Original Article

Grape Seed Proanthocyanidin Extract Alleviates Arsenic-induced Oxidative Reproductive Toxicity in Male Mice*

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Abstract

Objective To determine the ability of grape seed proanthocyanidin extract (GSPE) in alleviating arsenic-induced reproductive toxicity.

Methods Sixty male Kunming mice received the following treatments by gavage: normal saline solution (control); arsenic trioxide (ATO; 4 mg/kg); GSPE (400 mg/kg); ATO+GSPE (100 mg/kg); ATO+GSPE (200 mg/kg) and ATO+GSPE (400 mg/kg). Thereafter, the mice were sacrificed and weighed, and the testis was examined for pathological changes. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), heme oxygenase 1 (HO1), glutathione *S*-transferase (GST), NAD(P)H dehydrogenase, and quinone 1 (NQO1) expression in the testis was detected by real-time PCR. Superoxide dismutase (SOD), glutathione (GSH), total antioxidative capability (T-AOC), malondialdehyde (MDA), 8-hydroxydeoxyguanosine (8-OHdG), and reproductive indexes were analyzed.

Results ATO-treated mice showed a significantly decreased sperm count and testis somatic index and activity levels of SOD, GSH, and T-AOC than control group. Compared to the ATO-treated group, ATO +GSPE group showed recovery of the measured parameters. Mice treated with ATO+high-dose GSPE showed the highest level of mRNA expression of Nrf2, HO, NQO1, and GST.

Conclusion GSPE alleviates oxidative stress damage in mouse testis by activating Nrf2 signaling, thus counteracting arsenic-induced reproductive toxicity.

Key words: Grape seed proanthocyanidin extract; Arsenic; Reproductive toxicity; Nrf2 signaling

Biomed Environ Sci, 2015; 28(4): 272-280 *doi: 10.3967/bes2015.038* *ISSN: 0895-3988*

www.besjournal.com (full text) *CN: 11-2816/Q*

INTRODUCTION

Chronic exposure to arsenic (As) through drinking water from natural sources is associated with cancer, cardiovascular disease, diabetes, and developmental and reproductive effects[1-4]. Arsenic produces steroidogenic dysfunction in male rats and mice, which leads to infertility[5-8]. Chronic arsenic exposure may have contributed to male infertility in Comarca Lagunera, Mexico[9], impacted erectile function in a Taiwanese cohort[10], and reduced semen quality in a Chinese cohort of reproductive age[11].

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify reactive intermediates and repair the resulting damage. Some reactive oxidative species

*This work was supported by a grant from the Xinjiang Production and Construction Corps (2014BA039) and Shihezi University grant (RCZX201112).

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act as cellular messengers in redox signaling. Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signaling. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. In humans, oxidative stress is thought to be involved in the development of cancer and Parkinson's disease. Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defenses, such as glutathione. Oxidative injury is an important arsenic toxicity mechanism, which can be mediated by excess production of reactive oxygen species (ROS)[12-16]. Furthermore, inorganic arsenic produces toxicity by binding protein thiol groups and non-protein thiols such as glutathione (GSH)[17]. Arsenic also regulates the activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione reductase (GR), and catalase[18].

Proanthocyanidins are phenolic compounds found in fruits, vegetables, wine, tea, nuts, and seeds[19-20]. Grape seed extract from *Vitis* species (grapevines) is a rich source of plant flavonoids and proanthocyanidin oligomers[21-22]. Grape seed proanthocyanidin extract (GSPE) is primarily composed of dimers, trimers, and highly polymerized oligomers of monomeric catechins[23-24], and has a protective effect on oxidant-induced extracellular matrix production[25]. There are no reports on the effects of GSPE on reproductive toxicity induced by arsenic *in viv*o.

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a transcription factor that is encoded in humans by the *NFE2L2* gene. Nrf2 is a basic leucine zipper (bZIP) protein that regulates the expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation[26]. Several drugs that stimulate the Nrf2 pathway are being studied for treatment of diseases that are caused by oxidative stress. Under normal or unstressed conditions, Nrf2 is kept in the cytoplasm by a cluster of proteins, including Kelch-like-ECH-associated protein 1 (Keap1) and Cullin3, which degrade Nrf2 by ubiquitination[27-28]. Cullin3 ubiquitinates Nrf2[28], while Keap1 is a substrate adaptor that allows Cullin3 and Nrf2 to associate. Under oxidative stress, Nrf2 is not degraded, but instead travels to the nucleus, where it binds to DNA promoter regions and initiates transcription of antioxidative genes. When Nrf2 is ubiquitinated, it is transported to the proteasome, where it is degraded and its components are recycled. Under normal conditions, Nrf2 has a half- life of only 20 min. Oxidative stress or electrophilic stress disrupts critical cysteine residues in Keap1, preventing ubiquitination/ degradation by Keap1-Cul3. When Nrf2 is not ubiquitinated, it accumulates in the cytoplasm and translocates into the nucleus. In the nucleus, Nrf2 forms a heterodimer with a small Maf protein and binds to the antioxidant response element (ARE) in the upstream promoter region of many antioxidative genes, and thus initiates their transcription[29]. We hypothesized that GSPE would protect against arsenic-induced reproductive toxicity by counteracting oxidative injury via regulation of the Nrf2 pathway.

This study was designed to detect the combined effect of gavage GSPE and arsenic on male mice to elucidate the role of Nrf2 signaling in the combined effect. The findings in this study can provide empirical evidence for the mechanism of GSPE.

MATERIALS AND METHODS

Chemicals

Arsenic trioxide (ATO; As_2O_3; arsenite) was purchased from Beijing Chemical Reagent Corp. (Beijing, China). ATO was dissolved in 1 mol/L NaOH and brought to a pH of 7 with HCl according to published methods[30]. GSPE (>95% purity) was obtained from JF-Natural (Tianjin, China). Super oxide dismutase (SOD), glutathione peroxidase (GSH-Px), total antioxidative capability (T-AOC), 8-hydroxydeoxygua-nosine (8-OHDG), and malondialdehyde (MDA) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals were of analytical grade and obtained commercially.

Experimental Animals

All experiments were performed on 60 healthy KM (Kunming) male mice weighing 18-22 g with the approval of the Ethics Committee of Shihezi University. The mice were obtained from the laboratory center of XinJiang Medical University (Urumqi, China) (license numbers: SYXK 2011-0004). The animals were kept in well-ventilated cages in a temperature-controlled room at 22-25 °C under specific pathogen-free conditions on a 12-h light/dark cycle. The mice received a standard

laboratory diet composed of 60% ground corn meal, 15% ground beans, 10% bran, 10% corn oil, 3% casein, 1% mineral mixture, and 1% vitamin mixture, and supplied with water *ad libitum* throughout the experimental period. After 2 weeks of acclimation, animals were divided into 6 groups of 10 mice each. All mice were administered their respective treatments daily by gavage for 5 weeks.

Group 1 was a negative control group that received normal saline solution (NS). Group 2 was an arsenic-treated positive control group that received ATO solution (4 mg/kg). This dose was based on a previous report, in which it produced clinical effects, but did not affect survival[31]. Group 3 received high-dose GSPE (400 mg/kg). This dose was based on a previous report, in which it counteracted toxic effects induced by semicarbazide[32]. Group 4 received ATO (4 mg/kg) in the morning and low-dose GSPE (100 mg/kg) in the evening. Group 5 received ATO (4 mg/kg) in the morning and medium-dose GSPE (200 mg/kg) in the evening. Group 6 received ATO (4 mg/kg) in the morning and high-dose GSPE (400 mg/kg) in the evening.

At the end of the treatment period, mice were sacrificed by cervical dislocation. The testes were collected, blotted to remove blood, and used immediately in experiments.

Tissue Somatic Index

The body weight of the mice was recorded before they were sacrificed. Testes and accessory sex organs were weighed to the nearest milligram on a Shimadzu electronic balance (model BL-220H, Tokyo, Japan). The index weight of the organs was calculated by the following Formula:

index weight=organ weight/body weight×100. (1)

Evaluation of Sperm Quality

The cauda epididymal sperm suspension was prepared in normal saline at 37 °C. Sperm viability (live/dead ratio) and sperm density were calculated by the method of Nahas[33] and expressed as percentage viability and sperm $\times 10^6$/mL, respectively. The percentage of abnormal sperm was scored in 10 to 20 separate fields using 1% trypan blue by the method of Okamura[34].

Histopathological Examination of Testis Tissue

Sections were taken from the testis tissues from the mice in each group immediately after sacrifice. The tissues were washed with normal saline solution to remove blood, fixed in 10% neutral formalin for a period of at least 24 h, dehydrated in different grades of alcohol, and processed for paraffin embedding. Sections of 5-μm thickness were cut using a rotary microtome. The sections were processed and passed through a graded alcohol series, stained with hematoxylin and eosin, cleared in xylene, and examined microscopically according to previously described methods[35].

Tissue Homogenates

Tissue homogenates were prepared by soaking testis tissues in a lysis buffer containing 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, and the protein inhibitors sodium pyrophosphate, β-glycerophosphate, EDTA, Na_3VO_4, and leupeptin (Beyotime Biotechnology, Shanghai, China), and then homogenized with a TissueLyser (Qiagen, Valencia, CA, USA). The protein concentration in the homogenate was measured with a Bradford protein assay kit (Beyotime Biotechnology, Shanghai, China).

Lipid Peroxidation Assay

Malondialdehyde (MDA) in the tissue homogenates was measured with an MDA analysis kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. Briefly, the samples were treated with thiobarbituric acid (TBA), which produces a red compound with an absorption maximum at 532 nm in the presence of MDA. The concentration of MDA was calculated by comparing the absorbance to that produced by the control standard 1,1,3,3-tetraethoxypropane.

Enzyme Assays

GSH specifically deoxidizes dithio-bis-nitrobenzoic acid (DTNB) to form a yellow product (2-nitro-5-SH-benxoic acid), which can be assayed by colorimetry at 532 nm. SOD activity was determined by a hydroxylamine assay that was developed from a xanthine oxidase assay using chemical colorimetry. After the reaction, the absorbance at 560 nm was monitored using a spectrophotometer. The total antioxidative capacity (T-AOC) of the tissue homogenates was measured with a T-AOC analysis kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). This kit utilized antioxidants in the samples to reduce Fe^{3+} to Fe^{2+}, which was chelated with porphyrin to produce a purple complex that was quantified by measuring the absorbance at

550 nm. The T-AOC of the samples was determined by comparison with the control standard. 8-hydroxydeoxyguanosine (8-OHdG) in the testis tissue was assayed using a commercial enzyme-linked immunosorbent assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Results were normalized to the total amount of protein as measured by bicinchoninic acid (BCA).

Real-time PCR

Testis tissue was snap frozen in liquid nitrogen and total RNA was extracted by the TRIzol extraction method (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. Equal amounts of RNA (2 μg) were reverse-transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN, USA). Primers were synthesized by Sigma-Aldrich (St. Louis, MO, USA) for the following *Mus musculus* genes: heme oxygenase 1 (HO, HO1; 184 bp amplicon; F, 5′-CA GGTGATGCTGACAGAGGA-3′; R, 5′-ACAGGAAGCTGA GAGTGAGG-3′); glutathione *S*-transferase (GST; 199 bp amplicon; F, 5′-ATCGTTCCCTTTCTCGGCAT-3′; R, 5′-GCAGCCTGTAAGCCATTGAC-3′); NAD(P)H dehydrogenase, quinone 1 (NQO1; 112-bp amplicon; F, 5′-TGGCCGAACACAAGAAGCTG-3′; R, 5′-GCTACGAG CACTCTCTCAAACC-3′); nuclear factor (erythroid-derived 2)-like 2 (Nrf2, 173 bp amplicon; F, 5′-TTCCAT TTACGGAGACCCAC-3′; R, 5′-ATTCACGCATAGGAGCAC TG-3′); and beta-actin (240 bp amplicon; F, 5′-CACGA TGGAGGGGCCGGACTCATC-3′; R, 5′-TAAAGACCTCTAT GCCAACACAGT-3′).

The relative gene expression of targets was detected using a comparative cycle threshold (Ct) method[36]. All samples were tested in triplicate. Real-time PCR was performed on a mixture containing 10 μL of PCR Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 1 μL of forward and reverse primers (Sangon, Beijing, China), 1 μL of template DNA, and 8 μL of distilled water.

The qPCR conditions were: 1 cycle of initial denaturation (94 °C for 3 min), 30 cycles of amplification (94 °C for 30 s, 57 °C for 30 s, and 72 °C for 25 s), 1 cycle of melting curve measurement (95 °C for 5 s, 65 °C for 60 s, and a gradual increase in temperature to 97 °C), and a cooling period (40 °C for 30 s). The data presented were relative mRNA levels normalized to β-actin.

Statistical Analysis

The results were expressed as the mean± standard deviation. A general linear model was used to analyze the interactions of the effects of the combination of ATO and GSPE in a 2-factor, 2-level factorial design experiment. The model was as follows, in which a significant *P*-value for b_3 indicated an interaction between the effects of ATO and GSPE:

$$y=constant+b_1\times ATO+b_2\times GSPE+b_3\times(ATO\times GSPE) \quad (2)$$

where 'y' is a measured parameter such as body weight, testis weight, testis/body weight ratio, sperm count, etc. ANOVA was used to detect the differences among the low-, medium-, and high-dose GSPE groups, followed by Bonferroni's multiple comparison test to determine whether the differences were significant. The data were analyzed using SPSS software for Windows version 15.0 (SPSS Inc., Chicago, IL, USA), and a result of $P<0.05$ was considered to be statistically significant.

RESULTS

Indexes Related to Reproductivity in Mice

The effects of arsenic and GSPE on testis physiology are shown in Table 1. The body weight, testes weight, and testes somatic index of mice treated with ATO were significantly decreased in comparison with the control group ($P<0.05$). The body weight of the ATO group decreased gradually over the course of treatment (Figure 1). However, the body weights of the ATO+GSPE groups were between those of the ATO and control groups. In addition, there was a statistically significant interaction between the effects of ATO and GSPE on body weight ($P<0.001$). Similar results were obtained for testes somatic index, which also showed a significant interaction between the effects of GSPE and ATO ($P=0.001$).

There were no significant differences between the GSPE group and the control group for sperm count or sperm abnormalities, whereas the ATO group had a

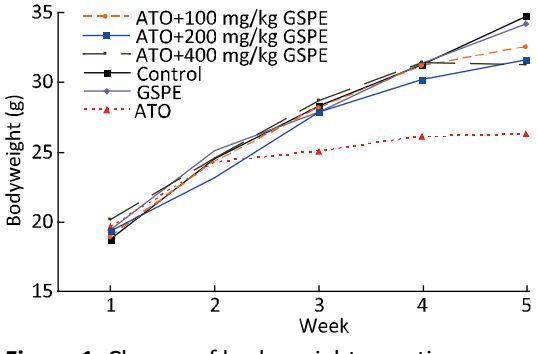

Figure 1. Change of body weight over time.

significantly lower sperm count (P<0.05) and a higher rate of sperm abnormalities (P<0.05) than the control group. A significant interaction was found between the effects of ATO and GSPE on sperm count (P<0.001) and sperm abnormalities (P<0.001). The group treated with ATO+GSPE (400 mg/kg) showed a significantly increased sperm count and a lower rate of sperm abnormalities in comparison with the ATO group (P<0.05) (Table 1).

Testicular Oxidative Damage Level and Antioxidant Enzymes

ATO treatment significantly increased oxidative damage (MDA and 8-OHdG levels) and decreased the activity of antioxidant enzymes (T-AOC, GSH, and SOD) in comparison with the control group (P<0.05) (Table 2). In comparison with ATO treatment, treatment with ATO+GSPE (400 mg/kg) significantly decreased MDA and 8-OHdG levels, and increased T-AOC and the activities of GSH and SOD (P<0.05). A significant interaction was found between the effects of ATO and GSPE on MDA level, 8-OhdG level, and GSH activity (all P<0.001). As the dose of GSPE was increased, MDA and 8-OHdG levels increased significantly (P<0.05), and GSH activity decreased significantly.

Morphological changes in the testis in the treatment groups are shown in Figure 2. As expected, ATO administration caused a reduction in germ cell number, necrosis of sperm, congestion of interstitial blood vessels, and degeneration and necrosis of germ cells lining the seminiferous tubules (Figure 2C). Treatment with GSPE alone did not cause morphological changes (Figure 2B), whereas the groups treated with ATO+GSPE showed some normal and some ectopic seminiferous tubules (Figure 2D, 2E, 2F).

Nrf2 *Signaling-related Gene Expression*

The effects of GSPE on genes related to the Nrf2 signaling pathway are shown in Figures 3 and 4. Expression levels of HO, GST, NQO1, and Nrf2 in the ATO group were significantly greater than those of the

Table 1. Effect of Arsenic and/or GSPE Administration on Indexes Related to Reproductivity in Male Mice (mean±SD, n=10)

Indexes	Control (NS)	GSPE (400 mg/kg)	ATO (4 mg/kg)	ATO+GSPE (4+100 mg/kg)	ATO+GSPE (4+200 mg/kg)	ATO+GSPE (4+400 mg/kg)
Initial body weight (g)	18.760±2.470	19.410±1.770	19.650±1.860	18.940±1.970	19.390±1.890	20.180±1.770
Final body weight (g)	34.730±2.150	34.170±1.240	26.290±1.850[a,c]	32.520±1.950[a,b]	31.640±1.890[a,b]	31.250±1.910[a,b]
Body weight gain (g)	18.500±3.490	14.470±2.600	7.240±2.610	12.830±2.710[a,b]	11.930±2.940[a,b]	11.680±3.100[a,b]
Testes weight (g)	0.262±0.019	0.245±0.036	0.211±0.025[a,c]	0.237±0.018[a,b]	0.221±0.021[a,b]	0.242±0.019[a,b]
Testes/body (%)	1.460±0.258	1.742±0.382	3.291±1.377[a,c]	2.133±0.541[a,b]	2.106±0.593[a,b]	2.203±0.546[a,b]
Sperm count (10^6/mL)	18.090±1.150	16.840±0.730	10.580±0.850[a,c]	13.520±1.060[a,b]	15.370±0.860[a,b]	16.640±0.960[a,b]
Sperm abnormalities (%)	1.400±0.750	1.700±0.890	16.900±3.920[a,c]	3.710±1.190[a,b]	3.190±1.040[a,b]	3.330±1.170[a,b]

Note. [a]significant difference from control (NS) group at P<0.05; [b]significant difference *vs.* ATO group (4 mg/kg) at P<0.05; [c]significant difference *vs.* ATO+GSPE group (4+400 mg/kg) at P<0.05.

Table 2. Effect of GSPE on MDA, 8-OHdG, T-AOC, GSH, and SOD in ATO-intoxicated Male Mice (mean±SD, n=10)

Experimental Group	MDA (nmol/L/mg protein)	8-OHdG (ng/mL)	T-AOC (U/mg protein)	GSH (mg/mg protein)	SOD (U/mg protein)
Control (NS)	2.71±0.48	0.61±0.04	0.47±0.17	38.37±5.91	71.70±19.50
GSPE (400 mg/kg)	2.94±0.54	0.71±0.06	0.56±0.15	46.32±2.43[b]	76.70±17.50
ATO (4 mg/kg)	5.13±0.62[a,c]	0.75±0.05[a,c]	0.68±0.18[a,c]	24.54±4.15[a,c]	98.10±23.50[a,c]
ATO+GSPE (4+100 mg/kg)	4.01±0.57[a,b]	0.72±0.04[a]	0.69±0.16[a]	26.24±3.19[a]	91.30±27.40[a,b]
ATO+GSPE (4+200 mg/kg)	3.62±0.47[a,b]	0.69±0.03[a,b]	0.62±0.23[a,b]	27.37±2.17[a,b]	90.80±29.13[a,b]
ATO+GSPE (4+400 mg/kg)	3.43±0.39[a,b]	0.68±0.03[a,b]	0.65±0.28[a,b]	30.13±3.11[a,b]	93.20±38.80[a,b]

Note. [a]significant difference from control (NS) group at P<0.05; [b]significant difference *vs.* ATO group (4 mg/kg) at P<0.05; [c]significant difference *vs.* ATO+GSPE group (4+400 mg/kg) at P<0.05.

control group (P<0.05). Interestingly, treatment with GSPE also significantly increased the expression levels of Ho, GST, and NQO1 in comparison with the control group (P<0.05). The groups treated with ATO+GSPE (100 mg/kg, 200 mg/kg, or 400 mg/kg) showed elevated expression of genes related to the Nrf2 signaling pathway in comparison with the other groups (P<0.05; Figure 3).

DISCUSSION

It has been shown that arsenic may exert an influence on the endocrine system[37-38]. Arsenic-treated mice exhibited decreased sperm counts, sperm motility, and testicular weight in comparison with untreated animals[39]. Arsenic disrupts meiosis and post-meiotic stages of spermatogenesis, and acute arsenic exposure causes rapid and extensive disruption of spermatogenesis in mice[8]. Our study suggested that exposure to arsenic reduced the quality of sperm in mice, and substantial pathological changes were clearly observed in the testis after 5 weeks of exposure to ATO. The MDA and 8-OHdG levels in the group treated with ATO were higher than the corresponding levels in the control group, while the activity of GSH was reduced. This result showed that arsenic induced significant oxidative damage in the testis. Testicular oxidative stress appears to be a common feature that underlies male infertility, which suggests that there may be benefits to the development of better antioxidant therapies for relevant cases of hypospermatogenesis[40-42].

GSPE is obtained from the seeds of *Vitis vinifera*, and contains biologically active compounds with protective effects against oxidative stress induced by free radicals and reactive oxygen spices[43]. Some research effort has focused on the protective effects of GSPE on reproductive toxicity induced by aluminum[44]. GSPE blocks arsenic-induced pathological changes and oxidative damage. Using a general linear model, our study found significant interactions between GSPE and ATO treatment effects on body weight and testis weight, sperm count, MDA level, 8-OHdG level, and GSH activity. In comparisons among the groups, we found that, in the ATO+GSPE groups, these parameters fell to levels between those observed in the ATO and control groups. These results indicate that GSPE produced an antagonistic effect on ATO.

Our study also demonstrated that GSPE activated Nrf2 to alleviate oxidative damage induced by arsenic. Few reports have focused on the relationship between GSPE and the Nrf2 signaling pathway. It has been suggested that activation of the transcription factor Nrf2 elicits a cellular defense system that detoxifies drugs and environmental pollutants[45-49], and the components of this system

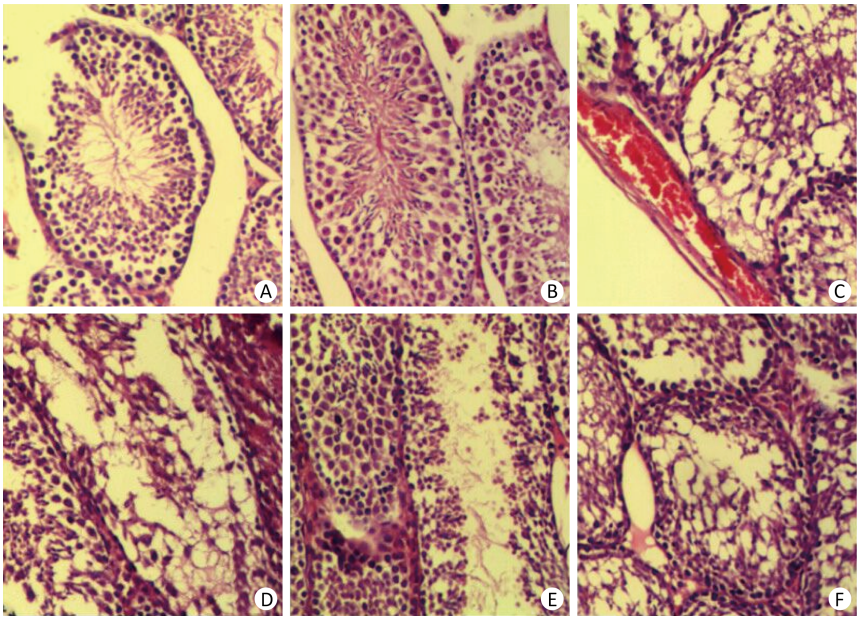

Figure 2. Histopathological examination of testis treated with ATO and/or GSPE. A: Control; B: GSPE (4 mg/kg); C: ATO (4 mg/kg); D: ATO+GSPE (4+100 mg/kg); E: ATO+GSPE (4+200 mg/kg); F: ATO+GSPE (4+400 mg/kg).

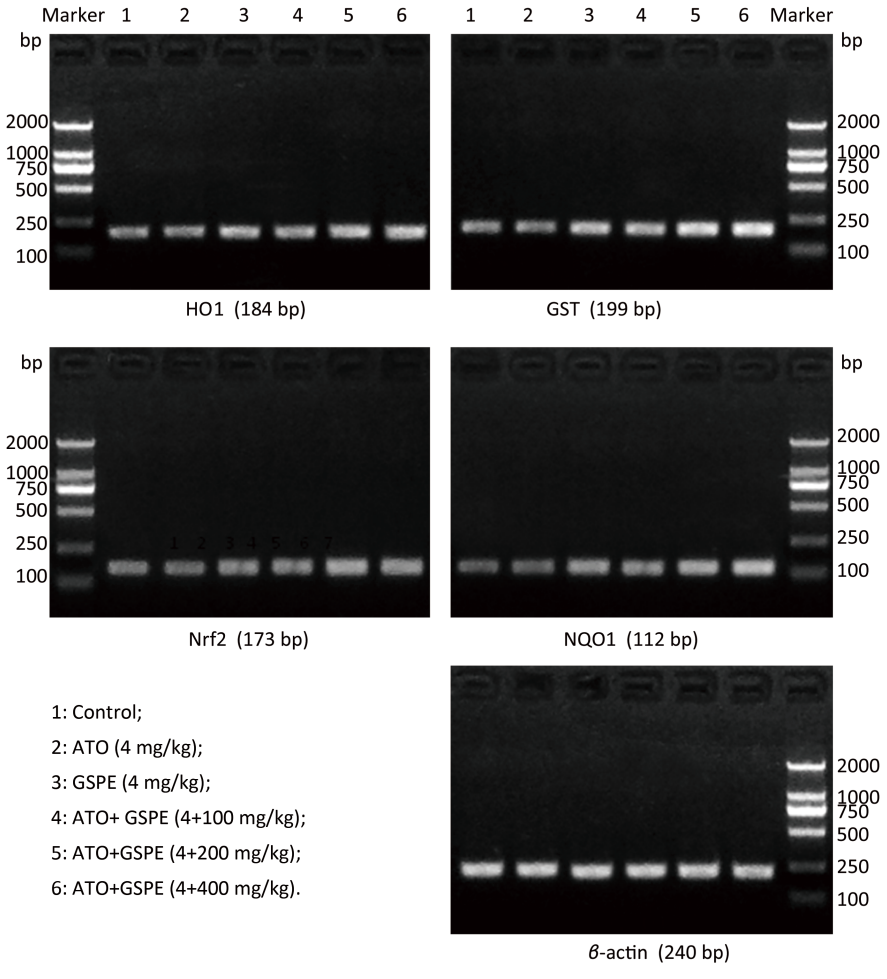

Figure 3. Electrophoregram of genes related to Nrf2 signaling.

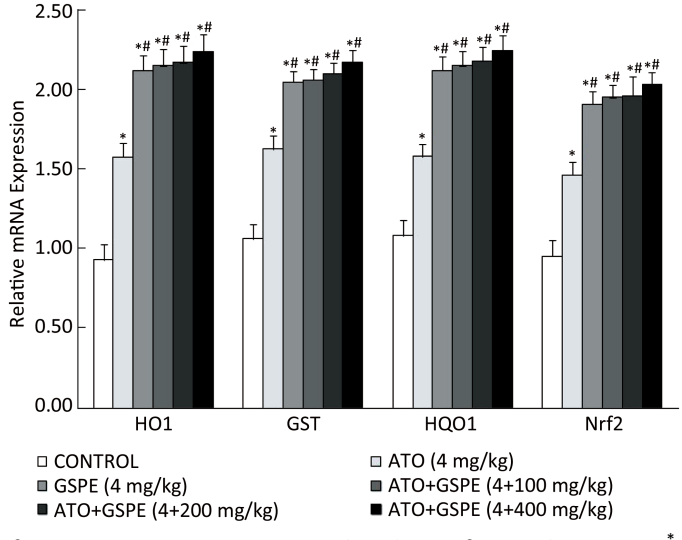

Figure 4. The effect of GSPE on mRNA expression related to Nrf2 signaling. *Note*: *significant difference *vs.* the control (NS) group at $P<0.01$; #significant difference *vs.* the ATO-treated group (4 mg/kg) at $P<0.05$.

include glutamate-cysteine ligase [composed of the glutamate-cysteine ligase catalytic subunit (GCLC) and the glutamate-cysteine ligase regulatory subunit (GCLM)], GST, NQO1, and HO1. This study showed that GSPE administration increased the mRNA expression levels of Nrf2, GST, HO1, and NQO1 in comparison with the control group, which suggested that GSPE activated Nrf2 signaling. Furthermore, GSPE significantly decreased MDA and 8-OHdG levels, and increased GSH activity. Based on these measured parameters and coordinated mRNA expression of Nrf2 signaling pathway components after GPSE exposure, our findings in mice suggest that GSPE activates Nrf2 signaling, which alleviates oxidative stress damage in the testis, and thus counteracts arsenic-induced reproductive toxicity. Future research should be performed with mice lacking the Nrf2 gene to validate the role of Nrf2 signaling in the effects of GSPE on arsenic-induced reproductive toxicity.

Received: November 9, 2014;
Accepted: March 6, 2015

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