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Dibutyl phthalate induces oxidative stress and impairs spermatogenesis in adult rat

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Abstract

Phthalates are abundantly produced plasticizers, and dibutyl phthalate (DBP) is the most widely used derivative in various consumer products and medical devices. This study was conducted to further explore the potential testicular toxicity of DBP in adult rats and to elucidate the underlying mechanisms. Adult male albino rats were treated orally with DBP at doses of 0, 200, 400, or 600 mg/kg/day for 15 consecutive days. Testicular weight, sperm count, and motility were significantly decreased. Treatment with DBP decreased serum follicle-stimulating hormone and testosterone levels and testicular lactate dehydrogenase activity. DBP treatment also decreased serum total antioxidant capacity and the activities of the testicular antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione reductase. Further, DBP treatment provoked degeneration with absence of spermatogenesis and sperms and necrosis in some of seminiferous tubules. These results indicated that oxidative stress and subsequent decrease in testosterone secretion were the potential underlying mechanism of DBP-induced testicular toxicity.

Keywords

Dibutyl phthalate, FSH, lipid peroxidation, testis, testosterone

Introduction

Recent studies have linked the declining reproductive health and fertility in men with toxicants found in the environment, in particular endocrine-disrupting chemicals such as phthalates (Nordkap et al., 2012; Wong and Cheng, 2011). One of phthalates, dibutyl phthalate (DBP), has attracted special attention from the scientific community and the general public due to its high production volume in millions of tons annually (Guerra et al., 2010). DBP is a ubiquitous plasticizer utilized in the production of flexible polyvinyl chloride (PVC) materials. As phthalates are noncovalently bound within PVC, they leach out, becoming available for biological exposure (Heudorf et al., 2007; Swan, 2008). Human exposure occurs primarily through contaminated food and water, especially high-fat foods, which may be in contact with plastic, adhesives, or other packing materials that contain DBP. Pharmaceutical formulations also result in significant human exposure because various plasticizers

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are used to coat medicines such as antibiotics, antihistamines, and laxatives (Schettler, 2006).

The majority of studies concerning DBP focused mainly on investigating *in utero* and lactational effects on the reproductive tract in experimental rodents (Scarano et al., 2010). DBP were shown in rats to interfere with normal development of the testis and reproductive tract when exposure occurs during gestation (Barlow et al., 2003; Scott et al., 2008), resulting in postnatal downstream disorders that are similar to those reported in human testicular dysgenesis syndrome, including cryptorchidism, hypospadias, impaired spermatogenesis, and reduced male fertility. On the testicular toxicity of DBP, previous studies have shown that sexually immature (prepubertal) rats are more sensitive to DBP than adults (Gray and Gangolli, 1986). Reported reproductive effects of DBP include alteration in activities of steroidogenic enzymes, alteration in testosterone metabolism, and decreased levels of plasma testosterone; they are suspected of acting as endocrine disruptors that have the potential to modify normal endocrine function (Hirosawa et al., 2006; Xiaofeng et al., 2009). The mechanism by which DBP exerts its toxic effect in reproductive system is not yet fully elucidated. Some of the effects of phthalate are related to its antiandrogenic potential (Ge et al., 2007; Noriega et al., 2009). This study was conducted to further explore the potential testicular toxicity of DBP in adult rats and to elucidate the underlying mechanisms.

Materials and methods

Chemicals

DBP was purchased from Sigma-Aldrich Chemical Company (St Louis, Missouri, USA). The follicle-stimulating hormone (FSH) and testosterone immunoassay kits were purchased from American Laboratory Products Co. Diagnostics (Salem, New Hampshire, USA) and BioVendor-Laboratorni medicina a.s. (Karasek, Czech Republic), respectively. The total antioxidant kit was purchased from Randox Laboratories Ltd. (County Antrim, UK). All other chemicals used are of analytical grade.

Animals and treatments

Twenty-four adult male Wistar rats (90 days; 180 $\pm$ 10 g) were housed in clean polypropylene cages (six/cage/group) and maintained on a 12-h light:12-h dark cycle at a temperature of 20–25°C with *ad libitum* access to food and water. For 7 days before beginning the experiment, the rats were handled daily for 5 min to acclimatize them to human contact and minimize their physiological responses to handling for subsequent protocols (Ma and Lightman, 1998; Vaithinathan et al., 2010). DBP was dissolved in corn oil and given to rats by gavage at doses 0, 200, 400, or 600 mg/kg/day for 15 consecutive days. Control group was given corn oil alone. Gavage volume was adjusted according to the weight of each rat. The doses and duration were selected as per previous publications (Gray et al., 2006; Nair et al., 2008; Scarano et al., 2010; Zhou et al., 2010, 2011). The control group of animals was maintained and gavaged corn oil vehicle alone.

Necropsy

Twenty-four hours after the last dose, blood samples were collected from the retro-orbital sinus under ether anesthesia. Samples were centrifuged, and the supernatant serum was separated from the clot as soon as possible and stored at −80°C until FSH, testosterone, and total antioxidant capacity (T-AOC) assay. Animals were euthanized and the testes were excised immediately, cleaned from adhering fat and connective tissues, and the weights were recorded in grams. The cauda epididymides from each animal were used for sperm count and motility assay. The testes were homogenized in ice-cold phosphate buffer (pH 7.0) using a glass–Teflon homogenizer. The homogenate was centrifuged at 10,000 *g* for 30 min at 4°C, and the supernatant was used for other biochemical assay and enzymes estimation as enzyme source. One testis from each group was used for histopathological examination. Protein content of the testicular homogenate was measured using crystalline bovine serum albumin as standard (Bradford, 1976).

Sperm count and motility

Cauda epididymides were dissected out, immediately minced in 5 ml of physiological saline, and then incubated at 37°C for 30 min to allow spermatozoa to leave the epididymal tubules. Total sperm number was determined by a Neubauer hemocytometer as described previously (Yokoi et al., 2003). To determine sperm motility, 100 sperms each were observed in three different fields, classified into motile and nonmotile sperms, and the motility was expressed as percentage incidence. The percentage of motile sperms

was recorded using a phase-contrast microscope at a magnification of 400× (Aly and Azhar, 2013).

Serum FSH and testosterone

Determination of serum FSH (ng/ml) and testosterone (ng/ml) was carried using enzyme-linked immunosorbent assay diagnostic kits.

Testicular LDH enzyme

The testicular homogenate was used to estimate testicular lactate dehydrogenase (LDH) enzyme (U/mg protein) spectrophotometrically using diagnostic kit.

Serum T-AOC

Twenty microliters of serum were incubated, in a cuvette, with 1 ml of chromogen composed of 610 mmol/l of 2,2′-azino-di-(ethylbenzthiazoline sulfonate) and 6.1 mmol/l of metmyoglobin. The reactants were mixed well, and the initial absorbance (A_1) was recorded. Then the substrate was added (200 μl of mmol/l hydrogen peroxide (H_2O_2)). The contents were mixed well, and the absorbance was recorded after 3 min (A_2). The ΔA was calculated for each of the sample and blank (Miller et al., 1993). The T-AOC (mmol/l) was calculated using the following equation:

$$\text{T}^{-}\text{AOC} = \text{Factor} \times \left(\Delta A_{\text{blank}} - \Delta A_{\text{sample}}\right) \quad (1)$$

$$\text{Factor} = \frac{\text{Concentration of standard}}{\left(\Delta A_{\text{blank}} - \Delta A_{\text{standard}}\right)} \quad (2)$$

Oxidative stress markers in the testis

Lipid peroxidation. Malondialdehyde (MDA), formed as an end product of the peroxidation of lipids, served as an index of the intensity of oxidative stress. MDA reacts with thiobarbituric acid to generate a colored product that can be measured optically at 532 nm. A break down product of lipid peroxidation (LPO), thiobarbituric acid reactive substance, was measured by the method described by Buege and Aust (1976). Briefly, the stock solution contained equal volumes of trichloroacetic acid 15% (w/v) in 0.25 N hydrochloric acid (HCl) and 2-thiobarbituricacid 0.37% (w/v) in 0.25 N HCl. One volume of the test sample (sperm suspension) and two volumes of stock reagent were mixed in a screw-capped centrifuge tube, vortexed, and heated for 15 min on a boiling water bath. After cooling on ice, the precipitate was removed by centrifugation at 1000*g* for 15 min, and absorbance of the supernatant was measured at 532 nm against blank containing all the reagents except test sample. The value is expressed as micromoles of MDA equivalent formed per minute per milligram of protein.

Antioxidant enzymes. The xanthine oxidase method was used to measure superoxide dismutase (SOD) activity in the testicular homogenate using purified bovine erythrocyte SOD (5000 U/mg solid) as a standard. The reaction between 50 mM xanthine, 50 mM xanthine oxidase 1000 U, and 0.1 mM ethylenediaminetetraacetic acid was used to generate superoxide radicals and uric acid at pH 7.8. The superoxide radicals produced reacted with 50 mM nitro blue tetrazolium (NBT) to produce a red formazan dye that was measured spectrophotometrically at 250 nm. The SOD present in the sample (0.1 ml enzyme source) competes with the NBT for superoxide radicals and so inhibits the production of formazan dye. The SOD activity was expressed as units per milligram of protein (Oynagui, 1984). Catalase (CAT) activity was determined using reaction mixture (2 ml) consisting of 1.95 ml of 10 mM H_2O_2 in 60 mM phosphate buffer (pH 7.0). The reaction was started by adding 0.5 ml of enzyme source and the absorbance was recorded at 240 nm after 3 min. One CAT unit is defined as the amount of H_2O_2 converted into water (H_2O) and ½ oxygen (O_2) in 1 min under standard conditions and the specific activity is reported as micromoles of H_2O_2 consumed per minute per milligram of protein (Aebi, 1984). Glutathione reductase (GR) activity was measured by mixing 0.1 ml enzyme source with 1 ml of 0.2 M sodium phosphate buffer, 1 mM glutathione disulfide (GSSG), and 0.1 mM nicotinamide adenine dinucleotide phosphate (NADPH). The absorbance was recorded at 340 nm. The enzyme activity was calculated as nanomoles of NADPH oxidized per minute per milligram of protein (McFarland et al., 1999).

Histopathological examination of the testes

Autopsy samples were taken from the testes of killed rats, fixed in 10% formalin solution for 10 h at least, and then washed in tap water for 12 h. Serial alcohols (methyl, ethyl, and absolute) were used for dehydration of the tissue samples. Tissue specimens were cleared in xylene and embedded in paraffin. The paraffin blocks were sectioned at 3-μm thickness by sledge microtome. The obtained tissue sections were collected on the glass slides and stained by

Table 1. Effect of DBP on testes weights and sperm characteristics.[a]

Parameter	Doses of DBP (mg/kg b.w./day)			
	Control	200 mg	400 mg	600 mg
Absolute testes weights (g)	2.93 ± 0.12	2.48 ± 0.17[b]	2.45 ± 0.29[c]	2.38 ± 0.31[c]
Cauda sperm count ($\times 10^6$/rat)	53.5 ± 3.21	47 ± 3.35[b]	45.17 ± 2.86[c]	42.67 ± 3.33[d]
Sperm motility (%)	86.33 ± 4.46	76.83 ± 5.08[b]	72.67 ± 5.57[c]	70 ± 5.83[d]

DBP: dibutyl phthalate; ANOVA: analysis of variance; b.w.: body weight.
[a]Data are expressed as mean ± SD ($n = 6$).
[b]$p < 0.05$: statistical analysis (ANOVA) for differences from corresponding control.
[c]$p < 0.01$: statistical analysis (ANOVA) for differences from corresponding control.
[d]$p < 0.001$: statistical analysis (ANOVA) for differences from corresponding control.

hematoxylin and eosin stain (Banchroft et al., 1996) for histopathological examination by the light microscope.

Statistical analysis

Differences between obtained values (mean ± SD, $n = 6$) were compared by one-way analysis of variance, followed by the Tukey–Kramer multiple comparison test. A p value less than 0.05 was taken as a criterion for a statistically significant difference.

Results

The weight of testes in animals treated with DBP (200, 400, or 600 mg/kg) revealed significant decrease ($p < 0.05$, $p < 0.01$, and $p < 0.01$, respectively) as compared to the control group (Table 1). Both sperm count and motility were significantly decreased ($p < 0.05$, $p < 0.01$, and $p < 0.001$), in a dose-related manner, in response to DBP (200, 400, or 600 mg/kg, respectively) as compared to the corresponding control (Table 1).

Serum FSH and testosterone levels were significantly decreased ($p < 0.001$) in response to DBP (200, 400, or 600 mg/kg) treatment as compared to the corresponding control (Figure 1(a) and (b), respectively). The testicular activity of LDH revealed a significant ($p < 0.001$) decrease in animals treated with 200, 400, or 600 mg/kg of DBP as compared to the control group (Figure 2). Treatment of animals with DBP (200, 400, or 600 mg/kg) significantly decreased ($p < 0.001$) the serum T-AOC as compared to the related control (Figure 3). In DBP-treated animals, the testicular LPO was significantly increased ($p < 0.001$), while the enzymatic activities of SOD, CAT, and GR were significantly decreased ($p < 0.001$) as compared to the related control (Table 2).

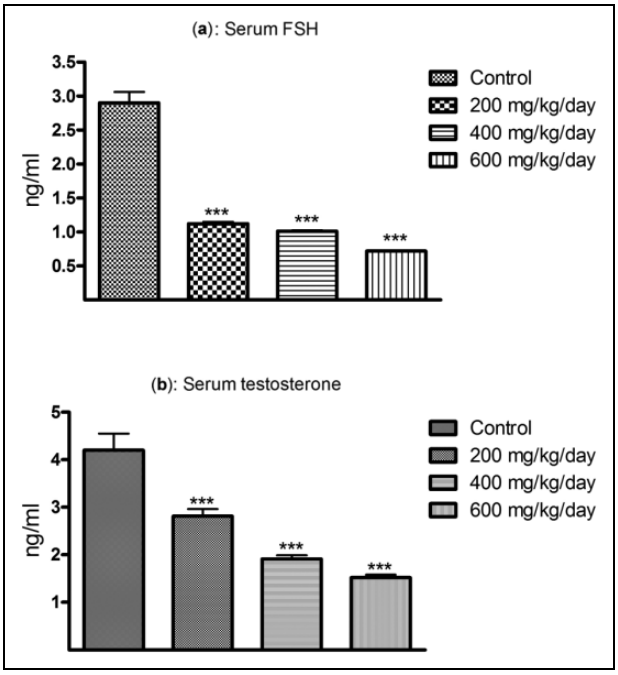

Figure 1. Effect of DBP on serum FSH and testosterone. Data are expressed as mean ± SD ($n = 6$). ***$p < 0.001$: statistical analysis (ANOVA) for differences from corresponding control. DBP: dibutyl phthalate; FSH: follicle-stimulating hormone; ANOVA: analysis of variance.

Histopathological observation of testes of control group showed normal architecture with an orderly arrangement of germinal cells and Sertoli cells (Figure 4(a)). Testes of DBP (200 mg/kg) treated animals showed degeneration with absence of spermatogenic series in the lumen of some seminiferous tubules (ds; Figure 4(b)). Animals treated with 400 mg/kg of DBP revealed degeneration with absence of spermatogenesis and sperms from most of the seminiferous tubules (ds; Figure 4(c)). Treatment of animals with 600 mg/kg of DBP revealed necrosis in some of seminiferous tubules (ns; Figure 4(d)).

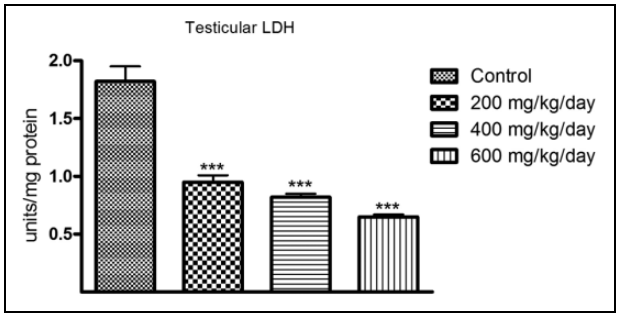

Figure 2. Effect of DBP on testicular LDH activity. Data are expressed as mean ± SD ($n = 6$). ***$p < 0.001$: Statistical analysis (ANOVA) for differences from corresponding control. LDH: lactate dehydrogenase; DBP: dibutyl phthalate; ANOVA: analysis of variance.

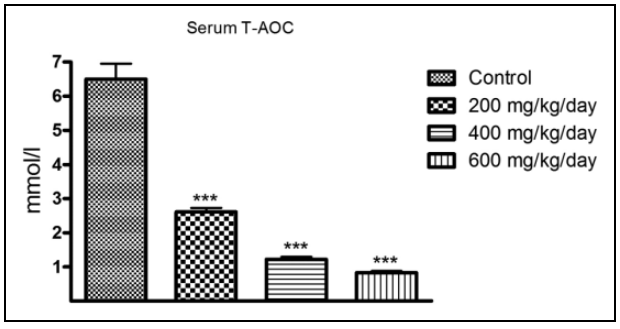

Figure 3. Effect of DBP on serum T-AOC. Data are expressed as mean ± SD ($n = 6$). ***$p < 0.001$: statistical analysis (ANOVA) for differences from corresponding control. T-AOC: total antioxidant capacity; DBP: dibutyl phthalate; ANOVA: analysis of variance.

Discussion

The decline in fertility of animals and humans over the last few decades potentially linked to environmental exposures has drawn global attention (Swan and Elkin, 1999). The results presented in this study clearly demonstrate that DBP induced testicular toxicity in rats. The evaluation of testicular weight is an integral component in the assessment of reproductive toxicity. The weight of the testis is largely depending on the mass of the differentiated spermatogenic cells (Ihsan et al., 2011). DBP caused a significant decrease in the absolute weight of the testes which could be attributed to the significant decrease in the sperm production in the testes or due to decreased gonadotropins release. Gonadotropins are prime regulators of testis weight (Goldman et al., 1989). The testicular sperm count is an important indicator of spermatogenesis (Wang et al., 2004). Sperm count in the epididymis decreased perhaps due to low level of sperm production in the testes. Such reduction in sperm count may be resulted from the direct effect of the DBP on testicular Leydig and Sertoli cells causing a decrease in testosterone production (Al-Thani et al., 2003), which is a prime regulator for sperm production (Steinberger, 1975). This could also be attributed to the reduced level of serum FSH, a hormone directly involved in maintaining spermatogenesis in conjunction with testosterone (Plant and Marshall, 2001). A decrease in sperm count and sperm motility is associated with decreased fertility (Narayana et al., 2002; Wyrobek et al., 1983). The decrease in sperm motility may be attributed to the reduction in serum testosterone level (Dirican and Kalender, 2012) or may be due to rapid loss of intracellular adenosine triphosphate (ATP) and damage of the sperm membrane caused by LPO (De Lamirande and Gagnon, 1992; Dokmeci, 2005). It is thus likely that the reduction of sperm number in the cauda epididymis in the DBP-treated rats reflects a state of inhibited and/or decreased spermatogenesis (Adesiyan et al., 2011).

Testicular activity is governed by gonadotrophic hormones, FSH and LH (Multinger et al., 1996). In this study, serum FSH and testosterone levels were significantly decreased in rats treated with DBP. Testosterone is produced in the Leydig cells of testis under the influence of LH secreted from the pituitary gland (Nilsson, 2000; Tahka, 1989). Alteration of Leydig cell function can adversely affect spermatogenesis (Senger, 1999). Decrease in serum testosterone might also contribute to the reduction of spermatogenesis (Nair and Shaha, 2003). FSH not only regulates spermatogenesis via Sertoli cell function but also exerts a steroidogenic function on Leydig cells (De Gendt et al., 2004; O'Shaughnessy et al., 2010; Willems et al., 2010). Alterations in this gonadotropin by DBP more likely lead to reproductive failure. The findings of this study suggest that DBP affects testicular function by affecting the functions of Sertoli and Leydig cells. Sertoli cells act as the so-called nurse cells, providing the structural and the metabolic support for developing germ cells (Bian et al., 2006). Since many factors essential for germ cell development are synthesized by Sertoli cells (Meehan et al., 2000), and since the number of spermatozoa produced per day is governed by the number of Sertoli cells in the seminiferous tubules (Amann, 1970), any agent that impairs the viability and the function of Sertoli cells may have profound effects on spermatogenesis (Bian et al., 2006).

Table 2. Effect of DBP on oxidative stress markers in the testis.[a]

Parameter	Doses of DBP (mg/kg b.w./day)			
	Control	200	400	600
LPO	3.2 ± 0.2	6.1 ± 0.42[b]	9.11 ± 0.48[b]	12 ± 0.71[b]
SOD	15.2 ± 1.89	9.52 ± 1.65[b]	7.32 ± 1.45[b]	5.92 ± 1.12[b]
CAT	0.82 ± 0.13	0.42 ± 0.05[b]	0.27 ± 0.01[b]	0.13 ± 0.002[b]
GR	32.7 ± 1.91	20.5 ± 1.95[b]	15.3 ± 1.45[b]	11.4 ± 1.15[b]

LPO: lipid peroxidation; SOD: superoxide dismutase; CAT: catalase; GR: glutathione reductase; ANOVA: analysis of variance; b.w.: body weight.

[a]LPO are expressed as micromoles of MDA equivalent formed per minute per milligram protein; SOD are expressed as units per milligram protein; CAT activity is expressed as micromoles of hydrogen peroxide consumed per minute per milligram protein; and GR as nanomoles of nicotinamide adenine dinucleotide phosphate oxidized per minute per milligram protein. Data are expressed as mean ± SD ($n = 6$).

[b]$p < 0.001$: statistical analysis (ANOVA) for differences from corresponding control.

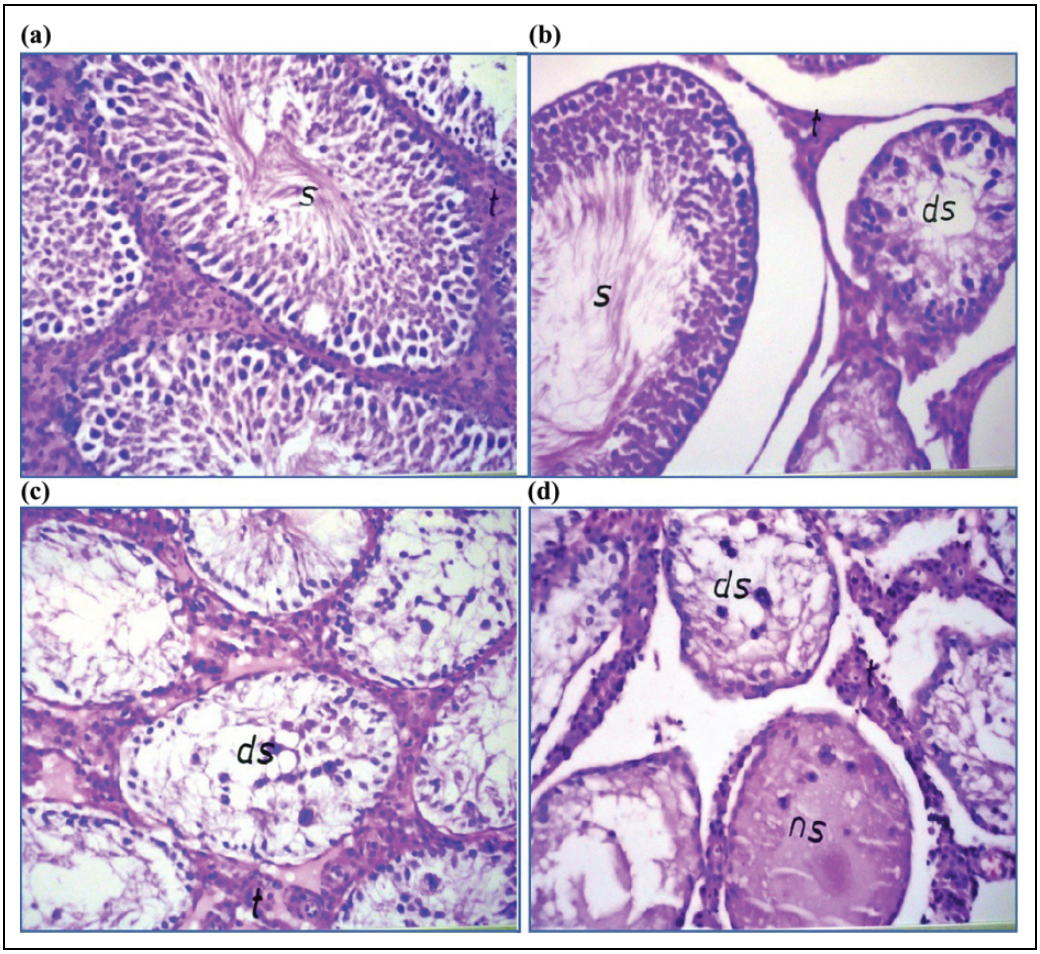

Figure 4. Representative illustrations of histological morphology of rat testes. (a) Testicular cross sections from control rats showing normal seminiferous tubules and spermatogenesis (H&E ×40). (b) Testicular cross sections from DBP (200 mg/kg/day) challenged rats showing degeneration with absence of spermatogenic series in the lumen of some seminiferous tubules (ds) (H&E ×40). (c) Testicular cross sections from DBP (400 mg/kg/day)-treated rats showing degeneration with absence of spermatogenesis and sperms from most of the seminiferous tubules (ds; H&E ×160). (d) Testicular cross sections from DBP (600 mg/kg/day)-challenged rats showing necrosis in some of seminiferous tubules (ns; H&E ×80). H&E: hematoxylin and eosin; DBP: dibutyl phthalate.

This study also exhibited decrease in testicular LDH activity in DBP-treated animals. LDH, a testicular germ cell marker enzyme, is the most active form of enzyme present in the mature sperm (Kumar et al., 2013). The spermatozoa require LDH for necessary metabolic activity during passage from testis to the site of fertilization in the oviduct (Mahi-Brown et al., 1990). Lactate is one of the compounds produced by the Sertoli cells and utilized primarily by the germ cells for ATP production in the mitochondrial oxidative phosphorylation (Riera et al., 2001). It is considered that testicular organs need great energy to continuously maintain spermatogenesis. Lactate deficiency induced apoptosis of testicular germ cells (Erkkila et al., 2002). The reduction in the LDH activity observed in the testis may contribute to low spermatozoa motility due to insufficient ATP generation (Adedara and Farombi, 2012). The alteration of lactate content in the testes has been attributed to perturbation in the hormonal control and supply (Yamamato et al., 2007). Further, the decreased activity of LDH enzyme in DBP-administered animals represents inhibition of spermatogenesis (Abarikwu et al., 2012).

Reactive oxygen species (ROS) is now well established to regulate normal sperm function; however, overproduction of ROS may result in oxidative stress causing significant adverse impact on semen quality and male fertility (Prakash et al., 2001; Shukla et al., 2009). The decreased activity of testicular antioxidants caused by DBP, together with increased LPO may explain the decreased T-AOC. This finding indicates that DBP induced oxidative stress in rat testes with remarkably increased MDA concentration, which may be due to ROS overproduction (Aitken and Baker, 2002). Plasma membranes of the sperms have a high content of polyunsaturated fatty acid; hence, they are highly sensitive to oxidative stress and LPO (Lenzi et al., 2000). LPO has been shown to be associated with reduction in sperm mobility, viability, and count (Kao et al., 2008). High levels of ROS are associated with poor sperm quality because ROS induce excessive apoptosis of spermatogenic cells and sperm by disrupting the inner and outer mitochondrial membranes or affecting the balance between pro- and antiapoptosis systems (Agarwal et al., 2003). The risk of oxidative damage from LPO is especially high for steroid-synthesizing tissues because these tissues, in addition to oxidative phosphorylation, use molecular oxygen for steroid biosynthesis (Murugesan et al., 2008). In this study, the declined serum testosterone level was accompanied by oxidative stress as evident by increased LPO. ROS can damage critical components of the steroidogenic pathway in Leydig cells, including steroidogenic acute regulatory protein (Diemer et al., 2003) and cytochrome P450 enzymes (Georgiou et al., 1987).

To prevent peroxidation of membrane lipids during conditions of elevated ROS, the rat testis has several antioxidant enzymes that scavenge and metabolize these free radicals (Peltola et al., 1992; Sikka, 2004). Among the antioxidant defense of testis, SOD, CAT, and GR are the first and most important lines of defense (Ourique et al., 2013). The increase in LPO was accompanied by concomitant decrease in the activities of antioxidant enzymes SOD, CAT, and GR. In mammalian testis, SOD plays a major role in male germ cell protection as well as differentiation (Bauche et al., 1994; Peltola et al., 1992). SOD catalyzed the dismutation of $O_2^{\bullet -}$ into H_2O_2, which is then degraded to water by the activity of CAT (Schmatz et al., 2012). A decrease in the activities of CAT may lead to an excess availability of H_2O_2, which in turn generates $OH^{\bullet}$, resulting in the initiation and propagation of LPO (Schmatz et al., 2012). GR mediates the reduction of oxidized GSSG to GSH, which utilizes NADPH (Bray and Taylor, 1993). The decreased activity of this enzyme in the testis may be an important factor for the depletion of GSH contents. Further, these results were well substantiated by marked alteration in the histopathological examination of testis. DBP treatment provoked degeneration with absence of spermatogenic series in the lumen of some seminiferous tubules, degeneration with absence of spermatogenesis and sperms from most of the seminiferous tubules, and necrosis in some of seminiferous tubules. We have observed in our study in parallel with the study by many researchers that the main target of phthalate esters is seminiferous tubules in rat testis (National Toxicology Program, 2003). Moreover, there was a dysfunction of Leydig cells, a significant decrease in the levels of testosterone, falling of gametes, and atrophy of the seminiferous tubules in rats treated with DBP (Mylchreest et al., 2002). Further, Güllen Ünal et al. (2013) reported apparent atrophy and deformity in the seminiferous tubules of DBP-treated rats. It has been found that the first targets of DBP are Leydig and Sertoli cells (Shirota et al., 2005), which may explain the degeneration and necrosis in some of seminiferous tubules observed in DBP-treated rats. These histopathological changes in the testis may be due to ROS generated in the testis and thus affect gonadal functions. The

decreased serum testosterone may have caused sperm and histopathological anomalies.

In summary, DBP induced decrease in testes weight, impairment of spermatogenesis, reduced serum FSH and testosterone level, altered testicular LDH, increased LPO, and decreased the levels of enzymatic antioxidants with histopathological anomalies. These results indicated that oxidative stress and subsequent decrease in testosterone secretion were the potential underlying mechanism of DBP-induced testicular toxicity.

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