Efficiency of naringin against reproductive toxicity and testicular damages induced by bisphenol A in rats

Soheila Alboghobeish [1], Masoud Mahdavinia [2*], Leila Zeidooni [3], Azin Samimi [3], Ali Akbar Oroojan [4], Saeid Alizadeh [3], Mohammad Amin Dehghani [3], Akram Ahangarpour [5], Layasadat Khorsandi [6]

[1] Department of Pharmacology, School of Medicine, Student Research Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[2] Department of Toxicology, School of Pharmacy, Jundishapur University of Medical Sciences, Ahvaz, Iran
[3] Department of Toxicology, School of Pharmacy, Student Research Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[4] Department of Physiology, Student Research Committee of Ahvaz Jundishapur University of Medical Science, Ahvaz, Iran
[5] Health Research Institute, Diabetes Research Center, Department of Physiology, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[6] Department of Histology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

ABSTRACT

Objective(s): Bisphenol A (BPA) as a synthetic compound is applied in many plastic industries. BPA has been reported to have endocrine-disrupting feature with cytotoxic effects. The study aimed to evaluate the efficiency of Naringin against testicular toxicity induced by BPA in adult rats.

Materials and Methods: The animals were assigned into six groups of control, BPA-treated (50 mg/kg), BPA+Naringin-administrated (40, 80, 160 mg/kg) and Naringin-treated (160 mg/kg) for 30 days. At the end of experiments, testicular weight, total testicular protein, epididymal sperm count, testicular enzymes, serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone and estradiol, testicular enzymatic and non-enzymatic antioxidants and histopathology of testis tissue were evaluated by their own methods.

Results: The results showed a reduction in testicular weight, total testicular protein, epididymal sperm count, testicular enzymes (alkaline phosphatase and lactate dehydrogenase) and decrease in the serum TSH, LH, testosterone and estradiol in BPA-administrated rats. Furthermore, BPA reduced the enzyme activities of glutathione peroxidase, superoxide dismutase, and catalase in testis tissue. Also, BPA caused an induction in lipid peroxidation and increase in reactive oxygen species levels, whereas it decreased the glutathione content of testis tissue. Histological findings exhibited seminiferous tubules vacuoles, atrophy and separation of the germinal epithelium in BPA-administrated rats. Oral administration of Naringin along with BPA normalized the biochemical, morphological and histological changes and reduced the testicular toxic condition.

Conclusion: These results demonstrated that Naringin significantly managed male reproductive toxicity by antioxidant capabilities, preventing morphological modifications and escalating defense mechanism, thereby reducing oxidative stress from BPA-induced damage.

Introduction

Statistical surveys show that 72.4 million couples in the world suffer from infertility problems (1), and about 90% of male infertility is due to low sperm count, a decrease in sperm quality, or both. There are many factors contributing to sperm defects, such as environmental pollutants, genetic abnormalities, alcohol or cocaine use, smoking and hormone deficiency (2). Bisphenol A (2, 2-bis (4-hydroxyphenyl) propane; BPA) is one of the most abundant chemical produced around the world (about 6 billion pounds a year) (3). This material that is used to make polycarbonate plastics and epoxy resins contains two unsaturated phenolic rings and is considered to be an estrogenic endocrine-disrupting chemical. Polycarbonate plastics and epoxy resins are used as coatings for metal cans and various plastic products such as toys, water pipes, drinking bowls, glasses, sports equipment, dental monomers and medical equipment (4). It has been reported that BPA may release from the walls of containers (5) and penetrate into the blood circulation and exert the genotoxic and cytotoxic effects (6). Also, heating of plastic cans exacerbates the BPA release (7). Given the high availability and side effects of the BPA on infertility, it is possible that increased contact with the BPA may be the underlying cause of increased infertility and breast cancer in industrialized countries over the past 50 years (3, 8, 9). In addition, *in vitro* studies have shown that the BPA binds to the receptors of estrogen (10), androgen, and thyroid hormones as well as peroxisome proliferator-activated receptor gamma (11). Most studies on male rodents reported that the BPA reduces fertility factors. For example, exposure to the BPA has been reported to reduce sperm count (7, 12, 13), and sperm motility (14), and increase sperm DNA impairment in rats and mice (15-17). Furthermore, the BPA exposure caused a decrease in testosterone level in rats (15, 18) and mice (19). It is known that the BPA produces reactive oxygen species (ROS), such as hydroxyl radical, hydrogen peroxide, and superoxide anion in the body (20). In addition, the BPA can exert reproductive toxicity and damage spermatogenesis

*Corresponding author: Masoud Mahdavinia. Department of Toxicology, School of Pharmacy, Jundishapur University of Medical Sciences, Ahvaz, Iran. Fax: +98-613-333-2036; Email: Mahdavinia-M@ajums.ac.ir

through production of ROS and impairment of natural antioxidant (21). The use of pharmaceutical properties of plants in the treatment of male reproductive toxicity is gaining more momentum due to its availability and affordability (22).

Naringin (NG) is a flavone found in citrus fruits, tomatoes, cherries, grapefruit, and cocoa (23). This compound acts as a nontoxic natural product with several functions, such as anticancer (24), anti-oxidative (25), anti-inflammatory (26), nephron-protective (27) and hepatoprotective activities (28). In one study, it was reported that NG restored normal testicular function, including sperm parameters in type 1 diabetic rats (29). With this background in mind, the present study aimed to assess the effect of BPA against the testicular function of Wistar rats and the therapeutic efficacy of NG in this regard. It is notable that the research was conducted using biochemical and histological methods.

Materials and Methods
Chemicals
BPA and NG were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).). Bovine serum albumin (BSA), 2,7- di chloro fluorescein diacetate (DCFH-DA), 3,4 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide(MTT),thiobarbituricacid(TBA),trichloroacetic acid (TCA), 1,1,3,3-tetramethoxypropane, reduced glutathione, oxidized glutathione, and Coomassie Brilliant Blue powder were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Sucrose 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB), dimethyl sulfoxide (DMSO), NaCl, KCl, $CaCl_2$, $MgCl_2$ and $NaHCO_3$ were obtained from Merck Company (Darmstadt, Germany). Glutathione peroxidase (GSH-Px; Cat No. S0058), and glutathione (GSH) assay kit (Cat. No. S0052) were purchased from Beyotime institute of Biotechnology (Jiangsu, China).

Study animals
This experimental study was performed on 36 male Wistar rats aged 44-48 days weighing 160-180 g prepared from the Animal Center of Ahvaz Jundishapur University of Medical Sciences (AJUMS). The rats were tested in this study according to the guidelines of the Animal Ethics Committee (IR.AJUMS.REC.1395.141). They were kept at a temperature of 20 ± 4 °C with a 12/12 hr light/dark cycle and free access to standard diet and water.

Study test design
The rats were divided into six groups with six rats in each group.

Group 1: The animals received 1 ml of olive oil emulsion for 30 days orally (control group).

Group 2: The animals received 1 ml of BPA (50 mg/kg body weight) in olive oil for 30 days orally.

Groups 3, 4 and 5: The animals received 1 ml of BPA (50 mg/kg body weight) in olive oil plus NG (40, 80 or 160 mg/kg body weight) for 30 days orally.

Group 6: The animals only received NG (at a concentration of 160 mg/kg body weight) for 30 days orally.

Overnight after the trial, the animals were sacrificed by cervical dislocation under mild anesthesia. Plasma samples were collected from the heart blood and centrifuged at 3500 rpm for 20 min and stored at -80 °C until the hormonal testing. The right and left testicles of all animals were immediately dissected and the testicular weight and morphology (width, length, and volume) were examined in each group. The testicular volume was calculated using the following equation: Volume = (D2/4 × π) L × K (length [L], width [D], K = 0.9, π = 3.14) (30).

Rat sperm count
Epididymis cauda was separated from the testicles of each rat, and after conversion into small pieces, 3 ml of normal saline was added. For sperm count, one drop of the above solution was transferred on a Neubauer chamber (depth 0.100 mm and area 0.0025 mm^2; HBG Henneberg-Sander GmbH, Gießen, Germany) and sperm count was performed manually using an optical microscope (Olympus Light Microscope; Olympus Corp, Japan). At the end, the data were calculated as the sperm count per ml (31).

Sample collection
In this stage, 0.1 mol/liter hydrochloric acid buffer (pH = 7.4) was added to the right testis and mixed with a homogenizer and centrifuged at 3000 rpm for 15 min.

Estimation of total testicular proteins
The total testicular proteins were determined using the Lowry method (32) and using BSA (1 mg/ml) as the standard. The absorbance was read by spectrophotometer at a wavelength of 750 nm.

Determination of alkaline phosphatase and lactate dehydrogenase enzymes in rat testicles
Diagnostic kits using spectrophotometer were applied to determine the alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) enzymes in testicular homogenization.

Measurement of the ROS level in the rat testicles
The ROS level in testicular tissue was measured using DCFDA, which was converted to DCF fluorescence by cell peroxides. In a nutshell, 2 ml of testicular homogenization was mixed with 4 ml of DCFDA in 1.25 mM of methanol and incubated at 37 °C for 15 min. Fluorescence level was measured using a fluorimeter (24, 33).

Measurement of glutathione in rat testicles
The GSH levels were measured using the method of Thomas and Screnska. Thus, 1 ml of testicular homogenization was incubated with 1 ml of 20% TCA and 1 ml of EDTA for 5 min and then centrifuged at 1000 rpm in 4°C for 30 min. Next, 200 μl of supernatant was mixed with 1.8 ml of DTNB. GSH reacts with DTNB and forms a yellow compound. The absorbance was read at a wavelength of 412 nm (34).

Measurement of malondialdehyde level in rat testicles
The level of lipid peroxidation was determined by measuring malondialdehyde (MDA). Thus, 1 ml of testicular homogenization was heated with 1 ml of 20% TCA and 2 ml of 0.67% TBA for 1 hr in boiling water. After cooling down, the mixture was centrifuged and

Table 1. Effect of bisphenol A (BPA) and naringin (NG) on final body weight, testis weight and testicular morphology (width, length and volume)

Parameters Treatment	Body weight (g)	Testis weight (g)	Testis length (mm)	Testis width (mm)	Testis volume (mm3)
Control	230.14±3.12	1.5±0.03	2.01±0.03	1.34±0.05	1.83±0.04
BPA 50 mg/kg	225.20±1.13	0.90±0.01*	1.85±0.1	1.07±0.15	1.368±0.12**
BPA 50 mg/kg + NG 40 mg/kg	224±5.7	1±0.03*	1.95±0.2	1.21±0.06	1.623±0.13#
BPA 50 mg/kg + NG 80 mg/kg	227±4.8	1.1±0.05	2±0.11	1.2±0.09	1.65±0.1#
BPA 50 mg/kg + NG 160 mg/kg	229.56±2.02	1.40±0.10#	2.08±0.09	1.2±0.07	1.716±0.08##
NG 160 mg/kg	230.44±4.30	1.5±0.22	2.12±0.07	1.3±0.07	1.895±0.07

Data are Mean±SDSEM; n= 6. Difference between control and other groups is significant at $P< 0.01$ (**) and $P<0.05$ (*). Difference between BPA-treated and other groups is significant at $P<0.01$ (##) and $P<0.05$ (#). P-values were from one-way ANOVA, followed by Tukey's test for multiple comparisons

the absorbance of supernatant was read at a wavelength of 532 nm. The MDA level was calculated using a molar coefficient of è = 1.56 × 105/M/cm (35).

Measurement of catalase in rat testicles
The catalase was measured using Goth method. Thus, 500 μl of Tris-HCl, 1 ml of H_2O_2 and 50 μl of the sample were mixed and incubated for 10 min and then 500 μl of ammonium molybdate 4-hydrate was added and the absorbance was read at a wavelength of 410 nm (36).

Measurement of Superoxide dismutase level in rat testicles
The superoxide dismutase (SOD) activity was measured using a xanthine/xanthine oxidase system to generate superoxide radical. Optical density was determined using spectrophotometer (UV-1601, Shimadzu) at a wavelength of 550 nm (37). The SOD activity was expressed as a unit per milligram of protein (U /mg protein).

Measurement of glutathione peroxidase activity
The GSH-Px activity was calculated by measuring the oxidation level of reduced GSH to oxidized GSH via H_2O_2 catalyzed by GSH-Px. A laboratory kit was used for this purpose (Jiancheng Bioengineering, China) (38).

Measurement of hormones
ELISA kits were used to measure the hormones of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol and testosterone (DRG Instruments GmbH, Marburg, Germany).

Histopathological examination of rat testicles
After blood sampling, the left testicles of the animals were separated and placed in the Bouin's fluid for histopathological examination. After overnight, the samples were cleaned by alcohol dehydration in benzene and subjected to paraffin wax. Sections of 5 to 7 micrometers were prepared from the tissue and stained with hematoxylin/eosin staining method. Six microscopic slides were explored to evaluate tissue changes in each rat, and their average was calculated. The diameters of tubules and lumen were measured using Motic Images plus 2.0 image analysis software (Motic, Hong Kong, China). The height of the epithelium was calculated by removing the diameter of the lumen from the diameter of the tubules. For each animal, 150 tubules were examined (37, 39).

Statistical analysis
All results were expressed as mean ± SEM using Graph Pad Prism software (version 5.04) and then analyzed by one-way ANOVA and then by Tukey's test. Kruskal-Wallis and then Mann-Whitney U tests were used for data with non-normal distribution and non-homogeneous variance. $P<0.05$ was considered as statistically significance level.

Results

Effect of BPA and NG on body weight, testis weight and testicular morphology (width, length and volume)
According to the results, there was no difference in body weight between the research groups. In addition, the weight of testes significantly decreased after BPA treatment, in comparison with normal control rats. Furthermore, an increase in testis weight was indicated in the group treated with NG plus BPA ($P<0.05$). On the other hand, an insignificant difference was observed between the NG and control rats ($P>0.05$). The morphological findings of the rat testicles represented a decrease in testicular volume after BPA treatment ($P>0.05$), and a significant increase was observed in the volume after administration of NG plus BPA ($P<0.05$) (Table 1).

Effect of BPA and NG on total testicular protein
By using BPA, total testicular protein was significantly reduced compared to normal rats ($P<0.01$). In contrast, in rats that used NG plus BPA, there was a significant increase in total testicular protein content compared to rats that used only BPA ($P<0.05$) (Figure 1A).

Effect of BPA and NG on sperm count
The results showed that the sperm count was decreased significantly with the use of BPA compared to the control group ($P<0.05$), but after NG administration, it was close to normal compared to BPA-treated group ($P<0.05$); however, no significant difference was found

Alboghobeish et al.
IJ**MS**
Effect of naringin on testicular toxicity of bisphenol
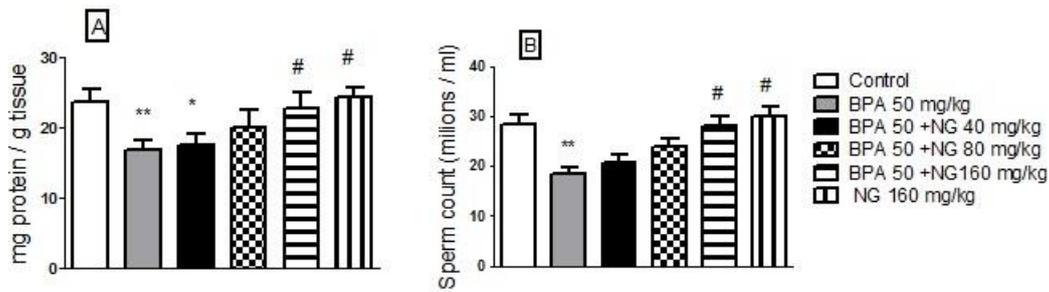
Figure 1. Effects of bisphenol A (BPA) and naringin (NG) on testis total protein (A) and sperm count (B). Each value was presented as means±SEM (n=6). *: Significantly different from control group (*P*<0.05), #: Significantly different from BPA-treated group (*P*<0.05), **: *P*<0.01. *P*-values were from one-way ANOVA, followed by Tukey's test for multiple comparisons

between the NG and the control groups (Figure 1B).

Effect of BPA and NG on testicular enzymes

Results demonstrated that the levels of ALP and LDH were significantly decreased in testis (*P*< 0.05) after BPA treatment. Furthermore, an increase (*P*<0.05) in ALP and LDH of testis was observed after the concomitant administration of NG plus BPA (Figure 2A, B).

Effect of BPA and NG on plasma levels of LH, FSH, testosterone and estradiol

The amount of FSH, LH, estradiol, and testosterone in plasma were significantly reduced due to BPA-induced toxicity (respectively, *P*<0.05, 0.05, 0.01), but after treatment with NG, there was a growth in the amount of these hormones. Furthermore, no change was

observed in this parameter in animals with independent administration with only NG (Table 2).

Effects on enzymatic and non-enzymatic antioxidants

After BPA intoxication, MDA and ROS amounts were significantly increased (*P*<0.05, 0.001), but their levels significantly decreased when NG was administered to BPA-intoxicated rats (respectively, *P*<0.05, 0.001). In addition we showed an induction in the GPx enzyme after BPA intoxication (*P*<0.05); however, this factor was elevated in NG-BPA co-treatment groups. The GSH level was reduced following the BPA intoxication, but incremented after NG-BPA intake (*P*< 0.01). On the other hand, SOD and CAT levels were significantly decreased after BPA intoxication (*P*<0.05), and increased after
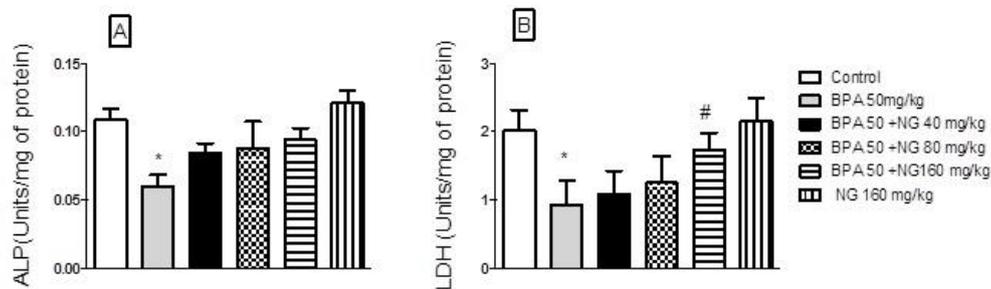
Figure 2. Effects of bisphenol A (BPA) and naringin (NG) on testicular homogenate enzymes: (A) Alkaline phosphatase (ALP), (B) lactate dehydrogenase (LDH). Each value was presented as means±SEM (n=6). *: Significantly different from control group (*P*<0.05), #: Significantly different from BPA-treated group (*P*<0.05). *P*-values were from one-way ANOVA, followed by Tukey's test for multiple comparisons

Table 2. Effect of bisphenol A (BPA) and naringin (NG) on hormonal levels in serum of control and experimental animals

Parameters / Treatment	Plasma FSH level (pg/ml)	Plasma LH level (pg/ml)	Plasma testosterone level (ng/ml)	Plasma estradiol level (ng/ml)
Control	33.30 ± 0.89	19.55 ± 2.10	3.98 ± 1.90	0.86 ± 0.07
BPA 50 mg/kg	12.42 ± 0.73**	9.18 ± 0.36**	1.07 ± 0.25*	0.58 ± 0.09*
BPA 50 mg/kg + NG 40 mg/kg	20.9±5.7##	10.76±0.03	1.95±0.2	0.68 ± 0.07
BPA 50 mg/kg + NG 80 mg/kg	19.75 ± 0.61##	15.27 ± 1.00##	2.04 ± 2.81	0.69± 0.04
BPA 50 mg/kg + NG160 mg/kg	21.95±2.02##	16.00±0.10##	3.08±0.09#	0.76± 0.06
NG 160 mg/kg	32.99 ± 1.24	19.09 ± 0.70	3.90 ± 3.03	0.83 ± 0.09

Data are Mean±SDSEM; n=6. Difference between control and other groups is significant at, *P*<0.01 (**) and *P*<0.05 (*). Difference between BPA-treated and other groups is significant at *P*<0.01 (##) and *P*<0.05 (#). FSH: follicle stimulating hormone; LH: luteinizing hormone. *P*-values were from one-way ANOVA, followed by Tukey's test for multiple comparisons

Table 3. Effect of bisphenol A (BPA) and naringin (NG) on enzymatic and non-enzymatic antioxidants

Groups / Variables	Control	BPA 50 mg/kg	BPA 50 mg/kg +NG 40 mg/kg	BPA 50 mg/kg +NG 80 mg/kg	BPA 50 mg/kg +NG 160 mg/kg	NG 160 mg/kg
MDA (nmol/mg protein)	6.65± 2.3	3.31± 0.7*	3.93± 0.4	4.83± 0.6	5.1±0.88#	6.6±0.81
ROS (% of Control)	100 ±10.1	197.87±12.61***	166.65±11.76	141.76±11,6##	117.64±14.7###	94.89±17.1
GSH (µg/ mg of protein)	0.7±0.09	0.2±0.01***	0.29±0.02	.042± 0.03	0.58±0.02##	0.8±0.01
Catalase (µmol/ mg of protein)	60.22±1.34	31.58±2.31*	42.3±3.8	53.7±2.9	53.90±1.72	61.28±1.87
SOD (U/mg of protein)	2.5±0.56	1.3±0.99*	1.54±0.29	1.72±0.59	2.02±0.48#	2.87±0.19
GSH-Px (µmol/ mg of protein)	2.3±0.47	1.2±0.17*	1.3±0.05	1.43±0.07	1.7±0.45#	2.4±0.99
MMP (% of Control)	100 ±16.7	64.87±12.61**	73.65±11.76	85.76±11,6#	95.64±14.7##	99.99±17.1

Data are Mean±SDSEM; n=6. Difference between control and other groups is significant at $P<0.001$(***), $P<0.01$ (**) and $P<0.05$ (*). Difference between BPA-treated and other groups is significant at $P<0.001$(###), $P<0.01$ (##) and $P<0.05$ (#). MDA: Malondialdehyde, ROS: Reactive oxygen species, GSH: Glutathione, SOD: Superoxide dismutase, CAT: Catalase, GSH-Px: Glutathione peroxidase, and MMP: Mitochondrial Mmembrane potential. *P*-values were from one-way ANOVA, followed by Tukey's test for multiple comparisons

treatment with NG ($P<0.05$) (Table 3).

Effects on testicular histopathology

The seminiferous tubule diameter and epithelial height were decreased in BPA-treated rats, compared to the control group ($P<0.001$). But co-administration of NG (80, and 160 mg/kg) with BPA significantly increased the tubular diameter, and epithelial height (respectively, $P<0.05$, 0.01, 0.001) (Figure 3 and Table 4). In the control and NG 160 mg/kg groups, testicular tissues appeared normal. In the BPA-treated group, atrophy and separation of the germinal epithelium was observed in the most of the seminiferous tubules vacuoles. In group BPA+NG 40 mg/kg, the separation was observed in some of the tubes vacuoles. In group BPA+NG 80 mg/kg, the testicular tissue damage was less than that of the BPA+NG 40 group. There was no separation in group NG
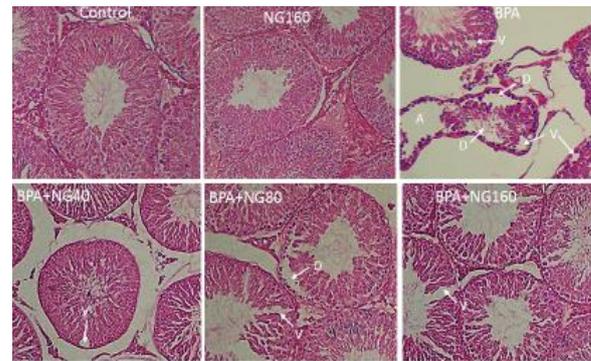
Figure 3. Effects of bisphenol A (BPA) and naringin (NG) on testicular histopathology (H&E, ×400). A: Atrophy, D: detachment of germinal epithelium, V: vacuoles in germinal epithelium
A: Atrophy; D: detachment; V: vacuoles in germinal epithelium

Table 4. Effect of bisphenol A (BPA) and naringin (NG) on tubular diameter and epithelial height

Treatment	Seminiferous tubule diameter (µm)	Epithelial height (µm))
Control	223.7±9.52	85.4±2.85
BPA 50 mg/kg	136.65±13.13***	56.92±0.61***
BPA 50 mg/kg + NG 40 mg/kg	139.65±5.7***#	49.58±1.33***#
BPA 50 mg/kg + NG 80 mg/kg	147.52±4.8**##	64.53±2.5**##
BPA 50 mg/kg + NG160 mg/kg	206.56±12.02*###	83.35±3.10###
NG160 mg/kg	219.59±4.30	86.64±2.22

Data are Mean±SDSEM; n=6. Difference between control and other groups is significant at $P<0.001$(***), $P<0.01$ (**) and $P<0.05$ (*). Difference between BPA-treated and other groups is significant at $P<0.001$(###), $P<0.01$ (##) and $P<0.05$ (#). *P*-values were from one-way ANOVA, followed by Tukey's test for multiple comparisons

160 mg/kg and only some tubes had vacuoles.

Discussion

Increased toxic effects of drugs and environmental substances have been recently reported on reproductive system (40). Plants and their bioactive components are the most extensively accessible materials that have the ability to scavenge free radical ions (41). Therefore, this context investigated the possible effects of NG treatment against BPA-induced reproductive toxicity and testicular damage in adult male rats. The significant decrease in testicular mass and volume may be due to reduced bioavailability of gender hormones, which points to the reproductive endocrine stipulation of the male. Testis weight and volume are directly related to the mass of the spermatogenic cells. Therefore, reduced testis mass and volume affects spermatogenesis activity with decreased germ cells in turn. In addition, it was noted that BPA intoxication decreases the testis weight and volume of the experimental rats by damaging the important molecules, such as proteins in the testis. These results were in line with studies reporting the testicular weight loss because of exposure to BPA (42). Furthermore, we showed an increase in the testes weight and volume in NG-BPA co-treated animals due to therapeutic efficacy and protective potential of NG.

In addition, we showed a reduction in amount of testicular total protein of BPA-treated rats. Previous studies have suggested that environmental testicular toxicants directly exert antagonistic effects on proteins and lipids of reproductive organs (43). Multiple stimulatory and inhibitory factors are in testicular fluid that selectively change the production of proteins (18). As a result, alteration in testicular protein level proposed a decrease in the synthetic activity of testes. In addition, we showed that NG can somewhat reduce the testicular total protein level.

The epididymal sperm count is among the most sensitive tests used to evaluate the spermatogenesis due to providing the result of all stages of meiosis, spermatogenesis, and transition in the epididymis. It is suggested that a reduction in sperm count is related to male infertility (44). In our study, NG treatment increased the sperm count, which might be due to suppression of the disturbances induced by free radicals in sperm.

LH, FSH and testosterone are essential hormones for spermatogenesis. The LH hormone is secreted from the pituitary gland and triggers the testosterone production. On the other hand, there is a need for testosterone to produce and maintain sperm (45). In addition, FSH and testosterone stimulate spermatid growth and sperm release (46). In our study, the BPA administration reduced the levels of LH, FSH and testosterone, possibly due to effects on Sertoli and Leydig cells. The results of our study were consistent with the findings of previous studies, in which the BPA caused a change in the hypothalamic-pituitary-gonadal axis and decreased sertoli cell function (47). In addition, previous studies suggested that reducing testosterone in rats treated with BPA may be due to interference with the proliferation and function of Leydig cells (48). According to Akingbemi *et al.* (49), the BPA has an effect on the pituitary and disrupts testicular activity, thereby reducing the LH release. The BPA has also been reported to reduce the testosterone production from Leydig cells by reducing the activity of 17α-hydroxylase or increasing aromatase activity (50).

However, the plasma levels of LH, FSH and testosterone were higher in animals treated with NG-BPA than in animals treated with BPA, which is consistent with increased spermatogenesis in the NG-treated group. This effect may be due to the direct or indirect effect of NG on the oxidative damage of cells. These results were consistent with previous reports in which NG treatment increased testosterone levels in diabetic rats (29). According to our results, estradiol level is reduced in BPA-exposed groups. Previous studies reported that low BPA doses have estrogen-inducing activity, while high BPA doses inhibit estrogen production (51). In addition, our results showed that NG was able to normalize the effects of changes caused by BPA on the estradiol levels.

Marker enzymes are sensitive indicators that are released when impairment happens in a tissue. It is an indicator used to evaluate the management of diseases. Most of the particular enzymes, such as LDH and ALP, are affected when there is an impact on homeostasis in the biological system. LDH is a cytoplasmic bi-directional enzyme capable of forming pyruvate and lactate in cells. In addition, LDH extensively exists in Sertoli and spermatogenic cells, which is importantly involved in testis energy generation, biotransformation and translocation of hydrogen from the cytoplasm to mitochondria by redox coupling α-hydroxyl acid/α-keto acid associated with spermatozoa metabolism (43). The LDH enzyme in testicles is associated with the maturation of germinal epithelial layer of seminiferous tubules and postmeiotic spermatogenic cells (18, 52). BPA-treated animals indicated a significant induction in LDH activity. In contrary, the administration of NG enhanced the activity of this enzyme. The testicular levels of ALP enzyme in rats declined after BPA administration, but increased by NG administration. The ALP enzyme in testicles is mainly of testicular and epididymal origin (53). In our study, reduced ALP activity revealed that the treatment with BPA may decrease the activity of testicular tissues.

Free radicals and lipid peroxidation are two leading factors in testicular pathology. The lipid peroxidation occurs in unsaturated lipids and is involved in the formation of active oxygen (54). On the other hand, the sperms are subject to increased lipid peroxidation because of the prevalence of unsaturated fatty acids in their membranes (55). In the present study, there was a significant increase in the incidence of testicular lipid peroxidation, probably due to the effect of BPA-induced oxidative damage. In this regard, previous study marked an induction in ROS generation due to the association of lipid peroxidation with abnormalities and decreased sperm counts (56). Spermatozoa are generally susceptible to injury, which is attributed to the deliberation of unsaturated fatty acids and also the ability of spermatozoa to generate ROS (57). BPA-motivated oxidative stress may involve in the destruction of intracellular ATP production, which directly displays the effect on declined sperm motility, improved cell penetrability, inactivation of biological enzymes and

manufacturing of spermicidal products (58).

In contrast, the effects of NG may be due to its protective effects on increased activity of lipid peroxidation or the production of free radicals. NG can protect the cell from oxidative damage by inhibiting intracellular ROS production and eliminating the produced ROS (23). To confirm this, previous studies have shown the protective role of NG against ROS-induced cytotoxicity by using arsenic (59), carbon tetrachloride (60), lead (61), cadmium (62), or cisplatin (63) in rats. It has been suggested that the NG may be coupled with Cu and Fe ions through the 5-hydroxy and 4-carbon groups in their C ring, thereby reducing the ROS (64). The NG has also been reported to stimulate expression of many antioxidant-related genes and inhibit the activity of ROS-forming enzymes such as NADPH oxidase (65).

Degradation and eventual rupture of the cellular membrane that leads to the release of cell organelle substances may be due to lipid peroxidation (66). In addition, it was reported that the BPA might be associated with the damage in mitochondrial membrane (67). Thereby, NG displayed anti-lipid peroxidation activity and protective effect on cell membrane against ROS by its free radical scavenging property.

The oxidative stress is an imbalance between the amount of free radicals production and the presence of antioxidants. The antioxidant defense system contains molecular antioxidants, antioxidant enzymes and metallic chemical agents (68). Antioxidant enzymes (e.g., SOD, CAT and GPx) protect the living system from the destructive effects of ROS and reduce their oxidative damage to testicular cell membranes (69). Reducing SOD activity results in the accumulation of superoxide radicals, which in turn inhibits the CAT enzyme (70). Reducing CAT activity decreases the ability of the testicles to eliminate H_2O_2 produced after exposure to BPA. The H_2O_2 can cause rapid oxidative damage to lipids, proteins and DNA (71). In addition, the GPx may act directly as an antioxidant enzyme, which is involved in inhibition of sperm lipid peroxides (72) and H_2O_2 (73). The low GPx level in BPA-exposed rats can lead to an increase in H_2O_2 production or a decrease in GSH concentration (18). In the present study, the antioxidant enzymes (such as SOD, CAT and GSH-Px) were reduced in BPA-exposed animals, which is consistent with the results of Chitra *et al.* study (74).

The GSH plays an important role in cellular function, including H_2O_2 destruction, lipid peroxidation, and the amino acid translocation in the cell membrane (75). In the present study, reduced testicular GSH level in BPA-exposed rats may be responsible for increasing ROS in the testes. In addition, reduced activity of antioxidant enzymes such as CAT, GPx, and SOD highlights the harmful effects of BPA. NG, on the other hand, reduces ROS-induced oxidative stress by stopping the lipid peroxidation in the testicle and increasing the enzymatic and non-enzymatic antioxidants.

According to the histological results, a decrease was observed in the seminiferous tubules diameter and the epithelial height in the BPA-administrated rats, which is in agreement with Tamilselvan *et al.* study (8). In contrast, treatment with NG shifted this change towards normal status, probably due to the medicinal property of this glycoside. The results of the current

research showed several vacuoles in the seminiferous tubule epithelium in the BPA-treated group. Meanwhile, there was a reduction in the count and size of these vacuoles in the BPA+NG-administrated group. In the BPA-treated group, atrophy and separation of the germinal epithelium was observed. On the contrary, the tissue was significantly protected against BPA-induced histopathological changes by NG administration.

Conclusion

Based on the findings from the present study, the BPA exposure resulted in structural and functional impairments in rat testes and epididymis, leading to reproductive toxicity, spermatogenesis impairment and disturbing of the hormonal balance. However, administration of NG reduced the BPA-induced reproductive toxicity through modulating the lipid peroxidation level, decreasing the free radicals and maintaining homeostasis, emphasizing the potential therapeutic role of NG against BPA-induced reproductive toxicity.

Acknowledgment

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

References

1. Gurunath S, Pandian Z, Anderson RA, Bhattacharya S. Defining infertility a systematic review of prevalence studies. Hum Reprod Update 2011;17:575-588.
2. Meeker JD. Exposure to environmental endocrine disrupting compounds and men's health. Maturitas 2010;66:236-241.
3. Mohapatra D, Brar S, Tyagi R, Surampalli R. Physico-chemical pre-treatment and biotransformation of wastewater and wastewater sludge fate of bisphenol A. Chemosphere 2010;78:923-941.
4. Vandenberg L, Hauser R, Marcus M, Olea N, Welshons W. Human exposure to bisphenol A (BPA). Reprod Toxicol 2007; 24: 139–177.
5. Geens T, Aerts D, Berthot C, Bourguignon J-P, Goeyens L, Lecomte P, *et al.* A review of dietary and non-dietary exposure to bisphenol-A. Food Chem Toxicol 2012;50:3725-3740.
6. Dobrzyńska MM, Jankowska-Steifer EA, Tyrkiel EJ, Gajowik A, Radzikowska J, Pachocki KA. Comparison of the effects of bisphenol a alone and in a combination with X-irradiation on sperm count and quality in male adult and pubescent mice. Environ Toxicol 2014;29:1301-1313.
7. Dobrzyńska MM, Radzikowska J. Genotoxicity and reproductive toxicity of bisphenol A and X-ray/bisphenol A combination in male mice. Drug Cheml Toxicol 2013;36:19-26.
8. Tamilselvan P, Langeswaran K, Vijayaprakash S, Revathy R, Balasubramanian MP. Efficiency of lycopene against reproductive and developmental toxicity of bisphenol A in male Sprague Dawley rats. Biomed Prevent Nutr. 2014;4:491-498.
9. Muñoz-de-Toro M, Markey CM, Wadia PR, Luque EH, Rubin BS, Sonnenschein C, *et al.* Perinatal exposure to bisphenol-A alters peripubertal mammary gland development in mice.

Endocrinology. 2005;146:4138-4147.

10. Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, Van Der Saag PT, *et al.* Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology 1998;139:4252-3263.

11. Richter CA, Birnbaum LS, Farabollini F, Newbold RR, Rubin BS, Talsness CE, *et al. In vivo* effects of bisphenol A in laboratory rodent studies. Reprod Toxicol. 2007;24:199-224.

12. Tainaka H, Takahashi H, Umezawa M, Tanaka H, Nishimune Y, Oshio S, *et al.* Evaluation of the testicular toxicity of prenatal exposure to bisphenol A based on microarray analysis combined with MeSH annotation. J Toxicol Sci 2012;37:539-548.

13. Tiwari D, Vanage G. Mutagenic effect of Bisphenol A on adult rat male germ cells and their fertility. Reprod Toxicol 2013;40:60-68.

14. Salian S, Doshi T, Vanage G. Neonatal exposure of male rats to Bisphenol A impairs fertility and expression of sertoli cell junctional proteins in the testis. Toxicology 2009;265:56-67.

15. D'Cruz SC, Jubendradass R, Jayakanthan M, Rani SJA, Mathur PP. Bisphenol A impairs insulin signaling and glucose homeostasis and decreases steroidogenesis in rat testis: an in vivo and in silico study. Food Chem Toxicol 2012;50:1124-1133.

16. Kabuto H, Hasuike S, Minagawa N, Shishibori T. Effects of bisphenol A on the metabolisms of active oxygen species in mouse tissues. Environ Res 2003;93:31-35.

17. Fang Y, Zhou Y, Zhong Y, Gao X, Tan T. Effect of vitamin E on reproductive functions and anti-oxidant activity of adolescent male mice exposed to bisphenol A. J Hygiene Res. 2013;42:18-22.

18. El-Beshbishy HA, Aly HA, El-Shafey M. Lipoic acid mitigates bisphenol A-induced testicular mitochondrial toxicity in rats. Toxicol Ind Health 2013;29:875-887.

19. Xi W, Lee C, Yeung W, Giesy JP, Wong M, Zhang X, *et al.* Effect of perinatal and postnatal bisphenol A exposure to the regulatory circuits at the hypothalamus–pituitary–gonadal axis of CD-1 mice. Reprod Toxicol. 2011;31:409-417.

20. Ho Y-S, Magnenat J-L, Gargano M, Cao J. The nature of antioxidant defense mechanisms: a lesson from transgenic studies. Environ Health Perspect 1998;106:1219-1225.

21. Hales DB, Allen JA, Shankara T, Janus P, Buck S, Diemer T, *et al.* Mitochondrial function in Leydig cell steroidogenesi: Ann N Y Acad Sci 2005;1061:120-134.

22. Abdel-Monaim M, Abo-Elyousr K, Morsy K. Effectiveness of plant extracts on suppression of damping-off and wilt diseases of lupine (Lupinus termis Forsik. Crop Protect 2011;30:185-191.

23. Podder B, Song H-Y, Kim Y-S. Naringenin exerts cytoprotective effect against paraquat-induced toxicity in human bronchial epithelial BEAS-2B cells through NRF2 activatio. J Microbiol Biotechnol. 2014;24:605-613.

24. Jain A, Yadav A, Bozhkov A, Padalko V, Flora S. Therapeutic efficacy of silymarin and naringenin in reducing arsenic-induced hepatic damage in young rats. Ecotoxicol Environ Safet. 2011;74:607-614.

25. Kannappan S, Palanisamy N, Anuradha CV. Suppression of hepatic oxidative events and regulation of eNOS expression in the liver by naringenin in fructose-administered rats. Europ J Pharmacol 2010;645:177-184.

26. Fang F, Tang Y, Gao Z, Xu Q. A novel regulatory mechanism of naringenin through inhibition of T lymphocyte function in contact hypersensitivity suppression. Biochem Biophys Res Commun. 2010;397:163-169.

27. Renugadevi J, Prabu SM. Naringenin protects against cadmium-induced oxidative renal dysfunction in rats. Toxicology 2009;256:128-134.

28. Renugadevi J, Prabu SM. Cadmium-induced hepatotoxicity in rats and the protective effect of naringenin. Exp Toxicol Pathol 2010;62:171-181.

29. Akondi RB, Kumar P, Annapurna A, Pujari M. Protective effect of rutin and naringin on sperm quality in streptozotocin (STZ) induced type 1 diabetic rats. Iran J Pharm res 2011;10:585-393.

30. Ahangarpour A, Oroojan AA, Khorsandi L, Arzani G, Afshari G. Effects of betulinic acid on the male reproductive system of a streptozotocin-nicotinamide-induced diabetic mouse model. World J Men's Health 2016;34:209-216.

31. Ahangarpour A, Oroojan AA, Heidari H. Effects of exendin-4 on male reproductive parameters of d-galactose induced aging mouse model. World J Men's Health 2014;32:176-183.

32. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-275.

33. Ahangarpour A, Zeidooni L, Rezaei M, Alboghobeish S, Samimi A, Oroojan AA. Protective effect of metformin on toxicity of butyric acid and arsenic in isolated liver mitochondria and langerhans islets in male mice: an in vitro study. Iran J Basic Med Sci 2017;20:1297-1303.

34. Ahangarpour A, Alboghobeish S, Oroojan AA, Zeidooni L, Samimi A, Afshari G. Effects of combined exposure to chronic high-fat diet and arsenic on thyroid function and lipid profile in male mouse. Biol Trace Elem Res 2017:1-12.

35. Ahangarpour A, Alboghobeish S, Rezaei M, Khodayar MJ, Oroojan AA, Zainvand M. Evaluation of diabetogenic mechanism of high fat diet in combination with Arsenic exposure in male mice. Iran J Pharm Res 2017;3:184-193.

36. Ahangarpour A, Zeidooni L, Samimi A, Alboghobeish S, Khorsandi LS, Moradi M. Chronic exposure to arsenic and high fat diet additively induced cardiotoxicity in male mice. Res Pharm Sci 2018;13:47-53.

37. Ahangarpour A, Oroojan AA, Rezaei M, Khodayar MJ, Alboghobeish S, Zeinvand M. Effects of butyric acid and arsenic on isolated liver mitochondria and pancreatic islets of male mouse. Gastroenterol Hepatol Bed Bench. 2017:134-139.

38. Lawrence RA. Reprint of "glutathione peroxidase activity in selenium-deficient rat liver". Biochem Biophysic Res Com 2012;425:503-509.

39. Talebi AR, Khorsandi L, Moridian M. The effect of zinc oxide nanoparticles on mouse spermatogenesis. J Assist Reprod Genet 2013;30:1203-1209.

40. Shalaby KA, Saleh EM. Ameliorative effect of honey bee propolis on the nonylphenol induced-reproductive toxicity in male Albino rats. Aust J Basic Applied Sci 2011;5:918-927.

41. Agarwal A, Gupta S, Sikka S. The role of free radicals and antioxidants in reproduction. Curren Opin Obstet Gynecol 2006;18:325-332.

42. Anjum S, Rahman S, Kaur M, Ahmad F, Rashid H, Ansari RA, *et al.* Melatonin ameliorates bisphenol A-induced biochemical toxicity in testicular mitochondria of mouse. Food Chem Toxicol 2011;49:2849-2854.

43. Revathy R, Langeswaran K, Ponnulakshmi R, Balasubramanian MP, Selvaraj J. Ipomoea batatas tuber efficiency on bisphenol A-induced male reproductive toxicity in sprague dawley rats. J Biol Active Prod Nature 2017;7:118-130.

44. Working P, Chellman G. The testis, spermatogenesis and the excurrent duct system. Reprod Toxicol Infertil 1993:55-76.

45. Mantovani A. Hazard identification and risk assessment of endocrine disrupting chemicals with regard to developmental effects. Toxicology 2002;181:367-370.

46. Chauhan A, Agarwal M, Kushwaha S, Mutreja A. Suppression of fertility in male albino rats following the administration of 50% ethanolic extract of *Aegle marmelos*. Contraception 2007;76:474-481.

47. Jin P, Wang X, Chang F, Bai Y, Li Y, Zhou R, *et al.* Low dose bisphenol A impairs spermatogenesis by suppressing reproductive hormone production and promoting germ cell apoptosis in adult rats. J Biomed Res 2013;27:135-139.

48. Nanjappa MK, Simon L, Akingbemi BT. The industrial chemical bisphenol A (BPA) interferes with proliferative activity and development of steroidogenic capacity in rat Leydig cells. Biol Reprod 2012;86:135, 1-12.

49. Akingbemi BT, Sottas CM, Koulova AI, Klinefelter GR, Hardy MP. Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig cells. Endocrinology 2004;145:592-603.

50. Kim JY, Han EH, Kim HG, Oh KN, Kim SK, Lee KY, *et al.* Bisphenol A-induced aromatase activation is mediated by cyclooxygenase-2 up-regulation in rat testicular Leydig cells. Toxicol Lett 2010;193:200-208.

51. Jahan S, Ain QU, Ullah H. Therapeutic effects of quercetin against bisphenol A induced testicular damage in male Sprague Dawley rats. Syst Biol Reprod Med 2016;62:114-124.

52. Sinha N, Narayan R, Saxena D. Effect of endosulfan on the testis of growing rats. Bull Environ Contam Toxicol 1997;58:79-86.

53. Turner R, McDonnell S. Alkaline phosphatase in stallion semen. Characterization and clinical applications: Theriogenology 2003;60:1-10.

54. Agarwal A, Prabakaran SA, Said TM. Prevention of oxidative stress injury to sperm. J Androl 2005;26:654-660.

55. Merker H, Günther T, Höllriegl V, Vormann J, Schümann K. Lipid peroxidation and morphology of rat testis in magnesium deficiency. Andrologia 1996;28:43-51.

56. Thiele J, Freisleben H, Fuchs J, Ochsendorf F. Ascorbic acid and urate in human seminal plasma: determination and interrelationships with chemiluminescence in washed semen. Hum Reprod 1995;10:110-115.

57. Kao S-H, Chao H-T, Chen H-W, Hwang TI, Liao T-L, Wei Y-H. Increase of oxidative stress in human sperm with lower motility. Fertil Steril 2008;89:1183-1190.

58. Chitra K, Latchoumycandane C, Mathur P. Induction of oxidative stress by bisphenol A in the epididymal sperm of rats. Toxicology 2003;185:119-127.

59. Mershiba SD, Dassprakash MV, Saraswathy SD. Protective effect of naringenin on hepatic and renal dysfunction and oxidative stress in arsenic intoxicated rats. Mol Biol Rep 2013;40:3681-1391.

60. Hermenean A, Ardelean A, Stan M, Herman H, Mihali C-V, Costache M, *et al.* Protective effects of naringenin on carbon tetrachloride-induced acute nephrotoxicity in mouse kidney. Chem Biol Interact 2013;205:138-147.

61. Wang J, Yang Z, Lin L, Zhao Z, Liu Z, Liu X. Protective effect of naringenin against lead-induced oxidative stress in rats. Biol Trace Elem Res 2012;146:354-359.

62. Prabu SM, Shagirtha K, Renugadevi J. Reno-protective effect of naringenin in combination with vitamins C and E on cadmium induced oxidative nephrotoxicity in rats. J Pharm Res 2011;4:1921-1926.

63. Badary OA, Abdel-Maksoud S, Ahmed WA, Owieda GH. Naringenin attenuates cisplatin nephrotoxicity in rats. Life Sci 2005;76:2125-2135.

64. Mostafa HES, Abd El-Baset SA, Kattaia AA, Zidan RA, Sadek A, Mona M. Efficacy of naringenin against permethrin-induced testicular toxicity in rats. Int J Exp Pathol 2016;97:37-49.

65. Ciz M, Denev P, Kratchanova M, Vasicek O, Ambrozova G, Lojek A. Flavonoids inhibit the respiratory burst of neutrophils in mammals. Oxid Med Cell Longev 2012:43-49.

66. Comporti M. Three models of free radical-induced cell injury. Chemi Biol Interact 1989;72:1-56.

67. Mínguez-Alarcón L, Hauser R, Gaskins AJ. Effects of bisphenol A on male and couple reproductive health. Fertil Steril 2016;106:864-870.

68. Sanja S, Sheth N, Joshi D, Golwala D, Patel Dhaval RM. Anti inflammatory activity of *Ipomoea reniformis* methanolic extract. Inter J Pharm Sci Drug Res 2009;1:34-39.

69. Qu J-H, Hong X, Chen J-F, Wang Y-B, Sun H, Xu X-L, *et al.* Fenvalerate inhibits progesterone production through cAMP-dependent signal pathway. Toxicol lett 2008;176:31-39.

70. Kono Y, Fridovich I. Superoxide radical inhibits catalase. J Biol Chem 1982;257:5751-5754.

71. Aitken RJ, Roman SD. Antioxidant systems and oxidative stress in the testes. Oxid Med Cell Longev 2008;1:15-24.

72. Mukherjee S, Mukhopadhyay P. Studies on arsenic toxicity in male rat gonads and its protection by high dietary protein supplementation. Al Ameen J Med Sci 2009;2:73-77.

73. Peltola V, Huhtaniemi I, Ahotupa M. Antioxidant enzyme activity in the maturing rat testis. J Androl 1992;13:450-455.

74. Chitra K, Latchoumycandane C, Mathur P. Effect of nonylphenol on the antioxidant system in epididymal sperm of rats. Arch Toxicol 2002;76:545-551.

75. Kaneko T, Iuchi Y, Kobayashi T, Fujii T, Saito H, Kurachi H, *et al.* The expression of glutathione reductase in the male reproductive system of rats supports the enzymatic basis of glutathione function in spermatogenesis: Eur J Biochem 2002;269:1570-1578.