Toxicology Reports 2 (2015) 664–676

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Supplemental dietary phytosterin protects against 4-nitrophenol-induced oxidative stress and apoptosis in rat testes

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ARTICLE INFO

Article history:
Received 2 March 2015
Received in revised form 27 April 2015
Accepted 27 April 2015
Available online 11 May 2015

Chemical compounds studied in this article:
4-Nitrophenol (PubChem CID: 980)
β-Sitosterol (PubChem CID: 222284)
Campesterol (PubChem CID: 457801)
Stigmasterol (PubChem CID: 5280794)

Keywords:
4-Nitrophenol
Phytosterin
Nrf2
Testis
Antioxidant gene
Apoptosis

ABSTRACT

4-Nitrophenol (PNP), is generally regarded as an environmental endocrine disruptor (EED). Phytosterin (PS), a new feed additive, possesses highly efficient antioxidant activities. The transcription factor, nuclear factor-erythroid 2-related factor 2 (Nrf2), is an important regulator of cellular oxidative stress. Using rats, this study examined PNP-induced testicular oxidative damage and PS-mediated protection against that damage. The generation of MDA and H_2O_2 upon PNP and PS treatment was milder than that upon treatment with PNP alone. This mitigation was accompanied by partially reversed changes in SOD, CAT, GSH and GSH-Px. Moreover, PNP significantly reduced the caudal epididymal sperm counts and serum testosterone levels. Typical morphological changes were also observed in the testes of PNP-treated animals. PNP reduced the transcriptional level of Nrf2, as evaluated by RT-PCR, but it promoted the dissociation from the Nrf2 complex, stabilization and translocation into the nucleus, as evaluated by immunohistochemistry and Western blotting. In addition, PNP enhanced the Nrf2-dependent gene expression of heme oxygenase-1 (HO-1) and glutamate–cysteine ligase catalytic subunit (GCLC). These results suggest that the Nrf2 pathway plays an important role in PNP-induced oxidative damage and that PS possesses modulatory effects on PNP-induced oxidative damage in rat testes.

1. Introduction

It is well established that the exposure of immature animals to chemicals during crucial developmental periods may result in growth alteration, structural abnormalities or functional deficits. 4-Nitrophenol (PNP), is in popular use worldwide for agriculture and industrial manufacturing [1]. PNP is a nitrophenol derivative that has been isolated from diesel exhaust particles (DEP) – 1 kg of DEP contains an average of 169 mg of PNP [2]. PNP is also a degradation product of the insecticide parathion [3]. Due to the stability of PNP, its non-biodegradable nature and its consequent persistence in the environment [4], increasing

Abbreviations: EED, environmental endocrine disruptor; PNP, 4-nitrophenol; DEP, diesel exhaust particles; ROS, reactive oxygen species; GSH, glutathione; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; ARE, antioxidant response element; Nrf2, nuclear factor erythroid 2-related factor 2; Keap1, kelch-like ECH-associated protein 1; PS, phytosterins; HO-1, heme oxygenase 1; GCLC, γ-glutamylcysteine synthetase; NQO1, NAD(P)H: quinone oxidoreductase 1; NF-κB, nuclear factor-κB.

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http://dx.doi.org/10.1016/j.toxrep.2015.04.007

attention has been focused on understanding the toxicology of this class of compounds. EPA regulations require the concentration in water to be less than 10 ng/L [5]. The general population can be exposed to PNP via inhalation of ambient air or via the ingestion of contaminated water. Occupational exposure to PNP can occur by both inhalation and dermal contact at workplaces where this compound is produced or used. Previous studies indicated that PNP had potential adverse effects, such as disturbing the endocrine and reproductive system [6,7]. Exposure to DEP is associated with oxidative damage [8], including protein oxidation and lipid peroxidation [9]. Reactive oxygen species (ROS) were thought to be the cause of oxidative stress following DEP exposure [10]. Therefore, the toxic influence of PNP is likely due to the formation of excessive free radicals, causing oxidative stress and leading to cell damage.

ROS have been known to cause a loss of membrane polyunsaturated fatty acids and an increase in the lipid-peroxides in spermatozoa [11]. Oxidative stress may induce free-radical-mediated decomposition of vital molecules, such as proteins and lipids, and ultimately cell death [12]. Oxidative stress is frequently cited to explain cell damage in various disease. Cells are endowed with an array of protective antioxidants, such as the glutathione (GSH), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT), which scavenge ROS to prevent possible cellular damage.

Expression of most antioxidant enzymes is controlled by the antioxidant response element (ARE) and activated by nuclear factor erythroid 2-related factor 2 (Nrf2) [13]. Nrf2 remains inactive in the cytoplasm through its interaction with Kelch-like ECH-associated protein 1 (Keap1) [14]. The release of Nrf2 from Keap1 can be induced via direct attack by multiple environmental factors and a variety of chemicals or via indirect actions, such as phosphorylation, allowing Nrf2 to translocate to the nucleus and activate the expression of ARE-containing genes [15]. Thus, a disruption in this pathway might affect organ toxicity caused by environmental chemicals.

Phytosterins (PS) are commonly found as minor constituents of edible vegetable oils and are natural constituents of the human diet. PS have attracted much attention in recent decades because of their health benefits for humans. There is a wide variety of PS structures but the most frequent PS found in nature are β-sitosterol, campesterol and stigmasterol [16]. Dietary intake of PS has been estimated to be 200–300 mg/day in northern European countries [17] and approximately 300–450 mg/day in Japan [18]. It is well established that certain PS reduce plasma cholesterol levels, ostensibly by inhibiting enterocytic cholesterol uptake through competition with dietary and biliary cholesterol for absorption [19]. In addition, PS are recognized to exert antioxidative actions [20,21].

To the best of our knowledge, PNP toxicity in gonads has been reported mainly based on endocrine mechanisms with low dosages of PNP, and the reproductive toxicity of high dose PNP has yet to be investigated. Because the consumption of PNP in industrial and agricultural activity is dramatically increasing and PNP is accumulating in water and soil, it is necessary to assess the toxicity of high doses of PNP. In the present study, we investigated the PNP-induced testicular oxidative damage in rats using a variety of assays. The attenuating effects of PS on PNP-induced testicular toxicity were also studied.

2. Materials and methods

2.1. Chemicals and antibodies

PNP monomer dry crystals ($C_6H_5NO_3$, >99.9% purity, CAS 100-02-7) were purchased from Chengdu Kelong Chemical Reagent Factory, China (Fig. 1A).

Commercial grade PS (mixtures of β-sitosterol, campesterol and stigmasterol, >90% purity) were provided by Jiangsu Chunzhigu Biological products company (Fig. 1B).

The primary antibodies used for tissue immunohistochemistry and Western blotting were anti-Nrf2 (Abcam, ab53019; rabbit anti-human), anti-caspase-3 (Cell Signaling, Asp175; rabbit anti-human), anti-β-actin (Beyotime Institute of Biotechnology, AA128; mouse antibody), anti-Histone3 (Beyotime Institute of Biotechnology, AH433; rabbit antibody). The secondary antibodies used in this study were goat anti-rabbit IgG (H + L) (Beyotime Institute of Biotechnology, A0208) and goat anti-mouse IgG (H + L) (Beyotime Institute of Biotechnology, A0216).

2.2. Animals

Twenty-one-day-old male Sprague-Dawley rats supplied by QingLongShan Laboratory Animal Company (NanJing, China) were maintained under controlled environmental conditions at room temperature (22 ± 2 °C) with 50 ± 10% humidity and an automatically controlled cycle of 12 h light and 12 h dark. Feed (purchased from a commercial supplier) and sterile distilled water were provided ad libitum. During the experiment, animals were allocated into polypropylene cages with laboratory-grade pine shavings as bedding. Animals were acclimatized to the experimental conditions for a period of one week prior to the beginning of the experiment. The experimental protocol was approved in accordance with the Guide for

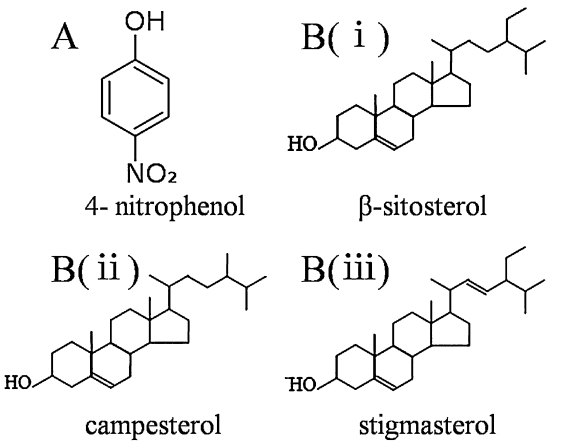

Fig. 1. Chemical structures of 4-nitrophenol, β-sitosterol, campesterol and stigmasterol.

the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee, Nanjing Agricultural University.

2.3. Experimental design

Twenty male rats were randomly assigned into four experimental groups (Control, PS, PNP, and PNP + PS). The vehicle was sterile phosphate-buffered saline (PBS, 0.1 M, pH 7.4) containing 0.05% Tween 80. PS were added to the basal diet supplied by the commercial supplier (50 ppm) [22] and pre-fed for one week. The PNP was dissolved in the vehicle, stored under refrigeration (approximately 4 °C), and replaced once per week to ensure the stability of dosing solutions. Rats were injected subcutaneously with PNP (100 mg/kg body weight) daily for 28 days at 9 am. Rats injected with vehicle alone (PBS containing 0.05% Tween 80) were used as the control group. Treatment regimen and dose selection were based on previous studies [23] with adaptations. Twenty-four hours after the final injection, rats were weighed, anesthetized with ethylic ether and sacrificed.

2.4. Sample collection

Blood was collected from the ruptured cervical vessels in plastic tubes and centrifuged at 3500 rpm for 15 min at 4 °C. Serum was separated and stored at −20 °C until it was assayed for testosterone and estradiol. Partial internal organs (liver, kidney and spleen) and reproductive/endocrine glands (testis, epididymis and adrenal) were excised and weighed immediately. A portion of the testis was fixed in 2.5% glutaraldehyde and 4% paraformaldehyde for transmission electron microscopy and immunohistochemical analysis; the remainder of the testes was stored in liquid nitrogen for RT-PCR, Western blot and biochemical assays.

2.5. Sperm analysis

Epididymal sperm counts in cauda epididymides were determined as described previously [24] with slight modifications. After sacrifice, the cauda epididymis were removed, weighed, and minced in 2.0 ml PBS. The minced material was filtered through a nylon mesh screen, and the filtrate was brought to a final volume of 10 ml with PBS. The number of sperm was directly counted by the standard hemo-cytometric method. Epididymal sperm morphology was observed under a microscope at high power after staining with Giemsa. The morphology of 200 sperm was evaluated per animal. The percentage of abnormal sperm was recorded and calculated.

2.6. Radioimmunoassay

The serum concentrations of testosterone were determined by Nanjing General Hospital of Nanjing Military Command using an ^{125}I-labeled radioligand double-antibody RIA Kit. The commercial RIA Kit was purchased from Beijing Beifang Institute of Biotechnology. The intra- and inter-assay coefficients of variation were less than 10% and 15%, respectively, for testosterone. The treatments were performed in triplicate.

2.7. Biochemical assay

Testes were homogenized in ice-cold physiological saline and centrifuged at 3500 rpm for 10 min. The supernatant and serum were used to measure the following biochemical parameters: SOD, MDA, CAT, H_2O_2, GSH and GSH-Px. All operations were performed at 4 °C. The activities of SOD, MDA, CAT, H_2O_2, GSH and GSH-Px were assayed using commercial reagent kits obtained from the Institute of Biological Engineering of Nanjing Jiancheng (Nanjing, China) according to the manufacturer's protocol [25,26]. Total proteins were quantified by the classical Bradford method with Coomassie Brilliant Blue G-250 [27].

2.8. Transmission electron microscopy

Transmission electron microscopy was based on the protocol described by a previous report [28]. The testes were fixed in 2.5% glutaraldehyde in PBS at 4 °C for 24 h. Next, the samples were washed with PBS for 12 h and post-fixed for 20 min in 1% OsO_4 in PBS. The samples were then washed with PBS, dehydrated in a series of increasing (20–100%) concentrations of ethanol, and embedded in a 1:1 Epon:alcohol mixture for 2 h, followed by 100% Epon for an additional 3 h. The samples were then incubated overnight in the oven. Thin sections (70 nm) were placed on copper grids and stained in a solution of 2% uranyl acetate and 1% lead citrate for 30 min. A JEM 100CX transmission electron microscope operated at 80 kV was used to visualize the ultrastructure of the testes samples.

2.9. Immunohistochemistry

The sections of testes were prepared for immunohistochemical analysis in a manner similar to that previously reported [29], with slight modulations. Briefly, the sections were deparaffinized with xylene and rehydrated in graded ethanol before being washed with twice-distilled water. To increase epitope exposure, the sections were heated for 15 min in sodium citrate buffer (0.01 M, pH 6.0) in a microwave oven. The sections were cooled, washed with PBS, and then blocked with 10% bovine serum albumin (BSA) in TBST (20 mM Tris-buffered saline, 0.05% Tween 20, pH 7.5) for 1 h at room temperature. The sections were incubated overnight at 4 °C with diluted (1:400) polyclonal antibodies against Nrf2 and Caspase-3. The antibodies were visualized with an SABC Kit Elite and 0.05% 3,3-diaminobenzidine tetrachloride (Sigma) in PBS, containing 0.01% H_2O_2 for 2 min. The sections were counterstained with hematoxylin and mounted with coverslips. Antibody specificity was examined with the use of 1% BSA instead of primary antibody.

2.10. Proteins extraction and Western blotting

Nuclear and cytosolic proteins were extracted with a protein extraction kit (Beyotime Institute of Biotechnology, P0027) according to the manufacturer's instructions.

The proteins extracted from the supernatants were measured with the BCA Protein Assay Protocol and subsequent quantification of Nrf2 was determined by Western blot analysis. Total protein (50 μg) was loaded in each lane of 12% PAGE with a MiniProtean Tetra System (Bio-Rad; Hercules, CA, USA) using Precision Plus Protein molecular weight standards (BioRad). The proteins were transferred to nitrocellulose membranes. Membranes were incubated with defatted milk powder for one hour at RT to block non-specific binding. Primary antibodies were incubated for overnight at 4 °C. Membranes were washed and incubated with HRP-labeled goat anti-rabbit IgG as a secondary antibody, followed by visualization using enhanced chemiluminescence (ECL) detection reagents. Bands were scanned in a computer, and their relative intensities were determined by densitometry using Scion Image v. 4.0.2 (Scion Corporation, Frederick, USA). Negative controls for Western blots were membranes incubated with the appropriate pre-adsorbed primary antibody or with blocking solution without primary antibodies. β-actin was used as the cytosolic control, and Histone3 was used as the nuclear loading control. Densitometry was performed with Image J software.

2.11. RNA extraction, reverse transcription (RT) and quantitative PCR

Total RNA was extracted from the testes using the RNeasy Mini kit® (Qiagen, China) according to the manufacturer's instructions. The concentration and purity of the isolated total RNA were determined spectrophotometrically at 260 and 280 nm with a Nanodrop® 8000 (Thermo Fisher Scientific, Wilmington, USA). The total RNA (1 μg) was reverse transcribed to cDNA with an Omniscript® Reverse Transcription kit (Takara) with Oligo-dT primers (Takara), according to the manufacturer's protocol. The target fragments were quantified by real-time PCR using a QuantiTect™SYBR Green® PCR Kit (Roche) with 100 ng of the cDNA template. Each sample was tested in duplicate. The gene expression data were normalized to β-actin expression. The specific primer sets, designed using Primer 5 Plus software, are shown in Table 1. For quantification of real-time PCR results, the threshold cycle Ct was determined for each reaction. Ct values for each gene of interest were normalized to the housekeeping gene; PCR amplification efficiencies were taken into account by amplifying various amounts of target cDNA for each reaction. Normalized values were used to calculate the degree of induction or inhibition expressed as a "fold difference" compared to normalized control values. Therefore, all data were statistically analyzed as "fold induction" between treated and control animals.

2.12. Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM) and were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. Statistical analysis was performed with GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Body and organ weights

The body weight gains before and after administration of PNP and/or PS were measured and compared. The weanling weight showed no significant differences ($P > 0.05$) across groups, and after administration of PNP and/or PS, there was still no significant difference in the body weight between the control group and the experimental groups ($P > 0.05$) (Table 2). No significant difference was observed in the organs' absolute weights (Table 2) or the relative organ weight (date not shown), which suggested that PNP did not manifest toxicity principally toward growth and development at the studied dosage.

3.2. Sperm parameters

The sperm count in the cauda epididymides of the PNP-treated group were significantly decreased ($P < 0.05$) compared with control; however, in combination with PS, this effect was obviously attenuated (Table 3). However, the percentage of normal morphology was not affected ($P > 0.05$) after treatment with either PNP alone or PNP combined with PS (Table 3).

3.3. Serum testosterone concentrations

Serum concentrations of testosterone are shown in Table 3. The mean serum concentrations of testosterone were significantly lower ($P < 0.05$) in the group treated with PNP alone compared with control. In the PS + PNP treatment, the normal levels of testosterone were retained.

3.4. Antioxidative status in the serum and testes

To determine whether PNP could induce oxidative stress in vivo, we first examined SOD, CAT and GSH-Px activities as well as the levels of MDA, H_2O_2 and GSH in the serum and testes. Compared with the control group, there was no difference in these antioxidative properties after administration of PS (Fig. 2). The results showed that SOD activity decreased significantly ($P < 0.05$) in both the serum (Fig. 2A) and the testes (Fig. 2B) in the PNP-treated group, and the MDA levels increased significantly in the serum ($P < 0.01$) (Fig. 2C) and testes ($P < 0.05$) (Fig. 2D). Furthermore, PNP treatment led to a decreased in the mean CAT activity in the serum ($P < 0.01$) (Fig. 2E) and testes ($P < 0.05$) (Fig. 2F) compared to the control, and much higher H_2O_2 levels were observed in the serum ($P < 0.05$) (Fig. 2G) and testes ($P < 0.01$) (Fig. 2H). In addition, after 4 weeks of consumption of the PNP, the GSH levels dramatically decreased in the serum ($P < 0.01$) (Fig. 2I) and testes ($P < 0.05$) (Fig. 2J) compared to the control, but GSH-Px activity did not change in either the serum (Fig. 2K) or the testes (Fig. 2L). It was note-worthy that 4 weeks of

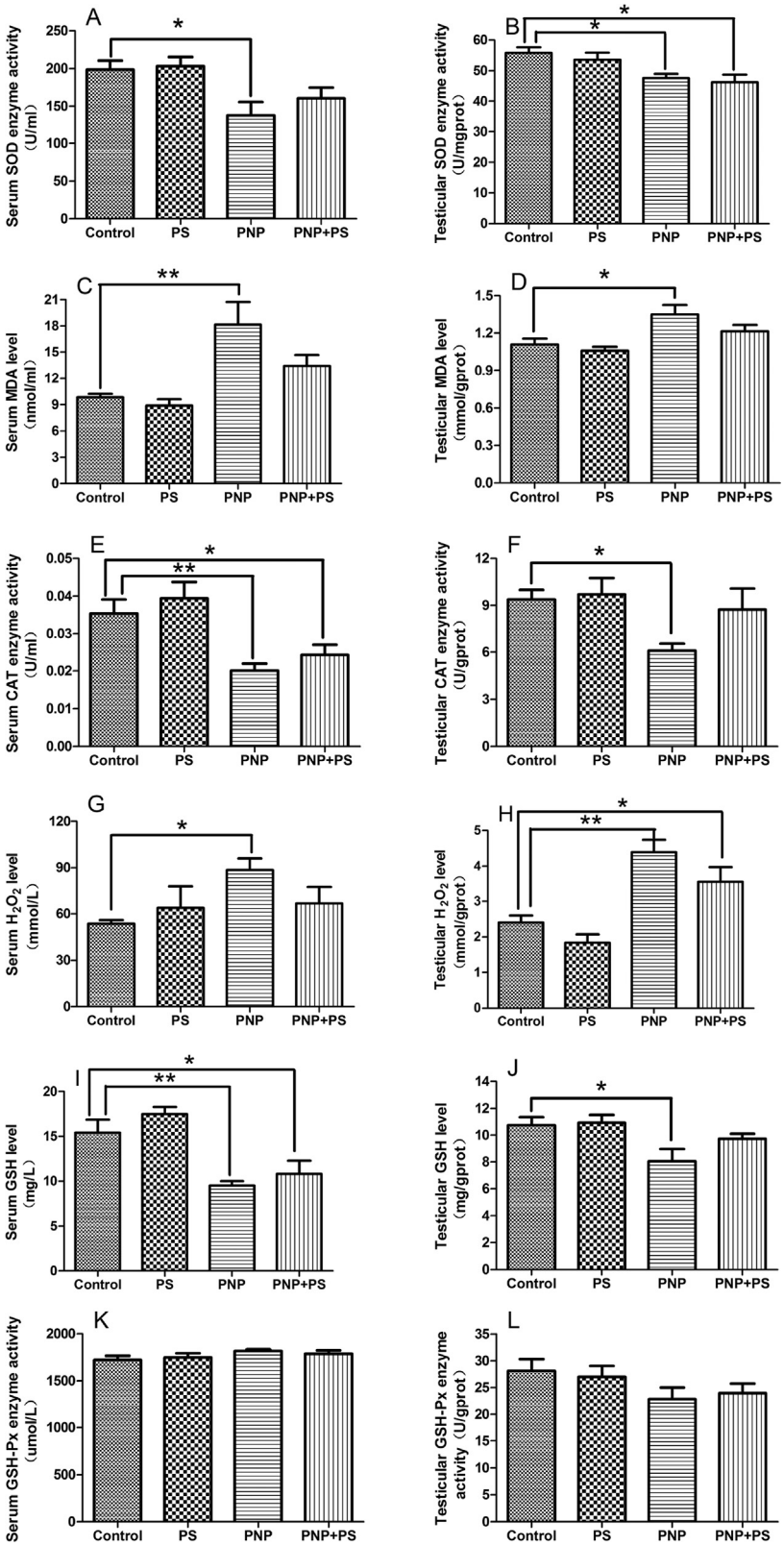

Fig. 2. The effects of PNP and PS exposure on antioxidative status in the serum and testes. The parameters were as follows: A, B: SOD; C, D: MDA; E, F: CAT; G, H: H_2O_2; I, J: GSH; K, L: GSH-Px; A, C, E, G, I, K: in serum; B, D, F, H, J, L: in testes. The values shown are the mean ± SEM of five animals per group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus corresponding control and $^{\#}P < 0.05$ versus corresponding PNP + PS group.

Table 1
Primers and qPCR details for analysis of mRNA levels.

Gene	Sequence (5′→3′)	Product size (bp)	40 PCR cycles
GCLC (BC081702)	F: AAATCAGTAAGTCTCGGTATG R: AAGGGTCAGTGGGTCTC	179	95 °C for 15 s 60 °C for 30 s 72 °C for 30 s
NQO1 (NM_017000)	F: CCATTCCAGCCGACAAC R: AGCCGTGGCAGAACTATC	199	95 °C for 15 s 60 °C for 30 s 72 °C for 30 s
Nrf2 (NM_031789)	F: TTTGGAGGCAAGACATAG R: TGGGCAACCTGGGAGTA	253	95 °C for 15 s 60 °C for 30 s 72 °C for 30 s
HO-1 (NM_012580)	F: TTCACCTTCCCGAGCAT R: GCCTCTTCTGTCACCCTGT	110	95 °C for 15 s 60 °C for 30 s 72 °C for 30 s
β-actin (NM_031144)	F: CGTTGACATCCGTAAAGACC R: GGAGCCAGGGCAGTAATCT	108	95 °C for 15 s 60 °C for 30 s 72 °C for 30 s

Table 2
Effects of PNP and PS on body and absolute organ weights in rats.

	Control	50 ppm PS	100 mg/kg body weight PNP	50 ppm PNP + PS
Body weight (g)	271.8 ± 5.34	275.0 ± 5.27	260.3 ± 6.76	284.2 ± 7.49
Liver (g)	13.09 ± 0.50	13.39 ± 0.50	13.12 ± 0.44	13.16 ± 1.29
Testes (g)	3.13 ± 0.09	3.33 ± 0.09	2.84 ± 0.10	3.05 ± 0.06
Epididymis (g)	0.58 ± 0.01	0.60 ± 0.01	0.54 ± 0.01	0.59 ± 0.02
Kidneys (g)	2.40 ± 0.07	2.60 ± 0.07	2.25 ± 0.09	2.56 ± 0.07
Adrenals (mg)	52.64 ± 3.77	54.64 ± 3.77	45.98 ± 0.98	51.50 ± 3.92
Spleen (g)	0.60 ± 0.03	0.62 ± 0.03	0.68 ± 0.06	0.76 ± 0.06

The values shown are the mean ± SEM of five animals per group.

Table 3
Effects of PNP and PS on sperm parameters and serum testosterone concentration in rats.

	Control	50 ppm PS	100 mg/kg body weight PNP	50 ppm PNP + PS
Sperm count ($\times 10^7$/g)	89.65 ± 4.49	90.25 ± 4.61	65.77 ± 4.68*	73.80 ± 9.14
Abnormal sperm (%)	2.10 ± 0.20	1.96 ± 0.18	2.87 ± 0.61	2.57 ± 0.45
Testosterone (ng/ml)	2.34 ± 0.10	2.60 ± 0.16	1.82 ± 0.13*	2.20 ± 0.28

The values shown are the mean ± SEM of five animals per group.
* $P < 0.05$ versus corresponding control.

consumption of the PS supplemented diet partially attenuated the changes caused by PNP, although there was not a complete return to normal levels (Fig. 2).

3.5. Ultrastructural analysis of testes

The number of testicular germ cells per a sectional area of seminiferous tubules were shown in Table 4. To investigate the effects of PNP exposure on testicular cell structure, we examined the ultrastructural changes in rats seminiferous tubules. As shown by electron microscopy (Fig. 3), the cell junctions in the control testes were intact, with no structural changes (Fig. 3A), and spermatogenic cells at all developmental stages were found. In the PNP-treated group, vacuoles between Sertoli cells (Fig. 3B) and spermatogonia (Fig. 3C) were observed in some sections (Table 4); the typical morphological changes of germ cells apoptosis were detected, including pyknosis (Fig. 3D) and apoptotic bodies (Fig. 3E). Moreover, mitochondrial sheath deletion of microscler spermatids (Fig. 3F) and lipid droplet

Table 4
Effects of PNP on morphology of testes in rats.

	Number of testicular germ cells (per seminiferous tubules)	Vacuoles (%)	Mitochondrial sheath deletion (%)
Control	317.0 ± 30.1	10.7 ± 1.4	5.5 ± 0.5
PNP	307.4 ± 29.3	35.3 ± 3.4**	27.6 ± 2.8**

The values shown are the mean ± SEM of five animals per group.
** $P < 0.01$ versus corresponding control.

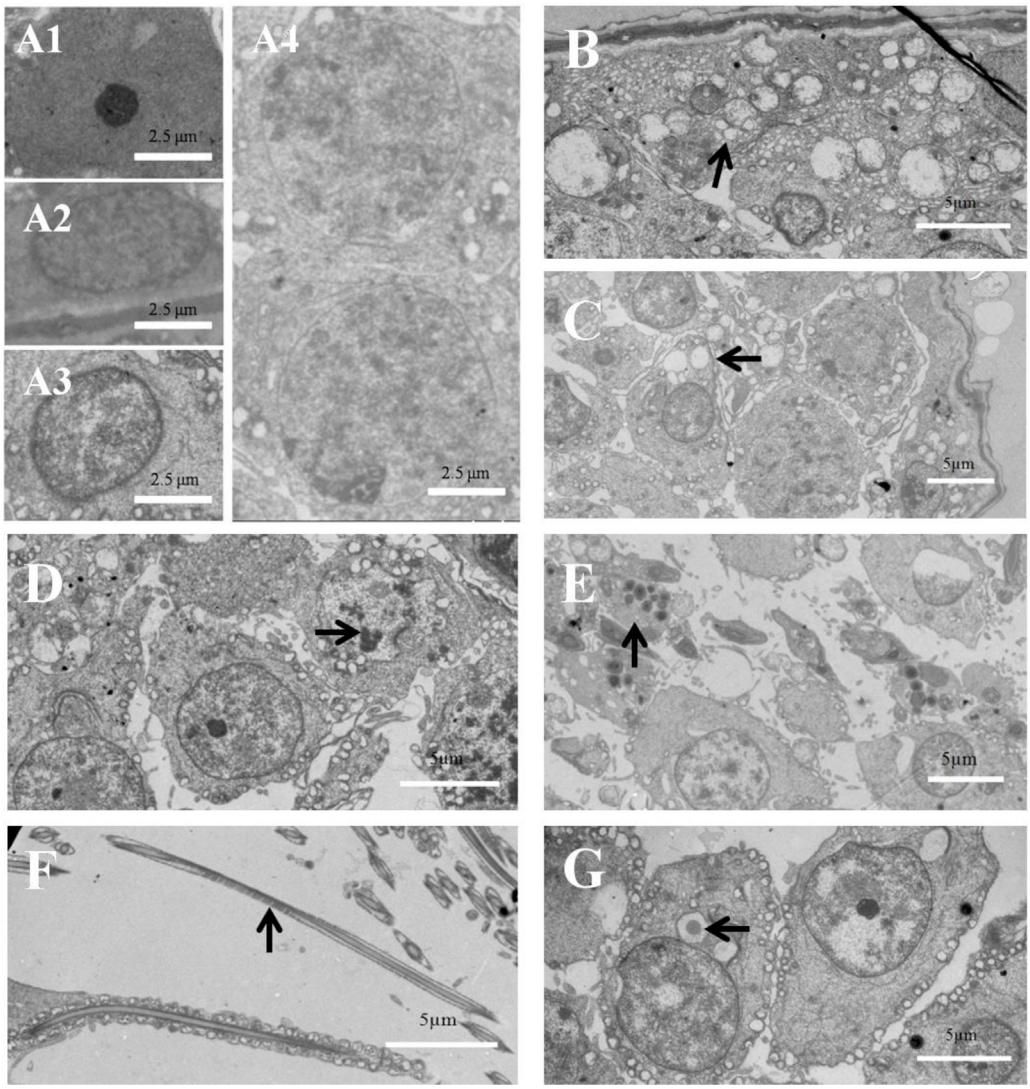

Fig. 3. Electron micrographs of testis tissues from control and PNP-treated rats. Tissues from the control rats appeared normal (A), but the PNP-treated rats showed salient histopathological features, including vacuoles between Sertoli cells (B) and spermatogonia (C), pyknosis (D) and apoptotic bodies (E) in germ cells. Moreover, mitochondrial sheath deletion of microscler spermatids (F) and lipid droplet deposition in spermatocytes (G) were also detected in the PNP-treated group.

deposition in spermatocytes (Fig. 3G) were also detected in the PNP-treated group.

3.6. Immunohistochemistry

Immunostaining for caspase-3, mainly distributed in spermatocytes and Leydig cells, was stronger in the testes of rats in the PNP-treated group (Fig. 4C) than in the control group (Fig. 4A). However, in combination with PS, this effect was mitigated compared with PNP treatment alone (Fig. 4D).

Nrf2 expression was low in the control group (Fig. 5A) and the PS group (Fig. 5B). In contrast, Nrf2 showed prominent nuclear accumulation in spermatids in all testes of the PNP-treated rats (Fig. 5C), and this effect was not significantly reversed in the PNP + PS group (Fig. 5D).

3.7. Western blotting

The primary control of Nrf2 transcriptional activation of target gene induction relies on subcellular distribution in response to oxidative stress, and activation of Nrf2 causes a translocation from the cytosol into the nucleus. To investigate whether the observed protective effect of PS was associated with nuclear translocation of Nrf2, we examined the localization of the Nrf2 protein in cytoplasm and nuclei in the testes following exposure to PNP with or without PS treatment. Immunoblotting for Nrf2 showed that there was increased nuclear translocation and decreased cytosolic expression in the PNP-treated groups with or without PS treatment (Fig. 6B). However, there was also increased nuclear translocation upon PS treatment alone (Fig. 6B).

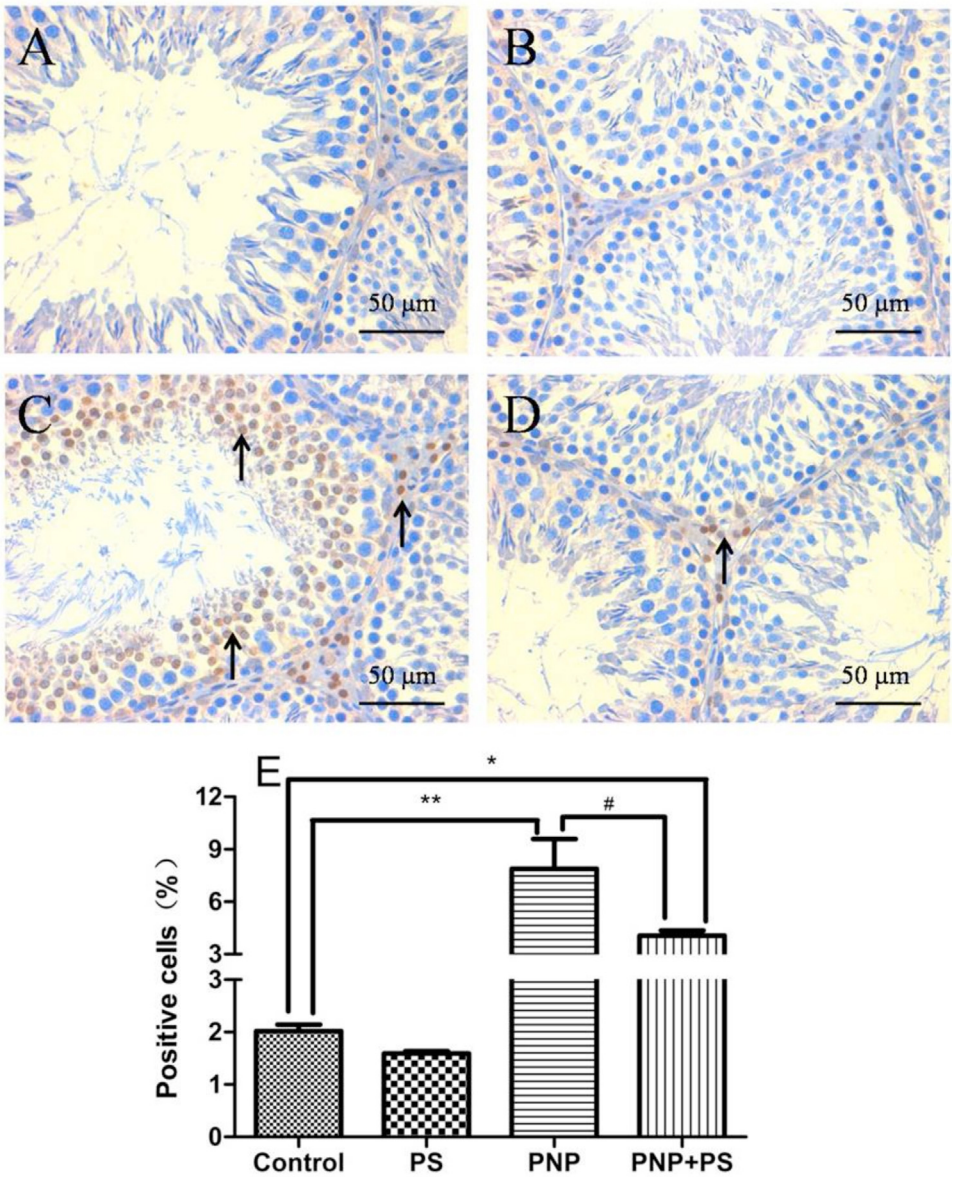

Fig. 4. Immunohistochemical detection of caspase-3 in the testis of control and experimental rats. Caspase-3, mainly distributed in spermatocytes and Leydig cells, was stronger in the testes of the PNP-treated group (C) than of the control group (A) or the PS group (B). However, this effect was mitigated in the PNP + PS group (D). analysis of cells positive for caspase-3 (E). $^{*}P < 0.05$, $^{**}P < 0.01$ versus corresponding control; $^{\#}P < 0.05$ versus corresponding PNP + PS group.

3.8. Expression of Nrf2-regulated genes

The mRNA transcript levels of Nrf2 were significantly decreased ($P < 0.05$) in PNP-treated testes compared with those in the control group; however, in combination with PS, the down-regulation of Nrf2 caused by PNP was attenuated (Fig. 7A). The mRNA transcript levels of heme oxygenase 1 (HO-1) and γ-glutamylcysteine synthetase (GCLC) were significantly upregulated ($P < 0.05$) in PNP-treated testes; both HO-1 and GCLC mRNA levels had a tendency to return to baseline levels with consumption of the PS-supplemented diet (Fig. 7B and C). There was no significant difference in NAD(P)H: quinone oxidoreductase 1 (NQO1) mRNA between the groups, although there was a slight increase in PNP-treated groups (Fig. 7D).

4. Discussion

The results of our present study clearly demonstrated that PNP treatment led to oxidative stress and germ cell damage in male rats, although subcutaneous injections of PNP did not manifest toxicity principally toward growth and development, as demonstrated by the unchanged body and organ weights.

Our previous reports suggested that PNP had estrogenic and anti-androgenic activities in vitro in a recombinant

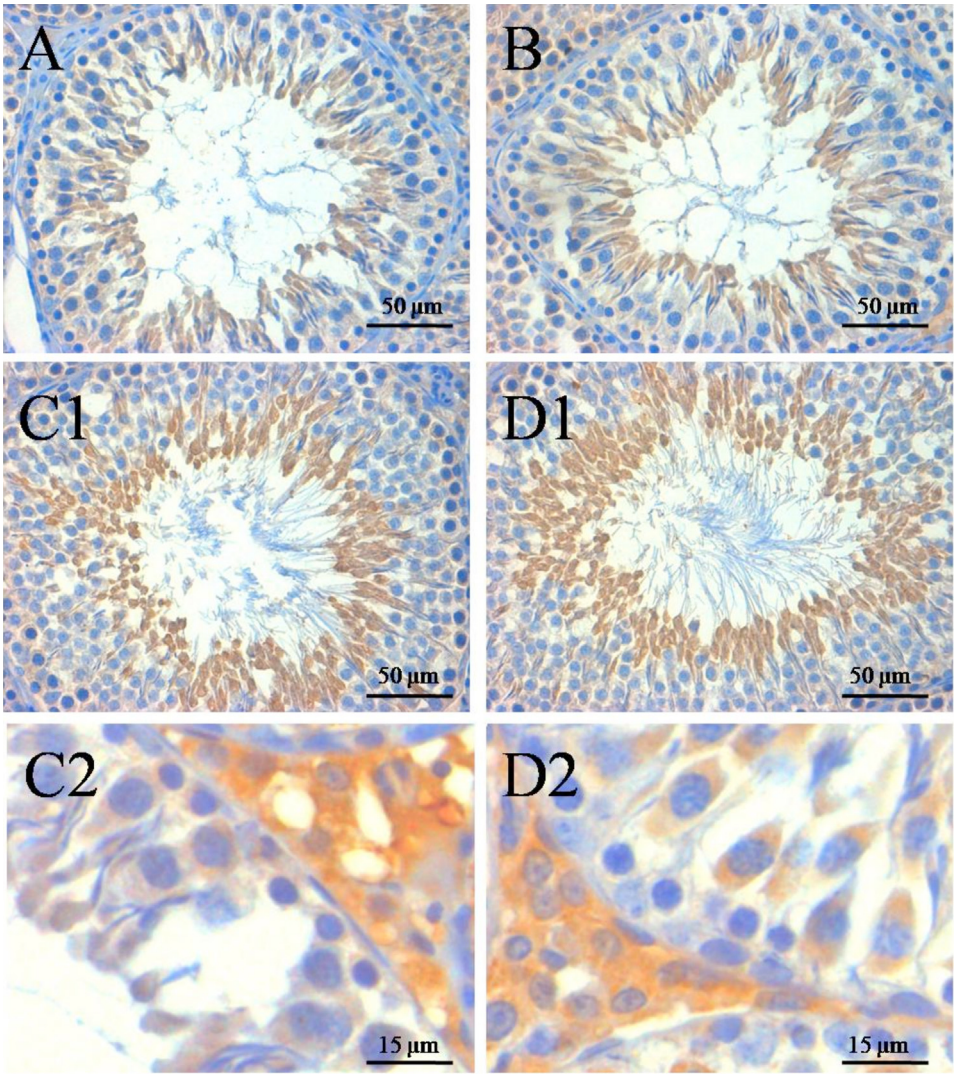

Fig. 5. Immunohistochemical detection of Nrf2 in the testes of control and experimental rats. Nrf2 expression was low in the control group (A) and the PS group (B). In contrast, Nrf2 showed prominent nuclear accumulation in spermatids in all testes of the PNP-treated rats (C), and this effect was not significantly reversed in the PNP + PS group (D).

yeast screening assay [30] and in vivo in immature rat uterotrophic and Hershberger assays [23]. In the present research, PNP treatment caused significant decreases in testosterone. The differences between these results and previous reports, which showed increased testosterone levels after PNP exposure [6,31], may be due to the differences in PNP dosage, i.e. the property of EEDs – endocrine effects in low dosage and toxic effects similar to general poison in high dosage. Testosterone is synthesized by Leydig cells of male rats [32] and is known to play crucial roles in the regulation of spermatogenesis [33]. Caspase-3 and caspase-activated deoxyribonuclease are associated with testicular germ cell apoptosis resulting from reduced intratesticular testosterone [34]. PNP induced increased expression of caspase-3 in male Japanese quail [7]. In the present study, upregulation of caspase-3 expression was also observed accompanied with decreased testosterone levels. Furthermore, significant reduction of epididymal sperm counts could be partly due to the disrupted testosterone production.

Transmission electron microscopy revealed ultrastructural changes in the PNP-treated testes. Vacuoles were visible between Sertoli cells and spermatogonia, with the corresponding typical morphological changes of germ cells apoptosis, including pyknosis and apoptotic bodies. Moreover, mitochondrial sheath deletion of microscler spermatids and lipid droplet deposition in spermatocytes were also detected in the PNP-treated group. Because control of spermatogenesis is mediated by Sertoli and Leydig cells [35], all of these phenomena can impair spermatogenesis, and can explain the decline in sperm counts.

Oxidative stress is a common phenomenon in the pathological processes of many toxic substances. ROS are products of normal cellular metabolism and play functional

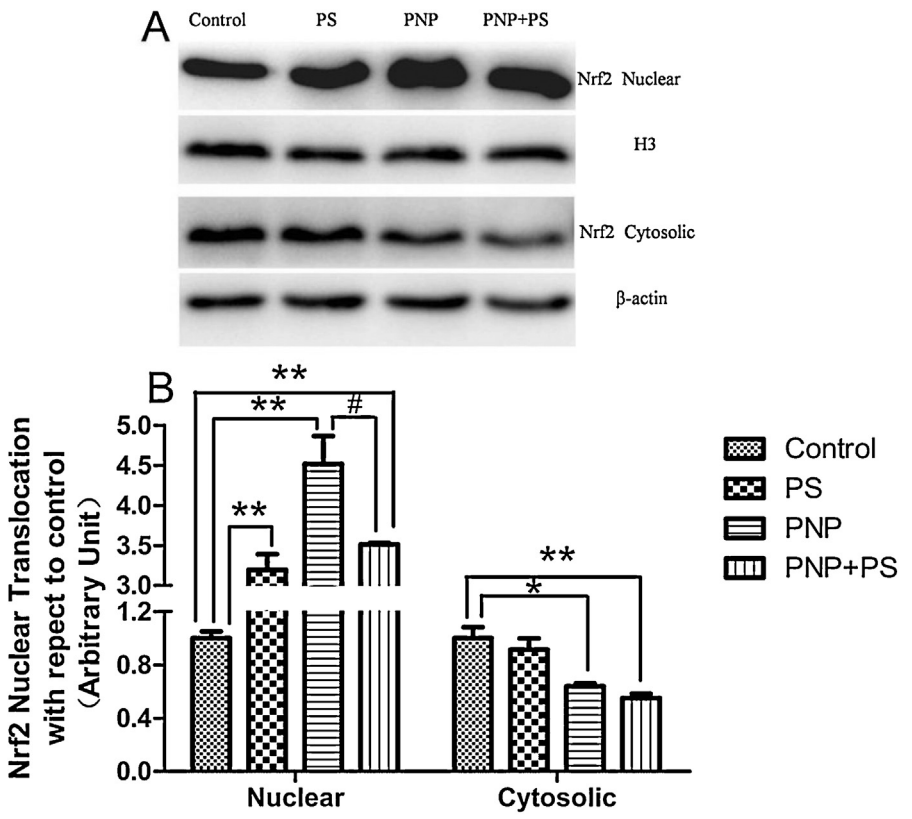

Fig. 6. Effects of PNP and PS on Nrf2 activation status. Immunoblot (A) and densitometric (B) analysis of Nrf2 protein in cytoplasm and nuclei. β-actin and H3 were used as cytosolic and nuclear loading controls, respectively. The test was repeated three times, representative blots are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the corresponding control; $^{\#}P < 0.05$ versus corresponding PNP + PS group.

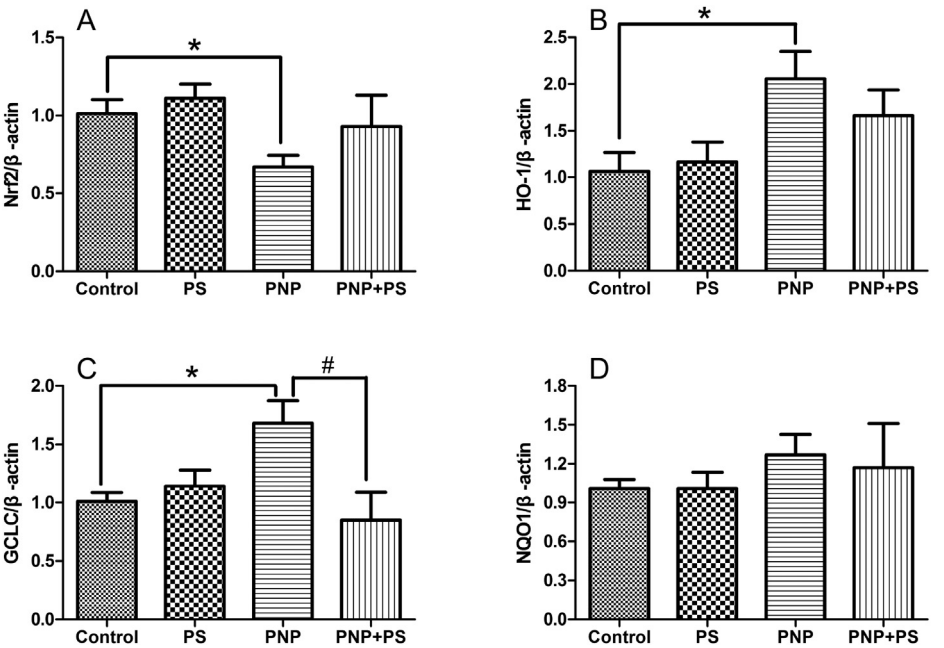

Fig. 7. Expression of Nrf2-regulated genes in the testes of control and experimental rats. Each mRNA was normalized to β-actin from the same preparation, and the mean of each experimental control was assigned a value of 1.0. A: Nrf2 mRNA; B: HO-1 mRNA; C: GCLC mRNA; D: NQO1 mRNA. The values shown are the mean ± SEM of five animals per group. $^{*}P < 0.05$ versus corresponding control and $^{\#}P < 0.05$ versus corresponding PNP + PS group.

roles as second messengers [36]. Low-level production of ROS by sperm supports physiological processes, such as capacitation and acrosome reactions [37]. Nevertheless, uncontrolled ROS production can cause aberrant sperm, and thus lead to infertility. Oxidative stress is an important factor in the etiology of oligospermia through peroxidative damage [38]. Our results demonstrated that PNP induced oxidative damage to the testes, characterized by increased production of MDA and decreased expression of antioxidants. These findings were similar to those of a previous study conducted in embryonic chicken testicular cells [39]. Thus, oxidative stress may be primarily responsible for the organ targeting as well as the testicular toxicity produced by PNP.

The expression of genes that encode most antioxidant enzymes is tightly controlled by Nrf2 activation [13]. Once the activation of Nrf2 is interrupted, those antioxidant enzymes cannot be expressed normally or in time. The Nrf2-antioxidant system is recognized as a prime molecular target against oxidative stress via accumulation in the nucleus to induce expression of antioxidant enzymes [40]. A previous study showed that the Nrf2-knockout mouse had high testicular lipid peroxidation levels and low antioxidant levels, resulting in lower epididymal sperm motility [41]. In this study, PNP induced Nrf2 accumulation in the nuclei of Leydig cells and germ cells. However, the decreased Nrf2 mRNA expression and cytosolic protein expression in the PNP-treated group suggested that Nrf2 synthesis was inhibited. This was likely to because the extended stress caused increased cytoplasmic activation and then increased nuclear accumulation of Nrf2. In addition, there were many factors antagonizing Nrf2 activity, such as nuclear factor-κB (NF-κB) and estrogen receptor α [42,43]. Therefore, the decreased Nrf2 expression might be attributed to other associated pathways.

HO-1, NQO1 and GCLC are cytoprotective genes regulated by Nrf2 [44]. HO-1 is expressed at a low level in testes, mainly in Sertoli and Leydig cells [45]. HO-1 derived from Leydig cells regulates apoptosis in premeiotic germ cells in response to stress [46]. NQO1 reduces quinones to hydroquinones to protect against oxidative stress [47]. GCLC combines Glu and Cys as the first step in GSH production [48]. Previous studies have reported that the activation of Nrf2 induced increased expression of HO-1, NQO1 and GCLC [49,50]. Indeed, consistent with the results mentioned above, the present study showed that PNP exposure significantly increased the expression of HO-1 and GCLC in testes, which confirmed the activation of the Nrf2 pathway and its role in protection against PNP-induced toxicity.

Although cytosolic protein expression of Nrf2 was not restored in the PNP + PS group, some parameters of antioxidants and antioxidant-related enzymes, including SOD, CAT, MDA and H_2O_2, showed trends toward partial elevation or reduction. Moreover, there was also increased nuclear translocation of Nrf2 upon PS treatment alone. Thus, the possibility of PS administration attenuating the effect of the PNP-induced oxidative damage to the reproductive system should not be disregarded, and further assessment is warranted. With respect to the mechanism of action of PS (Fig. 8), other associated pathways accompanied by Nrf2 pathways were thought to be related.

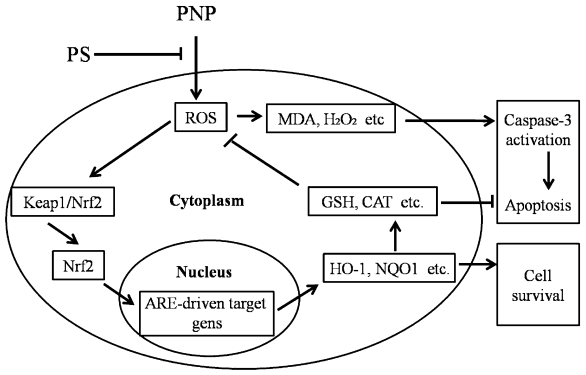

Fig. 8. Proposed model of PS-mediated protection against PNP-induced oxidative damage in rat testes.

To investigate the reproductive toxicity of PNP, a high-exposure dose (100 mg/kg) was used in the present study. Human exposure levels of PNP have not been reported clearly so far, and the dosage used in this study might be higher than for the overwhelming majority of people except for PNP workers. In fact, dose–response study of PNP had been done in the preliminary experiment, in which 0.1, 1,10 and 100 mg/kg PNP were selected, and significant reproductive effects occurred only in 100 mg/kg group. Both were the reasons that we choosed the dose of PNP, neither lower, nor higher. In any case, it is important and necessary to further clarify the relationship between rodent and human exposure levels to accurately estimate the health risk of PNP.

In conclusion, we discovered that PNP induced testicular oxidative damage, and demonstrated that supplementation with PS attenuated this effect in the present study. The mechanisms underlying these results support the idea that natural PS, as an antioxidant, protects against PNP-induced testicular oxidative damage, but these mechanisms require further investigation.

Conflict of interest

The authors do not have any conflicts to disclose.

Acknowledgments

This work was supported by the National "Twelfth-Five Year" Research Program of China (2012BAD39B02), The National Natural Science Foundation of Jiangsu Province (BK20131315), Qing Lan Project, National Nature Science Foundation of China (No. 31272485), Scientific Research Foundation for Returned Scholars, Ministry of Education of China ([2012]940), and Graduate Research and Innovation projects of Jiangsu Province (CXLX13-29).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxrep.2015.04.007.

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