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Original article

Chronic Effects of Di(2-ethylhexyl)phthalate on Stereological Parameters of Testis in Adult Wistar Rats

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Abstract

Objective(s)
In recent years concerns have been raised regarding the incidence of male reproductive disorders from exposure to endocrine disruptors. So, chronic effects of di(2-ethylhexyl)phthalate were studied on histological and stereological structure of testis in adult Wistar rats.

Materials and Methods
Thirty two adult Wistar rats were randomly divided in four equal experiment groups; oil vehicle group and three treated groups which received 10, 100 and 500 mg/kg/day di(2-ethylhexyl)phthalate by gavage for 90 days, respectively. At the end of exposure period the volume of testes was measured by Cavellieri method, testes weight was recorded and then fixed in Bouin's solution. Following tissue processing, 5 µm sections were stained with haematoxylin-eosin and evaluated with quantitative techniques. Seminiferous tubule diameter, germinal epithelium height, relative and total volumes of seminiferous tubules, tubular lumen and interstitial tissue were estimated.

Results
The results showed that mean weight and volume of testis were decreased significantly (35.2% and 23.9% respectively) in rats treated with 500 mg/kg/day DEHP for 90 days. Seminiferous tubules diameter reduced, 4.4% and 13.4% in 100 and 500 mg/kg/day DEHP-treated groups, respectively. Relative volumes of tubular lumen and interstitial tissue were increased significantly in 100 ($P< 0.05$) and 500 ($P< 0.01$) mg/kg/day doses groups. Also, testosterone serum levels were significantly higher ($P< 0.05$) in rats exposed to 500 mg/kg/day DEHP.

Conclusion
Present study indicated dose-dependent reductions of testicular parameters in adult male rats chronically exposed to di(2-ethylhexyl)phthalate.

Keywords: Di(2-ethylhexyl)phthalate, Spermatogenesis, Stereology, Testis

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Introduction

Over the last 30 years, studies have shown disturbing trends in male reproductive health (1). Endocrine disruptors are usually estrogen-like and/or antiandrogenic chemicals in the environment that have potentially hazardous effects on male reproductive axis resulting in infertility and other hormonal dependent reproductive defects. These chemicals mimic natural hormones by binding to hormone receptors, inhibit the action of hormones, and/or alter the normal regulatory function of the endocrine systems (2). Phthalates are one of the many classes of chemicals which have been implicated as having estrogenic properties. They are commonly found in many consumer products regularly used by humans, such as soap, shampoo, cosmetics, hairspray and also flexible plastics such as; blood transfusion bags, children's toys and food containers. Di(2-ethylhexyl)phthalate (DEHP) is one of the most abundant phthalates mainly used as a plasticizer of polyvinyl chloride (PVC) in the manufacturing of a wide variety of consumer products for building construction, automobiles, clothing, toys and medical devices (3).

The reproductive toxicity of DEHP in laboratory animals has been reported. It has been shown that DEHP disrupts the androgen biosynthesis by modulating of steroidogenic enzyme, 17b-hydroxysteroid dehydrogenase, activity and pituitary LH secretion (4), reduces fertility, decreases weight of male reproductive organs and changes histological structure of testis in juvenile and adult rats (5, 6). There are sufficient data to characterize DEHP as a reproductive toxicant in adult animals, at least in rodent (6-8).

However, some studies pointed to the necessity of considering dose when evaluating the consequences of phthalate exposures (9). The effects of acute exposure to DEHP may differ significantly from those associated with chronic exposures. Previous animal studies of phthalates were generally conducted with high doses and short exposure periods. Acute exposure paradigms do not approximate real-life situations for human populations who may be subjected to prolonged low-level exposures. Effects associated with chronic exposures of laboratory species to chemical agents, rather than acute exposures, are probably more related to the pattern of human exposures. Therefore, the present study was designed to investigate chronic effects of different doses of DEHP on histological and stereological structure of testis in adult Wistar rats.

Materials and Methods

Animals

A total of 32 adult male Wistar rats, 100 days old and 110±10 g body weight, were obtained from animal house of Jondishapour Medical Sciences University of Ahwaz, randomly divided to four equal experimental groups: control oil vehicle and three treatment groups which received 10, 100 and 500 mg/kg/day di(2-ethylhexyl)phthalate (Merk Co.) by gavages for 90 days. The animals were housed in stainless steel cages under standard animal house conditions with a 12 hr light/dark cycle and a temperature of 25±2 °C, received standard pellet food and distilled water was available *ad libitum*.

Microscopical analysis

One day after the end of exposure period, male rats were anesthetized with ketamine hydrochloride, the left testicular artery and vas deferens of each were clamped with a hemostat and the left testis was removed, separated from the epididymis, testicular weight was recorded and then was fixed in Bouin's solution for 24 hr. After tissue processing, 5 µm paraffin sections were prepared for histological studies. Seminiferous tubules diameter and germinal epithelium height were measured by using the Axiovision L.E. 4.5 software. For measuring of seminiferous tubule diameter and germinal epithelium height, 90 round or nearly round cross-sections of seminiferous tubules were randomly chosen in each rat. Then, two perpendicular diameters of each cross-section of seminiferous tubules were measured using an ocular micrometer of light microscopy (Olympus EH) at magnification of ×40 and their means were calculated. Also, germinal epithelium height was measured in 4 equidistance of each cross-section of

seminiferous tubules and their means were calculated.

Also, the right testes were fixed by perfusion (10) with Bouin's solution for 20 min and afterwards immersed in the same fixative for 24 hr. After fixation, right testis was embedded in paraffin and serial 5 µm sections, with a random start, were prepared along the long axis of the organ, stained with haematoxylin-eosin and used for quantitative analysis.

The testicular volume was estimated based on the principle of Cavalieri (11) from the cross-sectional areas of 10 sections of each testis. The area was estimated by superimposing a test point grid on tissue micrographs, taken from each section, and counting the points that fell on the testicular sectional area. The used formula was:

$V = N \times a \times t$

Where V is the calculated volume, N is the total number of points which hit the testicular tissue, a is the unit area associated with each test point and t is the thickness of each slice.

The volume densities (V_V) of seminiferous tubule, germinal epithelium, tubular lumen and testicular interstitium were determined by point counting method (11). Then, total volumes of the testicular parameters were calculated by multiplying corresponding volume densities by testis volumes.

Hormone assay

The blood serum samples were analyzed for testosterone with radioimmunoassay method employing a diagnostic kit (Immunotech, SA, France, PI-1119).

Statistical analysis

All data were analyzed using SPSS version 10.0 for windows. Testicular parameters in different groups were compared by one-way ANOVA and Tukey's test was used as a Post hoc test. The data were presented as the mean±SEM and differences considered to be significant when $P< 0.05$ and $P< 0.01$.

Results

Epithelial degeneration and atrophy of seminiferous tubules with the loss of spermatogenesis were observed in all DEHP treatment, particularly in 100 and 500 mg/kg/day, groups. Seminiferous tubules had focal areas of vacuolar degenerative changes in the spermatogenic epithelium and abnormal distribution of spermatogenic cells (Figure1A, B, C and D).

Mean weight and absolute volume of testis were lower significantly ($P< 0.05$) in rats exposed to 500 mg/kg/day DEHP compared to the control group (Table 1). There were no statistically significant differences in mean weight and absolute volume of testis among control and other DEHP-treated groups. Weight and volume of testis were decreased 35.2% and 23.9% in rats treated for a long-term exposure with 500 mg/kg/day of DEHP, respectively.

Seminiferous tubule diameter and germinal epithelium height showed significant ($P< 0.05$)

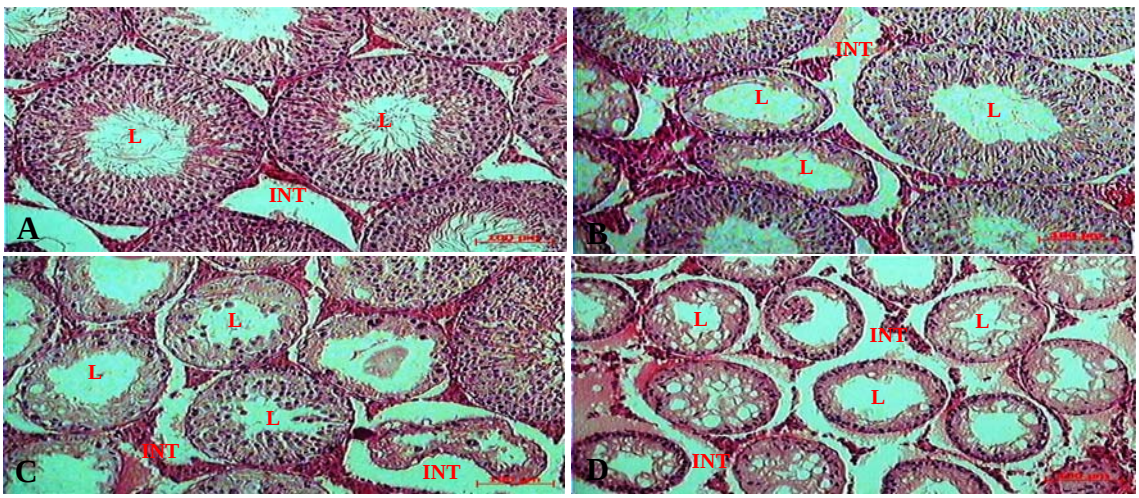

Figure 1. Histological sections of adult rat testis in experiment groups; control (A), 10 (B), 100 (C) and 500 mg/kg/day (D) DEHP groups (Scale bar: 100 µm, 350×, H&E). Degeneration and atrophy of most seminiferous tubules were seen with loss of spermatogenesis, decrease of germinal epithelium height, increase of tubular lumen and presence of vacuoles in epithelium in all treatment, particularly in highest dose, groups.

Table1. Mean (±SEM) of testis weight and volume, seminiferous tubules diameter and germinal epithelium height in DEHP-treated adult Wistar rats.

Variables	DEHP (mg/kg/day)			
	0	10	100	500
Testis weight (g)	1.05±0.02	0.96±0.01	0.93±0.01	0.68±0.11*
Testis volume (mm^3)	331.8±26.0	320.3±21.0	315.0±16.7	252.3±20.2*
STD (µm)	266.5±2.2	263.2±2.5	254.6±2.6*	230.7±3.3**
Epithelium height (µm)	74.0±1.3	72.0±1.2	70.1±1.1*	65.7±1.5**

STD: Seminiferous tubule diameter, *P< 0.05, **P< 0.01.

decrease in rats exposed to 100 and 500 mg/kg/day DEHP compared to the control group, so that these reductions were higher (P< 0.01) in highest dose group (Table 1). No significant differences were seen between 10 mg/kg/day DEHP and control groups. Seminiferous tubules diameter was reduced significantly (4.4% (P< 0.05) and 13.4% (P< 0.01) in 100 and 500 mg/kg/day DEHP-treated groups, respectively). Also, germinal epithelium height was decreased significantly (5.2% (P< 0.05) and 11.2% (P< 0.01) in 100 and 500 mg/kg/day DEHP-treated groups, respectively).

Testosterone serum levels were higher significantly (P< 0.05) only in rats exposed to 500 mg/kg/day DEHP (Figure 2). There were no significant differences in testosterone serum levels between low and moderate DEHP-treated and control groups.

Seminiferous tubules and germinal epithelium in control and DEHP-treated groups occupied 73.4 to 79.4% and 60.3 to 68% of the total testis volume, respectively. Relative and absolute volumes of seminiferous tubules and germinal epithelium were decreased in a dose-related manner compared with control group, but significant (P< 0.05) differences were only seen in 100 and 500 mg/kg/day DEHP treatment groups, particularly in highest dose (P< 0.01) (Table 2). There was no statistically significant difference in relative and absolute volumes of seminiferous tubules and germinal epithelium between low dose DEHP treatment and control groups. Relative volumes of seminiferous tubules and germinal epithelium decreased significantly (P< 0.05) in 100 mg/kg/day DEHP-treated group (2.49% and 3.5% respectively). These parameters decreased significantly (P< 0.01) in 500 mg/kg/day DEHP-treated group (6.07% and 7.7% respectively).

The interstitial space and tubular lumen occupied 20.5 to 26.5% and 11.4 to 13.1% of the total testis volume, respectively, in the controls and DEHP-treated groups. Significant (P< 0.05) increases were seen in volume densities of the interstitium and tubular lumen in 100 and 500 mg/kg/day DEHP treatment groups compared with control group (Table 2). Relative volumes of interstitium and tubular lumen were increased significantly (P< 0.05) 2.6% and 1.1%, respectively, in 100 mg/kg/day DEHP-treated group. These parameters were increased significantly (P< 0.01) in 500 mg/kg/day DEHP-treated group (6.0% and 1.7% respectively).

Table 2. Mean (±SEM) relative and total volumes of testicular parenchymal components in DEHP-treated adult Wistar rats.

Variables	DEHP (mg/kg/day)			
	0	10	100	500
Seminiferous tubule (V_V)	79.49±1.28	78.9±1.20	77.0±1.50*	73.42±1.33**
Seminiferous tubule (ml)	263.7±25.0	252.7±23.0	242.5±17.6*	185.2±10.4**
Germinal epithelium (V_V)	68.0±8.20	67.0±7.32	64.5±7.11*	60.3±7.45**
Germinal epithelium (ml)	225.6±17.3	214.6±15.5	203.2±22.7*	152.1±21.8**
Interstitium (V_V)	20.5±2.4	21.05±2.0	23.1±3.2*	26.5±2.8**
Interstitium (ml)	68.0±5.4	67.4±6.5	72.7±8.2*	66.8±7.6**
Lumina (V_V)	11.4±3.5	11.9±2.4	12.5±2.8*	13.1±4.2**
Lumina (ml)	37.8±8.1	38.1±7.5	39.3±6.1*	33.1±6.6**

V_V : 1

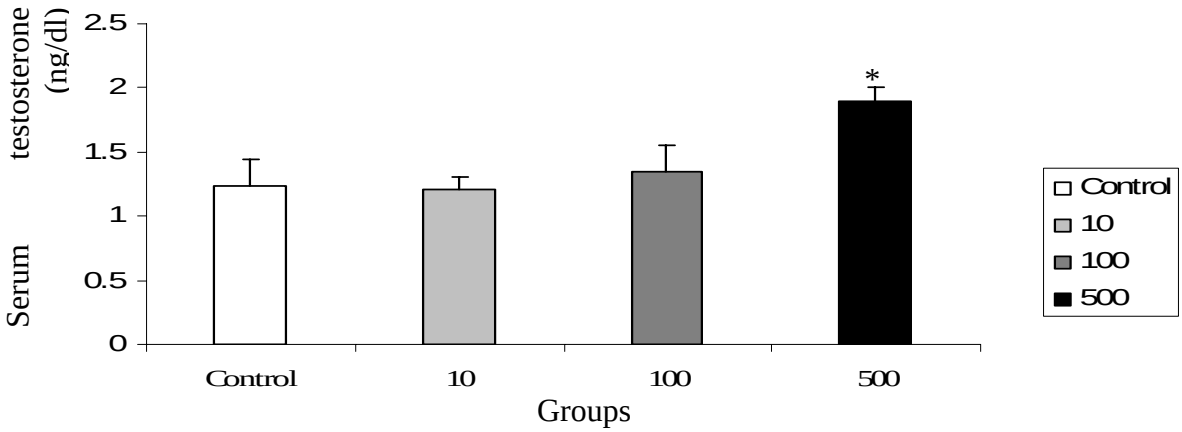

Figure 2. Comparison of mean (±SEM) of serum testosterone levels in DEHP-treated adult Wistar rats (* P <0.05).

Discussion

In the present study we have obtained quantitative stereological information about chronic effects of different doses of di(2-ethylhexyl)phthalate on testis of adult Wistar rats. The stereological data showed that there are marked variations in testicular parameters of adult Wistar rats in DEHP-treated groups. Significant reductions were seen in weight and volume of testis, seminiferous tubules diameter, germinal epithelium height and relative and absolute volumes of seminiferous tubules in testis of adult Wistar rats exposed to 100 and 500. mg/kg/day DEHP for 90 days. Oishi (1994) showed that in male Wistar rats, daily gavage administration of 2000 mg/kg/day DEHP for 7 days results in shrunken seminiferous tubules with necrotic debris and loss of spermatogenesis as well as a 38% decreases in testes weight (14). Decreased testes weight and increased testicular atrophy was also reported in Alderley Park rats at the same dose (15). Moreover, 2200 mg/kg/day DEHP for 10 days produces tubular atrophy in 15-week-old rats (13). Longer-term treatments with DEHP decrease testicular weight, change seminiferous tubule morphology, damage spermatogenic cells and reduce sperm counts in male Wistar rats dosed with 250 and 2000 mg/kg/day DEHP in the food for 1 to 13 weeks (16). In the 10-week-old rats, 500 mg/kg/day DEHP for 2 months produces histological changes in 5-50% of seminiferous tubules (13). However, DEHP was administered to Wistar rats via inhalation (1000 mg/m^3 daily for 6 hr), for 4 weeks also had no effect on any reproductive parameters (17). Also, it has been shown that DEHP-induced adverse effects in the reproductive system were not observed in all species and at all dose levels. Daily gavage administration of 500 to 2000 mg/kg/day DEHP for 4 to 14 days did not induce any significant reproductive deficits in Sprague-Dawley rats and Marmoset or Cynomolgous monkeys (15, 18-20).

It has been suggested that the site of action of DEHP is in the seminiferous tubules and Sertoli cells are primary targets of phthalates (13). Creasy *et al* (1983) reported that histological abnormalities developed in seminiferous tubules within a few hours after phthalate administration (12). So, it seems that statistically significant reductions in testicular parameters which were seen in 100 and 500 mg/kg/day DEHP-treated groups were due to germinal epithelium degeneration and atrophy of seminiferous tubules (Figure 1).

Present study demonstrates that chronic exposures of DEHP at 500 mg/kg/day induce significant increases in the serum testosterone. Creasy *et al* (1983) and Gray *et al* (1997) reported that changes in the Leydig cells develop only after repeated exposures of

DEHP (12, 21). Akingbemi *et al* (2004) showed that chronic exposure to DEHP induces Leydig cell hyperplasia (22). Although mature rat Leydig cells typically do not undergo mitosis beyond 60 days of age (23), a significant increase in DNA incorporation of thymidine by DEHP-treated Leydig cells was observed after treatment for about 2 months (22). Oishi and Hiraga (1979) reported a decrease in circulating testosterone in rats administrated with DEHP for 5 days (24). Akingbemi *et al* (2004) showed that oral exposures to 10 mg/kg/day DEHP during days 21 to 49 of pubertal development causes Leydig cell hyperplasia and persistently elevated testosterone levels (22). Similar results were obtained in rats treated by inhalation with a low dose of DEHP, comparable with 10 mg/kg/day oral treatment (3). Also, prepubertal rats exposed to DEHP at 10, 500 and 750 mg/kg/day form postnatal day 21 to 48, have higher levels of testosterone compared to the low doses (9) and, in contrast, testosterone levels decrease more significantly in highest dose group compared to control group. The boars received intramuscular DEHP-50 mg/kg/day twice a week- during puberty had elevated serum testosterone levels and Leydig cell hyperplasia (25). Akingbemi *et al* (2004) showed that basal and LH-stimulated testosterone production per Leydig cell decreases significantly ($P< 0.01$) in rats exposed to 10 and 100 mg/kg/day DEHP (22). So, they proposed that elevated serum testosterone levels are due to higher numbers of Leydig cells in DEHP-treated rats. Foster *et al* (2001) suggested that hyperplasia is associated with disturbances in paracrine relationships between Sertoli and Leydig cells due to germ cell loss and testicular atrophy (26). However, Shultz *et al* (2001) reported that di (n-butyl) phthalate, a compound closely related to DEHP, increases expression of two antiapoptotic genes, prostate message-2 and bcl-2, in testis (27).

Conclusion

Consequently, because of dose-response relationships in our quantitative microscopic data, present study show dose-related changes in testicular parameters of Wistar rat testis chronically exposed to di(2-ethylhexyl)phthalate (DEHP).

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