Effects of Di(2-Ethylhexyl)Phthalate on Testicular Oxidant/Antioxidant Status in Selenium-Deficient and Selenium-Supplemented Rats

Pınar Erkekoglu,[1] Belma Giray,[1] Walid Rachidi,[2] Isabelle Hininger-Favier,[3] Anne-Marie Roussel,[3] Alain Favier,[2] Filiz Hincal[1]

[1]Hacettepe University, Faculty of Pharmacy, Department of Toxicology, 06100 Ankara, Turkey

[2]CEA Grenoble, INAC/SCIB/LAN, 17 Rue des Martyrs, 38054 Grenoble Cedex 9, France

[3]LBFA, Universite Joseph Fourier, Grenoble F-38041, France

Received 25 April 2011; revised 25 August 2011; accepted 31 August 2011

ABSTRACT: Di(ethylhexyl)phthalate (DEHP), the most widely used plasticizer, was investigated to determine whether an oxidative stress process was one of the underlying mechanisms for its testicular toxicity potential. To evaluate the effects of selenium (Se), status on the toxicity of DEHP was further objective of this study, as Se is known to play a critical role in testis and in the modulation of intracellular redox equilibrium. Se deficiency was produced in 3-weeks-old Sprague–Dawley rats feeding them $\leq$0.05 mg Se /kg diet for 5 weeks, and Se-supplementation group was on 1 mg Se/kg diet. DEHP-treated groups received 1000 mg/kg dose by gavage during the last 10 days of the feeding period. Activities of antioxidant selenoenzymes [glutathione peroxidase 1 (GPx1), glutathione peroxidase 4 (GPx4), thioredoxin reductase (TrxR)], catalase (CAT), superoxide dismutase (SOD), and glutathione *S*-transferase (GST); concentrations of reduced glutathione (GSH), oxidized glutathione (GSSG), and thus the GSH/GSSG redox ratio; and thiobarbituric acid reactive substance (TBARS) levels were measured. DEHP was found to induce oxidative stress in rat testis, as evidenced by significant decrease in GSH/GSSG redox ratio ($>$10-fold) and marked increase in TBARS levels, and its effects were more pronounced in Se-deficient rats with $\sim$18.5-fold decrease in GSH/GSSG redox ratio and a significant decrease in GPx4 activity, whereas Se supplementation was protective by providing substantial elevation of redox ratio and reducing the lipid peroxidation. These findings emphasized the critical role of Se as an effective redox regulator and the importance of Se status in protecting testicular tissue from the oxidant stressor activity of DEHP. © 2011 Wiley Periodicals, Inc. Environ Toxicol 29: 98–107, 2014.

Keywords: di(ethylhexyl)phthalate; antioxidant enzymes; lipid peroxidation; oxidative stress; GSH/GSSG redox ratio; selenium deficiency; selenium supplementation

INTRODUCTION

There is a growing concern over the increasing incidence of male reproductive health problems and their possible association with exposure to endocrine disrupting chemicals (EDCs). Among the EDCs, phthalates are of high interest, because exposure to phthalates is ubiquitous and many phthalate derivatives are present in the environment ranging from food containers to toys and baby bottles, from intravenous tubings and blood storage bags to polyvinyl chloride (PVC) floorings and household dust (Graham, 1973; Rock et al., 1986; Bornehag et al., 2004).

Di(2-ethylhexyl)phthalate (DEHP) is the most widely used phthalate derivative and extensively present in flexible plastics such as PVC medical devices. It is found in

Correspondence to: B. Giray or F. Hincal; e-mail: bgiray@hacettepe.edu.tr or fhincal@tr.net

Contract grant sponsor: Hacettepe University Research Fund
Contract grant number: 0701301001

Published online 5 October 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/tox.20776

amniotic fluid, placenta, and fetal tissues of rats following ingestion, suggesting that DEHP might have an effect on the development of embryonic tissues (Singh et al., 1975; Calafat et al., 2006). The no-observed-adverse-effect level (NOAEL) and lowest observed adverse effect level (LOAEL) were reported as 3.6 and 37.6 mg/kg bw/day, respectively (Poon et al., 1997). Based on available animal studies, NOAEL of DEHP for humans was reported to be 3.7–14 mg/kg/day for reproductive/developmental effects (Kavlock et al., 2006). A tolerable daily intake of 0.05 mg/kg bw, based on a NOAEL of 4.8 mg/kg bw/day (Wolfe and Layton, 2003), was proposed by EFSA (2005). The typical human exposure to DEHP ranges from 3 to 30 μg/kg/day (Doull et al., 1999) but can be exceeded in specific medical conditions. In fact, for hemodialysis patients 1.5 mg/kg/day exposure, and for neonatal transfusion or parenteral nutrition between 10 and 20 mg/kg/day of DEHP exposures have been reported (Loff et al., 2000; Kavlock et al., 2006). Because phthalates (low-molecular weight) are also used to make coatings for oral medications, even two orders of magnitude higher than general population exposure level were reported in patients using phthalate containing drugs (Hauser et al., 2004; Hernández-Díaz et al., 2009).

DEHP and its main metabolite, mono(2-ethylhexyl)phthalate (MEHP) have been deleterious on the male reproductive system of the neonatal, prepubertal, and adult animals (Oishi, 1990; Li et al., 2000; Lyche et al., 2009; Noriega et al., 2009) and induced dramatic changes in germ cells (Richburg and Boekelheide, 1996; Kasahara et al., 2002). Researches over the three decades indicate that both Sertoli cells and Leydig cells are the primary testicular targets for phthalate derivatives (Dostal et al, 1988; Akingbemi et al., 2001). However, the mechanisms by which phthalates and particularly DEHP exert toxic effects in reproductive system are not yet fully elucidated. Some of the effects are related to their antiandrogenic potential (Ge et al., 2007). A peroxisome proliferator-activated receptor α-mediated pathway based on their peroxisome proliferating (PP) activity (Gazouli et al., 2002), and activation of metabolizing enzymes has also been suggested (O'Brien et al., 2005). Although the induction of an oxidative stress may represent a common mechanism in endocrine disruptor-mediated dysfunction, especially on testicular cells (Latchoumycandane et al., 2002), recent studies are also providing supporting evidences for such an effect with phthalates. Our recent studies conducted on LNCaP human prostate cells and MA-10 Leydig cells have shown the induction of an oxidative stress by DEHP exposure (Erkekoglu et al., 2010a,b, 2011a). This was evidenced by the changes in the enzymatic and nonenzymatic cellular antioxidants, increases in reactive oxygen species (ROS) production and induction of DNA damage.

Selenium (Se), the constituent of 25 selenoproteins in the body, is primarily involved in the modulation of intracellular redox equilibrium, thereby has a critical importance for the cellular antioxidant defense (Steinbrenner and Sies, 2009). Se-dependent glutathione peroxidases (GPxs) and thioredoxin reductases (TrxRs) protect the body from the endogenous products of cellular metabolism that have been implicated in DNA damage, mutagenesis, and carcinogenesis (Ganther, 1999; Jablonska et al., 2009). Moreover, Se is essential for the production of normal spermatozoa, thus, plays a critical role in testis, sperm, and reproduction (Flohé, 2007). 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., 1998).

Based on this background and taking into account the frequency of inadequate Se intakes, essentiality of Se in antioxidant system, and its potential for protection against testicular toxicity of DEHP exposure, this study was designed to investigate the effects of DEHP on oxidant/antioxidant parameters in the rat testis and to evaluate the possible changes by different Se status.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Colorimetric assay kit for TrxR was also from Sigma-Aldrich. Sep-Pak C18 cartridge was obtained from Waters (Milford, MA). All animal feed (A03/R03 base) were supplied by Scientific Animal Food and Engineering (SAFE) Laboratories (Augy, France).

Animals and Treatment

Male Sprague–Dawley rats, 3-weeks-old, supplied from Hacettepe University Experimental Animals Laboratory, were used in the experiments. Animals were divided randomly in six groups of six of each, 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-h light–dark cycle. Body weights were monitored weekly, including before the first dose of DEHP treatment. Feeding period was 5 weeks. The animals were treated humanely and with regard for alleviation of suffering, and the study was approved by Hacettepe University Ethical Committee.

Experimental Groups

(1) Control Group was fed regular diet (0.15 mg/kg Se); (2) Selenium-Supplemented Group (SeS) was fed Se-supplemented diet (1 mg/kg Se); (3) Selenium-Deficient Group (SeD) was fed Se-deficient diet ($\leq$0.05 mg/kg Se); (4) DEHP-Treated Group (DEHP) was fed regular diet (0.15

mg/kg Se) and received 1000 mg/kg DEHP during the last 10 days by intragastric gavage (i.g.); (5) Selenium-Supplemented DEHP Group (DEHPSeS) was fed Se-supplemented diet (1 mg/kg Se) and received 1000 mg/kg DEHP during the last 10 days by i.g.; (6) Selenium-Deficient DEHP Group (DEHPSeD) was fed Se-deficient diet ($\leq$0.05 mg/kg Se) and received 1000 mg/kg 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 and left testes were removed, frozen immediately in liquid nitrogen, divided into pieces, and stored at -80°C until the preparation of tissue homogenates.

Preparation of Testis Homogenates

Testis homogenates were prepared in a volume of ice-cold buffer containing Tris (10 mM), diethylenetriamine pentaacetic acid (1 mM), and phenylmethanesulphonyl fluoride (1 mM; adjusted to pH 7.4) using a Teflon pestle homogenizer to obtain 10% (w/v) whole homogenate. After centrifugation at 1500 $\times$ *g*, 4°C, for 10 min, thiobarbituric acid reactive substance (TBARS) concentration was measured in the supernatant. The rest of the supernatants were recentrifugated at 9500 $\times$ *g*, 4°C for 20 min, and the enzyme activities were determined in the supernatant. For the measurement of reduced glutathione (GSH), oxidized glutathione (GSSG), and total glutathione (tGSH) levels, the whole homogenate was diluted (5:1) with metaphosphoric acid (6%), centrifuged at 1500 $\times$ *g*, 4°C, for 10 min, and the supernatant was used.

Determination of Antioxidant Enzyme Activities

The activity of GPx1 was measured in a coupled reaction with glutathione reductase (GR) as described earlier (Günzler et al., 1974; Flohé and Günzler, 1984).

The assay of GPx4 activity was based on the same reaction as GPx1 determination, but freshly synthesized phosphatidylcholine hydroperoxide (PCOOH) was used as a substrate as described by Maiorino et al. (1990). One unit of enzyme was defined as the amount of GPx4 that transformed 1 μmol of NADPH to NADP per min at 37°C. PCOOH was synthesized using the method of Maiorino et al. (1990) and Weitzel et al. (1990) with some modification. Briefly, 10 mg L-phosphatidylcholine (Type III from soybean) was dried under nitrogen stream, dispersed in 4 mL of 3% deoxycholate, and diluted with 21 mL of 0.2 M sodium borate, pH 9.0. The reaction was started by the addition of 27.7 μl (5,000,000 U) of lipoxidase (EC 1.13.11.12, Type IV), bubbling with 99% O_2 using an oximeter (Hansatech, Norfolk, UK) and stirring on a heater bath (Julabo, Allentown, PA) at 37°C. The reaction mixture was then passed through a Sep-Pak C18 cartridge, which was previously washed with methanol and equilibrated with water. The cartridge was washed with 20 mL water, and PCOOH was then eluted with 2 mL of methanol, aliquoted, and kept at -80°C where it was stable for 4 weeks.

TrxR activity was determined colorimetrically using the Thioredoxin Reductase Assay kit. As described previously (Arnér et al., 1999), the method was based on the reduction of 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB) with NADPH into 5-thio-2-nitrobenzoic acid (TNB) the concentration of which was measured at 412 nm. One unit of TrxR activity was defined as the amount of enzyme that caused an increase in absorbance of 1.0 per min and per milliliter at pH 7.0 at 25°C.

Catalase (CAT) activity was determined according to Aebi (1974). The enzymatic decomposition of H_2O_2 was followed directly at 240 nm. One unit of CAT activity was defined as the amount of enzyme required to decompose 1 μmol H_2O_2 in 1 min.

The total superoxide dismutase (SOD) and Mn-SOD activities were determined by monitoring the auto-oxidation of pyrogallol at 420 nm (Marklund and Marklund, 1974). One unit of total SOD activity was defined as the amount of enzyme required to inhibit the rate of pyrogallol auto-oxidation by 50%. Potassium cyanide (3 mM) was used to inhibit both Cu,Zn-SOD, and extracellular SOD, resulting in detection of only MnSOD activity. Cu,Zn-SOD activity was then determined by substracting Mn-SOD activity from total SOD activity.

Glutathione *S*-transferase (GST) activity was determined using 1-chloro-2,4 dinitrobenzene as a substrate, monitoring the rate of production of 2,4-diphenyl glutathione at 340 nm, and the enzyme activity was expressed in μmol/mg protein/min (Habig et al., 1974).

Determination of Total, Reduced, and Oxidized Glutathione Levels

tGSH (GSH plus GSSG) contents of the samples were assessed using a kinetic assay in which catalytic amounts of GSH caused a continuous reduction of DTNB to TNB. GSSG formed was recycled by GR and NADPH, and the formation of TNB was monitored at 412 nm at 25°C (Akerboom and Sies, 1981). Quantification was achieved by parallel measurements of a standard curve of known GSH concentrations, and results were expressed in nmol/mg protein. For GSSG determination, reduced GSH was inactivated by the addition of 2-vinylpyridine. GSH content was calculated using the equation of GSH = (tGSH – 2 $\times$ GSSG).

Determination of Lipid Peroxidation

Lipid peroxidation (LP) in testis tissues was quantified measuring the concentration of TBARS by a

spectrofluorometric assay as described by Richard et al. (1992), and the level of TBARS was expressed as micromole per gram tissue.

Protein Determination

Protein concentrations were determined by the standard method of Lowry et al. (1951).

Statistical Analysis

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 (SPSS) version 17.0. *p*-values <0.05 were considered as statistically significant.

RESULTS

As we previously reported (Erkekoglu et al., 2011b), all animals appeared to remain healthy throughout the experiments. Significant differences in the food intake were not observed between the groups. There was no significant alteration in body weights in any of the groups before the DEHP treatment started. After 10 days of DEHP exposure, the weight gain in all DEHP-exposed groups was found significantly lower ($p < 0.05$) than the control group, and Se supplementation was found to be partially protective. At the termination of the experiments, neither absolute nor relative testis weights changed with DEHP exposure.

Under the experimental conditions used in the study, testicular GPx1 and GPx4 activities did not change significantly neither with DEHP exposure nor with Se supplementation or Se deficiency [Fig. 1(A,B)]. However, in DEHP-exposed Se-supplemented animals (DEHP/SeS group), GPx1 activity increased significantly (∼75%, $p < 0.05$), whereas GPx4 activity decreased markedly in DEHP/SeD group (∼45%, $p < 0.05$) compared to controls.

Testicular TrxR activity did not significantly alter by DEHP exposure [Fig. 1(C)], however, decreased both in Se-deficient animals and in DEHP/SeD group (∼30%, $p < 0.05$).

In none of the groups, total SOD and Mn-SOD activities changed markedly [Fig. 2(A,C)]. However, the activity of Cu,Zn-SOD was found as significantly decreased in DEHP and DEHP/SeS groups (∼45 and ∼50%, respectively; $p < 0.05$) compared to the control group [Fig. 2(B)]. It was also noted that Cu,Zn-SOD activity of the DEHP/SeD group was ∼30% higher than that of DEHP group ($p < 0.05$).

Except for an insignificant increase in SeD group, all experimental groups showed two to threefold increase in testicular CAT activity compared to control ($p < 0.05$) [Fig. 3(A)]. GST activity, on the other hand, did not change in any of the groups [Fig. 3(B)].

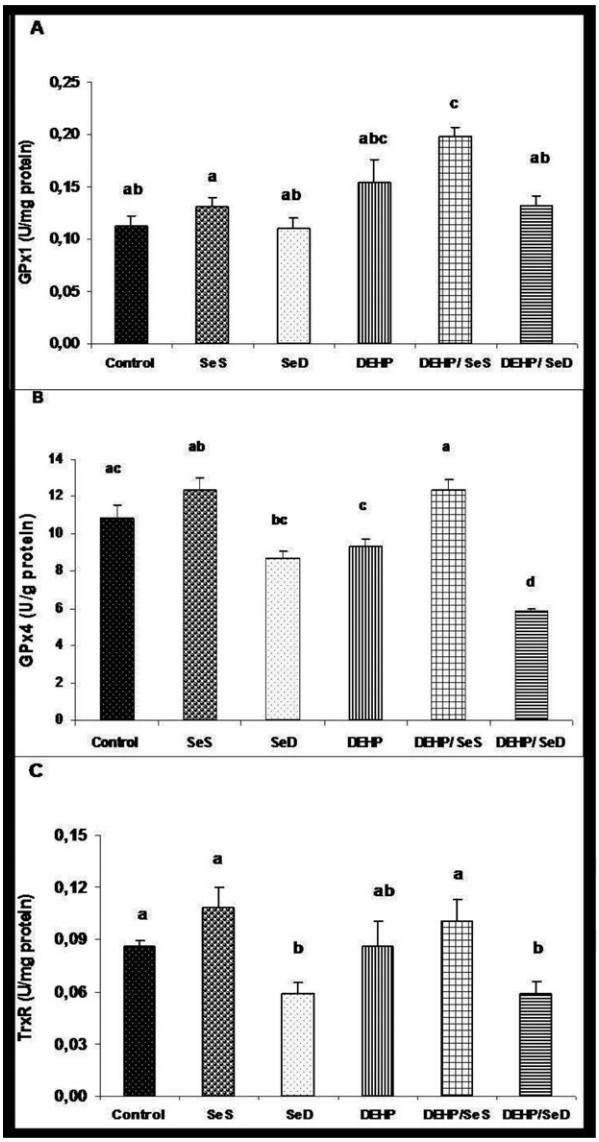

Fig. 1. Changes in testicular selenoenzyme activities in the study groups. A: Glutathione peroxidase 1 (GPx1) activity, (B) glutathione peroxidase 4 (GPx4) activity, (C) thioredoxin reductase (TrxR) activity. Experimental groups for 5 weeks were on: (Control) regular diet (0.15 mg/kg Se); (SeS) Se-supplemented 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; (DEHP/SeD) Se-deficient diet (≤0.05 mg/kg Se) and received 1,000 mg/kg, i.g. DEHP for the last 10 days. 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 ($p < 0.05$).

In comparison with the control group, testicular GSH levels decreased significantly ($p < 0.05$) in all DEHP-exposed groups (DEHP, ∼60%; DEHP/SeS, ∼30%; DEHP/

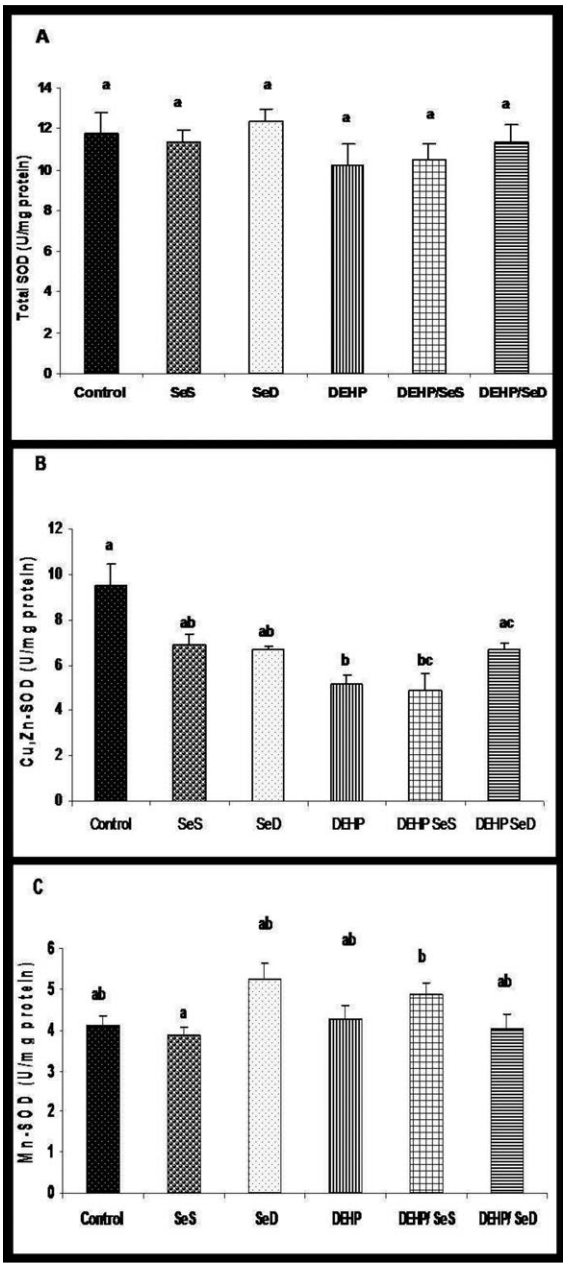

Fig. 2. Changes in testicular total superoxide dismutase, Cu,Zn-superoxide dismutase and Mn-superoxide dismutase activities in the study groups. A: Total superoxide dismutase (Total SOD) activity, (B) Cu, Zn-superoxide dismutase (Cu, Zn-SOD) activity, (C) Mn-superoxide dismutase (Mn-SOD) activity. Experimental groups for 5 weeks were on: (Control) regular diet (0.15 mg/kg Se); (SeS) Se-supplemented diet (1 mg/kg Se); (SeD) Se-deficient diet ($\leq$0.05 mg/kg Se); (DEHP) regular diet (0.15 mg/kg Se), and received 1,000 mg/kg, i.g. DEHP for the last 10 days; (DEHP/SeS) Se-supplemented diet (1 mg/kg Se) and received 1,000 mg/kg, i.g. DEHP for the last 10 days; (DEHP/SeD) Se-deficient diet ($\leq$0.05 mg/kg Se) and received 1,000 mg/kg, i.g. DEHP for the last 10 days. All results were given as mean $\pm$ SEM of $n = 6$ animals. Bars that do not share same letters (superscripts) are significantly different from each other ($p < 0.05$).

SeD, $\sim$65%), and Se supplementation provided significant protection for GSH as evidenced by a $\sim$40% difference between DEHP/SeS and DEHP groups [Fig. 4(A)]. Furthermore, highly significant increases (two to fivefold) in GSSG concentrations were found in all groups, except in SeS animals [Fig. 4(B)]. Thus, significantly decreased (3.5–18.5-fold) GSH/GSSG redox ratios were observed in all DEHP-exposed groups as well as Se-deficient animals (approximately fourfold). There was, however, a approximately threefold increase in the GSH/GSSG redox ratio of DEHP/SeS group in comparison with DEHP group indicating again the protective effect of Se supplementation against oxidative insult [Fig. 4(C)].

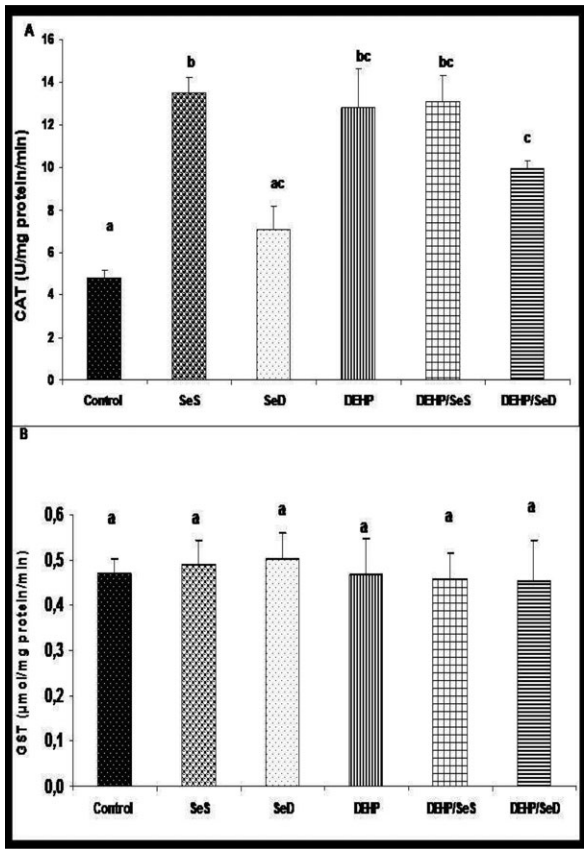

Fig. 3. Changes in testicular catalase and glutathione *S*-transferase activities in the study groups. A: Catalase (CAT) activity, (B) glutathione *S*-transferase (GST) activity. Experimental groups for 5 weeks were on: (Control) regular diet (0.15 mg/kg Se); (SeS) Se-supplemented diet (1 mg/kg Se); (SeD) Se-deficient diet ($\leq$0.05 mg/kg Se); (DEHP) regular diet (0.15 mg/kg Se) and received 1,000 mg/kg, i.g. DEHP for the last 10 days; (DEHP/SeS) Se-supplemented diet (1 mg/kg Se) and received 1,000 mg/kg, i.g. DEHP for the last 10 days; (DEHP/SeD) Se-deficient diet ($\leq$0.05 mg/kg Se) and received 1,000 mg/kg, i.g. DEHP for the last 10 days. All results were given as mean $\pm$ SEM of $n = 6$ animals. Bars that do not share same letters (superscripts) are significantly different from each other ($p < 0.05$).

As shown in Figure 4(D), both DEHP exposure and Se deficiency induced significant levels of oxidative stress in rat testis as evidenced by high TBARS levels (SeD, ~40%; DEHP, ~80%; DEHP/SeS, ~45%; DEHP/SeD, ~130%, $p < 0.05$) compared to control, whereas Se supplementation decreased basal level of TBARS markedly (~30%, $p < 0.05$); and Se supplementation in DEHP-treated animals was found to provide a ~20% ($p < 0.05$) decrease in TBARS levels compared to DEHP group.

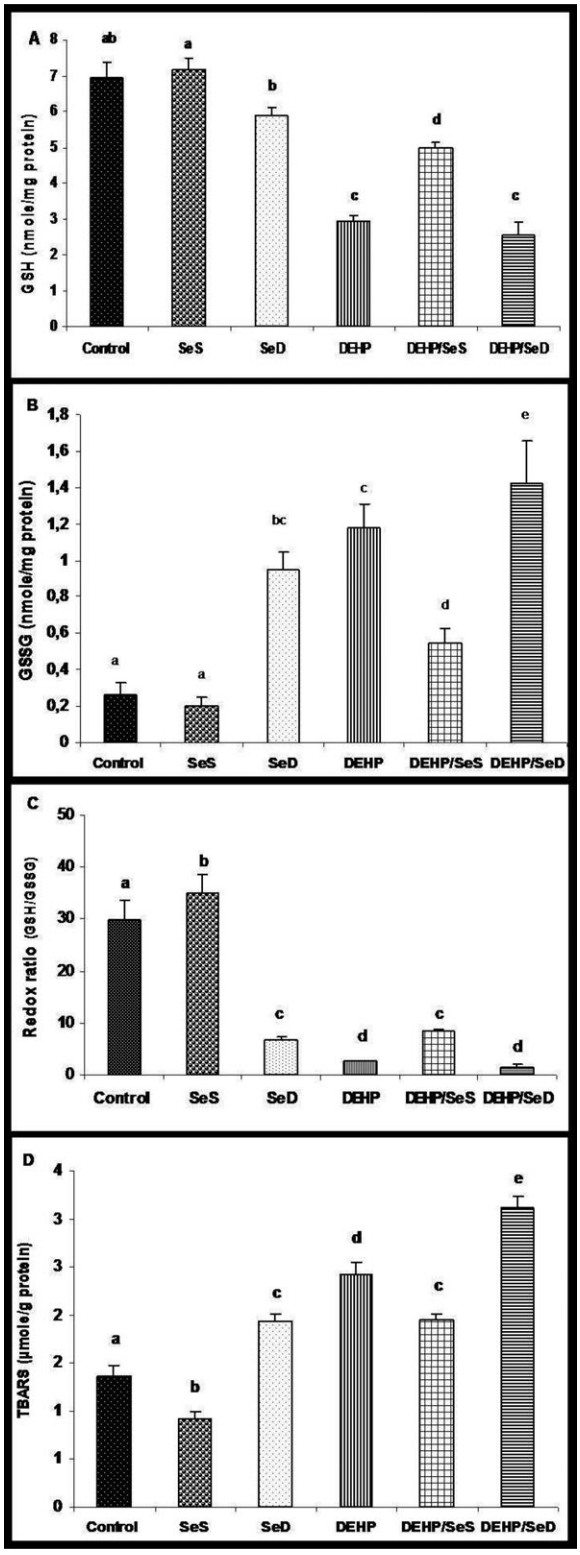

Fig. 4. Changes in testicular reduced glutathione levels, oxidized glutathione levels, and reduced/oxidized glutathione redox ratio in the study groups. A: Reduced glutathione (GSH) levels, (B) oxidized glutathione (GSSG) levels, (C) redox ratio (GSH/GSSG), (D) thiobarbituric acid reactive substance (TBARS) levels. Experimental groups for 5 weeks were on: (Control) 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; (DEHP/SeD) Se-deficient diet (≤0.05 mg/kg Se) and received 1,000 mg/kg, i.g. DEHP for the last 10 days. 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 ($p < 0.05$).

DISCUSSION

A leading hypothesis for the mechanism of PP-induced carcinogenesis in rodent liver has been an oxidative stress process (Rusyn et al., 2000). PPs were shown to cause significant induction in the activities of enzymes responsible for the production of H_2O_2, while leading to changes in the activity of enzymes involved in the degradation of H_2O_2 (Badr, 1992). For phthalates, a link between their PP activity and hepatocarcinogenic effect in rodents has also been a predominant theory (Rusyn et al., 2006). Along the same line of thinking, the possibility of oxidative damage to play a role in testicular toxicity of phthalates has been questioned. In fact, free radical production and LP are known as potentially important mediators in testicular physiology; cytochrome P450 enzymes of the steroidogenic pathway produce free radicals under physiological conditions, and the antioxidant defenses in rat testis play an important role in control of free radicals (Peltola et al., 1996; Hanukoglu, 2006). On the other hand, testicular oxidative stress was reported to play a role in a number of pathological conditions such as cryptorchidism, varicocel, testicular torsion, and as well as in toxicant exposures known to be detrimental to male fertility (Turner and Lysiak, 2008).

Although, the nature and mechanism underlying the action of phthalates on testicular development remain largely unknown, DEHP was reported to cause testicular atrophy, increase ROS generation, and cause depletion in antioxidant defenses (Kasahara et al., 2002). Our *in vitro* studies on MA-10 Leydig cells (Erkekoglu et al., 2010b) as well as LNCaP human prostate cells (Erkekoglu et al.,

2010a) have recently showed in the same line of data suggesting that at least one of the mechanisms underlying the reproductive toxicity of DEHP and MEHP is the induction of intracellular ROS, and alterations in the intracellular enzymatic and nonenzymatic antioxidants. The data of Fan et al. (2010) have also suggested oxidative stress as a new mechanism of MEHP action on Leydig cell streidogenesis via CYP1A1-mediated ROS stress.

Several strategies have been attempted to prevent the oxidative stress caused by toxic chemicals and the use of antioxidant vitamins has been the most common approach. Ishihara et al. (2000) showed that supplementation of rats with vitamin C and E protected the testes from DEHP-gonadotoxicity. Fan et al. (2010) reported that the increase in ROS generation with MEHP exposure in MA-10 cells was inhibited by *N*-acetylcysteine. In the above-mentioned *in vitro* studies (Erkekoglu et al., 2010a,b), we demonstrated that Se supplementation was highly protective against the cytotoxicity, genotoxicity, ROS producing, and antioxidant status-modifying effects of DEHP and MEHP in both MA-10 Leydig and LNCaP cells. Therefore, the main goals of the present *in vivo* study were to investigate whether an oxidative stress process is one of the underlying mechanisms of the testicular toxicity of DEHP and to determine whether Se status is a critical factor in DEHP toxicity.

The Effects of DEHP on Testicular Oxidant/Antioxidant Status

Our results demonstrating insignificant changes in the activities of GPx1, GPx4, TrxR, total SOD, and MnSOD by DEHP exposure indicated that the subacute (10 days) exposure of 1000 mg/kg DEHP was not effective to alter the activities of these cellular antioxidant enzymes in rat testis. We cannot argue for the possibility of induction in the expression of these enzymes, because we only measured their activities. However, significant ($\sim$80%) enhancement of cellular LP and substantial decrease ($>$10-fold) in GSH/GSSG redox ratio were the strong indicators for the production of an oxidative stress. Observations of approximately threefold increase in CAT activity, $\sim$45% decrease in Cu,Zn-SOD activity were the further evidences of the occurrence of an imbalance between the production of ROS and their efficient removal by available antioxidant system. Thus, as in the case of our *in vitro* studies, present *in vivo* study indicated the impairment of the cellular antioxidant status by DEHP exposure and the generation of testicular oxidative stress.

The Effects of Selenium on Testicular Oxidant/Antioxidant Status

In Se-supplemented rats, the only significant alterations we observed were approximately threefold increase in CAT activity, $\sim$30% decrease in LP and significant increase of GSH/GSSG redox ratio compared to control. Although in Se-deficient animals, GSH/GSSG redox ratio decreased more than fourfold and LP level enhanced $\sim$40%. Besides, Se deficiency caused a a significant decrease of $\sim$30% in testicular TrxR activity. These findings indicated a marked induction of oxidative stress as shown in earlier studies with animals fed on Se-deficient diet (Giray et al., 2004; Kaushal and Bansal, 2007, 2009). It has long been known that Se is essential for reproductive system, testosterone synthesis, normal production of protozoa, and spermatogenesis. Thus, Se status is critical particularly for male reproductivity (Ursini et al., 1999; Flohé, 2007). It was shown in rodent testis that Se concentration is typically higher than for any other tissue except kidney and generally do not decrease even with prolonged Se deficiency, so that it is preferentially maintained in the testis (Behne and Höfer-Bosse, 1984). When Se supply is limited due to Se deficiency, it is delivered preferentially to the Sertoli cells, thereby maintaining testis Se concentrations (Schriever et al., 2009).

The Effects of Selenium on the Testicular Oxidant/Antioxidant Status in DEHP Exposure

In DEHP-exposed Se-supplemented animals, significant enhancement of GPx1 and CAT activities was observed compared to control animals, and substantial elevation of GSH/GSSG redox ratio and significant reduction of LP levels compared to DEHP group were detected. On the contrary, in animals fed on Se-deficient diet and exposed to DEHP, LP levels increased and GSH/GSSG redox ratio decreased to a great extend compared to the all experimental groups, and except for an insignificant increase of GSH/GSSG redox ratio compared to DEHP group, all differences were significant. Moreover, the GPx4 activity of DEHP/SeD group was significantly lower than any of the other groups, and TrxR activity was significantly lower than that of control and DEHP/SeS groups. On the other hand, CAT activity did not significantly change in DEHP/SeD animals compared to DEHP or DEHP/SeS groups. These results clearly pointed out the protective effect of Se in modulating the redox status in testicular cells as the GSH/GSSG redox ratio of the phthalate exposed animals increased approximately threefold by Se supplementation and decreased $\sim$1.6-fold by Se deficiency, besides accordingly changed LP levels.

On the other hand, the reduction of GPx4 activity in DEHP/SeD group was particularly important, because the major role of Se in fertility is mediated by GPx4, which is the most abundant selenoprotein in testis (Ursini at al., 1999; Flohé, 2007). GPx4 is synthesized in spermatids under indirect control of testosterone, protects biomembranes from peroxidative damage, and is required for

proper sperm stability (Ursini et al., 1982). In fact, in a previous study of a similar experimental design, we reported that Se status affected spermatogenesis, testicular histology, and steroid hormone levels in rats (Erkekoglu et al., 2011b). The data demonstrated the toxicity and antiandrogenic effect of DEHP, which was evidenced by disturbed testicular histology and spermatogenesis, low testosterone level, and reduced sperm motility. The findings were compatible with the histological findings of the study where normal histology, intact seminiferous tubule epitelium, and acceleration of spermatogenesis were observed in SeS group, detachments in the seminiferous basal membrane was noted in SeD group, and impairment of the spermatogenic process was detected in DEHP group. Thus, the effects of DEHP were much more pronounced in Se-deficient rats, while Se supplementation was found to be protective particularly for sperm qualities.

In conclusion, the data we presented herein coincide very well with the results and conclusion of the above-mentioned study (Erkekoglu et al., 2011b), thereby corroborate the role of oxidative stress induction as one of the underlying mechanism for the testicular toxicity of DEHP. Furthermore, our findings emphasize the critical role of Se as an effective redox regulator and the importance of Se status in protecting the testicular structure and functions from oxidant stressor activity of DEHP.

The authors thank Mirelle Osman for her technical assistance in the measurement of TrxR activity.

REFERENCES

Aebi H. 1974. Catalase. In: Bergmeyer HU, editor. Methods of Enzymatic Analysis. New York: Academic Press. pp 673–677.

Akerboom TP, Sies H. 1981. Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. Methods Enzymol 77:373–382.

Akingbemi BT, Youker RT, Sottas CM, Ge R, Katz E, Klinefelter GR, Zirkin BR, Hardy MP. 2001. Modulation of rat Leydig cell steroidogenic function by di(2-ethylhexyl)phthalate. Biol Reprod 65:1252–1259.

Arnér ES, Zhong L, Holmgren A. 1999. Preparation and assay of mammalian thioredoxin and thioredoxin reductase. Methods Enzymol 300:226–239.

Badr MZ. 1992. Induction of peroxisomal enzyme activities by di-(2-ethylhexylphthalate in thyroidectomized rats with parathyroid replants. J Pharmacol Exp Ther 263:1105–1110.

Behne D, Höfer-Bosse T. 1984. Effects of a low selenium status on the distribution and retention of selenium in the rat. J Nutr 114:1289–1296.

Bornehag CG, Sundell J, Weschler CJ, Sigsgaard T, Lundgren B, Hasselgren M, Hägerhed-Engman L. 2004. The association between asthma and allergic symptoms in children and phthalates in house dust: A nested case-control study. Environ Health Perspect 112:1393–1397.

Calafat AM, Brock JW, Silva MJ, Gray LE Jr, Reidy JA, Barr DB, Needham LL. 2006. Urinary and amniotic fluid levels of phthalate monoesters in rats after the oral administration of di(2-ethylhexyl) phthalate and di-*n*-butyl phthalate. Toxicology 217:22–30.

Dostal LA, Chapin RE, Stefanski SA, Harris MW, Schwetz BA. 1988. Testicular toxicity and reduced Sertoli cell numbers in neonatal rats by di(2-ethylhexyl)phthalate and the recovery of fertility as adults. Toxicol Appl Pharmacol 95:104–121.

EFSA. 2005. Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on a request from the commission related to bis(2-ethylhexyl)phthalate (DEHP) for use in food contact materials. Question No. EFSA-Q-2003-191. EFSA J 243:1–20.

Doull J, Cattley R, Elcombe C, Lake BG, Swenberg J, Wilkinson C, Williams G, van Gemert M. 1999. A cancer risk assessment of di(2-ethylhexyl)phthalate: Application of the new U.S. EPA Risk Assessment Guidelines. Regul Toxicol Pharmacol 29:327–357.

Erkekoglu P, Rachidi W, De Rosa V, Giray B, Favier A, Hincal F. 2010a. Protective effect of selenium supplementation on the genotoxicity of di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate treatment in LNCaP cells. Free Radic Biol Med 49:559–566.

Erkekoglu P, Rachidi W, Yuzugullu OG, Giray B, Favier A, Ozturk M, Hincal F. 2010b. Evaluation of cytotoxicity and oxidative DNA damaging effects of di(2-ethylhexyl)-phthalate (DEHP) and mono(2-ethylhexyl)-phthalate (MEHP) on MA-10 Leydig cells and protection by selenium. Toxicol Appl Pharmacol 248:52–62.

Erkekoglu P, Rachidi W, Yüzügüllü OG, Giray B, Oztürk M, Favier A, Hincal F. 2011a. Induction of ROS, p53, p21 in DEHP- and MEHP-exposed LNCaP cells-protection by selenium compounds. Food Chem Toxicol 49:1565–1571.

Erkekoglu P, Zeybek ND, Giray B, Asan E, Arnaud J, Hincal F. 2011b. Reproductive toxicity of di(2-ethylhexyl) phthalate in selenium-supplemented and selenium-deficient rats. Drug Chem Toxicol 34:379–389.

Fan J, Traore K, Li W, Amri H, Huang H, Wu C, Chen H, Zirkin B, Papadopoulos V. 2010. Molecular mechanisms mediating the effect of mono-(2-ethylhexyl) phthalate on hormone-stimulated steroidogenesis in MA-10 mouse tumor Leydig cells. Endocrinology 151:3348–3362.

Flohé L. 2007. Selenium in mammalian spermiogenesis. Biol Chem 388:987–995.

Flohé L, Günzler WA. 1984. Assays of glutathione peroxidase. Methods Enzymol 105:114–121.

Ganther HE. 1999. Selenium metabolism, selenoproteins and mechanisms of cancer prevention: Complexities with thioredoxin reductase. Carcinogenesis 20:1657–1666.

Gazouli M, Yao ZX, Boujrad N, Corton JC, Culty M, Papadopoulos V. 2002. Effect of peroxisome proliferators on Leydig cell peripheral-type benzodiazepine receptor gene expression, hormone-stimulated cholesterol transport, and teroidogenesis: Role of the peroxisome proliferator-activator receptor alpha. Endocrinology 143:2571–2583.

Ge RS, Chen GR, Tanrikut C, Hardy MP. 2007. Phthalate ester toxicity in Leydig cells: Developmental timing and dosage considerations. Reprod Toxicol 23:366–373.

Giray B, Riondel J, Richard MJ, Favier A, Hıncal F. 2004. Oxidant/antioxidant status in relation to thyroid hormone metabolism in selenium- and/or iodine-deficient rats. J Trace Elem Exp Med 17:109–121.

Graham PR. 1973. Phthalate ester plasticizers—Why and how they are used. Environ Health Perspect 3:3–12.

Günzler WA, Kremers H, Flohé L. 1974. An improved coupled test procedure for glutathione peroxidase (EC 1-11-1-9-) in blood. Z Klin Chem Klin Biochem 12:444–448.

Habig WH, Pabst MJ, Jakoby WB. 1974. Glutathione *S*-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130–7139.

Hanukoglu I. 2006. Antioxidant protective mechanisms against reactive oxygen species (ROS) generated by mitochondrial P450 systems in steroidogenic cells. Drug Metab Rev 38:171–196.

Hauser R, Duty S, Godfrey-Bailey L, Calafat AM. 2004. Medications as a source of human exposure to phthalates. Environ Health Perspect 112:751–753.

Hernández-Díaz S, Mitchell AA, Kelley KE, Calafat AM, Hauser R. 2009. Medications as a potential source of exposure to phthalates in the U.S. population. Environ Health Perspect 117:185–189.

Ishihara M, Itoh M, Miyamoto K, Suna S, Takeuchi Y, Takenaka I, Jitsunari F. 2000. Spermatogenic disturbance induced by di-(2-ethylhexyl)phthalate is significantly prevented by treatment with antioxidant vitamins in the rat. Int J Androl 23:85–94.

Jablonska E, Gromadzinska J, Reszka E, Wasowicz W, Sobala W, Szeszenia-Dabrowska N, Boffetta P. 2009. Association between GPx1 Pro198Leu polymorphism, GPx1 activity and plasma selenium concentration in humans. Eur J Nutr 48:383–386.

Kasahara E, Sato EF, Miyoshi M, Konaka R, Hiramoto K, Sasaki J, Tokuda M, Nakano Y, Inoue M. 2002. Role of oxidative stress in germ cell apoptosis induced by di(2-ethylhexyl)phthalate. Biochem J 365:849–856.

Kaushal N, Bansal MP. 2007. Dietary selenium variation-induced oxidative stres modulates CDC2/cyclin B1 expression and apoptosis of germ cells in mice testis. J Nutr Biochem 18:553–564.

Kaushal N, Bansal MP. 2009. Diminished reproductive potential of male mice in response to selenium-induced oxidative stress: Involvement of HSP70, HSP70-2, and MSJ-1. J Biochem Mol Toxicol 23:125–113.

Kavlock R, Barr D, Boekelheide K, Breslin W, Breysse P, Chapin R, Gaido K, Hodgson E, Marcus M, Shea K, Williams P. 2006. NTP-CERHR expert panel update on the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate. Reprod Toxicol 22:291–399.

Latchoumycandane C, Chitra C, Mathur P. 2002. Induction of oxidative stress in rat epididymal sperm after exposure to 2, 3,7,8-tetrachlorodibenzo-*p*-dioxin. Arch Toxicol 76:113–118.

Li LH, Jester WF Jr, Laslett AL, Orth JM. 2000. A single dose of di-(2-ethylhexyl)phthalate in neonatal rats alters gonocytes, reduces Sertoli cell proliferation, and decreases cyclin D2 expression. Toxicol Appl Pharmacol 166:222–229.

Loff S, Kabs F, Witt K, Sartoris J, Mandl B, Niessen KH, Waag KL. 2000. Polyvinylchloride infusion lines expose infants to large amounts of toxic plasticizers. J Pediatr Surg 35:1775–1781.

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

Lyche JL, Gutleb AC, Bergman A, Eriksen GS, Murk AJ, Ropstad E, Saunders M, Skaare JU. 2009. Reproductive and developmental toxicity of phthalates. J Toxicol Environ Health B Crit Rev 12:225–249.

Maiorino M, Gregolin C, Ursini F. 1990. Phospholipid hydroperoxide glutathione peroxidase. Methods Enzymol 186:448–457.

Maiorino M, Wissing JB, Brigelius-Flohé R, Calabrese F, Roveri A, Steinert P, Ursini F, Flohé L. 1998. Testosterone mediates expression of the selenoprotein PHGPx by induction of spermatogenesis and not by direct transcriptional gene activation. FASEB J 12:1359–1370.

Marklund S, Marklund G. 1974. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 47:469–474.

Noriega NC, Howdeshell KL, Furr J, Lambright CR, Wilson VS, Gray LE Jr. 2009. Pubertal administration of DEHP delays puberty, suppresses testosterone production, and inhibits reproductive tract development in male Sprague-Dawley and Long-Evans rats. Toxicol Sci 111:163–178.

O'Brien ML, Spear BT, Glauert HP. 2005. Role of oxidative stress in peroxisome proliferator-mediated carcinogenesis. Crit Rev Toxicol 35:61–88.

Oishi S. 1990. Effects of phthalic acid esters on testicular mitochondrial functions in the rat. Arch Toxicol 64:143–147.

Peltola V, Huhtaniemi I, Metsa-Ketela T, Ahotupa M. 1996. Induction of lipid peroxidation during steroidogenesis in the rat testis. Endocrinology 137:105–112.

Poon R, Lecavalier P, Mueller R, Valli VE, Procter BG, Chu I. 1997. Subchronic oral toxicity of di-*n*-octyl phthalate and di(2-ethylhexyl) phthalate in the rat. Food Chem Toxicol 35:225–239.

Richard MJ, Portal B, Meo J, Coudray C, Hadjian A, Favier A. 1992. Malondialdehyde kit evaluated for determining plasma and lipoprotein fractions that react with thiobarbituric acid. Clin Chem 38:704–709.

Richburg JH, Boekelheide K. 1996. Mono-(2-ethylhexyl) phthalate rapidly alters both Sertoli cell vimentin filaments and germ cell apoptosis in young rat testes. Toxicol Appl Pharmacol 137:42–50.

Rock G, Labow RS, Tocchi M. 1986. Distribution of di(2-ethylhexyl) phthalate and products in blood and blood components. Environ Health Perspect 65:309–316.

Rusyn I, Rose ML, Bojes HK, Thurman RG. 2000. Novel role of oxidants in the molecular mechanism of action of peroxisome proliferators. Antioxid Redox Signal 2:607–621.

Rusyn I, Peters JM, Cunningham ML. 2006. Modes of action and species-specific effects of di-(2-ethylhexyl)phthalate in the liver. Crit Rev Toxicol 36:459–479.

Schriever SC, Barnes KM, Evenson JK, Raines AM, Sunde RA. 2009. Selenium requirements are higher for glutathione peroxidase-1 mRNA than GPx1 activity in rat testis. Exp Biol Med (Maywood) 234:513–521.

Singh AR, Lawrence WH, Autian J. 1975. Maternal-fetal transfer of 14C-di-2-ethylhexyl phthalate and 14C-diethyl phthalate in rats. J Pharm Sci 64:1347–1350.

Steinbrenner H, Sies H. 2009. Protection against reactive oxygen species by selenoproteins. Biochim Biophys Acta 1790:1478–1485.

Turner TT, Lysiak JJ. 2008. Oxidative stress: A common factor in testicular dysfunction. J Androl 29:488–498.

Ursini F, Maiorino M, Valente M, Ferri L, Gregolin C. 1982. Purification from pig liver of a protein which protects liposomes and biomembranes from peroxidative degradation and exhibits glutathione peroxidase activity on phosphatidylcholine hydroperoxides. Biochim Biophys Acta 710:197–211.

Ursini F, Heim S, Kiess M, Maiorino M, Roveri A, Wissing J, Flohé L. 1999. Dual function of the selenoprotein PHGPx during sperm maturation. Science 285:1393–1396.

Weitzel F, Ursini F, Wendel A. 1990. Phospholipid hydroperoxide glutathione peroxidase in various mouse organs during selenium deficiency and repletion. Biochim Biophys Acta 1036:88–94.

Wolfe GW, Layton KA. 2003.Multigeneration reproduction toxicity study in rats (unaudited draft): Diethylhexylphthalate: Multigenerational reproductive assessment by continuous breeding when administered to Sprague-Dawley rats in the diet. Ther Immune Research Corporation TRC Study No 7244–200. Gaithersburg, Maryland.