



Protective effect of quercetin on rat testes against cadmium toxicity by alleviating oxidative stress and autophagy

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Abstract

Cadmium (Cd), a highly toxic heavy metal, adversely affects human and animal health. Quercetin (Que) is a kind of flavonoid that can protect many tissues from the toxic effect of heavy metals. Although many studies have explored the adverse effects of cadmium on rats and other animals, the mechanism of Cd-induced testicular autophagy and the antagonistic effect of Que on cadmium remain unclear. In this study, Sprague–Dawley rats were treated with Cd, Que or Cd, and Que supplements to explore the mechanisms of Que-alleviated testis injury caused by Cd exposure. The rat body weight and relative testicular weight were measured. Morphological changes in testes and indices of oxidative stress were also examined. The expression levels of autophagy-related genes were detected as well. Results showed that Cd decreased the rat body weight and relative testicular weight and induced pathological changes in testes. Conversely, Que alleviated these changes. We also found that Cd increased the malondialdehyde content and decreased the contents of total superoxide dismutase, glutathione peroxidase, catalase, and glutathione. Moreover, the protein expression levels of P62 and LC3-II increased under Cd exposure conditions. Conversely, Que obviously alleviated these toxic activities induced by Cd. Overall, this study showed that Cd accumulated in rat testes, leading to oxidative stress and autophagy. Que can reduce cadmium toxicity by reducing oxidative stress and inhibiting autophagy. The specific mechanism of Que antagonizing Cd toxicity can provide new insights into countering cadmium toxicity.

Keywords Cadmium · Quercetin · Rat · Testes · Oxidative stress · Autophagy · P62 · LC3B

Introduction

Cadmium (Cd) is a highly toxic heavy metal that is extensively used in agriculture and industry. Cd discharged into the environment can cause air, water, and soil pollution. Cd pollution has become a common environmental problem in the

society because of the rapid development of industry and agriculture (Wang et al. 2019b; Wang et al. 2017a). Cd can accumulate in fish, birds, and mammals through the food chain, leading to acute and chronic poisoning (Bao et al. 2017; Liu et al. 2017a). Moreover, it can accumulate in animals for up to 30 years and can thus damage human or animal health for a long time. The liver, kidneys, pancreas, and other tissues and organs can be damaged by Cd (Wang et al. 2017b).

Low-dose Cd exposure can increase the mortality rate of prostate cancer and affect female reproduction and embryonic development (Min et al. 2014). Studies have shown that Cd has potential toxicity to reproductive organs, which can affect the reproductive hormone level, increase sperm-cell apoptosis, and even lead to male infertility (Akinloye et al. 2006; Li et al. 2016). Some researchers have proposed that oxidative stress is the main cause of Cd toxicity (Chen et al. 2015; Niknafs et al. 2015). Oxidative stress caused by reactive oxygen species (ROS) (Liu et al. 2017b; Zheng et al. 2019) is one of the main mechanisms of Cd-induced cytotoxicity. Organisms have acquired complex mechanisms to resist the

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ROS toxicity through adaptive evolution (Satoh et al. 2013). In these mechanisms, a series of antioxidant and detoxifying enzymes is activated to maintain the redox balance and weaken the oxidative damage in cells. These enzymes include heme oxygenase-1, glutathione reductase, glutathione peroxidase (GPx), and catalase (CAT). Cd can change the activities of these enzymes (Wang et al. 2019a).

The toxic effect of Cd is related to autophagy. Autophagy is the process of transferring part of the cytoplasm to lysosomes for degradation, removing damaged organelles, and misfolded proteins to maintain balance in the intracellular environment (Kesidou et al. 2013). Over-autophagy can damage cells, and autophagy death may lead to physiological damage (Yichao et al. 2015; Yu et al. 2013). Autophagy reportedly exerts a protective effect on rat osteoblasts. Microtubule-associated protein 1 light chain 3B (LC3B) is an autophagic protein marker (Ni et al. 2011). Under Cd exposure, the ratio of LC3-II to LC3-I increases (Liu et al. 2016). Cd can induce the autophagy of hematopoietic stem cells and progenitor cells (Gioacchino et al. 2008). Autophagy is increased in sea urchin embryos by Cd (Chiarelli et al. 2016). Low-dose Cd can also induce autophagy in renal cells and activate autophagy cell death in the liver (Chargui et al. 2011; Shi et al. 2015). Cd upregulates the expression of ATG3, ATG5, p62, Beclin1, and other autophagy-related genes. The expression levels of Beclin1 and LC3B in cells reflect its autophagy status (Liu et al. 2017a).

Quercetin (Que; 3, 3', 4', 5, 7-pentahydroxyflavone) is one of the most omnipresent flavonoids (Mao et al. 2018). Que is highly concentrated in apples, onion, potatoes, peanuts, soybeans, red wine, and other fruits and vegetables. Owing to its chemical structure, Que has strong anti-oxidative and cytoprotective effects on oxidant-induced endothelial cell apoptosis (Yean Jung et al. 2003). Additionally, Que prevents oxidative damage and cell death by preventing lipid peroxidation and scavenging oxygen free radicals. Que may cross the blood–brain barrier and exerts neuroprotective effect in many brain injury models (Cho et al. 2006; Elisabeth et al. 2005). Que has also been shown to protect germ cells from Cd-induced toxicity (Cüneyt et al. 2013).

Several studies have been conducted on the toxicity of Cd to the liver, kidneys, and spleen (Wang et al. 2019c; Xu et al. 2015). The testes are one of the main target organs of Cd. However, the mechanism of testicular damage caused by Cd is unclear. Moreover, the protective effect of Que on Cd-induced testicular injury remains to be further studied. In the present study, we suspected that Cd may damage the male reproductive system through oxidative stress and autophagy. We also examined the effects of Que on Cd-induced toxicity in rats. The purpose of this experiment is to provide a new method to detoxify Cd.

Materials and methods

Chemicals and antibodies

Cadmium chloride (CdCl₂) and Que were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Glutathione (GSH) assay kit, malondialdehyde (MDA) assay kit, total superoxide dismutase (SOD) assay kit, CAT assay kit, and GPx assay kit were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Enhanced chemiluminescence (ECL) kit was obtained from Thermo Fisher Scientific Pierce (Rockford, IL, USA). Bicinchoninic acid protein assay kit and Enhanced HRP-DAB Chromogenic Substrate kit were purchased from Sevicebio (Wuhan, China).

The primary antibodies P62, LC3B, and β -actin were purchased from Proteintech Group (Rosemont, USA). All of the secondary antibodies were bought from the Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China).

Animals and treatments

A total of 24 male SD rats weighing 180–200 g were purchased from the Henan Laboratory Animal Center (Zhengzhou, China). They were kept on 12 h light:12 h dark condition with controlled temperature (24 ± 2 °C) and fed with a commercial standard diet under standard laboratory conditions. They drink water freely. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The protocol of this study was approved by the Institutional Animal Care and the Ethics Committee of Henan University of Science and Technology (approval number HAUST 18010).

The rats were randomly divided into four groups after 2 weeks of adaptation in laboratory: a normal control group (received distilled water and 0.9% NaCl by oral gavage); a Que-treated group (the normal group with Que content of 50 mg/kg bw daily by oral gavage), a Cd-treated group (treated with 2 mg/kg bw CdCl₂ intraperitoneally), and a Cd+Que-treated group (received 2 mg/kg bw of CdCl₂ intraperitoneally and 50 mg/kg bw of Que intragastrically). The doses of CdCl₂ and Que used were selected from previous studies (Abdeen et al. 2019; Ahmed et al. 2019; Badr et al. 2019). Each treatment group consisted of six rats, which were placed in rat cages and received identical standard feeding and management.

Animal-based testing

The experiment lasted for 4 weeks. Every weekend, the rats' body weight was recorded. After the treatments, the rats were anesthetized with ether anesthesia. Then, all rats were sacrificed, and the testes were removed and washed using cold

phosphate-buffered saline (PBS) solution. The testes were immediately weighed and calculated the organ index according to the following formula: visceral index = visceral weight/body weight \times 100. The tissues were minced, homogenized (10%, w/v) in PBS solution (pH 7.4), and centrifuged (3000 rpm for 10 min). The clear supernatant was stored at $-80\text{ }^{\circ}\text{C}$ to measure the index of oxidative stress. Moreover, some testes were preserved in 10% neutral-buffered formalin for microscopic analysis.

Detection of testes-accumulated Cd

Testicular tissue of 1 g was weighed, mixed it with 8 mL concentrated nitric acid and 2 mL perchloric acid, and reacted in a fume hood overnight. The second day, the mixture was heated until they become transparent and colorless on a hot plate. Then, 5 mL of the total volume of the solution with distilled water was added and mixed well. Finally, the Cd concentration in the sample was determined by atomic absorption spectrophotometry (AA-6880; Kyoto Shimadzu Company, Japan). The accumulated Cd content in the testis was shown by Cd content (μg)/tissue weight (g).

Histological evaluation

The testicular paraffin section was stained with hematoxylin and eosin (H&E) dye. Histopathological changes of the testes were observed using a light microscope (Olympus, Japan).

Western blot method to detect P62 and LC3B expression

Proteins were extracted from testicular tissue (150 mg) using RIPA Lysis Buffer and quantified using the BCA protein assay kit (Sevicebio, China). SDS-PAGE gels (15%) and concentrated gel (5%) were prepared. Protein samples (30 μg) were pulsed into each sample hole, and the samples were electrophoretically analyzed at 120 V and transferred to the polyvinylidene fluoride (PVDF) membranes for 1 h (Bio-Rad, Hercules, CA, USA). Skim milk was dissolved in Tris buffer saline containing Tween-20 (TBST) to block the PVDF membranes for 1 h. Then membranes were then incubated with anti-P62 (1:1000; rabbit polyclonal, Proteintech), anti-LC3B (1:1000; rabbit polyclonal, Proteintech), and anti- β -actin antibodies (1:1000; rabbit polyclonal, Proteintech) at $4\text{ }^{\circ}\text{C}$ overnight, washed with TBST three times (10 min each time) at room temperature, incubated with appropriate antibodies (1:3000; Proteintech) at room temperature for 30 min, and then washed three times with TBST (10 min each time) (Abdeen et al. 2014). The ECL liquid

was added for exposure in the darkroom, and the images were fixed. Quantity One software (Bio-Rad) was used to analyze the grayscale values. The mean values of the repeated measurements were divided by the reference β -actin value, which represented the quantitative gray value of the target proteins.

Data analyses

All data analyses were performed in SPSS for Windows. One-way analysis of variance (ANOVA) and the least significant difference (LSD) post hoc test were used to analyze the data. Data are expressed as the mean \pm standard error (SE). $P < 0.05$ was considered statistically significant.

Results

Effect of Que on Cd-induced body weight

As shown in Fig. 1, the body weight significantly decreased ($P < 0.05$) by 15.7%, 18.1%, and 20.1% compared with that of the control group after Cd exposure for 2, 3, and 4 weeks, respectively, in rats treated with Cd alone. However, treatment with Que (50 mg/kg) alongside Cd significantly decreased the reduction in body weight ($P < 0.05$) by 10.7% and 10.4% after exposure for 3 and 4 weeks, respectively, compared with the control group.

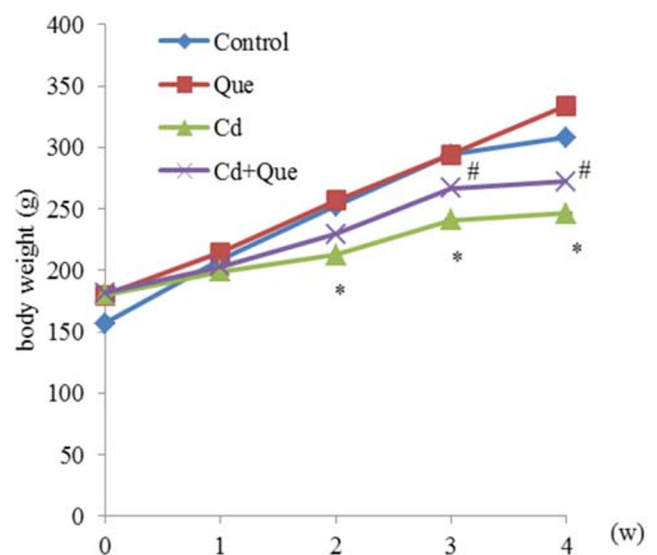


Fig. 1 Body weight (g). Rats were treated with CdCl₂ and/or Que for 4 weeks. Body weight of rats from each group at the end of the fourth week. Data are represented as mean \pm SE ($n = 6$). “*” indicates significant difference compared with the corresponding control ($*P < 0.05$). “#” indicates statistically significant difference between Cd and the Cd+Que group ($^{\#}P < 0.05$)

Effect of Que on Cd-induced relative weight of the testes in rats

As shown in Fig. 2, in rats treated with Cd alone, the relative weight of the testes significantly decreased ($P < 0.05$) by 36.9% compared with that of the control group. Moreover, the testicles were obviously shrunk and showed consolidation and yellow sections. Treatment with Que (50 mg/kg/day) alongside Cd improved the relative weight of the testes ($P < 0.05$) by 18.5% compared with the Cd-treated group.

Testicular Cd content

Cd was almost undetected in the control and Que-treated groups. The Cd content in the testicular tissue of rats exposed to Cd was significantly higher ($P < 0.01$) and was 49-fold the control group. However, the Cd content in the Cd+Que group significantly decreased ($P < 0.05$, Fig. 3) and was 62.6% of the Cd-treated group.

Effect of Que on testicular oxidative stress

Changes in oxidative status were assessed by enzymatic and nonenzymatic measures. As shown in Fig. 4, Cd administration significantly decreased the GSH level by 45.2% and increased the MDA content by 76.2% in the testicular tissue compared with the control group ($P < 0.05$). Treatment with Que induced a 46.1% increase in GSH level and 40.1% decrease in MDA content compared with the Cd-treated group. Cd administration significantly decreased ($P < 0.05$) the

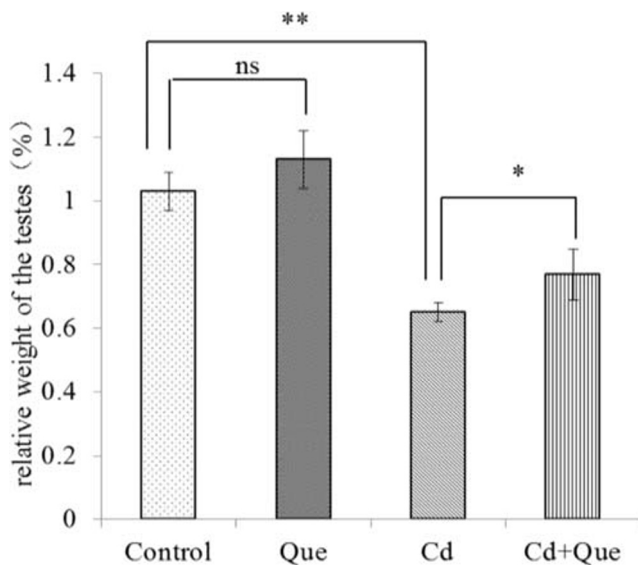


Fig. 2 Relative weight of the testes (%). Rats were treated with CdCl₂ and/or Que for 4 weeks. Relative weight of the testes obtained after the rats were sacrificed. Data are represented as mean ± SE ($n = 6$). ns, not significant; * $P < 0.05$, ** $P < 0.01$

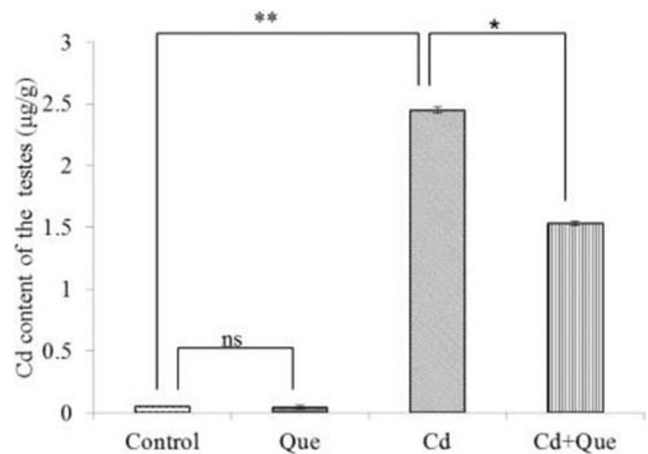


Fig. 3 Cd content of the testes (%). Rats were treated with CdCl₂ and/or Que for 4 weeks. Cd content of the testes from each group after being sacrificed. Data are represented as mean ± SE ($n = 6$). ns, not significant; * $P < 0.05$, ** $P < 0.01$

activities of SOD, CAT, and GPx by 23.5%, 48.3%, and 57.5%, respectively, compared with the control group. The Cd+Que group significantly increased CAT, SOD, and GPx activities by 25.5%, 58.0%, and 68.3%, respectively, compared with the Cd group ($P < 0.05$).

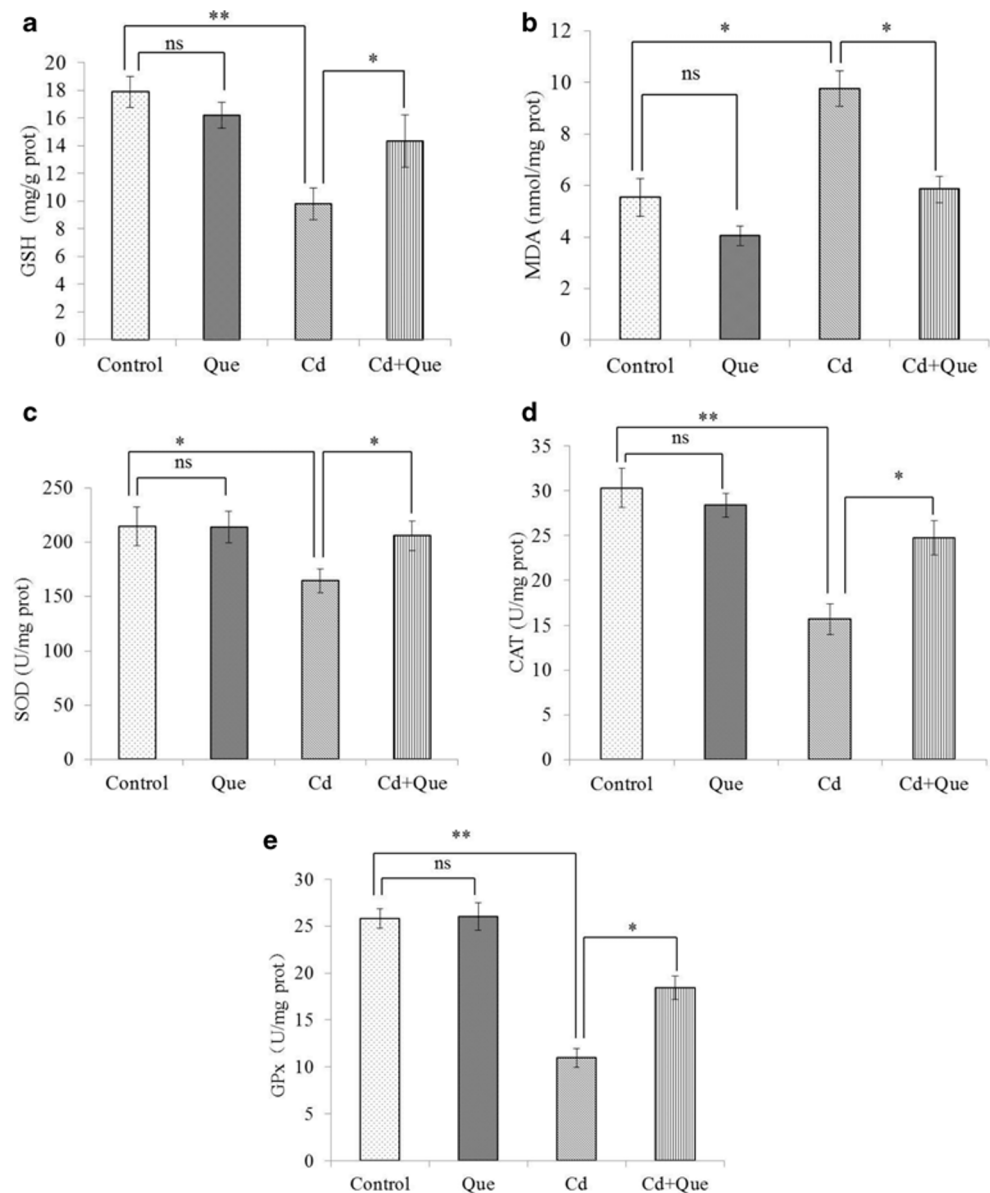
Histopathological observations based on H&E staining

Figure 5a and b show the results of H&E staining. In the control and Que-treated groups, the seminiferous tubules had normal shape, the inner cells were regular and closely connected with the inner membrane, and the seminiferous cells were clearly present in all stages and arranged in order. Numerous active spermatozoa were observed in the seminiferous tubules. Examination of the tissue sections confirmed the extreme morphological changes in the testes of Cd-treated rats (Fig. 5c). The spermatogenic cells in the testicular seminiferous tubules showed shrinkage, disordered in arrangement, decreased in the number of layers, and unclear in their levels. Relatively inflated and loose cells and few sperm are also noted in the tube cavity. In the CdCl₂+Que group (Fig. 5d), the atrophy and degeneration of rat testicular tissue structure were significantly improved, and the germ cell levels in the tubule of rats were basically in order with a large number of sperm.

Western blot analysis of P62 and LC3B expression

The expression of the autophagy-related proteins P62 and LC3B were next detected. The expression of P62 and LC3B-II increased significantly ($P < 0.01$) by 3.5- and 4.8-fold, respectively, compared with the control (Fig. 6). However, treatment with Que was efficient in restoring P62 and LC3B-II expression toward the normal level. P62 and LC3B-II decreased

Fig. 4 Oxidative stress assays in the testicular tissue. Rats were treated with CdCl₂ and/or Que for 4 weeks. Then, testicular tissues were applied to determine the levels of GSH (a), MDA (b), SOD (c), CAT (d), and GPx (e). Data are represented as mean ± SE (*n* = 6). ns, not significant; **P* < 0.05, ***P* < 0.01



by 96.2% and 63.7%, respectively, compared with the Cd-treated group.

Discussion

The toxic damage of Cd to the liver, kidney, and other organs has been studied, but the testes are also an important target organ affected by Cd. The harm of Cd exposure to the testes is of great clinical importance due to the important role of Cd in male reproduction. Researchers have found that exposure to different concentrations of Cd can lead to testicular damage in rats.

Our current results show that compared with the control group, the Cd-exposed rats are in poor general condition,

including timid, susceptible to shock, irritability, and eating and drinking less. However, the Cd+Que group has good spirit and eat and drink well. Data showed that testicular Cd concentrations in the Cd-exposed groups were significantly higher than that in the control group, and that the Cd concentrations in the Cd+Que group were much lower than that in the Cd-treated group. These results indicated that Que can antagonize the Cd toxicity by reducing the Cd accumulation in the rat testes.

Our histological results demonstrate that testicular morphology was abnormal in Cd-exposed rats compared with the control group. Histological examination of the testis tissue reveals that Cd intoxication showed the contracted spermatogenic cells in the testicular seminiferous tubules, arranged disorderly, the cell layer decreased, and the level was unclear.

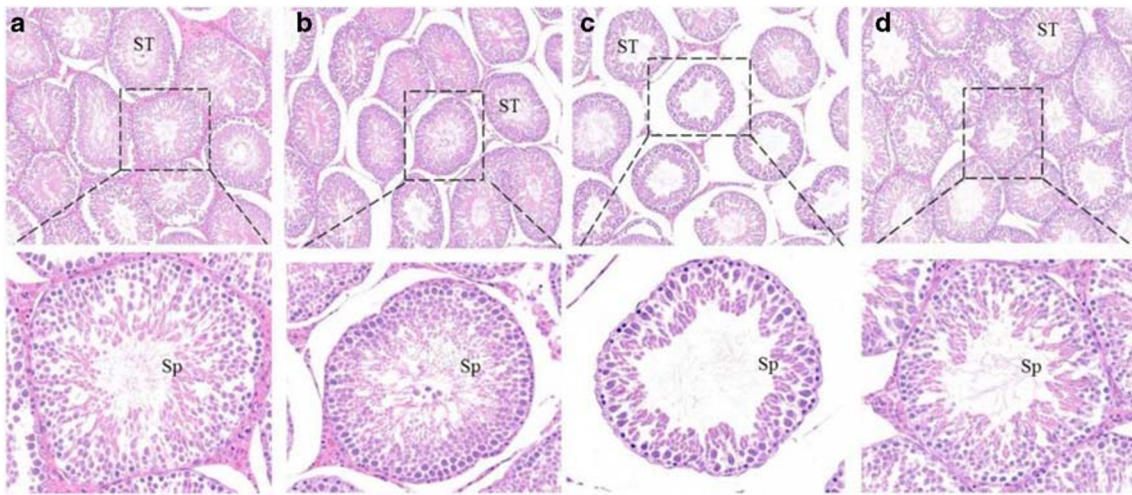


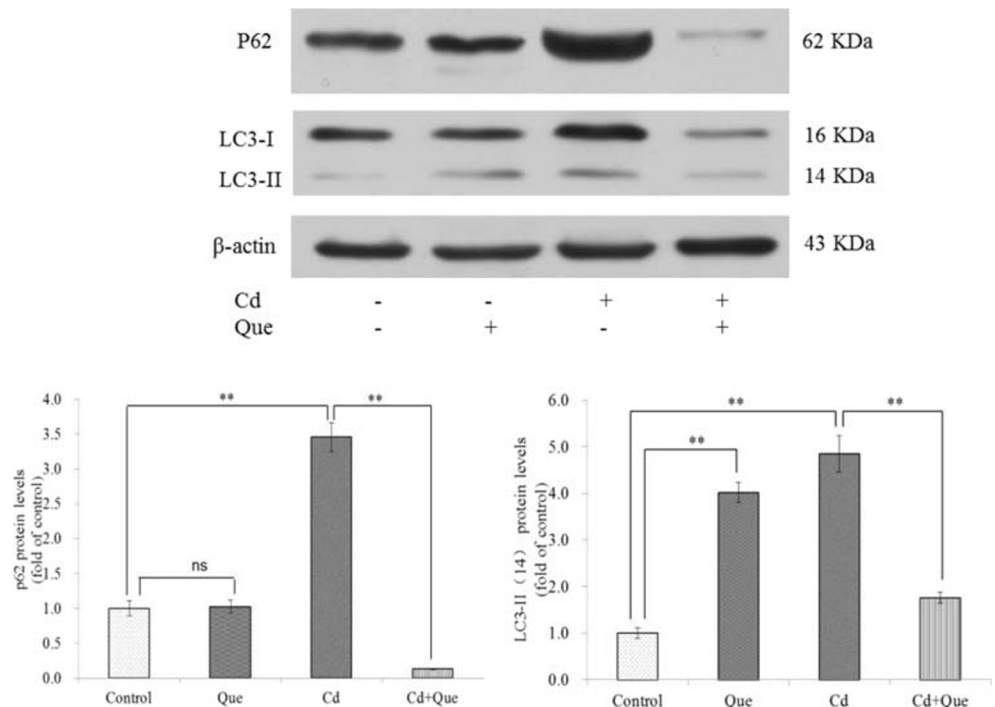
Fig. 5 Pathological changes of testicular tissues. Rats were treated with CdCl₂ and/or Que for 4 weeks, and the testis sections were stained with hematoxylin and eosin dye to assess the histomorphological changes. **a** Control showing normal morphology of seminiferous tubules, with regular inner cells, closely linked with the endomembrane and with all stages of spermatogenic cells clearly visible and regular. **b** Que alone-treated rats showing normal appearance of testicular structure. **c** Cd-treated rats

showing the spermatogenic cells in the testicular seminiferous tubule showed shrinkage, disordered arrangement, reduced layers, and their levels were unclear. Relatively inflated and loose cells and few sperm in the tube cavity were also noted. **d** The Cd+Que-treated group showing germ cell levels of in the tubule of the rats were basically in order. ST, seminiferous tubules; Sp, spermatozoa

The cells were relatively expanded and loose, and the sperm in the lumen was less, which undoubtedly affects the reproductive health. These results were consistent with the previous studies (Maria et al. 2014; Sakr and Nooh 2013). Que can restore the histological damage caused by Cd, indicating that Que can prevent Cd-induced testicular damage. Therefore, Que may inhibit Cd-induced testicular toxicity.

Previous studies have indicated that Cd exposure can seriously damage the liver, kidney, and brain, and that Que can antagonize Cd-induced toxicity (Halder et al. 2019; Nna et al. 2017). Vicente-Sánchez noted that compared with the control group, Cd poisoning markedly reduces GR and increases SOD activities, and MDA content in liver of rats significantly increased after 9 weeks of exposure. However, in the Cd+Que

Fig. 6 Inhibitory effect of Que on Cd-elevated expression of P62 and LC3-II in rat testes. SD rats were treated with Cd and/or Que for 4 weeks. Testis tissues were collected to analyze the protein levels of P62 and LC3B by using Western blot. Upper panel represents Western blot image; lower panel represents quantitative analysis. Data are represented as mean ± SE (*n* = 6). ns, not significant; **P* < 0.05, ***P* < 0.01



group, these Cd-induced changes were relieved (Vicente-Sánchez et al. 2008). In piglet Sertoli cells, the activities of SOD, T-AOC, and GPx significantly decreased, and the MDA content significantly increased when the Cd content in the cells was 40 μM CdCl₂ in the DMEM/F12 medium (Zhang et al. 2010). Bu reported that Cd treatment significantly decreased the GSH level, SOD, and GPx activities. Moreover, exposure to Cd resulted in the production of hydrogen peroxide and lipid peroxidation in mouse testes. However, Que exhibits an obvious antagonistic effect on the decrease in GSH, SOD, and GPx activities caused by Cd treatment (Bu et al. 2011).

In the present investigation, rats were intraperitoneally injected with Cd-evoked testis dysfunctions exhibited by a decrease in the relative weight of the testes along with histopathological alterations. Cd can induce oxidative damage in different tissues by enhancing membrane lipid peroxidation and modifying the antioxidant system of cells. Cd preferentially binds to sulfhydryl group (–SH)-containing cellular molecules such as GSH and SOD (Abdeen et al. 2019). The depletion of –SH is an important indirect mechanism of oxidative stress induced by Cd, especially those that participate in oxidative phosphorylation and detoxification. These events eventually induce the accumulation of ROS (such as superoxide radicals, hydroxyl radicals, and hydrogen peroxide) and further promote the formation of lipid peroxides. Cd also causes the dysfunction of mitochondrial respiratory regulation and oxidative phosphorylation coupling. This kind of respiratory dysfunction induces the consumption of a significant amount of oxygen and the overproduction of free radicals. Cd also competes with metals in antioxidant enzymes (SOD, CAT, and GPx) and inhibits the activities of these enzymes. For example, Cd can replace selenium in GPx or zinc in SOD, thereby reducing or causing the loss of the antioxidant activity of these enzymes (Wang et al. 2019a). The present results showed that in the Cd-treated group, the SOD, CAT, GPx activities, and GSH content decreased, whereas the MDA level increased. Thus, Cd reduced the activity of the testis antioxidant system, leading to free radical accumulation and thus increasing the oxidative damage to testis tissue. After Que (50 mg/kg) treatment, the MDA content significantly decreased and the antioxidant enzymes (SOD, CAT, and GPx) significantly increased. This finding indicated the remarkable protective effects of Que on Cd toxicity in rats.

Autophagy is related to many physiological and pathological processes, such as cell survival, cell death, cell metabolism, development, immunity, and aging (Chuang et al. 2014; Circu and Tak Yee 2012). Autophagy is an adaptive mechanism of the tissue and cells that respond to changing environmental stimuli, such as hunger and oxidative stress. Therefore, signal transduction can promote cell survival (Prozialeck and Edwards 2012). Autophagy plays an internal role in the cycling of cytoplasmic components and proteins under intracellular homeostasis (Chaabane et al. 2013). Under stress

conditions, cells remove harmful particles and protein aggregates by autophagy to prevent cell death. However, this ability is limited, and when a large number of cells are destroyed and cleared, the autophagic cells will die (Yuan et al. 2016). Studies have shown that autophagy is one of the potential mechanisms of Cd-induced hepatic and renal damage (Zou et al. 2015). However, the role of autophagy in Cd-induced testicular damage has not been fully elucidated. Autophagy can reduce the production of reactive oxygen and apoptosis, thereby reducing the damage caused by Cd (Dutta et al. 2013; Ko et al. 2016). However, autophagy exerts only a limited protective effect on the body because when the total dose and time of Cd exceed the safety threshold, it causes irreversible cell damage by inducing autophagy death.

Cd inhibits intercellular communication through gap junctions, thereby damaging cells. Autophagy can exacerbate this effect and lead to serious damage (Zou et al. 2015). Cd can also increase inflammatory responses of the testes (Takano and Inoue 2013). When rats are exposed to Cd, the testes cannot synthesize the functional metallothionein, which can reduce the inflammatory response. Thus, increased levels of inflammatory cytokines caused by Cd uptake lead to increased number of autophages (Lee et al. 2015). Some scholars also believe that Cd exposure is insufficient to cause cell damage and propose that autophages are highly sensitive to Cd, leading to aggravated organ damage induced by autophagy to cope with Cd-induced injury (Li et al. 2014). P62 and LC3B were widely used as markers of autophagic activity, and immunoblotting was widely used to monitor the progress of autophagy. P62 protein (SQSTM 1) is a common marker for studying autophagy activity. It accumulates when autophagy is inhibited and degrades when autophagy is induced. LC3-I and LC3-II are involved in the formation of autophagosome. LC3-I can be transformed into LC3-II during autophagy (Mizushima 2004). Wang reported that the expression levels of Beclin1, LC3-II, and calcium-sensing receptor levels increased with Cd exposure rats (Wang et al. 2017b). Our results showed an increase in the expression of P62 and LC3B, as well as its autophagy-active form LC3-II, in the testes. Significant differences were also observed between the Cd-treated and control groups. These results suggested that autophagy may lead to testicular damage induced by Cd. Furthermore, P62 and LC3B-II decreased in the Cd+Que group, suggesting that Que reduced Cd-induced autophagy.

In conclusion, Cd can induce oxidative stress and autophagy in rat testes. Que antagonizes the Cd toxicity in rat testes by reducing oxidative stress and inhibiting autophagy. This study revealed the specific mechanism of Que against Cd and provided a new clue to reduce Cd poisoning.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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