Oxidative Damage Induced by Di-(2-ethylhexyl) Phthalate and Protective Effects of Soybean Isoflavones in Mice

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Abstract: Di-(2-ethylhexyl) phthalate (DEHP) is widespread in the environment. As endocrine disruptors, it has seriously threatened human health and ecological environment safety. Male Kunming mice were treated daily with DEHP at the dosages of 250, 500 and 1 000 mg·kg⁻¹·d⁻¹, the relative organ weight, the contents of malondialdehyde (MDA) and L-Glutathione (GSH), and testis histology were investigated after 30-d DEHP treatment. Liver and lung enlargement and severe testicular atrophy were observed in a dose-dependent manner. The contents of hepatic MDA significantly increased, while the levels of hepatic and blood GSH both significantly decreased. In testis histological analysis, disruptions of spermatogenesis were observed such as necrosis and apoptosis of testicular cells, and massive sloughing of seminiferous tubule epithelium. The results showed that DEHP caused oxidative damage and reproductive toxicity in immature male mice, particularly at relatively high doses (500 and 1 000 mg·kg⁻¹·d⁻¹). To investigate the protective effect of soybean isoflavones (SI) on DEHP-induced oxidative damage and testicular injury, the mice were exposed to 1 000
mg·kg⁻¹·d⁻¹ DEHP and 100 mg·kg⁻¹·d⁻¹ SI. It is demonstrated that SI supplementation could significantly attenuate DEHP-induced oxidative stress and testicular injury in mice, probably due to the properties of SI as an antioxidant and estrogen regulator. **Keywords**: di-(2-ethylhexyl) phthalate (DEHP); oxidative damage; testicular injury; soybean isoflavones; antioxidant; estrogen regulator

Di-(2-ethylhexyl) phthalate (DEHP) accounts for a major part of phthalates that are a group of industrial chemicals widely used in consumer products, such as soap, shampoo, cosmetics and hairspray. DEHP is also used in flexible plastics, such as food and beverage packaging, children's toys and medical devices. Diet is the largest source of exposure of general population to DEHP, resulting from leaching of DEHP during processing, packaging and storing, and DEHP accumulation in certain foods. DEHP, dibutyl phthalate (DBP) and butyl benzyl phthalate (BBP) are found to be peroxisome proliferators which can cause oxidative damage, and therefore possess hepatocarcinogenic potential. Recently, it has been reported that mono-(2-ethylhexyl) phthalate (MEHP), the most active metabolite of DEHP, exerts deleterious effects on male reproductive system, especially neonatal and prepubertal males, and is suspected as endocrine disruptors. It has shown that DEHP is correlated to the prevalence of key features of the metabolic syndrome (e.g. abdominal adiposity, body mass index and insulin resistance), resulting in diabetes and obesity. Absolute and relative weights of androgen-dependent tissues or organs were affected by exposure to DEHP at 500 mg·kg⁻¹·d⁻¹ in male rats. Rats fed with a diet containing 1% (v/v) DEHP for 7-14 days showed severe testicular atrophy and oxidative stress. Studies also indicated that phthalate exposure might result in decline of human male fertility.

Soybean isoflavones (SI) occurs in large amounts in soybeans and soy products, chickpeas and other legumes, as well as clover, toothed medick and blue grass. As the two main isoflavones, genistein and daidzein are presented in the form of 7-O-glycosides, which are very similar to the chemical structure of mammalian estrogen (Fig. 1). According to the different types of estrogen receptor (ER) in cells, isoflavones can reduce or increase the activity of estrogen. Currently, SI is reported to effectively improve parameters related to aging, Alzheimer's disease, cardiovascular disease, cancer, gynecological problems and immune potentiation and has a neuroprotective effect on human cortical neurons, which may be due to its antioxidant capacity and phytoestrogenic activity. Long-term oral administration of isoflavones at 200 or 2 000 mg·kg⁻¹·d⁻¹ is unlikely to have adverse effect on reproductive function in male rats. Isoflavones are able to bind to the ER and act either as estrogen agonists or antagonists. As a selective estrogen receptor modulator (SERM), SI could be the potential therapeutic material for the treatment of metabolic disorders and the prevention of obesity and diabetes. Little research was conducted on the prevention of SI to DEHP-induced reproductive toxicity. The aim of the study is to investigate oxidative stress and injury in mice exposed to DEHP, and further to explore the therapeutic effects of SI on these damages.

**Fig. 1** Chemical structures of genistein and daidzein

### 1 Materials and methods

#### 1.1 Experimental animals

Male Kunming mice (aged 4 weeks, weighing approximately 20 g) were purchased from Laboratory Animal Center of Hubei (Wuhan, China). All the mice were fed with commercial diets, and given water
ad libitum. They lived in the pathogen-free cages at 20-25°C with 50%–70% relative humidity. The usage of animals was approved by the Office of Scientific Research Management of Huazhong Normal University with Certification on Application for the Use of Animals dated May 20th, 2007. All experimental procedures strictly adhered to the guidelines of National Committee of Animal Care and Use.

1.2 Chemicals

Di-(2-ethylhexyl) phthalate (DEHP) with purity of ≥ 99% was purchased from Sigma Chemical Co. (USA). Soybean isoflavones (SI) was purchased from Wuhan Yuancheng Technology Development Co., Ltd. (Wuhan, China). Its purity is 43.22% (genistin: 5.33%, genistein: 0.03%, daidzin: 23.64%, daidzein: 0.92%, glycitin: 13.14%, glycitein: 0.16%). GSH Kit was obtained from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). Any other chemicals were AR level.

1.3 Experimental design

The mice were randomly divided into six groups (A-F), at least five animals for every group. All the mice were orally administered DEHP reagent for 30 days. The control mice were administered saline only (denoted as Group A). The mice were administered 250, 500 and 1 000 mg·kg⁻¹·d⁻¹ DEHP in Group B, Group C and Group D. In Group E, the mice were administered 100 mg·kg⁻¹·d⁻¹ SI free of DEHP, while in Group F, the mice were administered 1 000 mg·kg⁻¹·d⁻¹ DEHP in the morning and 100 mg·kg⁻¹·d⁻¹ SI at night.

After 30 days, all the mice were sacrificed and their body weights were recorded. The liver, lung, kidney and testis were collected, weighted and processed according to the requirement of the following tests. The relative organ weight was expressed as organ weight per 100 g body weight.

1.4 Testis histological analysis

The testis were removed from the mice and immediately fixed in Bouin’s solution for 2 days until they were hard enough to be bisected. After being fixed for another day, the testis were washed by 5% (v/v) ammonia ethanol, and then processed for embedding in paraffin. After being stained with hematoxylin and eosin, the histopathological examination of the testis were conducted by light microscope (model XSP-2C, Shanghai Optical Instrument Factory, China).

1.5 Liver MDA analysis

The liver was made into 10% (v/v) homogenate. The amount of malondialdehyde (MDA) was used to reflect lipid peroxidation. 1.0 mL of 0.6% (v/v) thiobarbituric acid (TBA) was added to 1 mL samples and incubated at 100°C for 15 min. The treated samples were dissolved by 10% (v/v) trichloroacetic acid (TCA), then washed by tap water until cooling to room temperature, and centrifuged at 10 000 r·min⁻¹ for 10 min. The supernatant was measured at the wavelength of 450, 532 and 600 nm using a microplate reader (model ELx800, Bio-Tek Instruments, Inc. Vermont, USA).

1.6 GSH content analysis

The levels of L-Glutathione (GSH) were determined by using GSH assay kit. The application solution was added to 10% (v/v) homogenate of liver. After being centrifuged at 3 600 r·min⁻¹ for 10 min, a color reaction of the supernatant was performed. The samples were measured at the wavelength of 420 nm using a microplate reader (model ELx800, Bio-Tek Instruments, Inc. Vermont, USA). Results were expressed in mg GSH/g protein.

1.7 Statistical analysis

Data were analyzed using Origin 6.1. t test was conducted to analyze statistically significant differences between exposure groups and control groups. P<0.05 and P<0.01 were considered as significant difference and extremely significant difference, respectively. All the results were reported as mean ± SD.

2 Results

2.1 Body weight and organ relative weights

As shown in Table 1, there were significant differences in body weight between control group and five exposure groups (Group B, C, D, E and F). The testis relative weights of male mice administered by DEHP were found to decrease in a dose-dependent manner. The relative weights of testis in the
highest dose group (1 000 mg·kg⁻¹·d⁻¹ DEHP, Group D) dramatically decreased (P < 0.01) compared with control group (Group A), indicating severe testicular atrophy (Fig. 2). The testis relative weights increased in Group E (administered by 100 mg·kg⁻¹·d⁻¹ SI, P < 0.05). This is in agreement with the findings that a dose-dependent increase was observed in relative testis weights of male rats administered by SI (30-600 mg·kg⁻¹·d⁻¹) [17]. In contrast, the relative weights of liver and kidney significantly increased at 500 mg·kg⁻¹·d⁻¹ DEHP (Group C) and 1 000 mg·kg⁻¹·d⁻¹ DEHP (Group D) (P < 0.05 or P < 0.01). The degree of lung enlargement was more evident in the groups treated with dosage higher than 250 mg·kg⁻¹·d⁻¹ DEHP (Group B, C and D) (P < 0.01). Moreover, the relative weights of liver and testis in Group F were significantly different from those in Group D (P < 0.01) although significant difference was still observed between Group F and Group A (P < 0.05). The relative lung weight in Group F was lower than that in Group D (P < 0.05). The relative kidney weight in Group F exhibited no significant

<table>
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<th>Weight/g</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
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<td>Initial</td>
<td>21.89 ± 0.78</td>
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<td>32.44 ± 2.65**</td>
<td>36.68 ± 3.11*</td>
<td>34.22 ± 2.61**</td>
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</table>

Note: Group A (control group), Group B (treatment with 250 mg·kg⁻¹·d⁻¹ DEHP), Group C (treatment with 500 mg·kg⁻¹·d⁻¹ DEHP), Group D (treatment with 1 000 mg·kg⁻¹·d⁻¹ DEHP), Group E (treatment with 100 mg·kg⁻¹·d⁻¹ SI), Group F (treatment with 1 000 mg·kg⁻¹·d⁻¹ DEHP and 100 mg·kg⁻¹·d⁻¹ SI). * P < 0.05 and ** P < 0.01, compared with Group A. Data were expressed as mean ± SD, n = 7 or 8.

![Fig. 2 Relative organ weights of male mice after exposure to DEHP](image-url)

Note: Group A (control group), Group B (treatment with 250 mg·kg⁻¹·d⁻¹ DEHP), Group C (treatment with 500 mg·kg⁻¹·d⁻¹ DEHP), Group D (treatment with 1 000 mg·kg⁻¹·d⁻¹ DEHP), Group E (treatment with 100 mg·kg⁻¹·d⁻¹ SI), Group F (treatment with 1 000 mg·kg⁻¹·d⁻¹ DEHP and 100 mg·kg⁻¹·d⁻¹ SI). * P < 0.05 and ** P < 0.01, compared with Group A; # P < 0.05 and ## P < 0.01, compared between Group F and Group D.
difference compared with Group D. The above results showed that 1 000 mg·kg⁻¹·d⁻¹ DEHP could cause significant liver enlargement and testis atrophy, whereas SI could dramatically protect these organs from injury and damage.

2.2 Oxidative stress biomarkers

As presented in Fig. 3, hepatic MDA levels in Group B, Group C and Group D significantly increased compared with Group A (P < 0.01). However, the MDA level in Group F was markedly lower than that in Group D (P < 0.01), and exhibited no difference from that in control group (P > 0.05), which implied the treatment with 100 mg·kg⁻¹·d⁻¹ SI could suppress the production of MDA induced by 1 000 mg·kg⁻¹·d⁻¹ DEHP.

As can be seen in Fig. 4, the contents of both hepatic and blood GSH decreased in a dose-dependent manner after DEHP exposure, and they were significantly lower in Group C and Group D compared with Group A (P < 0.01). The reduction of GSH level induced by 1 000 mg·kg⁻¹·d⁻¹ DEHP was obviously prevented by the presence of 100 mg·kg⁻¹·d⁻¹ SI (hepatic GSH level, P < 0.05; blood GSH level, P < 0.01), as seen from the comparison between Group F and Group D. No significant difference was seen among Group E, Group F and Group A.

2.3 Histological analysis

A normal spermatogenesis was shown in the testis of control group. After 30-d DEHP exposure, disruptions of spermatogenesis in the testis were observed in Group C (Fig. 5C), and particularly in Group D (Fig. 5D). Severe testicular atrophy accompanied by aspermatogenesis and widespread apoptosis was seen within the seminiferous tubules. Necrosis and massive sloughing was also observed in...
seminiferous tubule epithelium. Group E exhibited complete spermatogenesis similar to that in Group A. An almost normal spermatogenesis was detected in Group F (Fig. 5F). Besides a marked recovery of testis weight was obtained in Group F (Fig. 2). Therefore, it was demonstrated that SI supplementation in the diet could protect the testis from the gonadotoxicity of DEHP.

3 Discussion

The present study showed that DEHP exposure at relative high dose (1 000 mg·kg⁻¹·d⁻¹) could induce oxidative stress in immature mice. It is unlikely that such a large dose of DEHP enters the human system. Because of its low vapor pressure and poor water solubility, concentrations of DEHP in outdoor air, water and indoor air are very low, in the range of 10 to 10² ng·m⁻³, 10⁻³ to 10 ng·m⁻³ and 10 to 100 ng·m⁻³, respectively²⁰. However, the bioaccumulation in organisms has the potential to help DEHP concentration to achieve the exposure level applied in present experiment. As seen in Group F, SI almost suppressed the liver and lung enlargement, MDA production and GSH decline which were induced by the oral administration of DEHP.

Former studies showed that DEHP caused significant reduction in the activities of enzymes dealing with the production of H₂O₂, and thus induced oxidative damage because of the accumulation of H₂O₂. The antioxidative agents such as d-psicose, vitamins C and vitamins E are potent scavengers of free radicals and terminators of free - radical chain reactions, so these antioxidants could prevent DEHP-induced testicular injury in rats.⁹²¹ The positive effect of genistein appeared to be mainly mediated by its antioxidative actions. Isoflavones could protect lipids from oxidative damage by scavenging the reactive species, such as lipid-based peroxy radicals.²² Isoflavones could protect lipids from oxidative damage by scavenging the reactive species, such as lipid-based peroxy radicals.²² Additionally, the oxidation from isoflavones towards low-density lipoprotein (LDL) involved stabilizing the structure of apoB and LDL.²⁵ GSH is a cosubstrate of glutathione peroxidase (cG-
SH-Px) and glutathione S-transferase (GST). GSH can scavenge $\text{O}^2^-\cdot$, $\text{H}_2\text{O}_2$ and lipid peroxyl radicals (LOO$^\cdot$), and then is converted to glutathione disulfide, thus protecting cellular thiol residues against oxidation $^{[24]}$. In this study, the levels of GSH significantly decreased after exposure to DEHP at relatively high doses ($500$ and $1\ 000\ \text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$). Meanwhile, the contents of MDA increased after DEHP exposure at doses higher than $250\ \text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$. Both of these indicated DEHP brought about lipid peroxidation in mice. $100\ \text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ of soybean isoflavones was able to effectively alleviate the DEHP-induced damage almost to the control group level, showing the decrease of MDA contents and the increase of GSH contents. The protective effect of SI for DEHP-treated mice may be due to its antioxidative property.

DEHP is well-known to be a reproductive toxicant that can cause the apoptosis and loss of spermatogenic cells, resulting in testicular atrophy. Zinc depletion might be another mechanism of DEHP-induced testicular toxicity $^{[26]}$. It has demonstrated that MEHP exerts suppressive effects on hCG-activated steroidogenesis in primary cultures of immature rat Leydig cells and suppresses 5-reductase activity $^{[27]}$. It is reported that the oxidative stress elicited by MEHP derived from DEHP is principally attributed to injuring mitochondrial function and inducing the release of cytochrome $c$, thereby causing spermatoocytes apoptosis and testis atrophy $^{[28]}$. The reasons mentioned above may partly explain the anti-androgenic effects of DEHP. In this study, isoflavones (e.g. SI) as phytoestrogens proved to be able to protect DEHP-treated mice from testicular atrophy, attenuating the adverse effects such as testis necrosis and apoptosis, and massive sloughing of seminiferous tubule epithelium. Isoflavones containing a phenolic ring can bind the ER, with a higher binding affinity to ER-$\beta$ than ER-$\alpha$ $^{[29]}$. The bioavailability and bioactivity of phytoestrogens vary depending on several factors, such as their exposure route, dosage, metabolism and interaction with other pharmacological substances $^{[30]}$. Isoflavones can elicit both estrogenic and antiestrogenic effects depending on tissue types and the levels of isoflavone and endogenous estradiol. In this study, results demonstrated that SI could ameliorate adverse effects induced by DEHP. This protective effect may be achieved though the SERM property of SI$^{[31-32]}$. After DEHP exposure, estradiol levels in mice remarkably increased. It is speculated that ER combined with SI activated transcription, protein synthesis and several other biological effects. Thereafter the DEHP-induced estradiol levels were lowered and organisms were recovered to the normal state.

In conclusion, DEHP exposure caused oxidative stress in male mice, characterized by the enlargement of liver, kidney and lung, the increased production of hepatic MDA, and the decline in hepatic and blood GSH levels. DEHP exposure decreased testicular relative weight, induced aspermatogenesis and widespread apoptosis within seminiferous tubules, and caused necrosis and massive sloughing of seminiferous tubule epithelium. Moreover, a moderate dose of isoflavones could alleviate DEHP-induced oxidative stress and testicular injury, due to the prevention of lipid peroxidation by free radicals scavenging.

**Biography of corresponding author**: Yang Xu (1954—), male, professor. His research field is environmental toxicology.

**References**


Chemical Toxicology, 2004, 42(1): 107 – 114


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