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ORIGINAL RESEARCH

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Effect of quercetin on the *IRE1-XBP1* apoptotic pathway in Cadmium-treated rat testes

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Abstract

Background: Cadmium (Cd) is a recognized toxic metal with serious reproductive toxicity in both animals and humans, while quercetin (Que) is considered the potent antioxidant among flavonoid compounds. However, little is known about the alleviating effect of Que on Cd-induced testicular cell apoptosis. This experiment aims to investigate the alleviating effect of Que on Cd-induced testicular cell apoptosis in rats. **Methods**: Twenty-four four-week-old male Sprague-Dawley rats were randomly divided into control group, Cd group, Cd + Que group and Que group. Following the assigned treatments, the testicular tissues of the rats were examined 28 days later. The levels of malondialdehyde (MDA) and reducing glutathione (GSH) in testicular tissue were measured via colorimetry, with HE (hematoxylin and eosin) and TUNEL (a TdT-UTP nick end labeling) staining employed to assess tissue damage and cell apoptosis. Total mRNA (messenger RNA) was extracted from testicular tissue using the Trizol method, and reverse transcription was followed by quantitative real-time PCR (Polymerase Chain Reaction) to measure the expression levels of relevant genes. The expression of related proteins was assessed using Western blot. **Results**: The data revealed that Cd exposure led to a decrease in body weight and a significant increase in MDA and GSH content in testicular tissue. In addition, histopathological examination of the tissue revealed extensive pathological changes, and TUNEL staining showed significant cell apoptosis in the tissue. Furthermore, Cd treatment promoted the expression of relevant genes, including *IRE1*α (inositol-requiring kinase 1), *Caspase-12*, *XBP1* (X box-binding protein 1), *GRP78* (glucose-regulated protein 78), *Bax* and *Caspase-3*, in the *IRE1-XBP1* apoptosis pathway. Meanwhile, the anti-apoptotic gene *Bcl-2* was significantly decreased. However, the application of Que significantly reduced these alterations and cellular damage induced by Cd. **Conclusions**: Our study suggests that Que can mitigate testicular tissue damage and cell apoptosis resulting from Cd exposure by suppressing oxidative stress and the *IRE1-XBP1* pathway.

Keywords

Cadmium; Testis; Toxicity; Apoptosis; Quercetin

Efecto de la quercetina en la vía apoptótica *IRE1-XBP1* en testículos de rata tratados con Cadmio

Resumen

Antecedentes: El cadmio (Cd) es un metal tóxico reconocido con grave toxicidad reproductiva tanto en animales como en humanos, mientras que la quercetina (Que) se considera el potente antioxidante entre los compuestos flavonoides. Sin embargo, poco se sabe sobre el efecto paliativo de Que en la apoptosis de células testiculares inducida por Cd. Este experimento tiene como objetivo investigar el efecto paliativo de Que en la apoptosis de células testiculares inducida por Cd en ratas. **Métodos**: Veinticuatro ratas macho Sprague-Dawley de cuatro semanas de edad fueron aclimatadas durante una semana y divididas aleatoriamente en grupo control, grupo Cd, grupo Cd + Que y grupo Que. Tras los tratamientos asignados, se examinaron los tejidos testiculares de las ratas 28 días después. Los niveles de malondialdehído (MDA) y glutatión reductor (GSH) en el tejido testicular se midieron mediante colorimetría, y se emplearon las tinciones HE (hematoxilina y eosina) y TUNEL (a TdT-UTP nick end labeling) para evaluar el daño tisular y la apoptosis celular. Se extrajo el ARNm total (ARN mensajero) del tejido testicular mediante el método Trizol y se realizó la transcripción inversa seguida de la PCR cuantitativa en tiempo real (reacción en cadena de la polimerasa) para medir los niveles de expresión de los genes relevantes. **Resultados**: Los datos revelaron que la exposición al Cd provocó una disminución del peso corporal y un aumento significativo del contenido de MDA y GSH en el tejido testicular. Además, el examen histopatológico del tejido reveló cambios patológicos extensos, y la tinción TUNEL mostró una apoptosis celular significativa en el tejido. Además, el tratamiento con Cd fomentó la expresión de genes relevantes, como *IRE1*α (inositol-requiring kinase 1), *Caspasa-12*, *XBP1* (X box-binding protein 1), *GRP78* (glucose-regulated protein 78), Bax y *Caspasa-3*, en la vía de apoptosis *IRE1-XBP1*. Mientras tanto, el gen anti-apoptótico *Bcl-2* disminuyó significativamente. Sin embargo, la aplicación de Que redujo significativamente estas alteraciones y el daño celular inducido por el Cd. **Conclusiones**: Nuestro estudio sugiere que Que puede mitigar el daño testicular y la apoptosis celular resultantes de la exposición al Cd mediante la supresión del estrés oxidativo y de la vía *IRE1-XBP1*.

Palabras Clave

Cadmio; Testículo; Toxicidad; Apoptosis; Quercetina

1. Introduction

Millions of people around the world are inevitably exposed to cadmium (Cd), a heavy metal that is ubiquitous in the Earth's crust, through occupational and non-occupational pathways [1]. In the occupational pathway, individuals working in an environment with cadmium levels above 0.005 $\mu g/m^3$ are at risk of many diseases, including lung cancer, prostate cancer, testicular impairment and bone disease [2–5]. In the non-occupational pathway, chronic low-level Cd intake is primarily attributed to smoking and the consumption of food and water contaminated with this heavy metal [6]. The elimination rate of Cd is extremely low in the organism [1]. After absorption, Cd slowly accumulates in high concentrations and persists in the body, mainly in the liver, kidneys and testes, gradually creating a potential health risk [7]. The literature extensively discusses the impacts of Cd accumulation on the reproductive system of animals [8–11]. Exposure Cd is known to decrease the density, volume and number of spermatozoa in semen, as well as to increase the proportion of immature spermatozoa [12]. In addition, a recent body of literature has indicated that cadmium can also lower the levels of testosterone in people's blood serum, and it can directly or indirectly reduce human libido and fertility [13–15].

Cd is known for its long half-life of 10 to 30 years and the difficulty of eliminating it from the body [16]. When present in low levels, Cd can suppress cellular respiration and oxidative phosphorylation [17]. However, high levels of Cd can lead to anemia and ataxia [18–20]. Cd itself cannot produce free radicals; however, it possesses the ability to catalyze the generation of different reactive species, such as hydroxyl radicals, nitric oxide radicals and superoxide radicals, consequently resulting in the production of reactive oxygen species (ROS) and, indirectly, oxidative stress [21, 22]. The production of ROS and free radicals generated by Cd are considered among the factors contributing to Cd's toxicity towards the testes [23]. When cells are exposed to Cd, the endoplasmic reticulum (ER) emerges as the primary target organelle [24]. High levels of ROS can trigger an unfolded protein response (UPR) in the ER, which acts as a protective mechanism against cellular damage and aims to restore normal cell function. However, if the accumulation of unfolded and misfolded proteins overwhelms this protective response, then it can lead to ER stress (ERS). ERS can activate various pathways such as inositol-requiring kinase 1 and X box-binding protein 1 (*IRE1-XBP1*), ultimately resulting in apoptosis [24, 25]. This mechanism helps explain the role of Cd in apoptosis [26]. Recent studies provide growing evidence that Cd induces ERS-induced apoptosis [25, 27, 28].

Quercetin (Que), a naturally occurring bioflavonoid, is abundantly found in commonly consumed fruits such as apples, blueberries, onions and leeks. It exhibits a wide range of significant biological properties, including antioxidant, anticancer, antiviral, antithrombotic, anti-ischemic, anti-inflammatory and anti-allergic activities [29], which contribute to overall health improvement and disease prevention. Que has been reported to utilize various mechanisms to combat ROS. These mechanisms include

its potent anti-free radical properties targeting hydroxyl radicals, peroxides and superoxide anions. It can also inhibit the activities of xanthine oxidase, cyclooxygenase and lipoxygenase enzymes, thereby reducing the generation of superoxide anions [30]. Moreover, it has the ability to form chelates with metals such as iron and copper. Thus when applied to Cd poisoning, Que can mitigate oxidative stress and degenerative diseases in different tissues and organs of the body [31]. Indeed, Que has been demonstrated to mitigate Cd-induced cellular toxicity by diminishing lipid peroxidation and augmenting intracellular antioxidant capabilities [32].

Cd, an extremely toxic heavy metal element, poses a significant threat to the health of humans and animals worldwide due to its ability to accumulate in the food chain. Cd poisoning can result in significant harm to testicular tissue, inducing widespread apoptosis of spermatogenic cells in male rats, ultimately leading to a marked reduction in both the quality and quantity of sperm, and potentially resulting in sterility [26]. Que, with its wide distribution and multiple biological activities, has gradually emerged as a novelty in medical research. In this experiment, we established an animal model of Cd-treated rats to investigate the effect of Que on the expression of signaling molecules related to the *IRE1-XBP1* apoptotic pathway in the testes of Cd-treated rats.

2. Materials and methods

2.1 Experimental materials

2.1.1 Laboratory animals

Twenty-four four-week-old male Sprague-Dawley rats, acquired from the Experimental Animal Center of Zhengzhou University.

2.1.2 Experimental reagents

Anhydrous $CdCl_2$ (purchased from Aladdin Industrial Corporation, analytical purity), Que (Shanghai Eon Chemical Technology Co., Ltd., purity >97%), malondialdehyde (MDA) kit, reducing glutathione (GSH) kit, hematoxylin-eosin staining solution, a TdT-UTP nick end labeling (TUNEL) staining kit, ethanol solution, methanol (Tianjin Comio Chemical Reagent Co., Ltd.), PCR primers, TRIzol isolation kit (all from Shanghai Sanko Biotechnology Co., Ltd., N1002-01, Shanghai, China), SYBR (Synergetic Binding Reagent) premix, Taq II kit (all from Takara, DRR041A, RR820A, Shiga, Japan), RIPA (Radio Immunoprecipitation Assay) lysis solution, ECL (Enhanced Chemiluminescence) solution, 0.9% saline, formaldehyde solution, skim milk powder (all from Beyotime Biotechnology Co., Ltd.).

2.2 Experimental methods

2.2.1 Grouping of experimental animals

After allowing the purchased rats to adapt to the feeding regimen for one week, they were randomly assigned to one of four groups, each comprising six rats. They were provided with sufficient food and water (the rats were provided with a standard commercial diet and were free to drink under standard laboratory conditions), and their weight was measured weekly. In addition, they were subjected to treatments for 28 days as follows:

A: Control group: Intraperitoneal injection of 0.9% NaCl (sodium chloride) and intragastric administration of 0.9% NaCl.

B: Cd group: Intraperitoneal injection of 2 mg/kg b.w. $CdCl_2$ and intragastric administration of 0.9% NaCl.

C: Cd + Que group: Intraperitoneal injection of 2 mg/kg b.w. $CdCl_2$ and intragastric administration of 100 mg/kg b.w. Que.

D: Que group: Intraperitoneal injection of 0.9% NaCl and intragastric administration of 100 mg/kg b.w. Que.

The dosages of $CdCl_2$ and Que utilized in this study were chosen based on prior research [33, 34].

2.2.2 Experimental sample collection and processing

After four weeks, the rats were fasted for 12 h and then euthanised by neck dislocation. The testicles were then removed, washed by 0.9% saline, allowed to dried and then weighed. Some of the samples were fixed in formalin for the preparation of testicles tissue pathological examination. A portion of testicular tissue weighing 0.500 g was taken and carefully ground in a grinder placed on ice. The ground tissue was mixed with the required ratio of phosphate-buffered saline (PBS) to testicular tissue (1:9). After thorough and complete grinding, the mixture was centrifuged at 12,000 r/min for 10 min. The supernatant was collected and used for the determination of MDA and GSH. The remaining samples were stored at −80 °C for further quantitative analysis.

2.2.3 Determination of antioxidant indicators

The content of MDA and GSH in testicular tissue homogenates was determined using the MDA and GSH assay kit, following the provided instructions.

2.2.4 Histopathologic analysis

Histological lesions were observed through hematoxylin and eosin (HE) staining. Paraffin sections were prepared from the left testis of rat using conventional methods to fix the samples [24, 35], followed by HE staining for observation under an optical microscope (E200 POL, Nikon, Tokyo, Japan).

2.2.5 TUNEL analysis

The TUNEL staining technique was utilized to observe apoptosis. Paraffin sections were prepared and subjected to decolorization using xylene. Subsequently, a new xylene dewaxing treatment was employed. The sections were then soaked in ethanol solutions of varying concentrations (100%, 95%, 85%, 70%, 50%), and DNase-free protease was added for labeling reaction. Following the reaction, the sections were washed with PBS and incubated with TUNEL detection solution for 1 h. After being rinsed with PBS, the sections were sealed using an anti-fluorescence quenching sealing solution and examined and photographed under fluorescence microscopy (Eclipse C1; Nikon, Tokyo, Japan).

2.2.6 Quantitative real-time polymerase chain reaction (RT-qPCR)

RT-qPCR was used to determine the relevant mRNA. The total RNA was extracted from rat testicular tissue using Trizol reagent. The concentration and purity of the RNA samples were analyzed using a nucleic acid concentration analyzer (Nanodrop2000c, Thermo, Waltham, MA, USA). The RNA samples were reverse transcribed to obtain sample cDNA. The cDNA samples were then subjected to RT-qPCR, where they were compared using the cycle threshold method ($2^{-\Delta\Delta ct}$). This method enables the relative quantification of gene expression levels by comparing the cycle threshold values of the target gene with a reference gene. The primers for *GRP78*, *IRE1α*, *XBP1*, *Caspase-12*, *Caspase-3*, *Bax*, *Bcl-2* and *β-Actin* are listed in Table 1.

2.2.7 Western blot analysis

Extract total protein from testicular tissue, used BCA (Bicinchoninic Acid) detection kit to determine and dilute to 5 μg/μL concentration. After the loading buffer was added, the protein was denatured in a boiling water bath. Subsequently, gel electrophoresis was performed, and the proteins were transferred to a methanol-activated PVDF (Polyvinylidene Fluoride) membrane. The membrane was then incubated with skimmed milk for 2 h to seal the PVDF membrane. At 4 ℃, the PVDF membrane, which had been washed with TBST (Tris Buffered Saline with Tween® 20), was incubated overnight with the primary antibody. After washing the membrane thrice with TBST, it was incubated with the secondary antibody for a duration of 1 h. After 2 min of incubation with the ECL chemiluminescent solution, the membrane was placed inside the exposure box (ChemiDoc Touch, Bio-Rad, Hercules, CA, USA), and a photograph was taken. The antibodies used and their dilution ratios are shown in Table 2 below.

2.3 Statistical methods

Statistical analysis was conducted using sing way ANOVA (Analysis of Variance) with SPSS (Statistical Package for the Social Sciences) for windows (version 26; SPSS Inc., Chicago, IL, USA). Results were expressed as mean ± standard deviation (SD). Bonferroni multiple comparisons were conducted to control for error rates at the overall significance level and differences were indicated as critically significant when $p < 0.01$.

3. Results

3.1 Body weight

As shown in Fig. 1, a significant decrease in body weight occurred in the second, third and fourth weeks of treatment with Cd in the Cd group compared with the control group during the same period ($p < 0.01$). At 21 days, the body weight of rats in the Cd + Que group was significantly higher than that of the Cd group ($p < 0.01$). When Cd and Que were administered concurrently, the body weights did not revert to those of the control group. Moreover, there was no statistically significant variation in body weight between the Que group and the control group over the 28-day period.

3.2 MDA and GSH

As shown in Fig. 2A, the MDA content in the Cd group was significantly higher than in the control group, and the difference was highly significant ($p < 0.01$); the MDA content in the Cd + Que group decreased compared with that in the Cd group and the difference was highly significant ($p < 0.01$). As shown in Fig. 2B, the GSH content in the Cd group was significantly higher than in the control group, and the difference was highly significant ($p < 0.01$); the GSH content in the Cd + Que group decreased compared with that in the Cd group and the difference was highly significant (p

TABLE 1. The primer sequence used in this study.

Genes	Serial number	Primers used for PCR
GRP78	NM_178021.1	F: ATGGTGTGGGAGATCCTGTTTTC R: CAAGACGCACAGGGATACGC
IRE1α	NM_001191926.1	F: GCGCAGGTGCAATGACATAC R: CATGCAAACTTCCGTCCAGG
XBP1	NM_001271731.1	F: CTGAGTCCGCAGCAGGTG R: GACCTCTGGGAGTTCCTCCA
Caspase-12	NM_130422.1	F: TCGGAGAAGGAGCGAGCTTA R: AGCTGTTTGTCGGAATTGGC
Caspase-3	NM_012922.2	F: GGAGCTTGGAACGCGAAGAA R: ACACAAGCCCATTTCAGGGT
Bax	NM_017059.2	F: CACGTCTGCGGGGAGT R: CATCCTCTCTGCTCGATCCTG
Bcl-2	NM_016993.2	F: AGCATGCGACCTCTGTTTGA R: TCACTTGTGGCCCAGGTATG
β-Actin	NM_031144.3	F: CACCCGAGAGTACAACCTT R: TCATCCATGGCGAACTGGTG

PCR: Polymerase Chain Reaction; GRP78: glucose-regulated protein 78; IRE1α: inositol-requiring kinase 1; XBP1: X box-binding protein 1.

TABLE 2. The antibodies used in this study.

Antibodies	Identifier	Sources	Dilution ratio
GRP78	PA1815	Proteintech Group	1:700
IRE1α	27528-1-AP	Proteintech Group	1:2000
XBP1	24868-1-AP	Proteintech Group	1:500
Caspase-12	55238-1-AP	ABclonal	1:1000
Caspase-3	A2156	Proteintech Group	1:3000
Bax	60267-1-Ig	Proteintech Group	1:10,000
Bcl-2	60178-1-Ig	ABclonal	1:1000
β-Actin	ACOO4	ABclonal	1:7000
Horseradish peroxidase-conjugated anti-mouse antibody	HA1006	HuaBio	1:20,000
Horseradish peroxidase-conjugated anti-rabbit antibody	HA1001	HuaBio	1:20,000

GPR78: glucose-regulated protein 78; IRE1α: inositol-requiring kinase 1; XBP1: X box-binding protein 1.

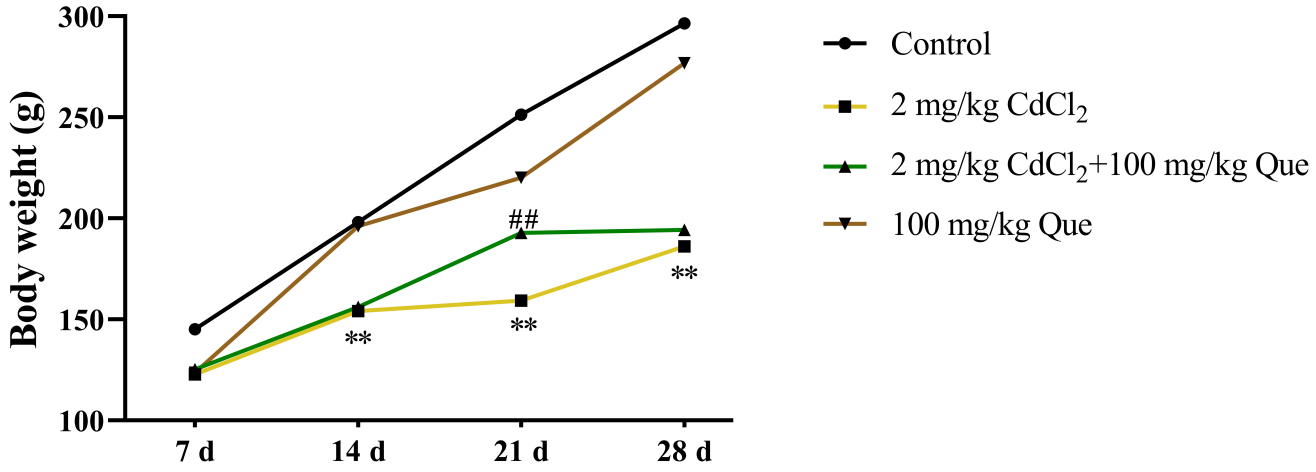


FIGURE 1. Effect of Que on body weight gain of Cd-treated rats. All measurement results were expressed as mean ± SD (n = 6). **indicates significant difference compared with control group at $p < 0.01$. ##indicates a significant difference of $p < 0.01$ compared with the 2 mg/kg Cd group. $CdCl_2$: cadmium chloride; Que: quercetin.

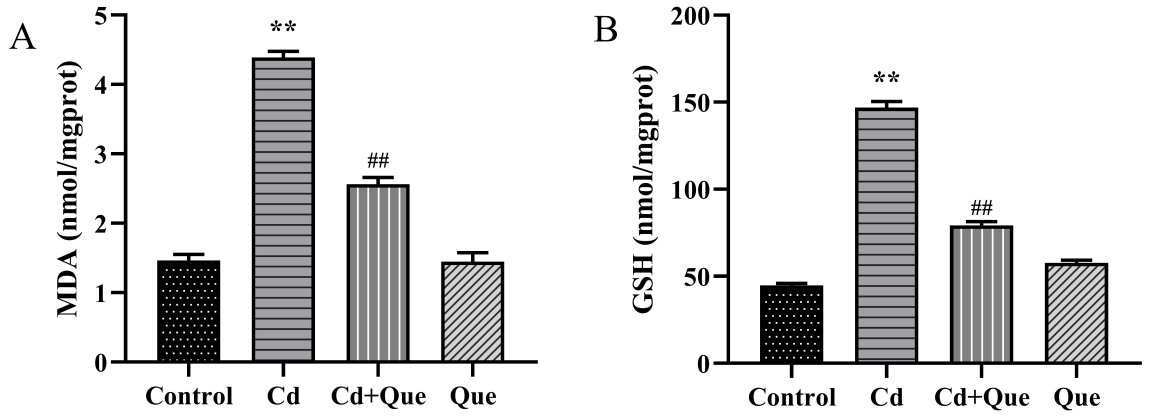


FIGURE 2. MDA and GSH levels assay in testicular tissues. The levels of MDA (A) and GSH (B) in rat testes. All measurement results were expressed as mean ± SD (n = 6). **indicates significant difference compared with the corresponding control $p < 0.01$. ##indicates statistically significant difference between the Cd and Cd + Que groups $p < 0.01$. MDA: malondialdehyde; GSH: glutathione; Cd: cadmium; Que: quercetin.

< 0.01). Furthermore, the levels of MDA and GSH in the Cd + Que group remained higher than those of the control group. No significant difference was observed between the quercetin-treated group and the control group.

3.3 Histological evaluation

As can be seen in the Fig. 3, in the control group (Fig. 3A), testicular tissue cells were lightly stained, structurally intact, with orderly cell arrangement and clear morphology. In the Cd group (Fig. 3B), the testicular histiocytes were densely stained and extensive liquefactive necrosis occurred. In the Cd + Que group (Fig. 3C), the degree of liquefying necrosis was reduced, and some of the spermatogenic tubules returned to normal, and the degree of damage was alleviated compared with that in the Cd group. Que group (Fig. 3D) testicular tissue cells were morphologically normal, and no significant difference from normal testicular tissue cells was found.

3.4 TUNEL assay

As shown in Fig. 4, compared with the control group, the Cd group indicated a significant increase in the number of apoptotic cells in the testicular tissue. A decrease in the number of apoptotic cells in the testicular cells of rats exposed to Cd and treated with Que relative to the Cd group.

3.5 RT-qPCR

As shown in Fig. 5, compared with the control group, the mRNA levels of apoptosis-related genes *IRE1*α, *Caspase-12*, *XBP1*, *GRP78*, *Caspase-3*, *Bax* were significantly increased in the Cd group ($p < 0.01$). In contrast, in the Cd + Que group, the mRNA levels of *IRE1*α, *Caspase-12*, *XBP1*, *GRP78*, *Caspase-3* and *Bax*, were significant decreased compared with the Cd group ($p < 0.01$). In the Cd + Que group, the mRNA expression levels of IRE1α, *Caspase-12*, *XBP1*, *GRP78*, *Caspase-3* and *Bax* genes still increased to the control group. Additionally, for the antiapoptotic gene *Bcl-2*, compared with the control group, its mRNA levels were significantly decreased in the Cd group ($p < 0.01$), while in the Cd + Que group, they were significantly increased compared to the Cd group ($p < 0.01$). In the Cd + Que group, *Bcl-2* genes still decreased to the control group. The mRNA expression levels of apoptosis-related genes in the quercetin group did not significantly differ from those in the control group.

3.6 Western blot

As depicted in Fig. 6, a significant upregulation of apoptosis-related proteins, including IRE1α, Caspase-12, XBP1, GRP78, Caspase-3 and Bax, was observed in the testis tissue of the Cd-exposed group when compared to the control ($p < 0.01$). Conversely, Bcl-2 expression levels were notably decreased ($p < 0.01$) in this group. However, the addition of Que to the Cd treatment led to a substantial decrease in the expression of these proteins ($p < 0.01$) and a concurrent increase in Bcl-2 expression ($p < 0.01$).

4. Discussion

The presence of the toxic heavy metal Cd has become a global problem [21] that poses an increasingly serious health risk to animal organisms. Testicular health is crucial, as the testes serve as the primary site for sperm production and are integral

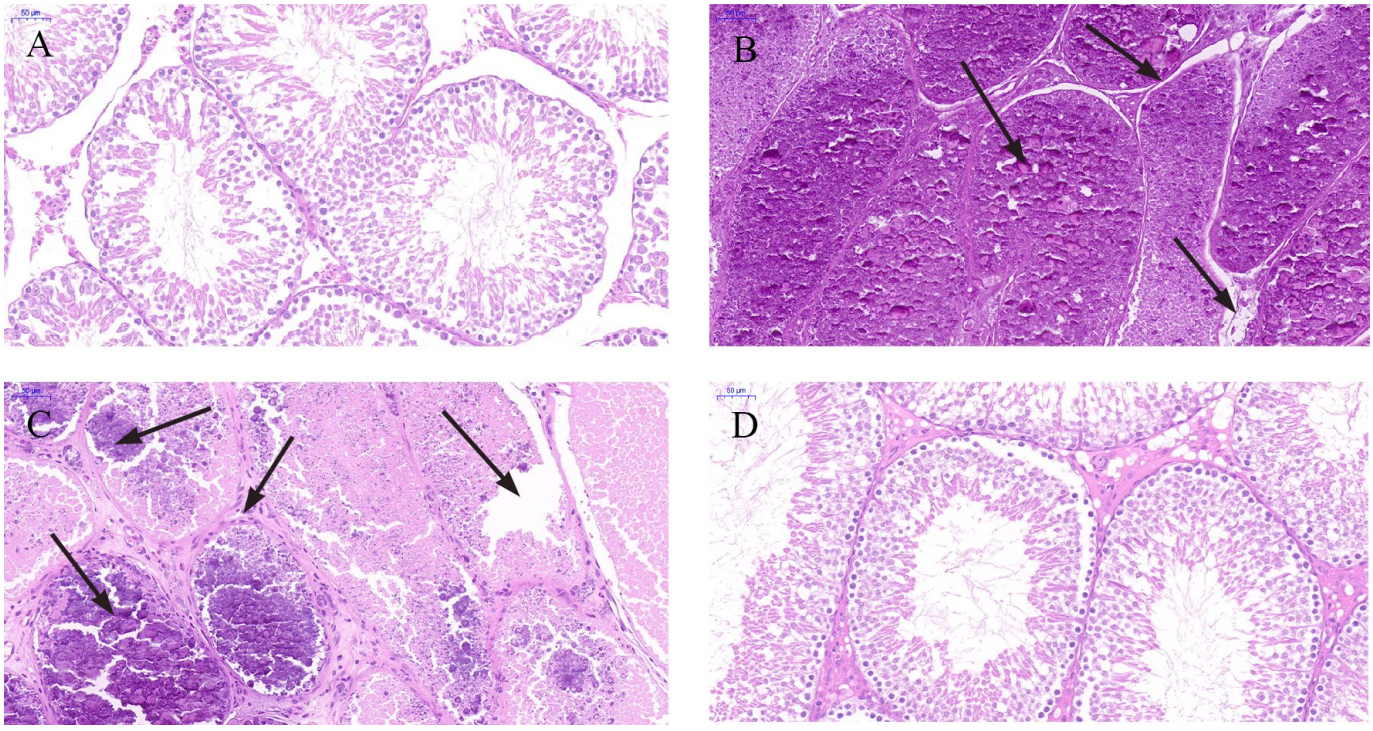


FIGURE 3. HE staining map of rat testes (HE, 40×, scale bar = 50 μm). (A) Control group; (B) Cd group ($CdCl_2$, 2 mg/kg b.w.); (C) Cd + Que group ($CdCl_2$, 2 mg/kg b.w. + Que, 100 mg/kg b.w.); (D) Que group (Que, 100 mg/kg b.w.). Black arrows indicate severe tissue damage.

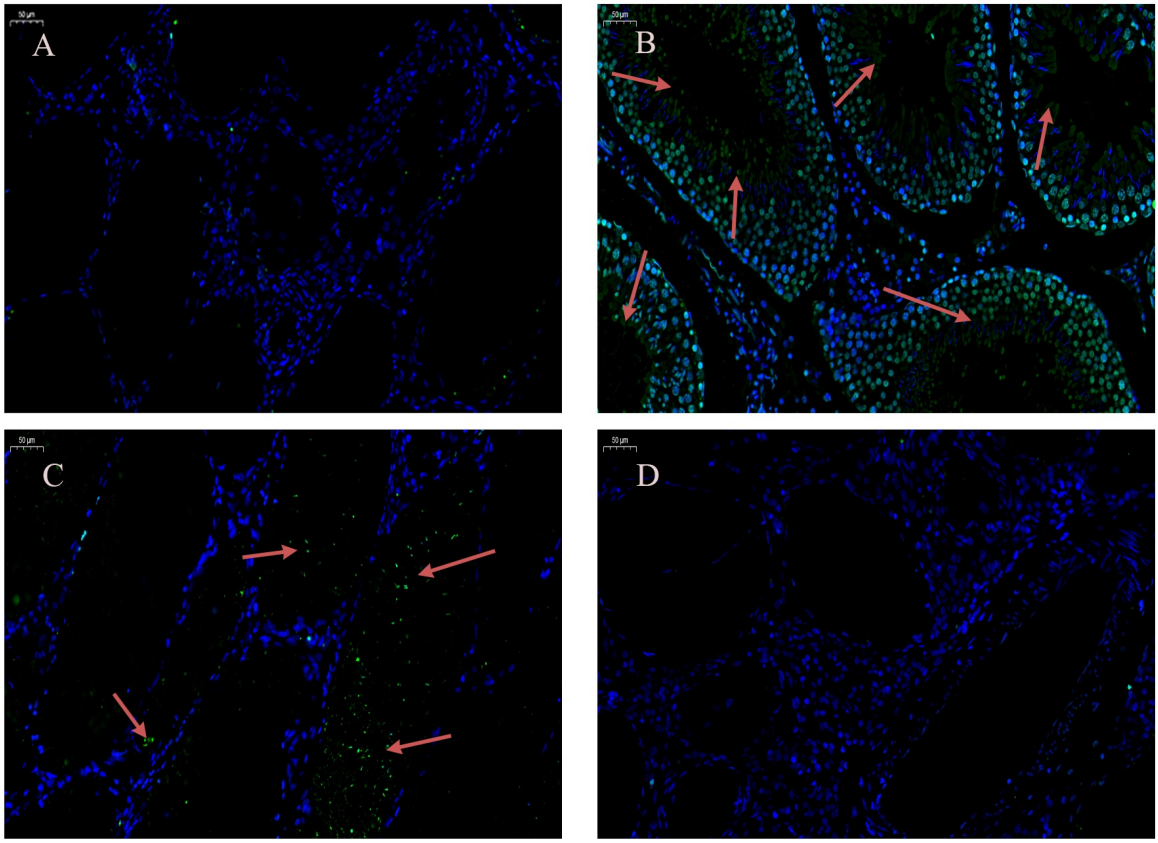


FIGURE 4. TUNEL assay of testis-tissue apoptosis. Rats were treated with $CdCl_2$ and/or Que for 4 weeks. Positive apoptotic cells are green (red arrows, 40×). (A) control group, (B) 2 mg/kg b.w. $CdCl_2$ treatment group, (C) 2 mg/kg b.w. $CdCl_2$ + 100 mg/kg b.w. Que co-treatment group, (D) 100 mg/kg b.w. Que co-treatment group.

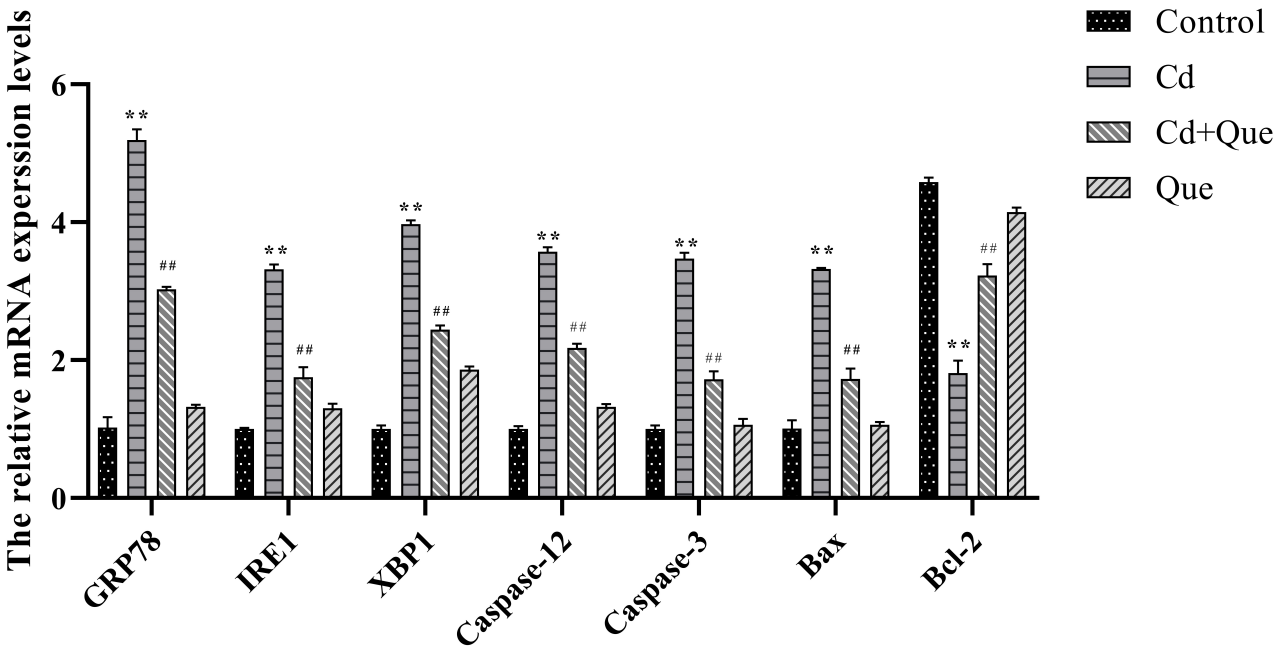
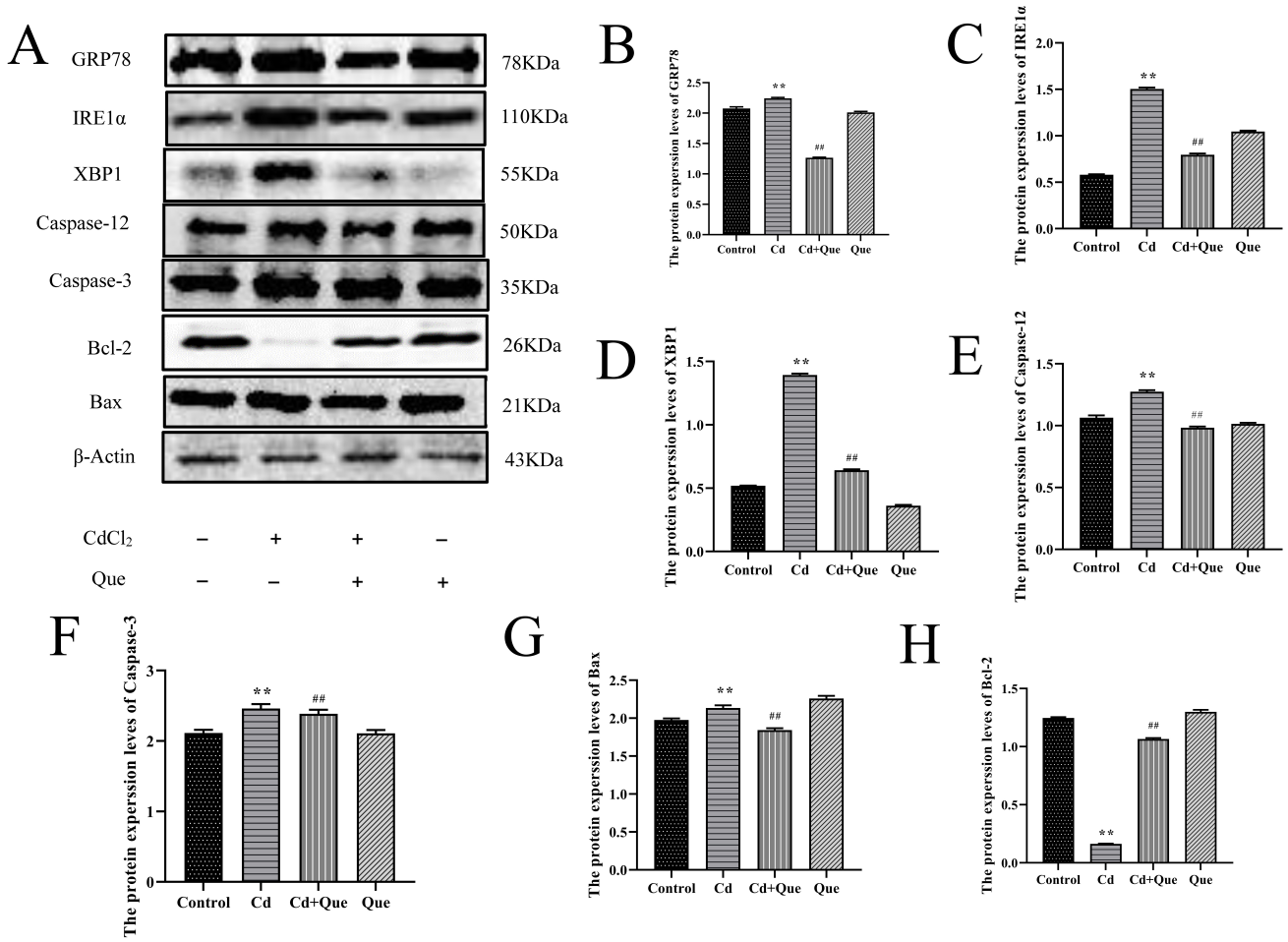


FIGURE 5. The mRNA expression of *GRP78*, *IRE1α*, *XBP1*, *Caspase-12*, *Caspase-3*, *Bax* and *Bcl-2* in the experimental group. Experimental groups included the control group, Cd group, Cd + Que group, Que group. All measurement results were expressed as mean ± SD (n = 6). **indicates significant difference compared with the corresponding control $p < 0.01$. ##indicates statistically significant difference between the Cd and Cd + Que groups $p < 0.01$. *GPR78*: glucose-regulated protein 78; *IRE1*: inositol-requiring kinase 1; *XBP1*: X box-binding protein 1; Cd: cadmium; Que: quercetin.



F I G U R E 6. The protein expression of GRP78, IRE1α, XBP1, Caspase-12, Caspase-3, Bax and Bcl-2 in the experimental group. Experimental groups included the control group, Cd group, Cd + Que group, Que group. The levels of GRP78, IRE1α, XBP1, Caspase-12, Caspase-3, Bax and Bcl-2 proteins (A) were measured by Western blot. The gray scale value analysis results of GRP78 (B), IRE1α (C), XBP1 (D), Caspase-12 (E), Caspase-3 (F), Bax (G) and Bcl-2 (H) in rat testis. β-Actin was used as a control. Data is presented as mean $\pm$ SD (n = 6). **indicates a significant difference compared to the control group at $p < 0.01$. ##indicates a significant difference of $p < 0.01$ compared to the 2 mg/kg Cd group. "–" means no specific treatment, "+" means special treatment. GRP78: glucose-regulated protein 78; IRE1α: inositol-requiring kinase 1; XBP1: X box-binding protein 1; Caspase-12: cysteine-containing aspartate hydrolase 12; Caspase-3: cysteine-containing aspartate hydrolase 3; Cd: cadmium; Que: quercetin; $CdCl_2$: cadmium chloride.

to animal reproduction. Experimental studies demonstrated that Cd is seriously toxic to the testicles, causing testicular damage that manifests as hemorrhagic inflammation, organ degeneration and dysfunction, and vacuolization of spermatogenic tubules [36]. The induction of apoptotic cell damage lacks clarity regarding the underlying mechanism. Que, the most extensively ingested dietary flavonoid, has been reported to possess formidable antioxidant properties, thereby instigating the inhibition of apoptosis through diverse anti-inflammatory and alternative pathways [37]. However, its mechanism of action is not clear. Therefore, in this experiment, we established a Cd toxicity model in rat testis, using Que to affect the toxicity of Cd, and detected the changes of apoptosis related changes to provide a theoretical basis for Cd-treated testicular toxicity and the protective effect of Que.

The body weight index of animals has an important role in toxicology experiments as a commonly observed index in toxicology experiments. In the study of Tariq Iqbal *et al.* [38], large dose of Cd (15 mg/kg) led to a decrease in body weight in rats, compared with the control group. The same as it is in our study, significant weight loss occurred in the Cd group compared with the control group, and the decreasing body weight became more obvious as the time of treated increased. In the Cd group, body weight significantly decreased during the second, third, and fourth weeks in comparison to the control group over the corresponding time period. Particularly, there was nearly no increase in body weight during the second week. However, the overall body weight of the rats remained elevated in comparison to their weight four weeks ago. In the study conducted by YJ Wang *et al.* [39], the variations in body weight resulting from the different Cd treatment concentrations were consistent with our findings. Compared with that in the Cd group, the body weight of the rats in the Cd + Que group increased significantly in the third week, indicating that the

toxic effect of Cd was suppressed due to the effect of Que, which has a protective effect and can significantly reduce the harm of Cd on the testicular tissue of rats. It is not uncommon to encounter instances where quercetin significantly mitigates impairment, thereby promoting weight gain [40, 41].

Histological analysis revealed significant pathological alterations in the testicular tissue cells of the Cd group when compared to the control group. These alterations included dense cytoplasmic staining and extensive liquefying necrosis of cells. Similarly, Burukoğlu and Bayçu [42] reported that Cd causes severe degeneration of rat testes, damage to spermatogenic tubules and necrosis, thus impairing reproductive capacity. In comparison with the Cd group, the Cd + Que group showed densely stained cells, and the level of damage was reduced compared with the Cd group. TUNEL staining demonstrated a significantly higher rate of cell apoptosis in Cd groups compared with the control group, and the number of apoptotic cells was significantly lower in the Cd + Que group than in the Cd group, Alizadeh and Flávia L Beltrame came to the same conclusion [43, 44].

Oxidative stress refers to the disturbance of homeostasis in the body as a result increased concentrations of ROS [21]. The introduction of Cd into the body disturbs the equilibrium between oxidation and antioxidant homeostasis, leading to oxidative stress [45]. GSH is a vital peptide compound involved in cellular defense against oxidative stress and serves as an indicator of oxidative stress. MDA, the end product of lipid peroxidation, induces harmful structural changes in macromolecules such as nucleic acids and proteins, and exhibits cytotoxic effects. Detecting the MDA content accurately reflects the degree of lipid peroxidation.

Cd induces oxidative stress in the tissues of different species, which is one of the main manifestations of its toxicity [23]. Our findings showed, that the toxic effects of Cd cause oxidative stress and lipid peroxidation in rat testis tissues, and Que significantly reduces the oxidative stress and lipid peroxidation induced by the toxic effects of Cd. While Santa Cirmi *et al.* [46, 47] found lower GSH content, the main reason for the significant increase in the GSH content in this experiment could be the initial stage of oxidative stress. With GSH as an anti-oxidative stress substance regulated by body homeostasis, its content increased to resist oxidative damage caused by Cd exposure [48]. While Francesca Capriglione and Jessica Maiuolo [49] reported that exposure to Cd resulted in elevated ROS, lipid peroxidation and ERS levels, indicating that Cd's harmful impact on thyroid cells may stem from the generation of free radicals. These radicals then induce cellular damage by triggering lipid peroxidation in biofilms. In studies of Cd on chicken testes and Cd on rat ovaries, it was similarly noted that Cd was found to cause oxidative stress [25, 50]. Cd-treated oxidative stress in the brain may be a risk factor for cognitive impairment [51]. What's more, in soybean studies, changes in Cd uptake were positively correlated with oxidative stress products [52].

IRE1, a transmembrane protein found on ER, comprises a serine and threonine kinase domain, along with a RNA endonuclease domain [53]. In its inactive state, IRE1 binds to the chaperone protein GRP78. However, during ERS, IRE1 dissociates from GRP78. Subsequently, the activation of IRE1 into phosphorylated IRE1α takes place, triggered by unfolded or misfolded proteins in the ER [54]. Upon activation, IRE1α induces splicing of *XBP1* mRNA, cleaving it into split *XBP1-S*. Once it has bound to ERS elements situated outside the nucleus, *XBP1-S* can traverse the nuclear membrane and gain entry into the nucleus where it initiates the activation of target genes [55]. The initiated target genes can coordinate the function of the ER to remove unfolded or misfolded proteins. However, when ERS is not resolved, cell degeneration and death will inevitably result [56]. When ERS cannot be resolved, IRE1 and XBP1 will activate Caspase-12 and Caspaes-3, which in turn will activate *Bax* to induce apoptosis, while *Bcl-2*, an apoptosis-suppressing gene, will shows an opposite trend to *Bax*. First, we found that the expression of *GRP78* mRNA and protein was significantly increased in the Cd group of rats, suggesting that Cd activates the ERS, and leads to UPR initiation. However, the mRNA and protein expression levels of *GRP78* were significantly reduced in the Cd + Que treated group, suggesting that Que can reduces the expression of ERS initiating proteins, thereby preventing the occurrence of ERS. In addition, the up-regulation of mRNA and protein levels of *IRE1*α, *Caspase-12*, *XBP1* and *Caspase-3* in the Cd group compared with the control group suggests that Cd treatment activates the *IRE1-XBP1* signaling pathway, which agrees with previous results [57]. Our study also found that mRNA and protein levels of *IRE1*α, *Caspase-12*, *XBP1* and *Caspase-3* were significantly decreased in the Cd + Que group. The ratio of *Bax* to *Bcl-2* ultimately determines whether apoptosis occurs or not [58]. We finally have conducted a comprehensive analysis of *Bax* and *Bcl-2* mRNA and protein expression in the testes of rats. The results obtained demonstrated a decrease in the mRNA and protein expression of the apoptosis inhibitor gene *Bcl-2* in the Cd group, along with an elevation in the pro-apoptosis gene *Bax*. Conversely, the Cd + Que group exhibited the opposite trend. The results indicate that Cd exposure leads to apoptosis in rat testicular tissue through the *IRE1-XBP1* cell apoptosis pathway, while Que can alleviate ERS and reducing cell apoptosis.

This study has limitations of research methods and sample numbers. In further research, we will add the methods of genetic knockout or pharmacological inhibition, and it is necessary to increase the sample size and explore other signaling pathways through which quercetin antagonizes cadmium induced testicular apoptosis.

5. Conclusions

In conclusion, this study showed that Cd exposure caused reduced body weight, severe oxidative damage to testicular tissue and increase of apoptosis mediated by the *IRE1-XBP1* pathway in rats, while Que could reduce the damage and apoptosis (Fig. 7). Regrettably, we did not conduct sperm profile assessment as an important indicator of testicular toxicity. Nevertheless, this study offers a theoretical foundation for mitigating Cd-induced oxidative damage and apoptosis in the testis.

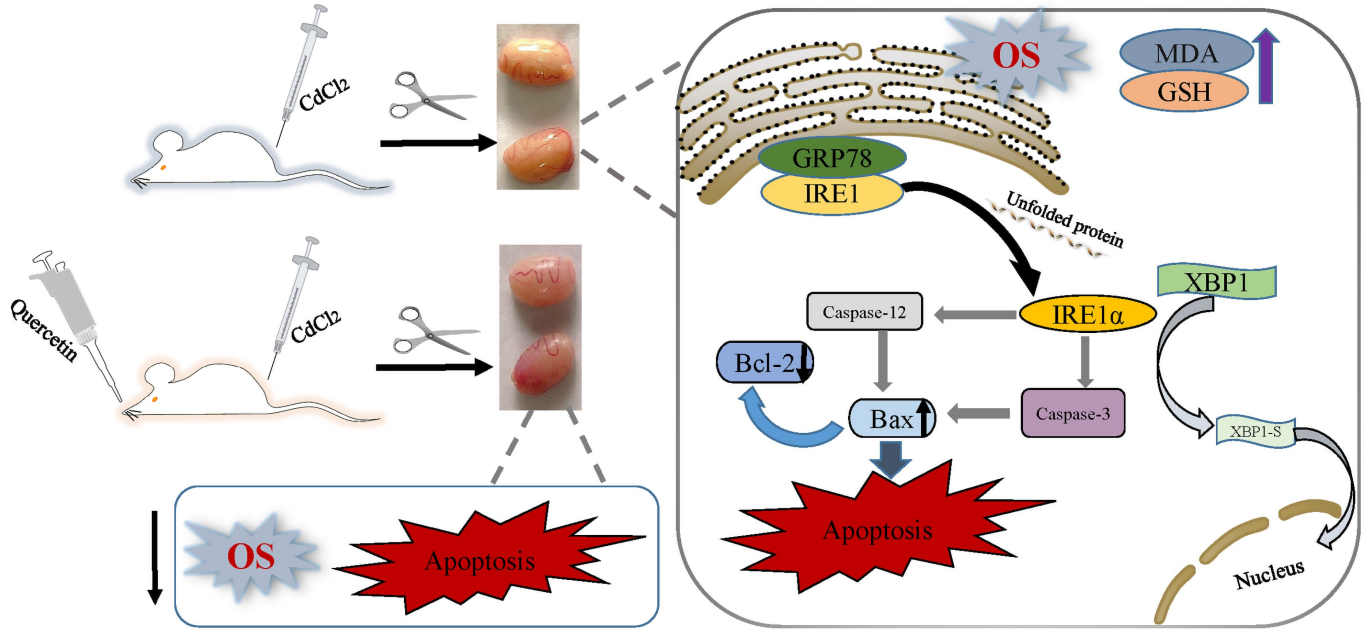


FIGURE 7. Effects of Que and Cd on the IRE1-XBP1 apoptotic pathway in rat testis. Cadmium intoxication induces oxidative stress in rat testis and activates the IRE1-XBP1 signaling pathway, which in turn leads to testicular cell apoptosis. Cadmium-induced oxidative stress and apoptosis were alleviated by the addition of quercetin. OS: Oxidative Stress; MDA: malondialdehyde; GSH: reducing glutathione; GRP78: ER chaperone-binding proteins; IRE1α: inositol-requiring kinase 1; XBP1: X box-binding protein 1; Caspase-12: cysteine-containing aspartate hydrolase 12; Caspase-3: cysteine-containing aspartate hydrolase 3; $CdCl_2$: cadmium chloride.

AVAILABILITY OF DATA AND MATERIALS

The data presented in this study are available on reasonable request from the corresponding author.

AUTHOR CONTRIBUTIONS

JBM—Conceptualization, formal analysis, data curation, writing–original draft. BX—Data curation, formal analysis. HLZ—Visualization, formal analysis. YNS—Methodology, formal analysis. WLZ—Methodology, resources. ZPL—Resources, validation, formal analysis. JCW—Conceptualization, project administration, software, writing–original draft, review, editing, funding acquisition. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The experimental protocol of this study complied with the ethical standards of the National Institutes of Health (NIH Publication No. 85–23, revised 1996) and was approved by the Animal Care and Ethics Committee of Henan University of Science and Technology (approval number: HAUST 2021018).

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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