band of RT-PCR and immunoblotting was scanned and its intensity ana- lyzed by Image J software [http://rsb.info.nih.gov/ij/]. For RT-PCR, the level of sXBP-1 mRNA was normalized to GAPDH mRNA level in the same samples. sXBP-1 mRNA level of the control was assigned as 1. For immunoblotting, the level of GRP78, pJNK, pCHOP, HO-1, pIRE1α and pβ was normalized to β-Actin level in the same samples. The level of the control was assigned as 1. All quantified data were expressed as means ± SEM. ANOVA and the Student–Newmann–Keuls post hoc test were used to determine differences among different groups.

3. Results

3.1. Ascorbic acid alleviates Cd-induced testicular histopathological damage

An obvious testicular edema was observed at 24 h after CdCl₂ injection. Consistent with testicular edema, the absolute and relative testis weights were significantly increased in mice injected with a single dose of CdCl₂. Pretreatment with ascorbic acid almost completely inhibited Cd-induced testicular edema. Correspondingly, ascorbic acid significantly alleviated Cd-induced elevation of the absolute and relative testis weights (Fig. 1). The effects of ascorbic acid on Cd-induced testicular histopathological damage were analyzed. As shown in Fig. 2, a slight necrosis of seminiferous tubules and a moderate hemorrhage in the interstitium were observed in testes of mice treated with CdCl₂. Ascorbic acid obviously alleviated Cd-induced histopathological damage in testes.

3.2. Ascorbic acid protects against Cd-induced germ cell apoptosis in testes

The effects of ascorbic acid on Cd-induced testicular germ cell apoptosis were analyzed. As shown in Fig. 3A, a single dose of CdCl₂ significantly increased the number of apoptotic cells in testes. Further analysis showed that most of TUNEL+ cells were observed mainly in germ cells of seminiferous tubules. Interestingly, pretreatment with ascorbic acid almost completely counteracted Cd-induced germ cell apoptosis in testes. Next, the percentage of tubules with TUNEL+ cells and the number of TUNEL+ cells per tubule were analyzed. As shown in Fig. 3B, the percentage of tubules with TUNEL+ cells was significantly increased in testes of mice injected with CdCl₂. In addition, the number of apoptotic cells per tubule was significantly increased in testes of mice injected with CdCl₂ (Fig. 3C). Ascorbic acid significantly reduced the percentage of tubules with TUNEL+ cells and the number of TUNEL+ cells per tubule (Fig. 3B and C).

3.3. Ascorbic acid alleviates Cd-induced ER stress in testes

The effects of ascorbic acid on Cd-induced testicular ER stress were then analyzed. As shown in Fig. 4, the level of testicular GRP78 protein, an ER chaperone, was significantly increased in testes of Cd-treated mice. Interestingly, pretreatment with ascorbic acid significantly attenuated Cd-induced upregulation of GRP78 in testes. Next, the effects of ascorbic acid on IRE1α signaling were analyzed. As shown in Fig. 5A, the level of phosphorylated
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IRE1α was significantly increased in testes of mice treated with CdCl2. Correspondingly, the level of spliced XBP-1 mRNA, a downstream target of IRE1α, was significantly increased in testes of Cd-treated mice (Fig. 5B). In addition, the level of phosphorylated JNK, another target of the IRE1 pathway, was significantly increased in testes of mice injected with CdCl2 (Fig. 5C). Ascorbic acid significantly alleviated Cd-induced IRE1α phosphorylation in testes (Fig. 5A). Moreover, pretreatment with ascorbic acid significantly attenuated Cd-induced XBP-1 splicing and JNK phosphorylation in testes (Fig. 5B and C). Finally, the effects of ascorbic acid on RNA-dependent protein kinase-like ER kinase (PERK) signaling were analyzed. As shown in Fig. 6A, the level of phosphorylated eIF2α, a downstream target of PERK signaling, was significantly increased in testes of Cd-treated mice. Moreover, CHOP, another downstream target of the PERK pathway, was upregulated in testes of mice injected with CdCl2 (Fig. 6B). Ascorbic acid significantly attenuated Cd-induced eIF2α phosphorylation in testes (Fig. 6A). In addition, ascorbic acid almost completely inhibited Cd-induced upregulation of CHOP in testes (Fig. 6B).

3.4. Ascorbic acid attenuates Cd-induced HO-1 upregulation in testes

The effects of ascorbic acid on Cd-induced upregulation of testicular HO-1 were analyzed. As expected, the level of HO-1 was significantly increased in testes of mice injected with CdCl2 (Fig. 7). Interestingly, pretreatment with ascorbic acid significantly alleviated Cd-induced upregulation of HO-1 in testes.

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**Fig. 1.** Ascorbic acid alleviates Cd-induced elevation of testis weights. Male mice were injected with CdCl2 (2.0 mg/kg). In AA + Cd group, mice were administered with ascorbic acid as described in Section 2. Testes were collected at 24 h after Cd injection. (A) Absolute testis weight. (B) Relative testis weight. All data were expressed as means ± SEM (n = 12). **P < 0.01 vs the control. ††P < 0.01 vs Cd group.

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**Fig. 2.** Effects of ascorbic acid on Cd-induced histopathological damage in testes. Male mice were injected with CdCl2 (2.0 mg/kg). In AA + Cd group, mice were administered with ascorbic acid as described in Section 2. Testes were collected at 24 h after Cd injection. Testicular cross sections were stained with H&E. Original magnification: 400×.
3. Ascorbic acid protects against on Cd-induced germ cell apoptosis in testes. Male mice were injected with CdCl₂ (2.0 mg/kg). In AA + Cd group, mice were administered with ascorbic acid as described in Section 2. Testes were collected at 24 h after Cd injection. (A) Testicular germ cell apoptosis was detected by TUNEL staining. Arrow showed TUNEL+ germ cells in seminiferous tubules. (B) The percentages of seminiferous tubules containing TUNEL-positive germ cells. (C) The number of TUNEL+ germ cells per seminiferous tubule. All data were expressed as means ± SEM (n = 12). **P < 0.01 vs the control. ‡‡P < 0.01 vs Cd group.

4. Discussion

In the present study, we investigated the effects of ascorbic acid on Cd-induced germ cell apoptosis in testes. As expected, a single dose of CdCl₂ obviously elevated the percentage of tubules with apoptotic germ cells. In addition, the number of apoptotic germ cells per tubule was significantly increased in testes of mice injected with CdCl₂. Pretreatment with ascorbic acid significantly alleviated Cd-induced germ cell apoptosis in mouse testes. These results are in agreement with those from a recent study, in which ascorbic acid protected against Cd-induced germ cell apoptosis in rat testes [10].

Several reports demonstrated that ER stress and the UPR were involved in the process of Cd-evoked apoptosis in tubular epithelial cells [31–33]. Our recent study showed that acute Cd exposure caused ER stress and UPR in testes [19]. Importantly, the UPR signaling contributes, at least partially, to Cd-evoked germ cell apoptosis in testes [20]. The UPR signaling is mediated by three transmembrane ER proteins: IRE1, activating transcription factor (ATF) 6, and PERK [16,17]. In the present study, we investigated the effects of ascorbic acid on Cd-induced ER stress and UPR in testes. Our results demonstrated that ascorbic acid counteracted Cd-induced ER stress and UPR in testes. First, pretreatment with ascorbic acid significantly attenuated Cd-induced upregulation of testicular GRP78, an ER chaperone and ATF6 target. Moreover, pretreatment with ascorbic acid significantly inhibited Cd-induced phosphorylation of IRE1α in testes. In addition, ascorbic acid significantly
counteracted Cd-induced XBP-1 splicing, indicating that Cd-evoked IRE1 signaling branch was repressed in testes when mice were pretreated with ascorbic acid before Cd injection. Finally, pretreatment with ascorbic acid significantly attenuated Cd-induced phosphorylation of testicular eIF2α, a molecule downstream of PERK signaling branch. Taken together, these results suggest that ascorbic acid alleviates Cd-induced ER stress and UPR in testes.

Increasing evidence demonstrates that CHOP, a downstream target of the PERK pathway [34], is one of the components in the ER stress-mediated apoptotic pathway [35–39]. According to an earlier study, CHOP overexpression could promote translocation of Bax from the cytosol to the mitochondria [40]. A recent study found that ER stress activated BH3-only protein Bim and triggered apoptosis through CHOP-mediated direct transcriptional induction [41]. In the present study, we showed that acute Cd exposure significantly upregulated the expression of CHOP in testes. Interestingly, pretreatment with ascorbic acid almost completely inhibited Cd-induced upregulation of CHOP in testes. These results suggest that CHOP might be one of the mediators in Cd-induced germ cell apoptosis in testes.

Numerous reports showed that JNK, a molecule downstream of IRE1 signaling branch, was involved in ER stress-mediated apoptosis [42,43]. According to several earlier reports, IRE1α could activate JNK and its downstream proapoptotic kinase apoptosis-signaling-regulating kinase (ASK)1 [44,45]. Further research found that ASK1-interacting protein 1 (AIp1) was critical in IRE1α/ASK1 activation and ER stress-mediated apoptosis [46]. Indeed, activation of IRE1α/JNK signaling has been demonstrated to play a role in Cd-initiated apoptosis of tubular epithelial cells [31]. In the present study, we showed that Cd exposure significantly attenuated Cd-induced phosphorylation of IRE1α and JNK in testes. Thus, IRE1α/JNK/ASK1 signaling pathway may also contribute to execution of Cd-triggered germ cell apoptosis in testes. Ascorbic acid could protect against Cd-induced testicular germ cell apoptosis through inhibiting activation of IRE1α/JNK/ASK1 signaling in testes.

HO-1, a stress-inducible enzyme, is expressed at a low level in testes, mainly in Sertoli and Leydig cells [47]. An earlier report showed that Leydig cell derived HO-1 regulated apoptosis of premeiotic germ cells in response to stress [11]. Indeed, the present study showed that acute Cd exposure significantly upregulated the expression of HO-1 in testes. Surprisingly, pretreatment with ascorbic acid significantly alleviated Cd-induced upregulation of HO-1 in testes. Thus, the present study does not exclude that ascorbic acid counteracts Cd-evoked germ cell apoptosis via inhibiting HO-1 expression in testes.
The protection of ascorbic acid against Cd-induced ER stress and germ cell apoptosis in testes may have preventive implications. According to a recent report, pretreatment with ascorbic acid significantly reduced the number of apoptotic cells in testes of rats treated with cisplatin [48]. In addition, supplementation with ascorbic acid significantly alleviated lead-induced germ cell apoptosis in mouse testes [49]. Several studies demonstrated that some antioxidants, such as melatonin and N-acetylcysteine, could effectively prevent from arsenite-induced neurotoxicity via the repression of ER stress mediated apoptosis [50–52]. Indeed, a recent study observed an increase in germ cell apoptosis in testes of mice unable to synthesize ascorbic acid [53]. Thus, ascorbic acid, as an antioxidant, may be therapeutically useful for the treatment of Cd-induced ER stress and germ cell apoptosis in testes.

In summary, the present study demonstrated that ROS-mediated ER stress and UPR signaling contribute, at least partially, to Cd-induced germ cell apoptosis in testes. Ascorbic acid, a well-known antioxidant, protects mice from Cd-triggered germ cell apoptosis via inhibiting ER stress and UPR in testes. Thus, antioxidants may be useful as pharmacological agents to protect against Cd-induced testicular injury.

Conflict of interest statement

The authors declare that there are no conflict of interest.

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References