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**RESEARCH ARTICLE**

# Reproductive toxicity of di(2-ethylhexyl) phthalate in selenium-supplemented and selenium-deficient rats

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**Abstract**

Phthalates are abundantly produced plasticizers, and di(ethylhexyl) phthalate (DEHP) is the most widely used derivative in various consumer products and medical devices. Animal studies show that DEHP and various other phthalates cause reproductive and developmental toxicity. Although the evidences are limited, it seems reasonable that DEHP may have a potential for similar adverse effects in humans. Such concerns are increasing, particularly for the developing reproductive system of male infants and children. By taking into account the essentiality of selenium (Se) in testicular structure and functions and the high prevalence of inadequate Se intake in various part of the world, this study was designed to investigate the testicular toxicity of DEHP in Se-deficient male rats and to examine the possible preventive effects of Se supplementation on phthalate toxicity. Se deficiency was generated by feeding 3-week-old Sprague-Dawley rats with a ≤0.05 Se mg/kg diet for 5 weeks. Supplementation groups were on a 1 mg Se/kg diet, and DEHP-treated groups received a 1,000 mg/kg dose by gavage during the last 10 days of the feeding period. Testicular histopathology, sperm count and motility, and sperm morphology were examined, and plasma levels of sex hormones were measured. Toxicity and antiandrogenic effects of DEHP were evidenced by disturbed testicular histology and spermatogenesis, diminished testosterone, leutinizing hormone (LH) and follicle stimulating hormone (FSH) levels, and sperm motility. The effects of DEHP were much more pronounced in Se-deficient rats, whereas Se supplementation was found to be protective, reflecting its regulating role in cellular redox equilibrium.

**Keywords:** Phthalates, di(ethylhexyl) phthalate (DEHP), selenium deficiency, selenium supplementation, testosterone, FSH, LH, sperm, testicular histopathology



## Introduction

Endocrine disrupting chemicals (EDCs) are defined as "exogenous agents that interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance, homeostasis, reproduction, development and/or behavior" (Kavlock et al., 1996). There is a growing concern over the signs of an increasing incidence of male reproductive health problems and their possible association with exposure to EDCs. Along with the increasing incidence of hormone-dependent cancers, including cancer of the breast and prostate, declining of sperm counts, poor semen quality, increased incidences of hypospadias, cryptorchidism, the change in pubertal timing, and testis cancers have all been reported extensively within recent decades, and the term testicular dysgenesis syndrome (TDS) is used for this range of male reproductive defects (Fisher et al., 2003; Yiee and Baskin, 2010). More than 200 chemicals meet the criteria for classification as EDCs; among them, attention has been paid recently to phthalic acid esters, because the prenatal exposure of rodents to phthalates can cause TDS-like syndrome in male offspring postnatally (Sharpe and Skakkebaek, 2008). Phthalates are one of the most abundant synthetic chemical contaminants in the environment, produced in high volume and used as plasticizers to impart flexibility and durability to plastics that are widely used in personal care products, food packaging and medical devices,

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cosmetics, baby feeding tubes, nipples, and toys. They leach out from the plastic matrix and generate extensive human exposures by various means. Phthalates are peroxisome proliferators (PPs) and hepatocarcinogens in rodents (Rusyn et al., 2006), and they target fetal and pubertal testis and lead to alterations in endocrine and spermatogenic functions (Lyche et al., 2009).

Di(2-ethylhexyl) phthalate (DEHP) is the most widely used phthalate derivative, ubiquitously present in flexible plastics, and the only phthalate currently used in polyvinyl chloride (PVC) medical devices. DEHP and its main metabolite, mono(2-ethylhexyl)phthalate (MEHP), were shown to induce testicular damage in both developing and adult animals (Lyche et al., 2009; Noriega et al., 2009) and decreased sperm motility (Kwack et al., 2009). Their exposures have also been found to result in decreased testicular testosterone (T) production in rodents (Jones et al., 1993), indicating that Leydig, along with Sertoli, cells are their targets. However, the mechanisms by which phthalates and, specifically, DEHP exert toxic effects in the reproductive system are not yet fully elucidated. Some of the effects are related to their antiandrogenic potential (Ge et al., 2007). A PP-activated receptor $\alpha$ (PPAR$\alpha$)-mediated pathway based on their PP activity (Gazouli et al., 2002) and activation of metabolizing enzymes leading to free radical production and oxidative stress have also been suggested (O'Brien et al., 2005). As to the effects in humans, available epidemiological data are not sufficient; therefore, there is no consensus on how phthalate exposure affects male reproductive toxicity in humans. Nevertheless, a recent study shows significant correlations between urine concentrations of phthalate monoesters, the primary metabolites of phthalates, in pregnant women and the incidence of anomalies, such as cryptorchidism and shortened anogenital distance, in their male infants (Swan et al., 2005). A recent study from our group also showed that plasma DEHP and MEHP levels were significantly high in pubertal gynecomastia patients (Durmaz et al., 2010).

The requirement for selenium (Se) and its beneficial effects in human health have been known for several decades. Low dietary Se intakes are associated with health disorders, including oxidative stress-related pathologies, reduced fertility and immune functions (Broadley et al., 2006), and increased risk of cancers (Clark et al., 1991). With its several forms of cellular selenoproteins, Se is primarily involved in the modulation of intracellular redox equilibrium and has a critical importance for cellular antioxidant defense (Steinbrenner and Sies, 2009). Se is also essential for the production of normal spermatozoa and thus plays a critical role in testis, sperm, and reproduction (Flohé, 2007). Testis Se is known to be remarkably and preferentially maintained in Se deficiency. It was reported that severe and prolonged deficiency in rodents results in sterility as spermatogenesis is arrested, whereas less severe Se deprivation reduces sperm motility, leading to impaired fertilization capacity and abnormal sperm morphology (Maiorino et al., 2006). Because soil Se content is the primary determinant of Se in food, Se deficiency in man occurs mainly in regions where Se soil content is low. Several parts of the world (e.g., Denmark, Finland, New Zealand, eastern and central Siberian Russia, a long belt from northeast to southcentral China, and Central Africa) are known for having very low amounts of Se in their soils and thereby in their food systems (Combs, 2001). However, low Se intakes have also been reported in parts of eastern Europe (Nève, 1995) and in Turkey (Giray and Hincal, 2004). Many of the plasma/serum Se levels reported (from 68 countries) are less than the minimum level associated with optimal plasma glutathione peroxidase (GPx3) activity (70 ng/mL) (Nève, 1995), suggesting that subclinical Se deficiency may affect at least 10% of residents in most countries and half of the population in some countries (Combs, 2001). Diseases directly linked to severe Se deficiency are destructive osteoarthritis (Kashin-Beck disease) and lethal myocarditis (Keshan disease) (Yang et al., 1988). Further, the clinical evidence that severe Se deficiency, in combination with other environmental factors, is deleterious for the thyroid was found in Central Africa, and children developed myxedematous cretinism in a region with endemic iodine and/or Se deficiency (Contempre et al., 1991).

Considering the essentiality of Se in testicular structure and functions but frequent availability of inadequate Se intakes and the high probability of DEHP exposure in humans, this study was designed to investigate the testicular toxicity of DEHP in Se-deficient male rats and to examine the possible protective effects of Se supplementation on phthalate toxicity.

## Materials and methods

### Chemicals and reagents

All chemicals, including Tris, diethylene triamine pentaacetic acid (DTPA), and phenylmethylsulfonyl fluoride (PMSF), were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Commercial chemiluminescence kits for rat plasma T and estradiol ($E_2$) were obtained from DPC (Los Angeles, California, USA). Follicle-stimulating hormone (FSH) immunoradiometric assay (IRMA) kits and luteinizing hormone (LH) radioimmunoassay (RIA) kits were purchased from MP Biochemicals (Asse-Relegem, Belgium). PureSperm Wash and the DiffQuik Stat III® Sperm Staining kit were from Nidacon International (Mölndal, Sweden) and MidAtlantic Diagnostics (Mt. Laurel, New Jersey, USA), respectively. All animal feed (A03/R03 base) was supplied by Scientific Animal Food and Engineering (SAFE) Laboratories (Augy, France).

### Animals and treatment

Male Sprague-Dawley (SD) rats, 3 weeks old, supplied from Hacettepe University Experimental Animals Laboratory (Ankara, Turkey), were used in the experiments. The animals were divided randomly in six groups, and each group was housed in plastic cages with stainless-steel grid tops. The cages were placed in a room

with controlled temperature (23°C), humidity (50%), and a 12-hour light-dark cycle. Body weights (BWs) were monitored weekly, including before the first dose of DEHP treatment. The feeding period was 5 weeks. The animals were treated humanely and with regard for alleviation of suffering, and the study was approved by the Hacettepe University Ethical Committee.

### Experimental groups

The control group (C) was fed a regular diet (0.15 mg/ kg Se); the selenium-supplemented group (SeS) was fed an Se-supplemented diet (1 mg/kg Se); the selenium-deficient group (SeD) was fed an Se-deficient diet (≤0.05 mg/kg Se); the DEHP-treated group (DEHP) was fed a regular diet (0.15 mg/kg Se) and received 1,000 mg/kg of DEHP during the last 10 days by intragastric gavage (i.g.); the selenium-supplemented DEHP group (DEHP/SeS) was fed an Se-supplemented diet (1 mg/kg Se) and received 1,000 mg/kg of DEHP during the last 10 days by i.g.; and the selenium-deficient DEHP group (DEHP/SeD) was fed an Se-deficient diet (≤0.05 mg/kg Se) and received 1,000 mg/kg of DEHP during the last 10 days by i.g. Animals were allowed to access *ad libitum* feed and drinking water.

Twenty-four hours after the last dose of DEHP treatment, overnight fasted animals were weighed and sacrificed by decapitation under thiopental anesthesia. Blood samples were collected into heparinized tubes, plasma was separated after centrifugation at 800 x *g* for 15 minutes, and the testis, epididymis, and liver were removed and weighed.

### Hormone measurements

Plasma FSH and LH levels were determined by IRMA and RIA, respectively, using commercial kits. Plasma T and $E_2$ concentrations were measured by commercial chemiluminescence kits.

### Sperm count, motility, and morphology

After removal, the right epididymis was trimmed and separated into the caput and cauda by obvious anatomical landmarks (i.e., the caput and corpus were separated at the neck, whereas the corpus and cauda were separated at the site where engorged tubules were first recognized). Weight of the cauda was recorded for the calculation of sperm counts, placed into a glass Petri dish containing PureSperm Wash supplemented with 0.5% bovine serum albumin, and minced with anatomic scissors. The suspension was centrifuged at 800 x *g* for 10 minutes. The pellet was diluted with PureSperm Wash, 10 µL of the suspension was applied to Neuber hemocytometer for sperm count and motility, and 100 sperms were assessed by manual counting for progressive sperm motility under a microscope at X200 magnification.

The same diluted samples were also used for sperm morphology. First, 10 µL of the suspension was applied to a slide and stained with the DiffQuik Stat III Sperm Staining kit. The abnormal and normal sperms were counted manually (100 sperms for each slide, duplicate counting) at X400 magnification, and abnormal sperms were classified as follows: tail without head, head without tail, big head, small head, amorphous head, other head abnomalies, midpiece anomaly, and tail anomaly.

### Histopathological evaluation

The right testis was divided into 2 pieces. One piece of the fresh tissue samples was rapidly fixed in Bouin's fixative solution, then dehydrated through graded alcohols and embedded in paraffin blocks. Sections (5-µm) were stained with hematoxylin and eosin according to standard protocols. The second piece of the fresh tissue samples was fixed in 2.5% glutaraldehyde solution in phosphate buffer (pH 7.4) for 4 hours and postfixed for 1 hour in 1% osmium tetroxide solution, dehydrated in alcohol, treated with propylene oxide, and embedded in araldite. Semithin sections were stained with methylene blue-azure II. The sections were examined and photographed with a Leica (DM6000B; Wetzlar, Germany) microscope with a DC490 digital camera (Leica).

### Selenium determination

#### *Sample preparation*

Left testis tissue was homogenized with homogenate buffer (10 mM Tris/1 mM DTPA/1 mM phenylmethylsulfonyl fluoride; PMSF) using a Teflon-glass homogenizer, then centrifuged at 2,000 x *g* at 4°C for 10 minutes. The supernatant was further centrifuged at 20,000 x *g* at 4°C for 20 minutes. The resulting supernatant was diluted to 1:25 with 1% (w/v) $HNO_3$ (ultrapure grade) prior to analysis. Galium (Ga, at 650 nmol/L) was used as an internal standard.

#### *Preparation of calibration solution and instrument configuration*

Standard addition calibration solution was prepared by the dilution of a parent stock Se solution (100 mg/L) with 1% (w/v) $HNO_3$ and the addition of 100 µL of homogenate buffer. Se measurements were performed by inductively coupled plasma mass spectrometry (ICP-MS) (PerkinElmer, Waltham, Massachusetts, USA). The instrument was operated with a Peltier cooled impact bead spray chamber, a single-piece quartz torch (1.5 mm i.d. injector) together with XT interface cones, and the collision cell option. A standard nebulizer was used. The instrument was run using the following parameters: radiofrequency (RF) power: 1,200 W; nebulizer gas flow: 0.90 L/min; auxiliary gas flow: 0.60 L/min; and cool gas flow: 13.5 L/min. Isotopes measured were $^{71}$Ga (internal standard) and $^{78}$Se. Dwell time per isotope were 20 ($^{71}$Ga) and 300 ms ($^{78}$Se). Sample uptake was 90 seconds, and wash time was 30 seconds. Two repeats were done per sample, and 20 repeats were done for the blank and the homogenate buffer.

### Statistical analysis

All the results were expressed as mean ± standard error (SEM). The differences among the groups were evaluated

with Kruskal-Wallis one-way analysis of variance, followed by Mann-Whitney U test using a Statistical Package for Social Sciences Program (version 17.0; SPSS, Inc., Chicago, Illinois, USA). *P*-values <0.05 were considered as statistically significant.

## Results

### Body and organ weights

Throughout the experiments, all animals appeared to remain healthy. No significant difference was observed in food intake between the groups. There was no significant alteration in BW in any of the groups before DEHP treatment started. After 10 days of DEHP exposure, weight gain in all DEHP-exposed groups was found to be significantly lower ($P<0.05$) than the control group, and Se supplementation was found to be partially protective (Figure 1).

Testis, epididymis, and liver weights were measured at the termination of the experiments, and their relative weights are shown in Table 1. Relative weights of livers of all DEHP-treated groups were found significantly higher than control, but neither absolute nor relative testis and epididymis weights changed with DEHP exposure.

### Plasma hormone levels

In DEHP- exposed rats, plasma T concentrations were found to be significantly (~35%; $P<0.05$) decreased (Figure 2A). Se-deficient animals (SeD) and the DEHP/SeD group also showed lower levels of T, compared to the control group (~60%; $P<0.05$). Se supplementation increased the circulating T by more than ~35%; however, possibly due to high variations, the difference was not significant ($P>0.05$). Compared to the DEHP group, the T level of the DEHP/SeD group was ~35% ($P < 0.05$) lower, whereas an ~83% ($P \sim 0.05$) increase was observed in DEHP/SeS animals, indicating the protective effect of Se. On the other hand, plasma E2 levels did not change significantly in any of the treatment groups, compared to the control group (Figure 2B).

High biological variations were noted in plasma LH levels, so that except for a marked decrease (~84%; $P<0.05$) in the DEHP/SeD group, LH levels of the study groups were not found to be significantly different than that of the control (Figure 2C).

Table 1. Effects of DEHP and selenium status on relative organ weights.

| | Relative testis weights (g/ 100 g BW) | Relative epididymis weights (g/ 100 g BW) | Relative liver weights (g/ 100 g BW) |
|---|---|---|---|
| Control | 0.81 ± 0.03[a] | 0.15 ± 0.01[a] | 4.70 ± 0.38 [a] |
| SeS | 0.78 ± 0.02[a] | 0.17 ± 0.01 [a] | 4.99 ± 0.07 [a] |
| SeD | 0.79 ± 0.02[a] | 0.15 ± 0.01 [a] | 4.82 ± 0.14 [a] |
| DEHP | 0.70 ± 0.01[a] | 0.14 ± 0.01 [a] | 6.38 ± 0.09 [b] |
| DEHP/SeS | 0.79 ± 0.01[a] | 0.14 ± 0.01 [a] | 6.39 ± 0.09 [b] |
| DEHP/SeD | 0.70 ± 0.02[a] | 0.15 ± 0.01 [a] | 6.35 ± 0.08 [b] |

Experimental groups for 5 weeks were on (C) regular diet (0.15 mg/ kg Se); (SeS) Se- supplemented diet (1 mg/kg Se); (SeD) Se- deficient diet (≤ 0.05 mg/kg Se); (DEHP) regular diet (0.15 mg/ kg Se) and received (1,000 mg/kg, i.g). DEHP for the last 10 days; (DEHP/SeS) Se- supplemented diet (1 mg/kg Se) and received (1,000 mg/kg, i.g.) DEHP for the last 10 days; and (DEHP/SeD) Se- deficient diet (≤ 0.05 mg/kg Se) and received (1,000 mg/ kg, i.g.) DEHP for the last 10 days.

Rows that do not share same *letters* (superscripts) are significantly different from each other (P < 0.05).

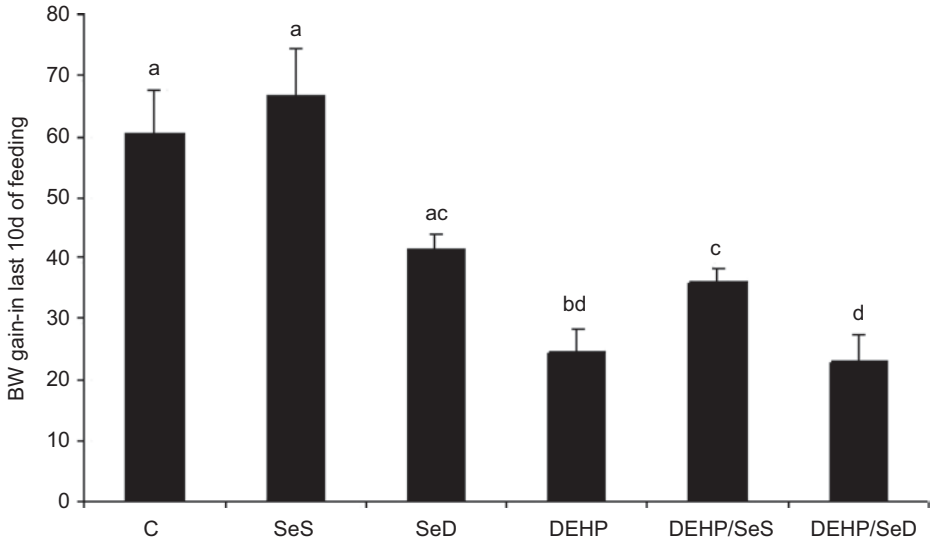


Figure 1. Effects of DEHP and Se status on BW gain in pubertal male rats. BW gains are shown as the difference of the BWs measured on the initial [postnatal day (PND) 46] and terminal (PND 55) days of DEHP treatment. All results were given as mean ± SEM of $n=6$ animals. Bars that do not share same *letters* (superscripts) are significantly different from each other (P < 0.05). Experimental groups for 5 weeks were on the following: (C) regular diet (0.15 mg/kg Se); (SeS) Se- supplemented diet (1 mg/kg Se); (SeD) Se- deficient diet (≤ 0.05 mg/kg Se); (DEHP) regular diet (0.15 mg/kg Se) and received (1,000 mg/kg, i.g.) DEHP for the last 10 days; (DEHP/SeS) Se- supplemented diet (1 mg/kg Se) and received (1,000 mg/kg, i.g.) DEHP for the last 10 days; and (DEHP/SeD) Se- deficient diet (≤ 0.05 mg/kg Se) and received (1,000 mg/kg, i.g.) DEHP for the last 10 days.

Significantly decreased FSH levels were found by DEHP exposure (~20% in DEHP and ~41% in DEHP/SeD groups; $P < 0.05$), but there were no alterations in the other groups (Figure 2D).

### Sperm count and motility

Compared to control rats, epididymal sperm count markedly increased in Se-supplemented animals (~94%; $P < 0.001$) and decreased in DEHP-exposed, Se-deficient animals (~14%; $P < 0.05$). The rest of the alterations were insignificant (Figure 3A).

DEHP exposure decreased progressive sperm motility significantly (~38% in DEHP and ~53% in DEHP/SeD groups; $P < 0.05$), compared to the control group. Se deficiency itself showed a significant motility-decreasing effect (~18%; $P < 0.05$), but Se supplementation did not cause any alteration. However, in DEHP-exposed animals Se supplementation provided such a protection that a ~47% increase in sperm motility was observed in the DEHP/SeS, compared to DEHP/SeD, group ($P < 0.05$) (Figure 3B).

### Sperm morphology

The percentage of sperms with normal morphology were diminished in the SeD (10%), DEHP (13%), and DEHP/SeD (17%) groups, compared to controls, but the decrease was significant only in the DEHP/SeD group (Figure 4A).

Significant increase of sperms with "tail anomalies" were observed in the DEHP and DEHP/SeD, compared to both control and SeS, groups ($P < 0.05$) (Figure 4B). The percentage of sperms with "tail without head" anomaly also increased significantly in the SeD, DEHP, and DEHP/SeD groups. In DEHP/SeS animals, Se supplementation prevented the induction of sperm tail without head by DEHP exposure (Figure 4C). In DEHP and/or SeD groups, several other types of morphologic abnormalities were observed, but none of them were significant (data not shown).

### Testicular histopathology

Normal testicular histology was observed in the control group (Figure 5A). The seminiferous epithelium was well organized with Sertoli cells and normal germ

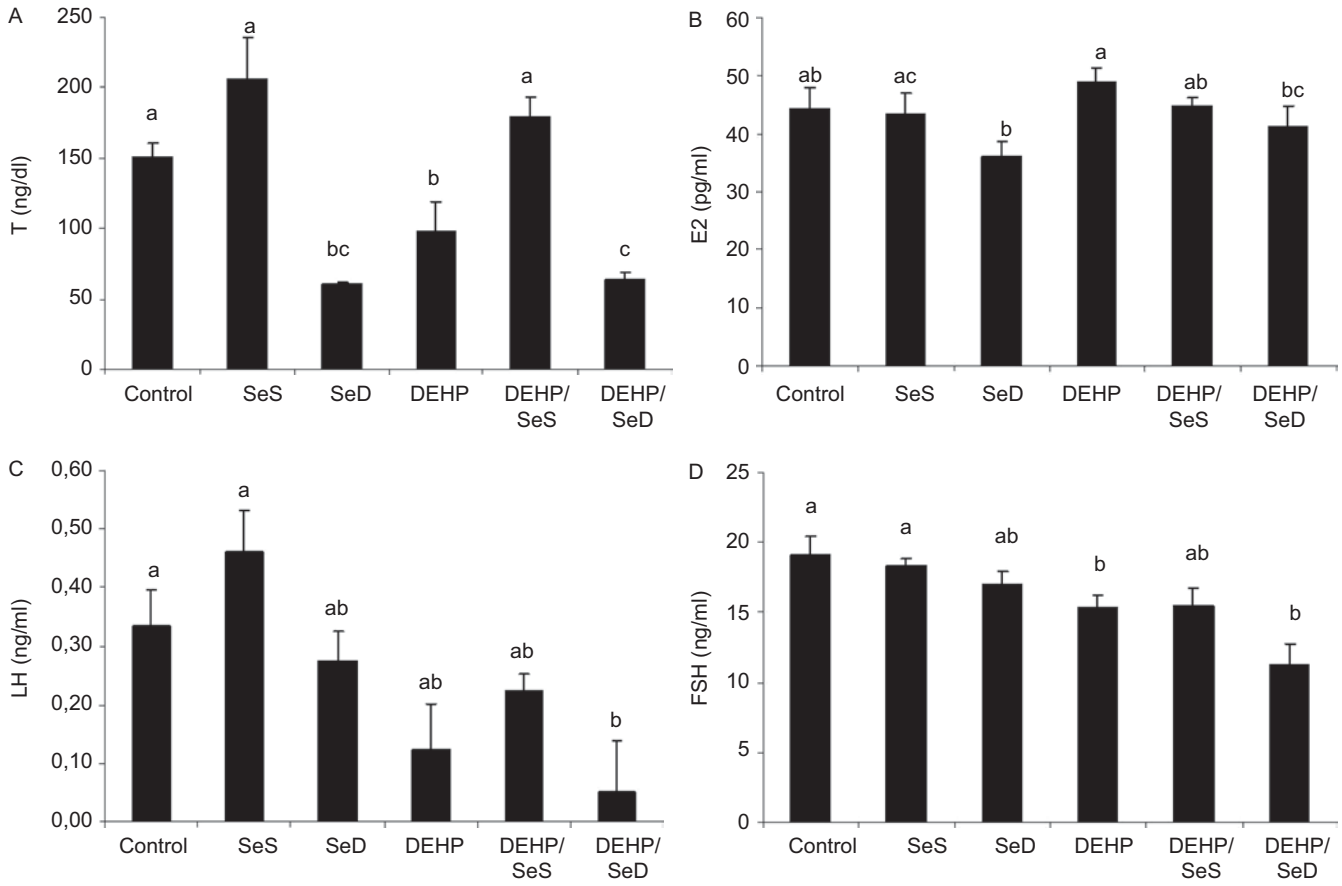


Figure 2. Effects of DEHP and Se status on circulating hormone levels in pubertal male rats. (A) Testosterone (T); (B). Estradiol ($E_2$); (C). luteinizing hormone (LH); (D). follicle- stimulating hormone (FSH). All results were given as mean ± SEM of $n = 6$ animals. Bars that do not share same *letters* (superscripts) are significantly different from each other ($P < 0.05$). Experimental groups for 5 weeks were on the following: (C) regular diet (0.15 mg/kg Se); (SeS) Se- supplemented diet (1 mg/kg Se); (SeD) Se- deficient diet (≤ 0.05 mg/kg Se); (DEHP) regular diet (0.15 mg/kg Se) and received (1,000 mg/kg, i.g.) DEHP for the last 10 days; (DEHPSeS) Se- supplemented diet (1 mg/kg Se) and received (1,000 mg/kg, i.g.) DEHP for the last 10 days; and (DEHPSeD) Se- deficient diet (≤ 0.05 mg/kg Se) and received (1,000 mg/kg, i.g.) DEHP for the last 10 days.

cells at various stages. Normal histology and an intact seminiferous tubule epithelium was also found in the SeS group, and spermium heads were observed in the seminiferous tubule epithelium, suggesting that spermatogenesis was accelerated with Se supplementation, compared to the control group (Figure 5B). In the SeD group, detachments were observed between germ and Sertoli cells in some tubules, but most of the tubules were normal (Figure 5C). In spite of the presence of small vacuoles and lipid droplets in the cytoplasm, normal Sertoli cell histology as well as normal Leydig cells were observed.

In the DEHP group, the structure of the seminiferous tubule epithelium was disturbed, the basal membrane of the tubules was uneven, and significant detachments were detected between the spermatogenic cells and Sertoli cells. Both spermato- and spermiogenesis were observed to be disturbed. Cellular debris was seen in the lumen of the seminiferous tubules consisting of spermatogenic cells being differentiated, but not reached to spermium stage (Figure 5D). Large spaces were observed between Sertoli and germ cells, indicating that the normal relation between these two cell types was disturbed. Further, marked increases in the number and volume of the Leydig cells and lipid droplets were noted.

In the DEHP/SeS group, the structure of the seminiferous epithelium was normal in most of the tubules, although not a full restoration was observed, compared to the DEHP group (Figure 5E). Distruption of spermatogenesis was noted in some of the tubules; however, most of the spermatogenic cells were well preserved. Leydig cells were observed between seminiferous tubules in the interstitial zone and their numbers increased, but not as high as in the DEHP/SeD group.

In the DEHP/SeD group, the structure of seminiferous tubules were severely degenerated (Figure 5F). Tubule diameters decreased, the spermatogenic epithelium detached from the basement membrane, an increased number of empty spaces between the basal membrane and spermatogonia, and a slight intertisiel edema between seminiferous tubules were observed. Cell loss and exfoliation were more evident than in the DEHP group, and the exfoliated epithelium formed patches of homogenous necrotic cell residues. Very few spermatids were seen in close association with Sertoli cells, and loss of spermatogonia was observed in some of the tubules, indicating that the spermatogenesis was severely disrupted. Besides, the number of Leydig cell was increased even more than seen in the DEHP group, and there were profound lipid droplets.

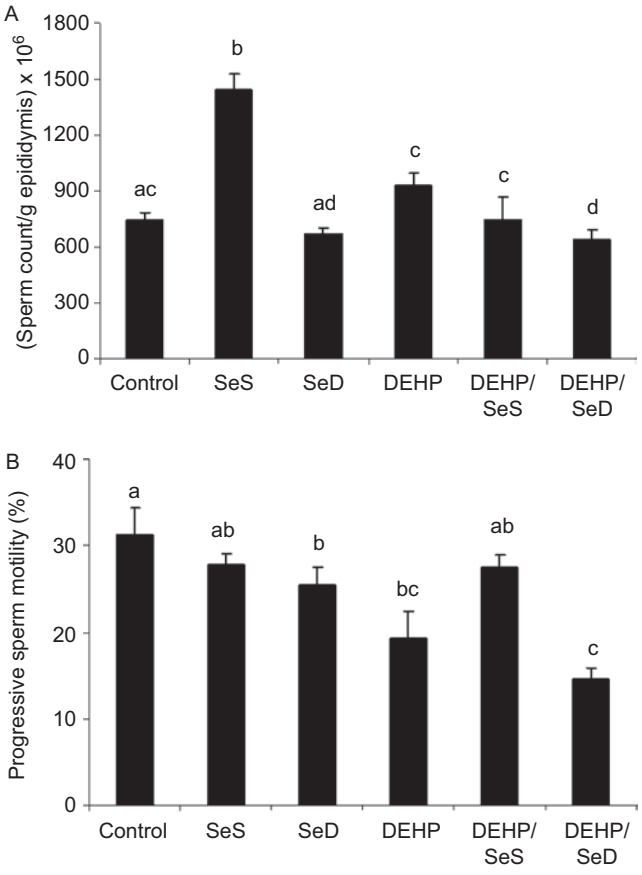


Figure 3. Effects of DEHP and Se status on epididymal sperm count and progressive sperm motility in pubertal male rats. Sperm counts were determined using a hemocytometer, and 100 sperms were used for progressive sperm- motility assessment. All results were given as mean ± SEM of $n = 6$ animals. Bars that do not share same *letters* (superscripts) are significantly different from each other ($P < 0.05$). Experimental groups for 5 weeks were on the following: (C) regular diet (0.15 mg/kg Se); (SeS) Se- supplemented diet (1 mg/kg Se); (SeD) Se- deficient diet (≤ 0.05 mg/kg Se); (DEHP) regular diet (0.15 mg/kg Se) and received (1,000 mg/kg, i.g.) DEHP for the last 10 days; (DEHP/SeS) Se- supplemented diet (1 mg/kg Se) and received (1,000 mg/kg, i.g.) DEHP for the last 10 days; and (DEHP/SeD) Se- deficient diet (≤ 0.05 mg/kg Se) and received (1,000 mg/kg, i.g.) DEHP for the last 10 days.

### Testis selenium levels

Se deficiency was evident in the SeD group, with a significant decrease of testicular Se concentration (0.27 ± 0.01 vs. 0.43 ± 0.01 μg/g in control), and in SeS animals testis Se level was found to be increased markedly (0.5 ± 0.11 μg/g tissue), compared to the control group ($P < 0.05$).

## Discussion

The main aims of the present study were to investigate the testicular toxicity of subacute DEHP exposure in normal and Se-deficient male rats and to examine the possible protective effects of Se supplementation. Results are discussed below.

### Effects of DEHP as a testicular toxicant

The present study showed a significant level of decrease in circulating T in pubertal SD rats that received (1,000 mg/kg, i.g.) DEHP for 10 days. Besides a significant decrease in FSH, there was also a marked tendency of reduction in LH levels. Because the hypothalamic–pituitary–testicular (HPT) axis regulates reproductive function and gonadal steroids modulate the HPT axis, our results suggest that

along with a direct effect on T production in the testis, the responsiveness of the negative feedback system might be inhibited, suggesting also the impairement of pituitary-hypothalamic function. This suggestion is in line with the results of previous studies that have demonstrated the adverse effects of DEHP on the HPT axis in neonatal female rats, as well as on *ex vivo* steroidogenesis in granulosa cells and secretion of LH by gonadotropes (Supornsilchai et al., 2007). However, the direct effect of DEHP on the testis and its modulation of fetal T production, testicular physiology, and mammalian reproduction and fertility were clearly shown earlier by several studies (Akingbemi et al., 2001; Borch et al., 2006; Jones et al, 1993), and the decrease we observed in circulating T level was in accord with most of the previously reported data for several phthalate derivatives (Jones et al., 1993; Ge et al., 2007). DEHP and other phthalates are universally categorized as prototype antiandrogens and affect T

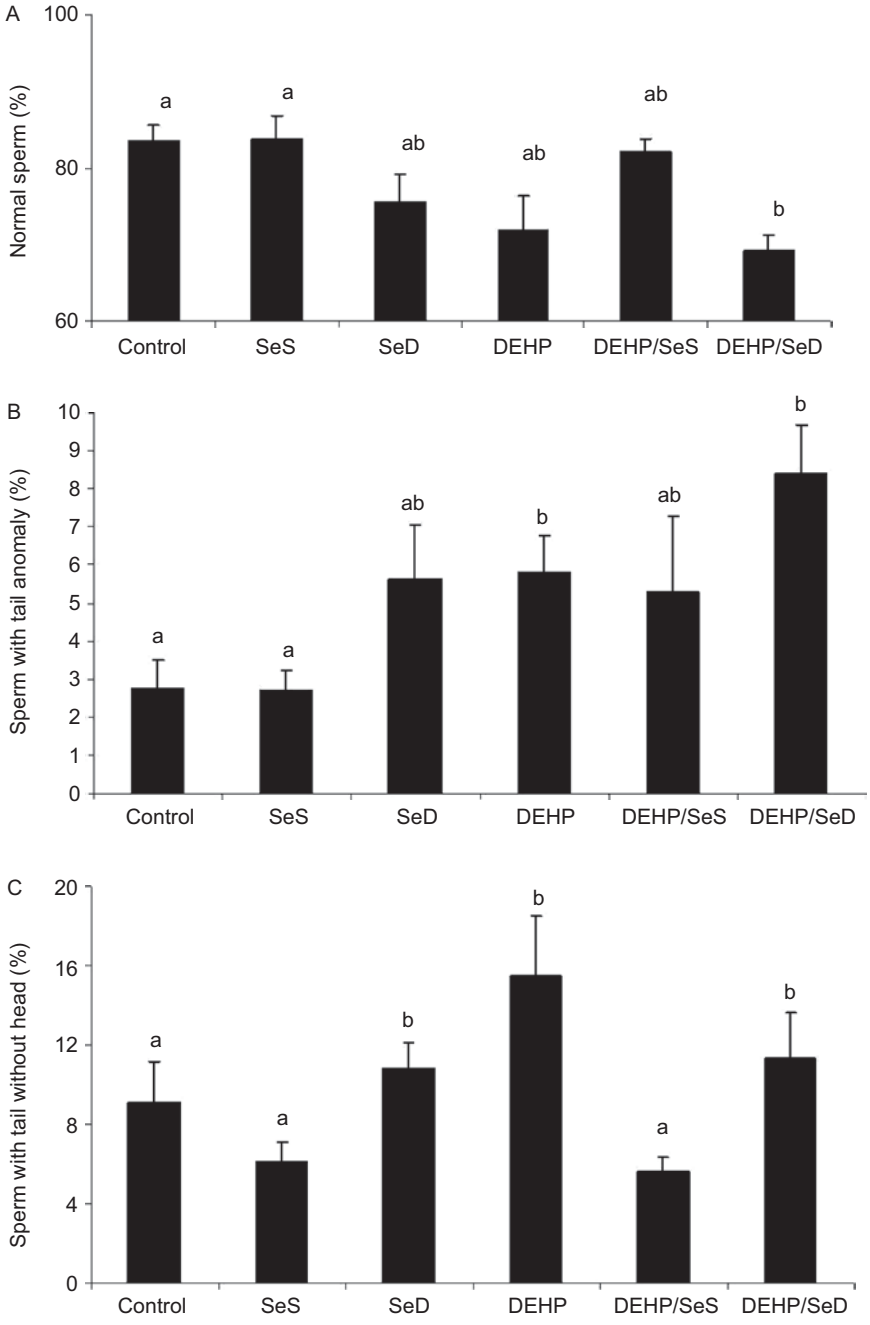


Figure 4. Effects of DEHP and Se status on epididymal sperm morphology in pubertal male rats. 100 sperms were used for the assessment of sperm- morphology abnormalities. All results were given as mean ± SEM of $n = 6$ animals. Bars that do not share same *letters* (superscripts) are significantly different from each other ($P < 0.05$). Experimental groups were (for 5 weeks) on the following: (C) regular diet (0.15 mg/kg Se); (SeS) Se- supplemented diet (1 mg/kg Se); (SeD) Se- deficient diet (≤ 0.05 mg/kg Se); (DEHP) regular diet (0.15 mg/ kg Se) and received (1,000 mg/kg, i.g.) DEHP for the last 10 days; (DEHP/SeS) Se- supplemented diet (1 mg/kg Se) and received (1,000 mg/kg, i.g.) DEHP for the last 10 days; and (DEHP/SeD) Se- deficient diet (≤ 0.05 mg/kg Se) and received (1,000 mg/kg, i.g.) DEHP for the last 10 days.

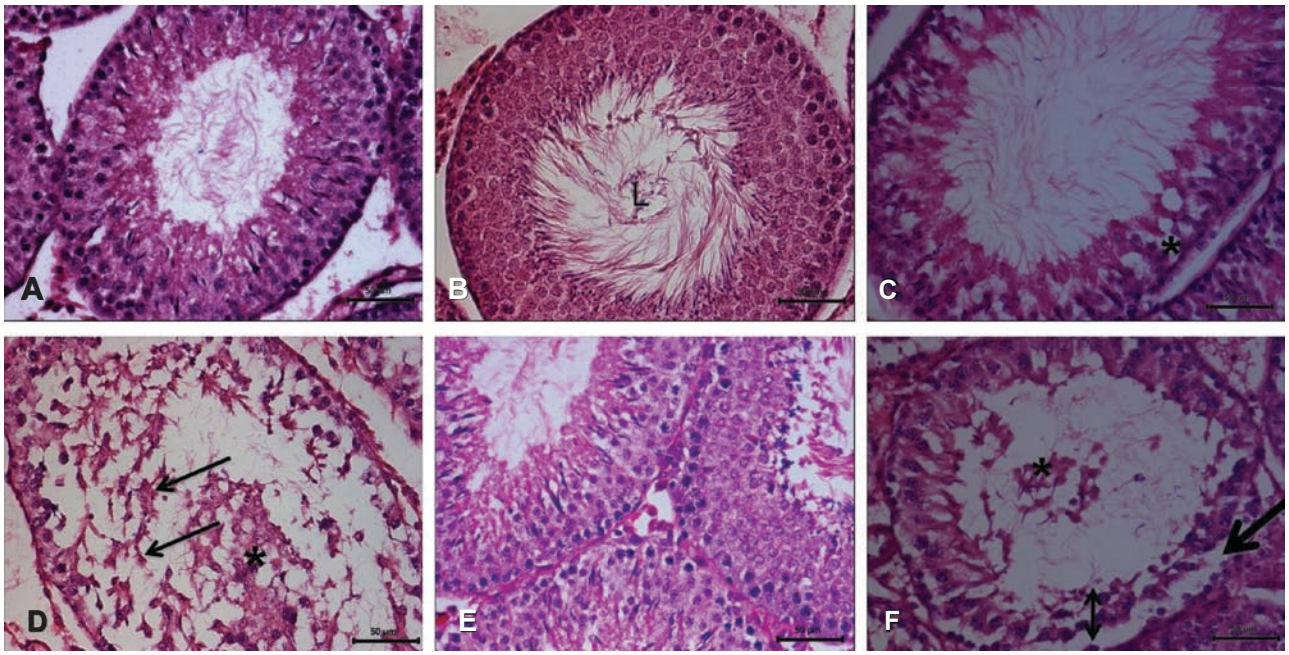


Figure 5. Histological sections of seminiferous tubules from pubertal male rats with different Se status and DEHP exposure (stained with hematoxylin- and eosin, magnification X400). (A). Normal seminiferous tubule histology in the C group. (B). Normal seminiferous tubule histology containing abundant spermium in the lumen (L) in the SeS group. (C). Large spaces between Sertoli cells and germ cells in the SeD group (ASTERIX). (D). Degeneration in tubules, detachments between spermatogenetic cells, and spilling of the spermatogenetic germ cells (arrow) and cellular debris (asterix) in the lumen in the DEHP group. (E). Normal tubule epithelium in the DEHP/SeS group. (F). A severely degenerated tubule in the DEHP/SeD group. Exfoliated and detached germ cells (asterix). Detachment between seminifereous epithelium and basement membrane (arrow). Thinning of the seminiferous epithelium (double- headed arrow).

production by affecting the development of Leydig cells, the steroidogenic capacity of which is required for normal testis descent and spermatogenesis (Ge et al., 2007). The effect of phthalates on the reproductive system and Leydig cells is mainly dependent on the term of exposure. *In utero* exposures cause a decrease in fetal Leydig cell function, lower testicular levels of T (Fisher et al., 2003; Parks et al., 2000), and insulin-like growth factor 3 (INSL3) mRNA (Wilson et al., 2004) and induce postnatal changes, such as cryptorchidism, hypospadias, impaired spermatogenesis, and reduced male fertility in rats (Parks et al., 2000). In neonatal exposure *via* the mother's milk, a correlation between exposure levels and adverse reproductive health outcomes was reported (Main et al., 2006). Pubertal exposures to phthalates produce complex results dependent on both duration and dose; acute exposures may differ significantly from chronic exposures, and it appears that phthalates have biphasic effects on both fetal and adult Leydig cells (Akingbemi et al., 2001; Jones et al., 1993; Culty et al., 2008). However, the results of a recent study have not provided evidence of a biphasic dose response to DEHP during puberty, and suggested that DEHP may be acting on the pubertal male rat testis *via* two modes of action: one *via* the Leydig cells and the other *via* the Sertoli cells.(Noriega et al., 2009).

In the present study, toxicity of DEHP in the testis was further evidenced with the observed histologic alterations, which include degeneration in seminiferous tubule structure, Sertoli cell vacuolation, germ-cell detachment, hypospermatogenesis, and Leydig cell hyperplasia. These findings were in line with various previous data reported for high doses of DEHP and MEHP. The observed lipid droplet accumulation in the DEHP group was also in accord with earlier reports, which were described as a morphologic evidence of the inhibition of lipid utilization (Dees et al., 2001; Jones et al., 1993).

We observed significantly low weight gain and increased liver weight in DEHP-exposed animals, but in contrast to most previous reports, there was no change in testis weight. This might be mainly due to the differences in dose, term and duration of exposure, species, and strain, as suggested earlier (Noriega et al., 2009; Vo et al., 2009).

A reduction in sperm production after DEHP exposure was reported previously (Dalsenter et al.., 2006; Kwack et al., 2009). In the current study, although we did not find a significant decrease in sperm count, we observed a significant reduction in sperm motility and abnormal maturation of the sperms, as evidenced by the increased number of sperms with abnormal morphology. The regulation and cellular interactions occuring in the testis are so intricate that toxicants can disrupt spermatogenesis by affecting multiple distinct targets. Adult mammalian spermatogenesis is a T-dependent process (O'Donnell et al., 1996). T, secreted by Leydig cells under the control of LH, exerts its effect to control the fate of developing germ cells (Sun et al., 1990; McLachlan et al., 1994). Differentiation of round

spermatids to elongated spermatids requires the action of T, whereas lower T levels cause premature detachment of round spermatids and thus cause a failure to complete spermiogenesis. FSH, the other pituitary hormone, is also vital for normal spermatogenesis and plays an essential role in spermatogonial development (McLachlan et al., 2002; O'Donnell et al., 2005). Lack of functional FSH causes impaired fertility and defected elongated spermatids, and Sertoli cells, which are the important regulators of germ-cell differentiation (Krishnamurthy et al., 2000; Grover et al., 2004). Thus, our study, with its data showing significant decreases in both T and FSH levels and demonstrating disturbances in the testis histology, such as vacuolation of Sertoli cells and germ-cell detachment from them, strongly indicated the impairment of the spermatogenic process by DEHP exposure.

Multiple mechanisms of action were suggested for phthalate effects in the reproductive system, including PP-activated or estrogen receptor-mediated mechanisms (Gazouli et al., 2002), dysregulation of gene-expression pattern (Borch et al., 2006; Fan et al., 2010), and affecting spermatogenesis by altering the activities of enzymes responsible for the maturation of sperms (Barlow et al., 2003). On the other hand, our recent studies on MA-10 Leydig (Erkekoglu et al., 2010a) and LNCaP human prostate cells (Erkekoglu et al., 2010b) have produced data suggesting that at least one of the mechanisms underlying the reproductive toxicity of DEHP and MEHP might be the induction of intracellular reactive oxygen species (ROS) and causing alterations in intracellular enzymatic and nonenzymatic antioxidants, thereby producing oxidative stress. In fact, these two studies showed that both DEHP and MEHP caused significant decreases in cell viability, altered antioxidant status, particularly decreasing glutathione peroxidase 1 (GPx1) and thioredoxin reductase (TrxR) activities, and induced DNA damage as measured by the alkaline Comet assay. The data of Fan et al. (2010) have also suggested a new mechanism of MEHP action on Leydig cell streidogenesis *via* CYP1A1-mediated ROS stress.

### Effects of selenium on testis and testicular biomarkers

It has long been known that Se is essential for the reproductive system (Flohé, 2007). In the rodent testis, Se concentrations are typically higher than for any other tissue except the kidney and generally do not decrease even with prolonged Se deficiency, showing that it is preferentially maintained in the rodent testis (Behne and Höfer-Bosse, 1984). When Se supply is limited due to Se deficiency, incoming Se as selenoprotein P (Sepp1) would be delivered preferentially to the Sertoli cells, thereby maintaining testis Se concentrations (Schriever et al., 2009). Se is known to be involved in the modulation of intracellular redox equilibrium, and the major role of Se in fertility is mediated by phospholipid hydroperoxide glutathione peroxidase (GPx4), which is the most abundant selenoprotein in the testis, is synthesized in spermatids under indirect control of T, and protects biomembranes from peroxidative damage as required for proper sperm stability (Ursini et al., 1999).

In our experimental conditions, Se status did not affect either body or organ weights, but affected steroid hormone levels, testicular histology, and spermatogenesis. Se deficiency was evident with a significant decrease of testicular Se concentration and caused significant diminution of T, decreased motility, and increased morphologic abnormalities in epididymal sperms, but did not affect sperm count. Whereas Se supplementation did not cause alterations in motility and morphology of sperms, but increased their number markedly and although not significant, a tendency of increase in T and LH levels was observed. All these findings were in the same line as the histological findings of the study where normal histology, intact seminiferous tubule epitelium, and accelaration of spermatogenesis was observed in the SeS group, whereas detachments in the seminiferous basal membrane was noted in the SeD group.

### Effects of selenium on the testicular toxicity of DEHP

As expected from the testicular-damaging effect of DEHP and the critical importance of Se in the testis, in DEHP-exposed, Se-deficient rats, marked alterations in hormone levels and sperm qualities and worsening of testicular damage were found, compared to DEHP-exposed normal rats, as well as compared to control animals. In addition to the much higher decreases in T, LH, and FSH levels and motility and number of sperms, higher morphologic abnormalities were observed in DEHP/SeD animals, compared to both the DEHP and SeD groups. When Se-supplemented animals received DEHP, however, T and FSH concentrations remained as those of the control group and thus provided significant improvement, compared to the DEHP group. Sperm motility was also protected, and the effects on sperm morphology seemed to be reduced. Compared to the DEHP group, BW gain increased in the DEHP/SeS group, but still remained lower than the control. All these findings indicated that Se was protective against the effects of DEHP, whereas Se deficiency increased the damage produced in testis structure and functions.

It is known that the seminiferous epithelium and mature sperms require a particularly efficient protection against oxidative stress (Tramer et al., 1988). Antioxidants can be beneficial to minimize the detrimental effects of oxidative stress-producing toxicants. In fact, whereas ROS can inhibit Leydig cell steroid producion (Diemer et al., 2003), this can be partially prevented by antioxidants (Hanukoglu, 2006; Sen Gupta et al., 2004). Fan et al. (2010) demonstrated that the increase in ROS generation with MEHP exposure in MA-10 cells is inhibited by *N*-acetylcysteine (NAC). In the above-mentioned *in vitro* studies (Erkekoglu et al., 2010a, 2010b), we also demonstrated that Se supplementation with either sodium selenite (SS) or

selenomethionine (SM) was highly protective against the cytotoxicity and genotoxicity of DEHP and MEHP in both MA-10 Leydig and LNCaP cells. Further, 30 nM SS and 10 μM SM produced almost the same level of protection against antioxidant status-modifying effects, ROS, and p53-inducing potentials, and DNA-damaging effects of these phthalate derivatives on MA-10 Leydig, as well as LNCaP, cells. It was thus shown that DEHP produced oxidative stress in MA-10 Leydig cell cultures, and Se supplementation appeared to be an effective redox regulator in the experimental conditions used, implicating its critical role in the modulation of redox state in the testicular cells. The results of the current *in vivo* study showing the influence of Se status on the adverse effects of DEHP in rats similarly suggest that DEHP exposure may cause alterations in the cellular redox state, and that Se provides protection by the same mechanisms.

## Conclusions

In conclusion, this study demonstrated that the testicular toxicity of DEHP exposure in pubertal rats is modified by Se status, suggesting an oxidative stress induction by DEHP as a possible mechanism. Our results emphasize the importance of Se status for reproductive health; however, the bimodal nature of Se as being a pro- and antioxidant, depending on the dose (Steinbrenner and Sies, 2009), should be carefully considered.

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## Decleration of interest

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