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Alleviative effect of quercetin on germ cells intoxicated by 3-methyl-4-nitrophenol from diesel exhaust particles*

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Abstract: As a component of diesel exhaust particles, 3-methyl-4-nitrophenol (4-nitro-*m*-cresol, PNMC) is also a metabolite of the insecticide fenitrothion and imposes hazardous effects on human health. In the present study, the alleviative effect of a common antioxidant flavonoid quercetin on mouse germ cells intoxicated by PNMC was investigated. Results showed that a single intraperitoneal injection of PNMC at 100 mg/kg induced severe testicular damage after one week. PNMC-treated mice showed a significant loss of germ cells (approximate 40% loss of round germ cells). PNMC caused an increase of hydroxyl radical and hydrogen peroxide production and lipid peroxidation, as well as a decrease in glutathione level, superoxide dismutase and glutathione peroxidase activities. Furthermore, treatment of PNMC increased expression of the pro-apoptotic protein Bax and decreased expression of the anti-apoptotic protein Bcl-XL in germ cells. In addition, testicular caspase-3 activity was significantly up-regulated and germ cell apoptosis was significantly increased in the PNMC-treated mice. In contrast, combined administration of quercetin at 75 mg/kg significantly attenuated PNMC-induced testicular toxicity. These results indicate that the antioxidant quercetin displays a remarkable protective effect on PNMC-induced oxidative damage in mouse testes and may represent an efficient supplement to attenuate reproductive toxicity by environmental toxicants to ensure healthy sperm production.

Key words: Quercetin, 3-Methyl-4-nitrophenol, Oxidative damage, Apoptosis, Germ cell, Testis
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1 Introduction

There is a growing international concern about hazardous effects of air pollution, which has become an important public health problem. Vast amounts of diesel exhaust particles (DEPs) are released into the atmosphere in many countries. In Japan, 58902 t of DEPs were emitted each year, ranking third, behind only the European Union (240000 t) and the United States (111530 t). Noya *et al*. (2008) revealed that 1 kg of DEPs contain an average of 79.1 mg 3-methyl-4-nitrophenol (4-nitro-*m*-cresol, PNMC) and PNMC was present in a significantly higher concentration than those reported previously (Miyabara *et al*., 1998). As a consequence of the use of diesel engines, DEP imposes hazardous effects on human health that include lung cancer, allergic rhinitis, and bronchial asthma-like diseases by polluting the atmosphere. Different studies have also reported that DEP pollutants exert toxic effects on the male reproductive system in vivo (Watanabe and Oonuki, 1999; Yoshida *et al*., 1999).

As a component of DEP, PNMC is also a major breakdown product of the insecticide fenitrothion, which is a broad-spectrum insecticide used extensively throughout the world (Wright *et al*., 1982). Once used, fenitrothion is rapidly metabolized by microorganisms, plants, and animals, with PNMC as

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a major metabolite. Several research studies have been performed to elucidate the diverse toxic effects induced by PNMC on vertebrates. Mori *et al*. (2003) isolated PNMC from DEPs and showed that it has a vasodilatation activity, while Huang *et al*. (2010) reported that PNMC induced an acute cardiac dysfunction after short-term DEPs exposure of rats. Similarly, exposure of DEPs in vitro impaired early embryonic development and quality in mice (Januario *et al*., 2010). PNMC has been reported to exhibit estrogenic (Furuta *et al*., 2004; 2005) and antiandrogenic activity (Li *et al*., 2006) in rats both in vitro and in vivo, and induced impairment of testicular function in adult male Japanese quail (*Coturnix japonica*) as well as in male rats by reduced daily sperm production. PNMC has also been reported to inhibit the activity of ribonucleotide reductase (Wright *et al*., 1982).

DEP has been reported to be able to generate reactive oxygen species (Ross and Kasum, 2002) and induce oxidative stress and inflammation in the lung and respiratory tract (Park *et al*., 2006). Han *et al*. (2001) reported that DEP generated hydroxyl radical (·OH) in the lung of mice. Chemicals in DEPs have also been reported to generate reactive oxygen radicals and induce apoptosis in macrophages (Hiura *et al*., 1999). Our previous study in vitro showed that PNMC induced oxidative stress on the spermatogonial cells in chicken embryo (Mi *et al*., 2009). Though PNMC-induced reproductive toxicity has been reported, its detailed mechanism is not well known.

Quercetin, as an effective antioxidant flavonoid, is ubiquitously distributed in vegetables and fruits. Quercetin can prevent oxidative injury and cell death by chelating metal ions, scavenging oxygen radicals, and protecting against lipid peroxidation. It has been reported that quercetin has alleviative effects against male reproductive toxicity induced by DEP (Izawa *et al*., 2008). Previous results also indicate that quercetin showed protective effects on male reproductive toxicity caused by PNMC in embryonic chicken and cadmium in mice (Mi *et al*., 2009; Bu *et al*., 2011). However, the effect of quercetin on PNMC-induced testicular toxicity in vivo is not well known.

Therefore, the present study was performed to reveal the relationship between oxidative stress and PNMC-induced testicular toxicity and to explore the protective effect of quercetin on PNMC-induced reproductive toxicity in male mice.

2 Materials and methods

2.1 Animals

Adult male Institute of Cancer Research (ICR) mice were purchased from the Laboratory Animal Center of Zhejiang University (Hangzhou, China). Animals were housed at conditions of (24±2) °C, (45±5)% humidity, and 12-h light/12-h dark cycle, with free access to food and water. The animals were acclimatized for one week prior to beginning the experiments. The experimental procedures were carried out in accordance with the Guidelines for Care and Use of Laboratory Animals of Zhejiang University.

2.2 Treatments

In the first experiment, mice received a single intraperitoneal (i.p.) injection of PNMC (Alfa Aesar Co., Massachusetts, USA) at 1, 10, or 100 mg/kg body weight (BW). The control received the vehicle (phosphate buffered saline containing 0.5% (v/v) dimethyl sulfoxide and 0.05% (v/v) Tween 80). There were six animals in each group. The mice were sacrificed by cervical dislocation one week after injection. The toxic effect was estimated by conventional histological hematoxylin and eosin (HE) staining of the testis sections. Since the dose of 100 mg/kg induced significant germ cell damage, this dosage was selected for the subsequent studies.

In the second study, mice were randomly divided into four groups with six animals per group. The control received vehicle only. The animals in Group II received PNMC (100 mg/kg, i.p.). The animals in Group III received quercetin (75 mg/kg, i.p.) for 3 d. The animals in Group IV received PNMC (100 mg/kg) plus quercetin (75 mg/kg, i.p.) for 3 d. After one week the right testes were immediately collected and fixed in 4% (v/v) paraformaldehyde for histological studies, and the left testes were stored at −80 °C for analysis of biochemical parameters including ·OH, hydrogen peroxide (H_2O_2), malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidation (GSH-Px), and caspase-3.

2.3 Histological studies

The fixed testes were dehydrated and embedded in paraffin, sectioned at 5 μm for HE staining and immunohistochemical staining. The morphological

changes were examined under a microscope (Eclipse 80i, Nikon, Kanagawa, Japan) and images were captured with a digital camera (DS-Fi1, Nikon, Kanagawa, Japan).

2.4 Determination of biochemical parameters

2.4.1 Preparation of testicular homogenate

Frozen testicular tissue was homogenized with ice-cold 9 g/L NaCl solution. The homogenate was centrifuged at 1700×*g* for 15 min at 4 °C and the supernatant was used for further measurements.

2.4.2 Biochemical analysis of antioxidant status

·OH and H_2O_2 were measured by detecting the absorbance at 405 nm according to the commericial kit's instruction. MDA as an endpoint of lipid peroxidation was calculated by detecting the absorbance of thiobarbituric acid reactive substances (TBARSs) at 532 nm. Reduced GSH was determined by measuring the absorbance at 412 nm. SOD activity was determined by measuring the absorbance at 560 nm. GSH-Px activity was assayed by the method based on the reaction between GSH remaining after the action of GSH-Px and 5,5′-dithiobis-2-nitrobenzoic acid to form a complex with the maximal absorbance at 412 nm (Rotruck *et al*., 1973). One unit of GSH-Px activity was defined as the decrease of 1 μmol/L of GSH per min per mg protein deducting the effect of nonenzyme-catalyzed reaction. The biochemical parameters (·OH, H_2O_2, MDA, GSH, SOD, and GSH-Px) were determined using commercial kits, which were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.5 Immunohistochemistry of Bax and Bcl-XL

The pro-apoptotic protein Bax and anti-apoptotic protein Bcl-XL were evaluated by immunohistochemical staining using commercial kits (Boster Bio-engineering Co., Ltd., Wuhan, China). Histological sections were treated with 3% (v/v) H_2O_2 to block endogenous peroxides and antigen retrieval was carried out in nitrate buffer at 95 °C for 20 min. Fetal bovine serum (FBS, 10% (v/v)) was used to block nonspecific staining. Then, the sections were respectively incubated with a rabbit anti-Bax or Bcl-XL polyclonal antibody (Boster Bioengineering Co., Ltd., Wuhan, China) at a dilution of 1:200 (v/v) overnight at 4 °C. The immunoreaction was achieved with the goat anti-rabbit antibody (Boster Bioengineering Co., Ltd., Wuhan, China) at a dilution of 1:200 (v/v) and developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. The images of the seminiferous epithelium were captured with a digital camera. Germ cells with a brown cytoplasm were considered positive, and the number of positive cells was counted in each image using the Imaging Software (NIS-Elements BR 2.30, Nikon, Kanagawa, Japan).

2.6 Determination of caspase-3 activity

The testicular activity of caspase-3 was determined using caspase-3 assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). The activity was expressed as the activity relative to the control group.

2.7 Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay

Apoptotic cells were detected with a Promega apoptosis detection TUNEL kit in the seminiferous epithelium. Cells with a brown nucleus were considered positive. Germ cells with a brown cytoplasm were considered positive, and the number of positive cells was counted in each image using the Imaging Software (NIS-Elements BR 2.30, Nikon, Kanagawa, Japan).

2.8 Statistical analysis

All data are expressed as mean±standard error mean (SEM) and analyzed by one-way analysis of variance (ANOVA). The statistical analysis was performed using the generalized linear model (GLM) procedure of Statistical Analysis System (SAS) software (Version 6). Significance was declared at $P<0.05$.

3 Results

3.1 Morphological changes of testes

After one week of treatment, control mice showed normal morphology of seminiferous tubules (Fig. 1a). Treatments with PNMC at doses of 1 or 10 mg/kg induced no significant changes of the seminiferous tubules compared with the control

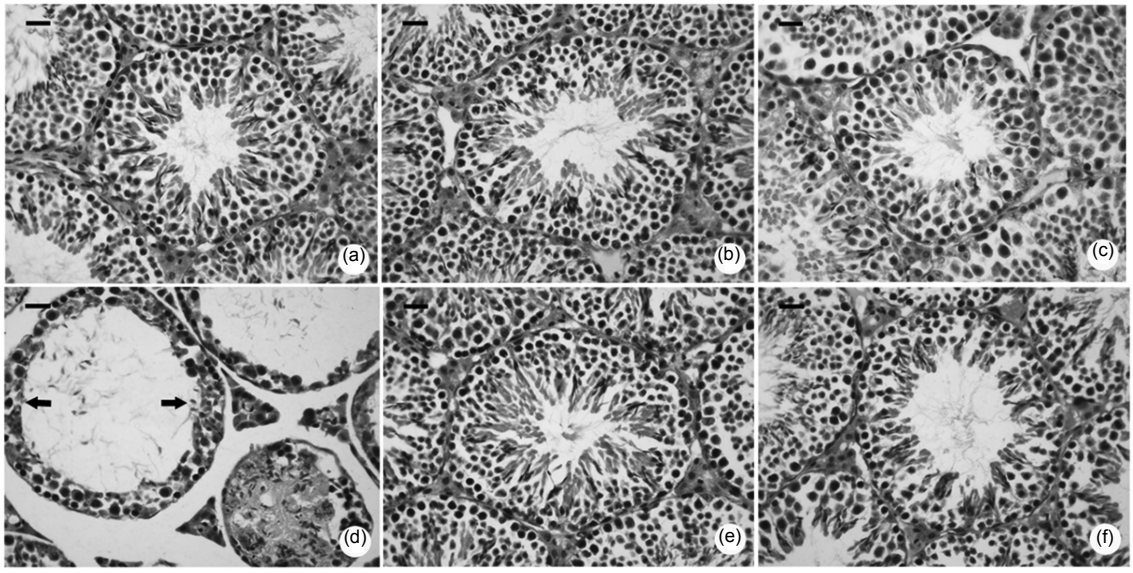

Fig. 1 Morphological changes of the seminiferous tubules one week after treatments of PNMC and quercetin
(a) Control; (b−d) PNMC treatments (1, 10, and 100 mg/kg, respectively); (e) Quercetin (75 mg/kg); (f) PNMC (100 mg/kg)+quercetin. Scale bar: 20 μm

(Figs. 1b and 1c). However, PNMC at the dose of 100 mg/kg resulted in remarkable damage to the seminiferous tubules. As shown in Fig. 1d, PNMC-treated mice showed approximately 40% loss of round germ cells. Almost no elongated spermatozoa could be found and the diameters of seminiferous tubules were markedly increased (approximately half that of control). Moreover, atrophy of testes was also detected.

Histological results showed that in the quercetin (75 mg/kg)-treated group, the mice had normal seminiferous epithelia (Fig. 1e). After combined treatment with quercetin, the PNMC-induced damage was attenuated significantly. There was no germ cell decline in the seminiferous tubules of the co-treated mice as shown in Fig. 1f.

3.2 Antioxidant status

Treatment with PNMC (100 mg/kg) markedly destroyed the antioxidant system of the testes. PNMC significantly induced increases of ·OH and H_2O_2 levels (Fig. 2a; $P<0.05$). MDA level was also significantly increased (Fig. 2b; $P<0.05$). Administration of quercetin to PNMC-treated mice significantly reduced ·OH and H_2O_2 levels, as well as MDA level. PNMC significantly depleted testis GSH level (Fig. 3; $P<0.05$), while treatment with quercetin caused a significant increase in GSH level ($P<0.05$). The activities of SOD and GSH-Px were significantly reduced in mice treated with PNMC ($P<0.05$); however, there was a tendency for treatment with quercetin to enhance the activities of both enzymes ($P>0.05$).

3.3 Bax and Bcl-XL expression

As shown in Fig. 4, PNMC significantly up-regulated Bax expression and down-regulated Bcl-XL expression in the germ cells, compared to the control, while quercetin up-regulated Bcl-XL expression in germ cells compared with the control. Moreover, in combination with PNMC, quercetin significantly down-regulated PNMC-induced increase of Bax expression, and further up-regulated the expression of Bcl-XL (Fig. 5a; $P<0.05$).

3.4 TUNEL-positive cell number

The immunohistochemical results showed that, in the control and quercetin-treated group, mice showed almost no apoptotic cells (Figs. 4i–4l). However, TUNEL-positive cell number increased significantly in the PNMC-treated mice testes compared to the control (Fig. 5b; $P<0.05$). The apoptotic cells mainly included spermatocyte and round spermatid. Moreover, in combination with PNMC, quercetin markedly reduced the PNMC-induced increase of TUNEL-positive cell number ($P<0.05$).

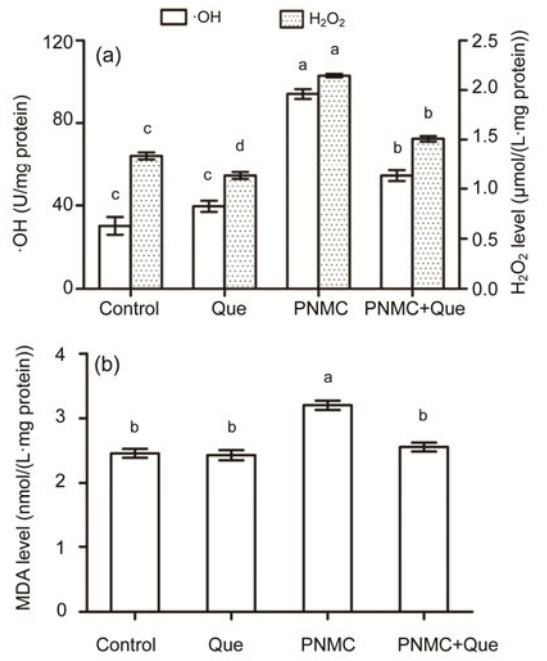

Fig. 2 Changes of ·OH, and H_2O_2 (a) and MDA (b) production in testes after treatments of PNMC and quercetin (Que)

Columns represent mean±SEM (n=6). Bars with different letters were statistically different (P<0.05)

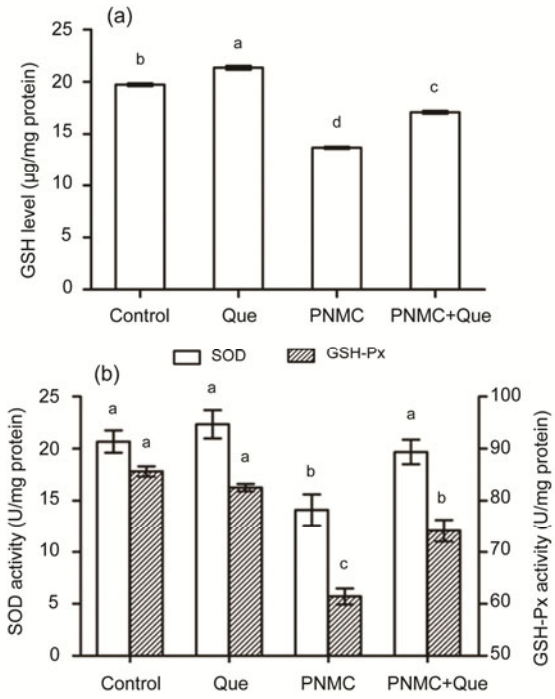

Fig. 3 Changes of GSH level (a) and SOD and GSH-Px activities (b) of testes after treatments of PNMC and quercetin (Que)

Columns represent mean±SEM (n=6). Bars with different letters were statistically different (P<0.05)

3.5 Caspase-3 activity

The activation of caspase-3 plays a pivotal role in the execution of apoptosis. Our results showed that PNMC induced a significant increase of caspase-3 activity compared to the control (Fig. 6; P<0.05). There was no significant change in caspase-3 activity of quercetin-treated mice relative to the control. However, in combination with PNMC, quercetin was demonstrated to effectively suppress caspase-3 activity.

4 Discussion

Our previous in vitro studies have reported that PNMC induced oxidative damage to chicken embryonic spermatogonial cells (Mi *et al*., 2009). Our present study found that PNMC treatment at 1 or 10 mg/kg (one week after a single i.p. injection) showed no significant changes of seminiferous tubules in mature male mice; however, at the dose of 100 mg/kg, it induced focal severe damage to the seminiferous epithelium. Moreover, germ cell loss was observed in PNMC-treated mice and almost no elongated spermatozoa were found.

It is known that reactive oxygen species play a key role in the pathophysiological processes (Rodrigo and Bosco, 2006). A previous study in vitro has reported that oxidative stress plays a critical role in PNMC-induced cytotoxicity (Mi *et al*., 2009). Our biochemical results showed that PNMC treatment induced oxidative stress on testes. With significant increases of ·OH and H_2O_2 levels and lipid peroxidation, the antioxidant system of testis was significantly disturbed, and at the same time the antioxidant GSH, enzymatic antioxidants SOD and GSH-Px were also significantly inhibited. The antioxidant GSH functions as a direct reactive free-radical scavenger (Romão *et al*., 2006). SOD and GSH-Px are two main components of antioxidant enzymes. As an important superoxide radical scavenger, SOD converts superoxide anions to hydrogen peroxide, which then can be detoxified by GSH/GSH-Px to yield reduced GSH.

There is a close relation between oxidative stress and apoptosis. The Bcl-2 family consists of pro-apoptotic (e.g., Bax) and anti-apoptotic (e.g., Bcl-XL) members, which play an important role in the mitochondrial pathway of apoptosis. The altered

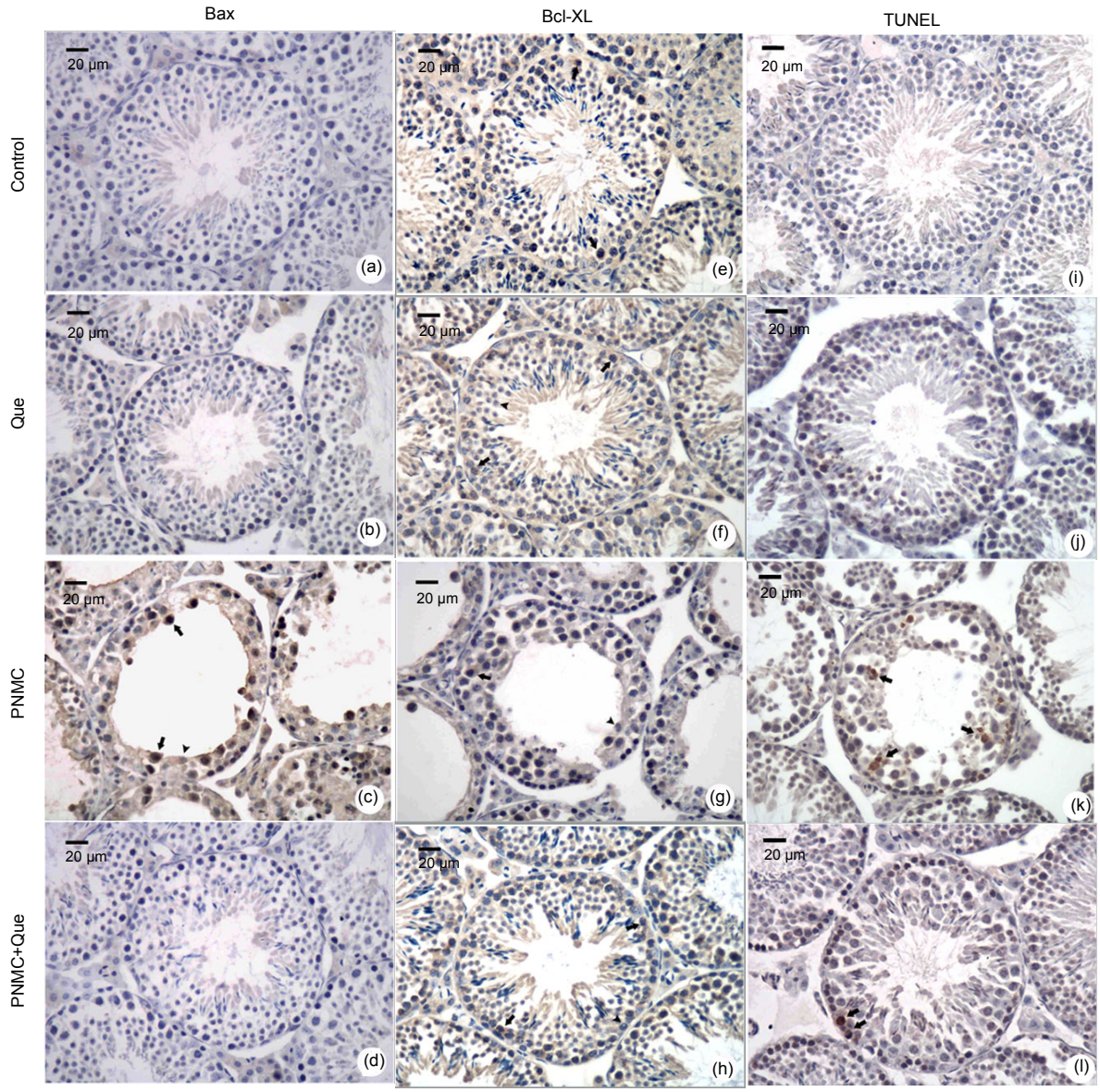

Fig. 4 Changes of Bax (a–d) and Bcl-XL (e–h) expression and TUNEL staining (i–l) of germ cells in seminiferous epithelia
(a, e, i) Control; (b, f, j) Quercetin (75 mg/kg); (c, g, k) PNMC (100 mg/kg); (d, h, l) PNMC+quercetin. Arrows: positive cells; Arrowheads: negative cells

expression of Bax and Bcl-XL may occur before the activation of apoptosis (Adams and Cory, 1998; Hengartner, 2000; Gustafsson and Gottlieb, 2007). There are many factors which can alter the expression of Bcl-2 proteins, such as hypoxia, oxidative stress, and DNA damage (Gustafsson and Gottlieb, 2007). DEP induce down-regulation of Bcl-XL (Landvik *et al*., 2007) and the change of Bcl-2 family proteins may trigger caspase cascade activation. Activation of caspase-3 is known to be an important step in the progress of apoptosis. Our results showed that the activity of caspase-3 in PNMC-treated mice was significantly up-regulated.

It is known that reactive oxygen species are able to induce DNA damage. The most potent reactive oxygen species to react with DNA is ·OH, which generates damage to both DNA bases and deoxyribose residues. It has been reported that exposure to DEP extracts induced apoptosis in different cell types (Baulig *et al*., 2003). As shown in our results, one week of PNMC exposure (100 mg/kg) induced significant DNA damage of germ cells in the mice testis. TUNEL-positive cells in PNMC-treated mice were significantly increased.

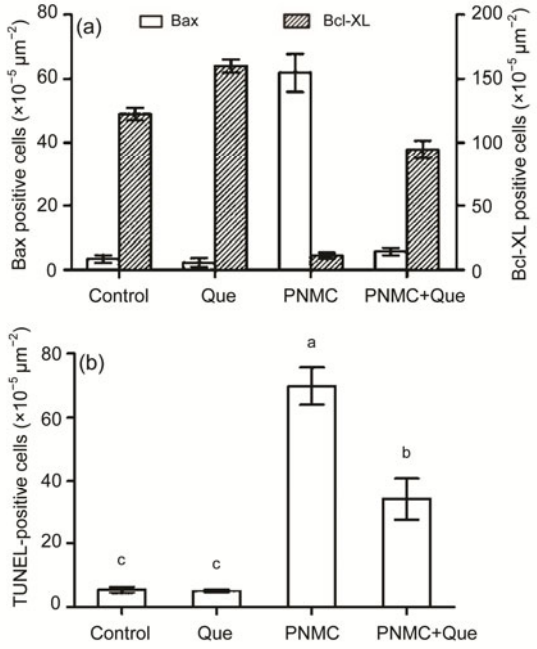

Fig. 5 Changes of Bax and Bcl-XL expression (a) and TUNEL-positive germ cells (b) after treatments of PNMC and quercetin (Que)

Columns represent mean±SEM (n=6). Bars with different letters were statistically different (P<0.05)

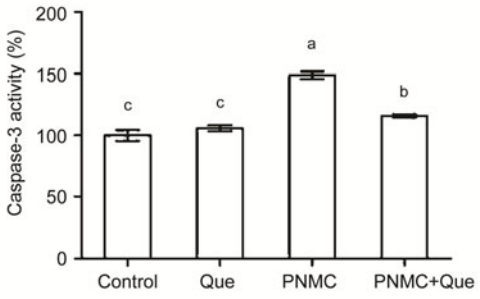

Fig. 6 Changes of caspase-3 activity in testicular homogenate after treatments of PNMC and quercetin (Que)

Columns represent percents of control (mean±SEM, n=6). Bars with different letters were statistically different (P<0.05)

Quercetin, as a natural antioxidant, is ubiquitously distributed in fruits and vegetables. Our previous study found that flavonoid quercetin and daizein have protective effects on cadmium or polychlorinated biphenyls-induced oxidative damage in mice testes (Zhang *et al*., 2008; Bu *et al*., 2011). In the present study, we found that quercetin supplement attenuated PNMC-induced increase of ·OH and H_2O_2, and further decreased lipid peroxidation, suggesting that superoxides ·OH and H_2O_2 are the main damage factors in PNMC-induced oxidative stress in mice testis. Furthermore, quercetin supplementation significantly restored the depletion level of antioxidant GSH and the activities of enzymatic antioxidants SOD and GSH-Px. These results are in agreement with our studies using chicken testicular germ cells (Mi *et al*., 2009).

Structure-activity relationships of quercetin were investigated by Cao *et al*. (1997) and the antioxidant activities of quercetin may depend upon its hydroxyl groups. It is supposed that flavonoids prevent the progression of the radical chain reaction by traping free radicals at the interface of the membranes (Ross and Kasum, 2002). In addition to its free-radical scavenging properties, quercetin can also chelate those transition metal ions responsible for the generation of reactive oxygen species (Rice-Evans *et al*., 1996). Our present study also revealed that quercetin exerts antioxidant abilities by enhancing the activities of endogenous antioxidant and enzymes.

It is reported that quercetin also has antiapoptotic function (Chao *et al*., 2009; Kim *et al*., 2009). Our previous studies have shown that quercetin has effectively inhibited pro-apoptotic member Bax and up-regulated anti-apoptotic member Bcl-XL (Bu *et al*., 2011; Jia *et al*., 2011). In the present study, quercetin treatment effectively down-regulated the expression of pro-apoptotic protein Bax and up-regulated the expression of Bcl-XL induced by PNMC. The change of the ratio of Bax/Bcl-XL would prevent apoptosis by suppressing caspase-3 activity. Furthermore, suppression of apoptosis was demonstrated by the decreased number of TUNEL-positive cells in quercetin-treated mice.

In conclusion, the present study suggests that PNMC has reproductive toxic effects on mouse germ cells involving mitochondrial oxidative damages via the inhibition of Bcl-XL expression, activation of Bax expression and caspase-3 activity. However, as an effective antioxidant, quercetin manifested a protective effect against PNMC-induced toxicity through attenuating lipid peroxidation, renewing the activities of antioxidant enzymes, and alleviating apoptosis by modulating Bax and Bcl-XL expression and inhibiting caspase-3 activity. Therefore, these observations suggest that the dietary antioxidant quercetin potentially reduces environmental PNMC-induced toxicity and improves reproductive health.

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