

Genistein attenuates di-(2-ethylhexyl) phthalate-induced testicular injuries via activation of Nrf2/HO-1 following prepubertal exposure

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Abstract. Di-(2-ethylhexyl) phthalate (DEHP) and genistein (GEN) are of the most common endocrine disrupting chemicals (EDCs) present in the environment or the diet. However, investigation of the effects of acute exposure to these two EDCs during prepuberty has been lacking. In this study, DEHP and GEN were administered to prepubertal male Sprague-Dawley rats by gavage from PND22 to PND35 with vehicle control, GEN 50 mg/kg body weight (bw)/day, DEHP50, 150 and 450 mg/kg bw/day, and combined treatment. Reproductive parameters including testis weight, anogenital distance and organ coefficient were evaluated on PND36. Enzyme activity involved in the regulation of testicular redox state as well as expression of genes and proteins related to anti-oxidative ability and apoptosis were also investigated. The results revealed that by PND36, DEHP treatment had significantly decreased the testis weight, organ coefficient, testicular anti-oxidative enzyme activities and caused tubular vacuolation; however, co-administration of GEN partially alleviated DEHP-induced testicular injuries and enhanced testicular anti-oxidative enzyme activities and upregulated the expression of NF-E2 related factor 2 and heme oxygenase-1, which indicated that GEN partially attenuated DEHP-induced male reproductive system damage through anti-oxidative action following acute prepubertal exposure to DEHP. Thus, GEN may have use in attenuating the damaging effects of other EDCs that lead to reproductive disorders.

Introduction

Evidence has confirmed that endocrine disrupting chemicals (EDCs) have potentially deleterious effects on development,

growth, metabolism and reproduction, as they can interfere with the production, release, transport, metabolism, binding action or elimination of the natural hormones in the body (1). The effects of exposure to multiple EDCs on reproduction is of great concern as various EDCs are present at high levels in the environment, and how the combination of the compounds these impacts the reproductive system is largely unknown. The multiple exposure effects may function in independent, dose addition or interaction manners (2). However, evaluation of mixture toxicity is not simple because of the complexity of the mechanisms of action of EDCs, and uncertain interference under different doses and exposure durations (3).

The prepubertal male reproductive system is highly response to sex steroids and how EDC exposure would affect prepubertal development has been scarcely studied. Exposure to EDCs during this stage may account for a major change in the total activity of the hormone involved, resulting in adverse consequences that may be apparent during puberty or even during adult life due to interference in the developmental programming (4,5). From the time of conception through to adulthood, humans are exposed to countless anthropogenic and naturally-occurring EDCs, among which di-2-(ethylhexyl) phthalate (DEHP) is the most widely used plasticizer in polyvinylchloride (PVC) plastics, which is prevalent in cosmetics, personal care products and medical devices, accounting for ~80% of the total phthalates consumption worldwide (6). As DEHP is not chemically bound to PVC, it easily leaches, migrates and evaporates into indoor air and the atmosphere, foodstuff and other materials. The mechanism by which DEHP exerts toxic effects in the male reproductive system has not been fully elucidated (7). Previous evidence revealed that DEHP exerts its anti-androgen effect by suppressing fetal testosterone biosynthesis via activation of peroxisome proliferator-activated receptors (PPARs) (8), and subsequent inhibition of anti-oxidant enzymes, leading to free radical production and oxidative stress, which contributed to oxidative DNA damage (9). Following exposure to DEHP during prepuberty and puberty, significant decreases in glutathione (GSH)/glutathione disulfide (GSSG) redox ratio and a marked increase in the levels of thiobarbituric acid reactive substances (TBARS) were observed (10). Epidemiological analysis also demonstrated that urinary oxidative stress marker malondialdehyde (MDA) concentrations were

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significantly associated with DEHP metabolite levels in prepubertal children (11).

Genistein (GEN), a weak estrogenic phytoestrogen, is widely present in the Asian diet (12) and is considered to be a potent anti-oxidant. Notably, GEN may enhance fertility at lower doses by promoting the acrosome reaction, however, GEN potentially suppresses male fertility via inhibiting the acrosome reaction at higher doses, with no significant effect on sperm morphology (13). Furthermore, isoflavones can alleviate the oxidative stress induced by other EDCs, cadmium and tetradecanoylphorbol acetate, via modulating the activity of anti-oxidative defenses in different systems (14,15), however, the estrogenic and anti-oxidative effects seem largely dependent on its concentration (16).

Previous studies have predominantly focused on the reproductive effects following exposure to a single EDC *in vitro*, *ex vivo* and *in vivo* (17-19); however, few studies have examined how multiple EDCs alter mammalian reproductive development, particularly for those that act via different mechanisms. Our most recent study demonstrated that GEN normalized reactive oxygen species-induced neonatal effects of DEHP through an anti-oxidant action, and also revealed that co-administration of the two EDCs did not follow classical dose-response effects, which highlighted the importance of assessing effects across a range of doses and ages (20).

Oxidative stress is a common pathological process involved in the mechanism of EDC-induced testicular injury, which makes oxidative stress monitoring an informative method for investigating interactions between numerous toxicants and the reproductive consequences (2,21). We hypothesized that low-dose GEN exposure would exert its anti-oxidative role in the reproductive system during prepuberty, which may alleviate the toxic effects in the reproductive system induced by different doses of DEHP. The current study examined reproductive parameters, including testis weight, anogenital distance (AGD), gene and protein expression associated with anti-oxidative ability and apoptosis, enzyme activity involved in the regulation of testicular redox state, to gain an insight into the early cellular and molecular events that may drive long term changes caused by EDCs.

Materials and methods

Chemicals, animals and treatment. Di-2-(ethylhexyl) phthalate (DEHP; CAS no. 117-81-7) was obtained from Tianjin Kermel Chemical Reagent Co., Ltd. (Tianjin, China); GEN (CAS no. 446-72-0) was obtained from Shaanxi Huike Botanical Development Co., Ltd. (Xi'an, China). Corn oil was obtained from Longda Co., Ltd. (Yantai, China).

Prior to study initiation, the experimental protocol was reviewed and approved by the Committee on Animal Research and Ethics of Xi'an Jiaotong University (Xi'an, China). Specific pathogen free Sprague-Dawley rats (21 days old) were obtained following weaning from the Experimental Animal Center of Xi'an Jiaotong University and housed in 12-h light/dark cycle at $21\pm 2^\circ\text{C}$ with relative humidity of $50\pm 5\%$. Soy-and alfalfa-free diet and purified water were provided *ad libitum*. Male rats ($n=48$) were housed 3 per cage on arrival. On the subsequent day, all the rats were treated by daily gavage from postnatal day 22 (PND22) to PND35 with corn oil (vehicle control), GEN 50 mg/kg body weight (bw)/day (G50), DEHP50, 150

and 450 mg/kg bw/day (D50, D150 and D450) and the combination of GEN + DEHP at different doses (G+D50, G+D150, G+D450), $n=6$ per group. DEHP and GEN were dissolved in corn oil and corn oil was administered at a dose of 2 ml/kg. The dose of each chemical was calculated daily according to body weight of each rat prior to dosing.

The dose of GEN was chosen on the basis of previous reports (22). Serum concentration of phytoestrogen under a classical Asian diet is equivalent to that of rat at the dose of 40-50 mg/kg (23,24) and the no observed adverse effect level of GEN is considered to be 50 mg/kg/day (22). Additionally, 50 mg/kg/day is considered to be the cut-off for low-dose effects of GEN based on the weight-of-evidence evaluation of *in vivo* studies (25).

Body weight, AGD, testis weight and organ coefficient. Body weight of each rat was measured on PND36 and AGD was measured using a vernier caliper by a single investigator in a blinded manner on the same day. The AGD of each animal was divided by the cube root of body weight (AGD/body weight^{1/3}) as the adjusted AGD to avoid errors caused by differences in body size. On PND36, all the rats were anesthetized with 400 mg/kg chloral hydrate. The right testis of each rat were removed and stored in -80°C refrigerator for subsequent analysis of testicular redox state.

The left testicle of each rat was removed and weighed separately using an electronic balance and the organ coefficient was calculated as (organ weight/body weight). The left testis was immediately placed in Bouin's fixative solution (75 ml saturated picric acid solution; 25 ml 40% formalin aqueous solution; 5 ml glacial acetic acid) for 12 h and routinely processed for histology.

Evaluation of testicular redox state. Testis tissue (200 mg) was cut into small pieces and homogenized in 1.8 ml ice-cold saline buffer (1:9, wt/v) using an Ultra-Turrax (T8; IKA®-Werke GmbH & Co., KG, Staufen, Germany) to obtain testicular homogenates at a concentration of 0.1 g/ml. Subsequently, testicular homogenates were centrifuged at $3,500 \times g$ for 5 min at 4°C and the supernatants were collected for further testicular redox state analysis. Total anti-oxidant capacity (T-AOC) (26), superoxide dismutase (SOD) (26), GSH peroxidase (GSH-PX) (27), total GSH (28), GSSG (28) and MDA (29) were evaluated using clinical chemistry assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions to monitor testicular redox state.

T-AOC was determined by the ferric reducing/anti-oxidant power assay and detected at 520 nm using a spectrophotometer, the final concentration is expressed as U/mg protein.

SOD activity was determined by water soluble tetrazolium salts assay, which monitors the inhibition rate of SOD to the process of formazan dye formation from tetrazolium salt mediated by the superoxide anion. The absorbance was scanned at 450 nm using a microplate reader.

GSH-PX activity was detected by determination of the reduction of GSH. The GSH reacts with 5,5-dithiobis-(2-nitrobenzoic acid) and produces yellow colored compounds detected at 412 nm using a spectrophotometer and the final result are presented as U/mg protein.

T-GSH and GSSG content were measured using dithionitrobenzoic acid reagent and the absorbance was scanned at

Table I. Primer sets used for reverse transcription-quantitative polymerase chain reaction.

Gene name	Accession no.	Forward primer	Reverse primer
β -actin	NM_031144.2	5'-CTATCGGCAATGAGCGGTTCC-3'	5'-TGTGTTGGCATAGAGGTCCTTACG-3'
Nrf2	NM_031789.2	5'-ACGGTGGAGTTCAATGAC-3'	5'-TGTTGGCTGTGCTTTAGG-3'
HO-1	NM_012580.2	5'-GAAGAGGAGATAGAGCGAAAC-3'	5'-TGTGGCTGGTGTGTAAGG-3'
Caspase-3	NM_012922.2	5'-TGGAACGAACGGACCTGTG-3'	5'-CGGGTGCGGTAGAGTAAGC-3'

Nrf2, NF-E2 related factor 2; HO-1, heme oxygenase-1.

405 nm using microplate reader. GSH content was calculated as T-GSH-2X GSSG, the final results are expressed as the ratio of GSH/GSSH.

MDA was analyzed using the TBARS method and the absorbance was measured with the ultraviolet spectrometer at 532 nm against blanks prepared with distilled water. The result is expressed as nmol/mg protein.

Testicular histology. Following fixation in Bouin's fixative solution for 12 h at 4°C, testes collected on PND36 were transferred to 75% ethanol, embedded in paraffin and cut to 5 μ m sections. Sections were stained with 0.2% hematoxylin for 2 min and 0.5% eosin for 10 min and evaluated under light microscopy. Evaluations were performed by an experienced investigator blind to the treatment groups.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Testis RNA was extracted using total RNA extraction kit according to the manufacturer's instruction (Fastagen Biotech Co., Ltd., Shanghai, China; <http://www.fastagen.cn/aboutus/lxwm.htm>) (30). cDNA was synthesized from isolated RNA using RevertAid™ First Strand cDNA synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) (31). Total RNA (5 μ l) and oligo(dT)18 (0.5 μ g) were added together and incubated at 70°C for 5 min, and then 4 μ l 5X Reaction Buffer, 0.5 μ l RiboLock RNase Inhibitor, 2 μ l dNTP Mix and 2 μ l RevertAid Reverse Transcriptase were added and incubated at 42°C for 60 min. The reaction was terminated at 70°C for 10 min. qPCR was performed using the Bio-Rad Real-Time PCR system (IQ5; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The cycling conditions were as follows: Hot-start DNA Polymerase activation at 95°C for 10 min; PCR (40 cycles) at 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec; melt curve (1 cycle) at 95°C for 15 sec, 60°C for 60 sec and 95°C for 15 sec. β -actin was used as an endogenous control and for normalization of gene targets. The relative gene expression was analyzed using the $2^{-\Delta\Delta C_q}$ algorithm (32). The genes and primer sequences are listed in Table I.

Western blot analysis. Total protein was extracted using a Total Protein Extraction kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) (33). Protein concentration was determined using NanoDrop2000c microvolume spectrophotometer (Thermo Fisher Scientific, Inc.) Proteins (loaded sample, 50 μ l at 1 μ g/ μ l) were separated on a 12% polyacrylamide gel and then transferred to a polyvinylidene fluoride membrane. Following blocking at room temperature with 5% milk in 1X

Tween-20-PBS (PBST), the membranes were incubated with specific primary antibodies against rat NF-E2 related factor 2 (Nrf2; 1:200; BS1258; Bioworld Technology, Inc., St. Louis Park, MN, USA), heme oxygenase-1 (HO-1; 1:500; ab13248; Abcam, Cambridge, UK) and cleaved caspase-3 (1:300; 9661; Cell Signaling Technology, Inc., Danvers, MA, USA) and β -actin (1:10,000; HC201-01; Beijing Transgen Biotech Co., Ltd., Beijing, China) diluted in PBST overnight at 4°C, followed by incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:2,000; HS101-01) or HRP-conjugated anti-mouse IgG (1:10,000; HS201-01; Beijing Transgen Biotech Co., Ltd.) for 1 h at room temperature. Substrate Chemiluminescence kit (EMD Millipore, Billerica, MA, USA) was used to detect proteins. Images were captured using the Alpha FluorChem E gel documentation system (ProteinSimple, San Jose, CA, USA). The density of the lanes was measured using Bio-Rad QuantityOne software (version 4.6.2; Bio-Rad Laboratories, Inc.).

Statistical analyses. Data were expressed as the mean \pm standard error and analyzed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Normality and homogeneity of variances were evaluated prior to statistical analysis. Data were analyzed by one-way analysis of variance and multiple comparison were done between combined exposure groups and control, and single exposure groups by least significance difference tests when equal variances were assumed, otherwise followed by Games-Howell test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Body weight, AGD, testis weight and organ coefficient. Body weight, AGD, testis weight and organ coefficient of each rat on PND36 are presented in Fig. 1. No significant changes of body weight were observed among the groups. Examination of PND36 body weight, AGD and adjusted AGD revealed no significant differences between control and treated animals. Exposure to 150 and 450 mg/kg/day DEHP caused a significant decrease in testis weight and the testis organ coefficient compared with the control group ($P < 0.05$), and the combined exposure of GEN + 150 or 450 mg/kg/day increased the testis weight and testis organ coefficient compared with the corresponding DEHP single exposure groups ($P < 0.05$), thus, although not completely normalized, GEN may have a protective effect in prepubertal testis development.

Testicular redox state. The testicular redox state in each group at PND36 is presented in Fig. 2. The consecutive DEHP treatment resulted in a significant reduction of intratesticular T-AOC,

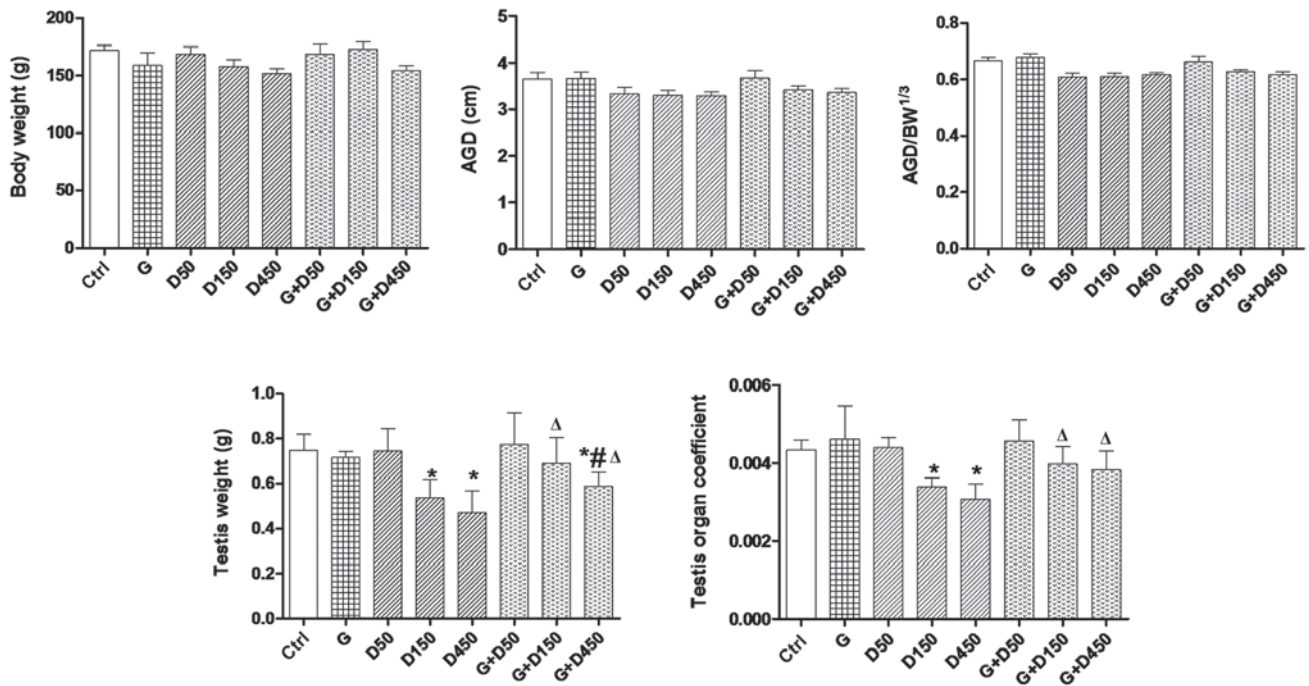


Figure 1. Body weight, AGD, adjusted AGD, testis weight and organ coefficient comparison between groups on postnatal day 36. Rats were treated with G (50 mg/kg), D (50, 150, 450 mg/kg) or combined treatment. *P<0.05 vs. control; #P<0.05 vs. G; ΔP<0.05 vs. corresponding D group. AGD, anogenital distance; BW, body weight; G, genistein; D, di-2-(ethylhexyl) phthalate.

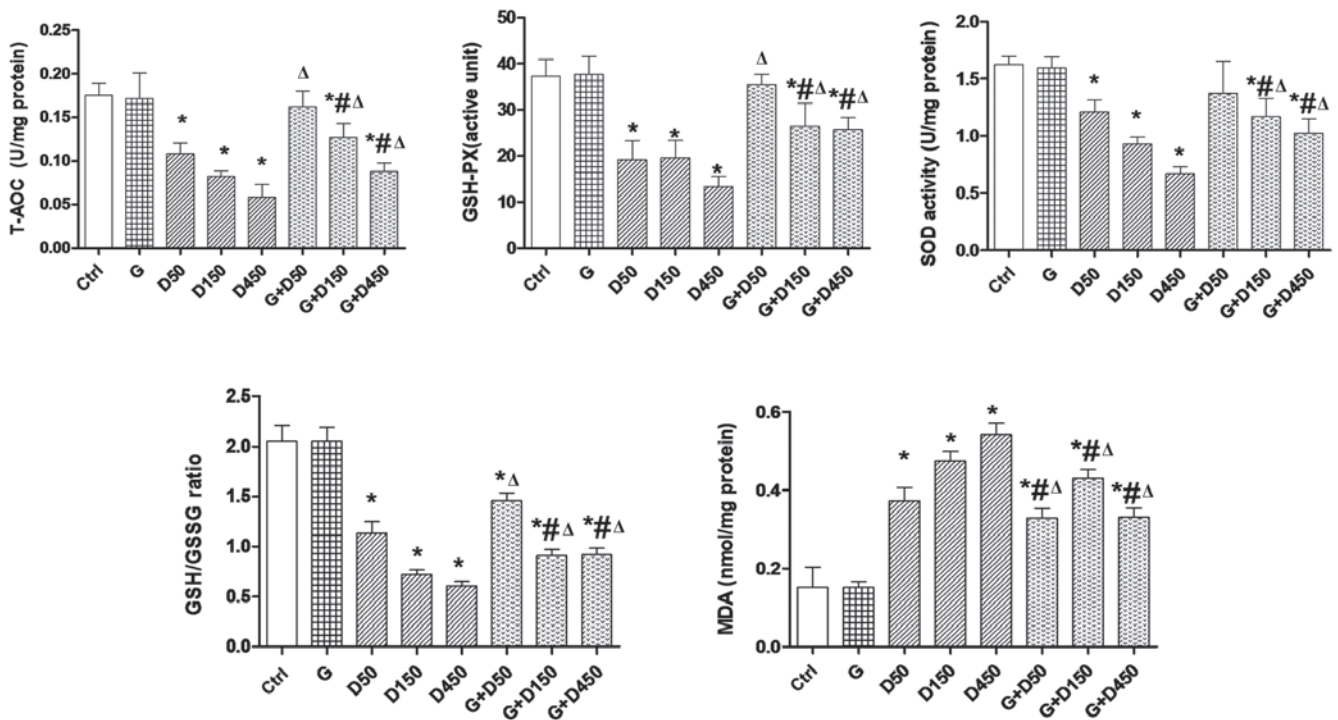


Figure 2. Testicular redox state comparison between groups at postnatal day 36. Rats were treated with G (50 mg/kg), D (50, 150, 450 mg/kg) or combined treatment. *P<0.05 vs. control; #P<0.05 vs. G; ΔP<0.05 vs. corresponding D group. T-AOC, total anti-oxidant capacity; AGD, anogenital distance; BW, body weight; G, genistein; D, di-2-(ethylhexyl) phthalate; GSH-PX, glutathione peroxidase; SOD, superoxide dismutase; GSH/GSSG, glutathione/glutathione disulfide; MDA, malondialdehyde.

SOD activity and GSH-PX level, and the ratio of GSH/GSSG compared with the control group (P<0.05), whereas combined treatment with GEN and DEHP significantly increased these parameters compared with corresponding single DEHP exposure

groups (P<0.05), exhibiting similar trends to the changes in testis weight and testis organ coefficient. This indicated that the anti-oxidative role of GEN and the enhancement of testicular anti-oxidative ability may contribute to the recovery of testis

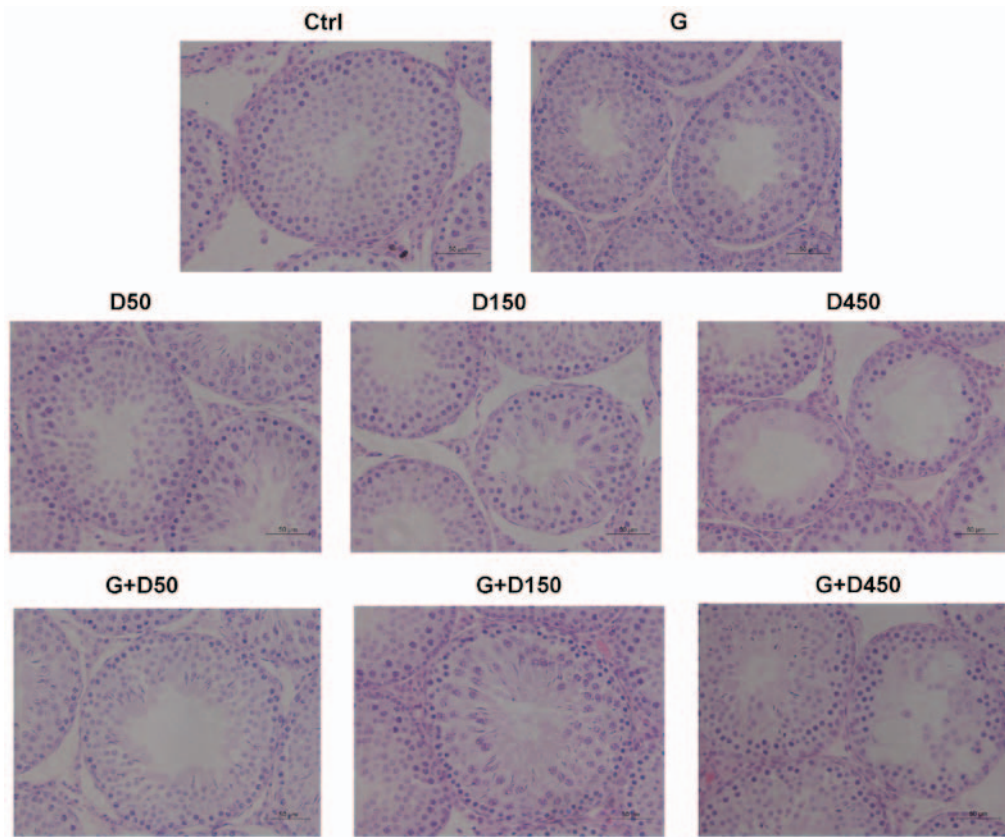


Figure 3. Testicular sections of male rats on postnatal day 36 (hematoxylin-eosin staining). Rats were treated with G (50 mg/kg), D (50, 150, 450 mg/kg) or combined treatment. Note decreased cell layers (in D150), decreased cell layer and tubule diameter, the loosely arranged germ cells and severe tubular vacuolization (in D450) and improved testicular histology (G + D150, G + D450). Magnification, x400. Scale bars indicate 50 μ m. G, genistein; D, di-2-(ethylhexyl) phthalate.

weight. By contrast, the MDA level in each DEHP-treated group was increased significantly compared with the control group ($P < 0.05$). Co-administration of GEN and DEHP resulted in a significant decrease in the MDA level compared with the corresponding single DEHP exposure groups ($P < 0.05$), which may be largely dependent on the increased anti-oxidative capacity following co-administration with GEN.

Testicular histology. Testicular sections from each group at PND36 are presented in Fig. 3. No obvious histological damage was observed following treatment with GEN, D50, or combined GEN and D50; the tubule diameter and formation of tubule lumen were normal, and the number of cell layers in each seminiferous tubule was >5 . However, exposure to DEHP at 150 mg/kg/day induced a decrease in the thickness of seminiferous epithelium and a decrease of the number of cell layers, which was 3-4 layers; single vacuolization was also presented in part of the seminiferous tubules. The combined treatment with GEN + DEHP at 150 mg/kg/day increased the cell layer numbers compared with D150 single exposure group and no obvious tubular vacuolization was observed.

The D450 group exhibited shorter tubule diameter and loosely arranged germ cells, severe tubular vacuolization was visible in part of the seminiferous tubules and in those tubules no spermatids were observed, indicating prepubertal exposure to high dose of DEHP delayed tubule development of the testis during puberty and had deleterious effects on testis development, which may lead to adult spermatogenesis

arrest. Combined exposure in the G + D450 group increased the cell layer number and the tubule diameter compared with the D450 group; vacuolization was still present in several tubules, which demonstrated that prepubertal coexposure to GEN partially alleviated DEHP-induced testicular development disruption, however further testicular damage may not be reversible.

Gene expression of Nrf2, HO-1 and caspase-3. Gene expression of Nrf2, HO-1 and caspase-3 of each group on PND36 is presented in Fig. 4. No significant changes were observed between control and group G. Exposure to 50, 150 and 450 mg/kg/day DEHP caused significant decrease in Nrf2 and HO-1 expression compared with control ($P < 0.05$), while the combined exposure of G + D50, G + D150 and G + D450 increased Nrf2 and HO-1 expression compared with the corresponding DEHP single exposure groups ($P < 0.05$), which indicated that although not completely normalized, GEN may exert its protective effects in prepubertal testis development by increasing Nrf-2 and HO-1 expression. Caspase-3, the marker of cell apoptosis, was significantly increased in D150 and D450 groups compared with the control group ($P < 0.05$), while the combination of G + D150 and G + D450 decreased caspase 3 mRNA compared with DEHP single exposure although the expression remained higher than the control ($P < 0.05$).

Protein expression of Nrf2, HO-1 and cleaved caspase-3. Alterations in Nrf2, HO-1 and caspase-3 mRNA at PND36

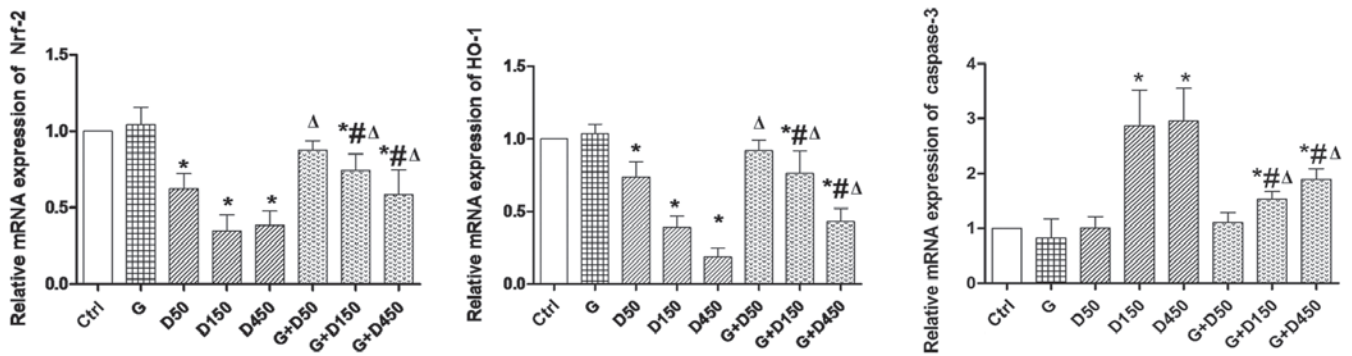


Figure 4. Gene expression of Nrf2, HO-1 and caspase-3 on postnatal day 36. Rats were treated with G (50 mg/kg), D (50, 150, 450 mg/kg) or combined treatment. *P<0.05 vs. control; #P<0.05 vs. G; ^ΔP<0.05 vs. corresponding D group. Nrf-2, NF-E2 related factor 2; G, genistein; D, di-2-(ethylhexyl) phthalate; HO-1, heme oxygenase-1.

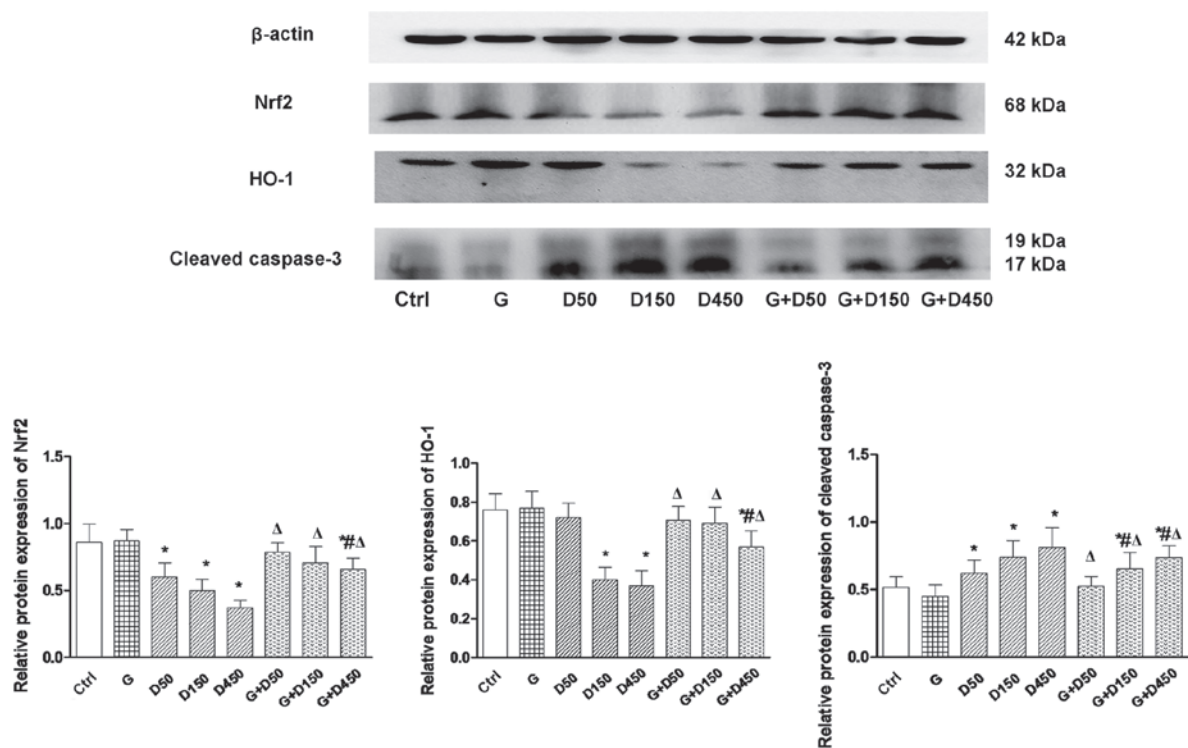


Figure 5. Protein expression of Nrf2, HO-1 and cleaved caspase-3 on postnatal day 36. Rats were treated with G (50 mg/kg), D (50, 150, 450 mg/kg) or combined treatment. *P<0.05 vs. control; #P<0.05 vs. G; ^ΔP<0.05 vs. corresponding D group. Nrf-2, NF-E2 related factor 2; G, genistein; D, di-2-(ethylhexyl) phthalate; HO-1, heme oxygenase-1.

were further validated at the protein level by western blot analysis and subsequent densitometry image analysis (Fig. 5). Consistent with gene expression, protein expression of Nrf2 and HO-1 exhibited similar trends. No significant changes were observed between the control and group G. With the increase in DEHP doses, a significant decrease in Nrf2 and HO-1 expression relative to control was observed (P<0.05), while the G + D50, G + D150 and G + D450 groups exhibited an increase in Nrf2 and HO-1 expression compared with the corresponding DEHP single exposure groups (P<0.05).

Cleaved caspase-3, the activated form of caspase-3, was elevated significantly in the three DEHP-treated groups (P<0.05), while the combination with GEN significantly reduced cleaved caspase-3 expression, although the level

remained increased compared with the control group (P<0.05), which further demonstrated that may GEN partially attenuate testis apoptosis in prepubertal testis, and that Nrf-2 and downstream HO-1 upregulation may have a vital role in alleviating DEHP-induced oxidative stress.

Discussion

Prepuberty is a critical period for male reproductive system development; during this stage the process of testicular spermatogenesis and steroidogenesis is highly responsive to EDC-induced endocrine disorders (5), which may result in disturbed spermatogenesis and higher incidence of testicular germ cell cancer (34). The acute effects following prepubertal

EDCs exposure include compromised development of androgen-dependent sex organs due to impaired testosterone production, ultimately contributing to decreased sperm motility and fertilizing ability at adulthood (10,35). However, the relative scarcity of studies on this developmental stage and the imminent concern of mixed EDCs exposure mean it is important to investigate the potential acute effects on testis development and other reproductive parameters (5).

It is well established that DEHP causes endocrine disruption in males, through its action as an androgen antagonist, and may have lasting effects on reproductive function, following childhood and adult exposure. Several toxic effects have been identified, including activation of PPAR- α gene and oxidative damage (8,9). Similar to various other isoflavones, GEN acts as an anti-oxidant, and thus may alleviate the damaging effects of free radicals in tissues (14,15). GEN also influences multiple biochemical functions in living cells, including activation of PPARs, inhibition of several tyrosine kinases, activation of Nrf2 anti-oxidative response and stimulation of autophagy (19,28). Theoretically, we can predict the effects of exposure to multiple EDCs and the mechanisms involved based on previous studies, however the co-administration multiple EDCs may not follow classical dose-responses and the health effects may differ from what would be expected by simply adding or subtracting the effects of individual components. Thus, it is necessary to elucidate the combined effects following exposure to EDCs, particularly for the most widely used plasticizer and the most common phytoestrogen.

The present study demonstrated that single exposure to the most common plasticizer, DEHP, particularly at high doses, decreased testis weight, and altered testis histology and development, of which the most significant histological abnormality was the tubular vacuolation. Typically, the tubular vacuoles are within or between Sertoli cells. In the prepubertal period Sertoli cells are relatively quiescent and seminiferous tubules grow slowly. Vacuolation of Sertoli cells is believed to be a common early feature of morphological injury to Sertoli cells prior to any germ cell degeneration, indicative of a breakdown in Sertoli-germ cell junctions (36). Alternatively, vacuoles often accompany generalized germinal cell degeneration, where they probably represent spaces left by the missing germ cells (37). All those effects indicate that prepubertal exposure to a high dose of DEHP may significantly delay tubule development of the testis during puberty, of which the Sertoli cell may be the main target of EDC-induced injuries. As Sertoli cell are capable of supporting a finite number of germ cells through cell-cell contact and maintaining the integrity of the epithelium, the decreased Sertoli cell number and functional deterioration established during prepuberty will inevitably lead to disruption of germinal cells differentiation or even spermatogenesis arrest. Notably, combined exposure to GEN and DEHP significantly increased the testis weight compared with DEHP-single exposure, testis cross sections also exhibited minor abnormality in the combined exposure, however tubular vacuolation was still observed in the G + D450 group; it is reasonable to speculate that GEN may exert protective effects when in combination with DEHP, which acts differently from the classical dose-responsive effect, highlighting the importance of assessing impacts across a range of doses, ages and mixtures.

AGD or adjusted AGD is a non-invasive index of masculinization and it has been confirmed that in humans AGD is positively correlated with testis size, sperm count/fertility, penis length and testosterone levels, consistent with experimental data from rats (38), which was associated with EDCs-induced male reproductive disruption during the masculinization programming window (39,40). However, the majority of published studies exclusively focused on EDCs exposure during gestation, as conditions associated with testicular dysgenesis syndrome are considered to initiate from aberrancies during fetal development. Other studies revealed that prepubertal rats exposed to phthalates at 0.4-2.2 g/kg/day exhibited altered seminiferous epithelium and delayed spermatogenesis (41,42); however no changes in AGD were reported in the previous reports, even with the highest dose of DEHP and its combination with GEN. In the current study, there was no significant alternation in AGD and adjusted AGD among the different treatment groups; a potential explanation is that the effects of DEHP, single exposure or in combination with GEN, were age-specific in this species. Additionally AGD or adjusted AGD may not be a sensitive parameter to reflect the prepubertal or even subsequent masculinization process.

It has been confirmed that controlled and low levels of oxidative stress are essential for normal testicular function, which is generated by spermatogenesis and steroidogenesis that are high energy-demanding processes. In a normal physiological state, testes are equipped with a potent anti-oxidant system that protects it against damage caused by reactive oxygen species (ROS) (43). Previous studies revealed that exposure to EDCs caused an imbalance in pro-oxidant/anti-oxidant levels and thus, promotes the generation of ROS (44,45). DEHP has been reported to have deleterious effects on the male reproductive system by inducing dramatic changes in germ cells, Sertoli cells and Leydig cells (46,47). However, the underlying mechanism by which DEHP exerts toxic effects on the reproductive system has not yet been fully elucidated. The results of the current study demonstrated that exposure to three doses of DEHP caused impairment of testicular anti-oxidative enzyme activities, alteration of the ratio of GSH/GSSG and an increase of MDA. The testicular anti-oxidative enzyme activities were associated with the dose of DEHP, which revealed aggravation of oxidative stress as the doses increased. Previous studies reported that DEHP exerted anti-androgenic potential critically mediated by suppressing fetal testosterone biosynthesis via PPARs activation (8), however, more recently DEHP was reported to induce oxidative stress in DEHP-mediated testicular dysfunction (7,10), in which alterations in the testicular enzymatic and nonenzymatic cellular anti-oxidants were involved, and accompanied by elevated level of ROS production and DNA damage. Kasahara *et al* (48) reported that DEHP enhanced the generation of ROS and selectively decreased GSH and ascorbic acid in the testis, thus inducing apoptosis of spermatocytes to cause atrophy in testes. In a study conducted to examine sperm function in adult rats following exposure to low-dose DEHP during adolescence, Hsu *et al* (49) reported a significant increase in hydrogen peroxide generation in the sperm following exposure to 1,000 $\mu\text{g}/\text{kg}$ DEHP, accompanied a higher percentage of sperm with tail abnormalities and increased sperm DNA fragmentation index.

For combined or mixed exposures, the health effects may differ from what would be expected from simply adding or

subtracting the effects of individual components, which leads to concern that combined exposures may exhibit aberrant effects on the male reproductive system, particularly for those possessing 'low-dose effect' (2,25). The combined exposure of GEN and DEHP resulted in upregulated activity of anti-oxidative enzymes and reduced MDA production compared with the DEHP single treatment group. Even though GEN appears to help testis recover from DEHP-induced testicular injuries, the enzyme activities were still reduced and the MDA level increased in the G + D150, G + D450 groups compared with the control group, suggesting that prepubertal GEN exposure partially attenuated DEHP-induced acute alterations in prepubertal testes and enhanced testicular anti-oxidative ability. Dietary intake of isoflavones has an important role in various physiological processes in the body. It is well established that GEN has both estrogenic (50) and anti-oxidative effects (51). A recent study revealed that GEN improved T-AOC, and decreased protein carbonyl and MDA levels in nephrotic rats (52). Similar findings were also reported in other *in vitro* and *in vivo* studies (53,54). Although other reported have investigated the combined effects of GEN with other EDCs in early studies, the information on the combination effects of DEHP and GEN is limited. Gong *et al* (14) revealed that GEN exerted anti-oxidant and cytoprotective effects in a neurotoxic animal model induced by another EDC, cadmium. Our most recent study [a collaboration with Martine Culty's group (20)] examined testicular effects following gestational DEHP exposure at a relative low dose, and revealed that the redox markers [NAD(P)H quinone dehydrogenase 1, SOD2, SOD3, thioredoxin, glutathione S-transferase and catalase] were altered significantly at PND3, whereas these effects were attenuated when combined with GEN, demonstrating the involvement of cellular stress in the short-term effects of DEHP and the protective effect of GEN.

The expression of genes and proteins involved in the process of testicular anti-oxidative defense and apoptosis was also examined in the present study. Exposure to DEHP, particularly at high doses, induced the downregulation of Nrf2 and HO-1 expression, whereas caspase-3 was upregulated. By contrast, cotreatment with GEN attenuated these effects. The kelch like ECH associated protein 1 (Keap1)-Nrf2-anti-oxidant responsive element (ARE) pathway is one of the most important defense system against oxidative stress, in which Nrf2 is constitutively controlled by repressor protein Keap1. In the event of oxidative stress, stress-sensing cysteine in cytoplasmic Keap1 changes conformation and subsequently dissociates from Nrf2, followed by Nrf2 translocation to the nucleus where it heterodimerizes with small Maf bZIP transcription factor protein and binds to AREs, resulting in the transcriptional regulation of target genes (55). Of all the target genes, HO-1 has been reported to be exclusively expressed in the Sertoli cells in rat testes and have a role in normal spermatogenesis (56). Different from the other isomers, including HO-2 and HO-3, HO-1 is an inducible type of HO and is detectable under normal conditions. Furthermore, HO-1 is reported to have the most AREs on its promoter, making it a highly protective anti-oxidant enzyme by degradation of its pro-oxidant substrate, heme, and enhancing the production of the anti-oxidants, biliverdin and bilirubin. The results of the current study suggest that GEN functions as an anti-oxidant in the testis and suppresses apoptosis by ROS scavenging via activation of Nrf2 and HO-1 expression, exerting protection against DEHP-induced testicular toxicity,

which is consistent with the morphological and histological changes in the PND36 rats.

The current study demonstrated that the natural phytoestrogen GEN partially normalizes ROS-induced acute effects of DEHP when administered prepubertally. Yousef *et al* (57) previously reported the beneficial effects of isoflavones in reducing the negative effects of cypermethrin on the reproductive characteristics of mature male New Zealand white rabbits when administered every other day for 12 weeks. However, redox state evaluation was not a focus in previous studies. In the current study, redox state alteration was evaluated following administration of three increasing doses of DEHP and their combination with GEN. The results suggested that prepubertal exposure to GEN and DEHP may lead to acute alterations, which were different from their individual effects. Thus, assessing reproductive risk based on the effects of individual chemicals may not accurately represent the true outcome of combined exposure during critical periods of male reproductive development. Future experiments will involve detailed analysis of cellular and molecular events contributing to acute effects on testis development, and epigenetic aberrations that may exert long-term perturbations in gene expression.

In conclusion, GEN partially attenuated DEHP-induced male reproductive system damage through the anti-oxidative activity following acute prepubertal exposure to DEHP. Treatment with GEN to reduce endocrine-disrupting side effects may have promising future for attenuating other EDC-induced reproductive disorders.

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Competing interests

The authors declare that they have no competing interests.

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