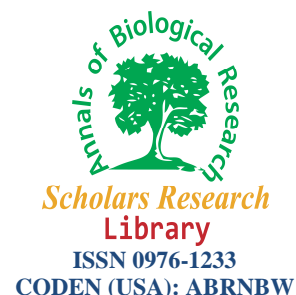




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Annals of Biological Research, 2014, 5 (1):80-87  
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## Adverse effects of di (n-butyl) phthalate on reproduction in adult male rabbits (*Oryctolagus Cuniculus*)

Lamia Rihani\*, Kamel Khelili and Cherif Abdennour

Laboratory of Animal Ecophysiology, Department of Biology, Faculty of Sciences, University  
Badji Mokhtar, Annaba, Algeria

### ABSTRACT

*Di-n-butyl phthalate (DBP) is an environmental pollutant, widely used as a plasticizer in various products including plastics and cosmetics. Previous studies have revealed that DBP has potential reproductive toxicity in animals and humans. The present study was designed to investigate the effect of DBP on histological, hematological and reproductive markers in adult male rabbits. Thus, 24 adult male rabbits *Oryctolagus cuniculus* were divided into four groups. DBP was administered to each group by gavage at doses of 0 (control), 250, 500 and 750 mg/kg/day for a period of 4 weeks. The results revealed a significant reduction in the weight of testes, epididymis and also in the number, speed, mobility and vitality of sperm, accompanied with a significant decrease in morphological changes of flagellum in the treated groups compared to control group. Moreover, a significant decline was observed in the mean of red blood cells counts, haematocrit and hemoglobin in all treated groups compared to the control. The DBP has decreased the level of testosterone and caused histological alterations in the seminiferous epithelium and the epididymal tubules. In conclusion, DBP exposure disturbed reproduction of adult male rabbits, which was dose-dependent.*

**Key words:** DBP, toxicity, reproduction, sperm, rabbit, testosterone

### INTRODUCTION

Phthalates are a high production volume group of chemicals used as plasticizers in an extensive range of products that we use in everyday life. They have been detected in many biological fluids including saliva and urine, also in cord blood and amniotic fluid of the general population [1- 2]. One of phthalates, Di (n-butyl) phthalate (DBP), is a ubiquitous environmental pollutant, used extensively as a plasticizer in many products and as a solvent and is suspected to be an endocrine disrupter [3]. The major route of human exposure to Di-ester phthalates is through the ingestion, and other routes including inhalation and dermal contact [4-5]. Mono-butyl phthalate (MBP), the active metabolite of DBP, is formed by intestinal hydrolases upon ingestion and is quickly absorbed in the gut [6]. It has been found to be the active toxic metabolite of DBP [7]. Previous studies have shown that the toxic effects in animal models have been characterized by changes in blood cell and sperm counts and in provoke testicular atrophy.

DBP is known to affect male fertility, cause testicular atrophy in young rats [8-9]. In adult rodents, exposure to DBP and other phthalates caused pathological and biochemical changes in the testis [10]. Oral administration of DBP to male rats can produce testicular lesions characterized by sloughing of germ cells, vacuolization of Sertoli cells, and testicular atrophy [11-12]. Although abnormal maturation and death of germ cells are the most common adverse responses in the rat testis exposed to phthalates, the Sertoli cell appears to be a primary target for a toxic action of these chemicals [13]. DBP also induces histopathological changes within seminiferous epithelium in rats and rabbits, including widespread germ cell loss, vacuolization of Sertoli cell cytoplasm, Leydig cell hyperplasia, dysgenetic seminiferous tubules, and atypical germ cells resembling carcinoma *in situ* cells [14].

DBP has well defined anti-androgenic effects by interfering with the production of testosterone [15]. However, many studies have suggested that the phthalates induce significant morphological and biochemical alterations prior to the loss of zinc in the testis [16]. Recently, there are growing concerns regarding the toxicity of DBP exposure on male reproduction [17].

The objectives of this study were to determine the reproductive effects of DBP on adult male rabbits *Oryctolagus cuniculus*. It is still relevant with the fact that the levels of human exposure to DBP ranged from 0.84 to 113 mg/ kg [18]. In this study, we selected the rabbits as species of experiment because the function of rabbit's reproductive is better approximate the comparable function of human reproductive. Furthermore, use of rabbit facilitates multiple evaluations semen motility and quality. The use of this species belonging to an order of mammals (lagomorpha) different from that of rodents (rodentia), also addresses the issue that phthalate-induced effects on reproductive function may only be rodent-specific. Thus, male rabbits were exposed to DBP at a dose level known to adversely affect testicular function in rodents without causing systemic toxicity.

## MATERIALS AND METHODS

### Animals

Twenty four male adult rabbits *Oryctolagus cuniculus* aged between 6 to 9 months, weighing  $1800 \pm 130$  g, from Annaba region (eastern Algeria) were used in the current study. Animals were housed in the Department of Animal Biology, Annaba. The room was maintained at a 12-h light-dark cycle at approximately 19-21°C and 40%-60% humidity. Animals were kept in special cages (50 x 60 x 53 cm); they provided food and water *ad libitum* until the time of sacrifice.

### Study Design

The rabbits were divided into four groups, each comprising 06 individuals; all animals acclimated for 1 week before the period of treatment. Dosing solutions of DBP were prepared by diluting DBP (CAS 84-74-2, 99.8% pure PM: 278.345, density: 1.047) in deionized water. Rabbits in DBP-exposed groups were given DBP at a dose of 250, 500 and 750 mg/kg/day (5 ml/ kg body weight), by oral gavage for 4 consecutive weeks. Rabbits in the control group were orally administered corn oil in the same volume for 4 consecutive weeks. The total body weight was recorded weekly throughout the study period.

At the end of the exposure, the rabbits were sacrificed. Blood samples were collected by decapitation using EDTA as an anticoagulant for determination of selected haematological parameters. The testes and epididymis were immediately removed and weighed. One side testes and epididymis of each rabbits was used for histological study.

### Assay of Testosterone

Serum was prepared by centrifuging at 2000 g at 4 C° for 10 min and was stored at -20 C° prior to analysis. The serum concentration of testosterone was determined by using enzyme-linked immunosorbent-colorimetric method using (testosterone ELIZA kit) [19].

### Sperm Analysis

Rabbit's sperm were assessed in cauda epididymis by the method given in (OMS, 1993) [20]. The left epididymis removed at the sacrifice of each rabbit, and a droplet (1µl) of the sperm is added to 49µl of 0.9% NaCl to obtain a solution in order which is used to study the concentration, speed, motility, vitality and malformations of sperm as follow:

**Sperm concentration:** a droplet of solution was introduced in Malassez cell and then sperm was counted under optical microscope.

**Sperm motility:** a droplet of sperm is deposited on a slide and covered with a coverslip, then 100 sperms counted to record the percentage of sperm motility.

**Sperm speed:** the travel time of a sperm was calculated between 02 lines of Malassez cell using a chronometer to calculate the speed.

**Sperm vitality:** vital staining technique is determined by the count of colored and colorless sperms in three fields of view, and then the percentage of each category (colored and colorless sperm) was calculated.

**-Hypo-osmotic swelling of sperm technique:** a droplet of sperm was added into solution of fructose and sodium citrate. The percentage of sperm showing changes in the form of flagellum were calculated.

### Histological Evaluations

After sacrifice, testes and epididymis were fixed in Bouin's solution and dehydrated with 70% ethanol. The tissues were fixed in paraffin, and then 5 mm sections were cut and mounted onto slides. The slide sections were stained with haematoxylin and eosin. The histological features of the testes and epididymis were determined by optical microscopy [21].

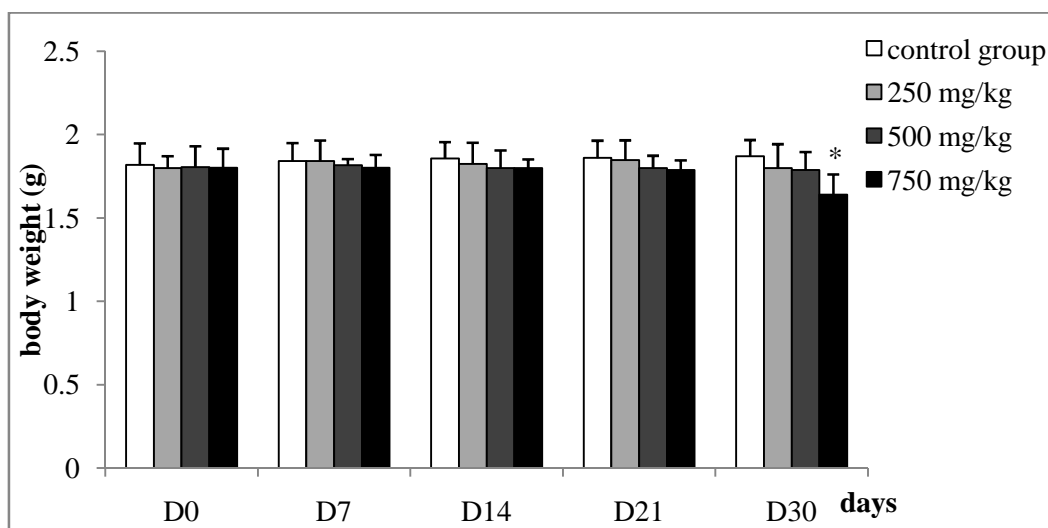
### Statistical Analysis

All values tested in this study were expressed as mean  $\pm$  S.E (n=6). Differences between the control and the treatment groups were determined using a one-way ANOVA.  $p \leq 0.05$  was considered statistically significant.

## RESULTS AND DISCUSSION

DBP is a ubiquitous environmental contaminant, human are constantly exposed to DBP through food, water or contact with a many products [22]. Although the doses used in our study were higher than the levels found in the general environment, it should be considered that people are exposed to this chemical throughout his entire life-time. The lipophilicity and long half-life of DBP could result in his accumulation to ultimately high levels in the body. This chemical was administered orally in this study, because human exposure to this chemical principally occurs by this route [23].

In the actual study, no obvious signs of toxicity were observed throughout the experiment. Also, the DBP treatment has no adverse effect on total body weight or food consumption. However, the body weight is decreased at 750 mg/kg of DBP in the last week compared to control group (Figure 1). The weights of testes and epididymis were decreased after treatment with DBP at 500 and 750 mg/kg/day compared to the control (Table 1). According with these results, Srivastava et al. (1990) [12] have investigated that DBP exposure in male rat induced a reduction in testes weights. However, the weight of the testes in rat is largely dependent on the mass of the differentiated spermatogenic cells, and the reduction in the weight of the testes may be due to the reduced tubule size, spermatogenic arrest and inhibition of steroid biosynthesis of Leydig cells [24]. In addition, the changes of epididymal weight were consistent with the changes of epididymal histological structure. Epididymal weight is largely dependent on the mass of the epididymal tubules and sperm; the observed reduction in the epididymal weight might be due to the atrophy of epididymal tubules and decreased number of sperm in its lumen. Fan et al. suggested that the reduction in the weight of accessory reproductive organs is an indicative of reduced availability of androgens in the rat male [25].



**Figure 1: Total Body weight (g) of male rabbits after administration of DBP for 30 days is shown**

Results are expressed as the means  $\pm$  S.E.M. (n=6). Statistically significant differences were determined by one-way ANOVA; (\*) Indicates value differs from control by  $p \leq 0.05$

**Table 1: The effect of DBP on the organ weight (testes, epididymis) of male rabbits after 30 days of treatment**

Organs weight	DBP (mg/kg/day)			
	Control group	250 mg/kg	500 mg/kg	750 mg/kg
Paired testes (g)	3,245 $\pm$ 0,148	3,053 $\pm$ 0,175	2,678 $\pm$ 0,298*	2,566 $\pm$ 0,256**
epididymis (g)	0,6552 $\pm$ 0,0501	0,6354 $\pm$ 0,042	0,5996 $\pm$ 0,0262	0,557 $\pm$ 0,047*

Abbreviation: DBP, Di-n-butyl phthalate. All values are expressed as mean  $\pm$  S.E (n=6). (\* $P \leq 0,05$  versus control, \*\* $P \leq 0.01$  versus control)

Recent interest in phthalates has focused on both the toxic effects to male gonads and hemolysis of red blood cells during blood storage. It was proposed that the testicular toxicity might be associated with hypoxia due to hemoglobin deprivation induced by the metabolite of DBP in the male rat [26]. In the present study, administration of DBP for 30 days caused a reduction in red blood cells counts, haemoglobin and haematocrit concentration (Table 2). It is conceivable the MBP may decompose HB directly after crossing the erythrocyte membrane and the MBP treated erythrocyte by reducing the binding affinity of haemoglobin for oxygen causing anoxia [27].

**Table 2. The effect of DBP on haematological parameters of male rabbits after 30 day of treatment**

parameters	DBP (mg/kg/day)			
	Control	250 mg/kg	500 mg/kg	750 mg/kg
RBC ( $10^{12}/l$ )	6,514 ± 0,61	6,31 ± 0,53	5,908 ± 0,277	5,73 ± 0,292*
HB (g/dl)	118,6 ± 15,8	105 ± 11,8	94 ± 11,8*	90 ± 12,9**
HT (%)	36,99 ± 2,04	36,29 ± 1,8	35,08 ± 1,09	34,11 ± 1,56*
WBC ( $10^9/l$ )	12,29 ± 0,414	12,738 ± 0,543	12,942 ± 0,561	13,018 ± 0,643
PL ( $10^9/l$ )	432,8 ± 62,4	439,6 ± 63,6	454,8 ± 60,8	465,4 ± 62,8
MCV (fl)	57,6 ± 2,07	56,8 ± 1,92	56,2 ± 1,79	55 ± 1,58
MCH (pg)	20,02 ± 1,04	19,62 ± 0,746	19,36 ± 0,817	19,16 ± 1
MCHC (g/dl)	414 ± 11,4	42,4 ± 14,8	340,6 ± 8,38	337,2 ± 9,63

Abbreviation: DBP, di-n-butyl phthalate; RBC, red blood cells; HB, haemoglobin; HT, haematocrit; WBC, white blood cells; PL, platelets; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean cell haemoglobin concentration. All values are expressed as mean ± S.E. (n=6). (\*P ≤ 0.05 versus control, \*\*P ≤ 0.01 versus control)

Sperm evaluation is a useful indicator for assessing the effects of environmental pollutants on the reproductive function of the testis in experimental animals and humans. The parameters of sperm (concentration, motility, vitality, speed) are presented in (Table 3). The results are shown a significant reduction in the number, motility, vitality and speed of sperm between treatment groups compared to the control group. It has been regarded this as a consequence of damage to the seminiferous epithelium. Previous to this work, it was known that some phthalate esters affect adult testes and reduce sperm production in rodents [28]. The reduction in the sperm count might be due to the reduction in gonadotropin levels. Moreover, adult male hamsters given diethylstilbestrol showed a significant decrease in gonadotropin levels, leading to the increased spermatogenic cell apoptosis through the suppression of testosterone level [29]. The wide-spread germ-cell loss in the seminiferous epithelium of the testis appears to result from Sertoli cell dysfunction [30, 31]. An increased of apoptosis of germ cells has also been found to be associated with the toxicity of Phthalate monoester [32], which may be responsible for loss of spermatogenic cells in the testes. However, in adult rodents, the Sertoli cell is the likely cellular target for testicular injury mediated by the monoester of this molecule [13].

The motility of sperm decreased after DBP administration in the male rabbits (Table 3). According with this result, Duty et al., (2003) [33] found an inverse dose–response relationship between monobutyl phthalate (MBP), a metabolite of DBP, and sperm motility. A significant reduction of sperm vitality is also investigated in this study (Table 3). This result may be explained by the dysfunction of Sertoli cell. Boekelheide (2004) investigated that phthalates exposure to rats inducing abnormal maturation and death of germ cells and the Sertoli cell appears to be a primary target for a toxic action of these chemicals [10]. In young rats, exposure to mono-(2-ethylhexyl) phthalate resulted in collapsed filaments of vimentin cytoskeleton in Sertoli cells and germ cell sloughing [32].

**Table 3. The effect of DBP on sperm parameters in male rabbits after 30 days of treatment**

Parameters of sperm	DBP (mg/kg/day)			
	Control	250 mg/kg	500 mg/kg	750 mg/kg
Concentration (X106/ml)	409,2 ± 12,4	368,8 ± 18,6**	291,8 ± 13,5***	197,5 ± 11,6***
Mobility (%)	62,5 ± 5,24	50,33 ± 2,88**	30 ± 3,58***	18,5 ± 3,62***
Speed of sperm (µm/sec)	49,3 ± 2,74	42,61 ± 2,6**	22,24 ± 1,84***	15,24 ± 2,02***
Vitality (%)	68,71 ± 31,71	34,29 ± 65,71***	25 ± 75***	16,57 ± 83,43***

Abbreviation: DBP, Di-n-butyl phthalate. All values are expressed as mean ± S.E (n=6). (\*P ≤ 0,05 versus control, \*\*P ≤ 0.01 versus control, \*\*\*P ≤ 0.001 versus control)

The morphological changes characteristic of sperm exposed to hypo-osmotic stress are presented in (Table 4). We founded a significant reduction in the modification of flagellum of sperm category (A, B) between the treatment groups compared with the control group. However, the normal sperm was increased in the treatment groups compared to control group. The reduction in morphological changes of flagellums in the treatment groups indicates the adverse effect of DBP in sperm vitality by causing injury in the membrane of flagellum.

Table 4: The morphological changes characteristic of sperm exposed to hypo-osmotic stress in male rabbits after 30 days of treatment

Morphological changes	DBP(mg/kg/day)			
	Control	250 mg/kg	500 mg/kg	750 mg/kg
A	26, 21± 6,74	14,04± 6,7*	6,87± 4,44**	5,81±3,91***
B	27,86 ±3,34	18,76±4,68*	13,09± 7,92*	9,68±6,48**
C	11,54 ± 3,75	9,34±8,69	9±2,9	5,04±4,9
N	34,36 ±7,14	57,85±6***	70,98±7,1***	79,11±5,33***

Abbreviation: DBP, di-n-butyl phthalate; A, low modification in flagellum; B, significant modification in flagellum; C, modification important of flagellum and middle piece; N: normal (death) sperm. All values are expressed as mean±S.E. (n=6). (\*P≤0,05 versus control, \*\*P≤0.01 versus control, \*\*\*P≤0.001 versus control)

Testosterone is the principal hormone required for normal spermatogenesis and for inhibition of germ cell apoptosis [34]. Previous studies reported that adult male rats exposed to repeat doses of DBP have been shown a reduction blood testosterone level [24]. Our results revealed that DBP could reduce the concentration of testosterone in treatment rabbits after 30 days of treatment (Figure 2). Because LH secreted by the pituitary gland is the primary regulator of testosterone synthesis in testes, one possible explanation for the changes in the levels of serum testosterone is that DBP may decrease testosterone production in the testes by decreasing the LH secretion in the pituitary gland. Exposure to high doses of DBP decreased the levels of the serum hormones testosterone and LH, while E2 and FSH levels were increased. The changes in serum LH and FSH levels indicate that pituitary function may be affected by DBP exposure as DBP was reported to affect pituitary hormone-producing cells at adult stages in rat [35]. In another study, administration of high dose of estradiol-3-benzoate (EB) to male rats causes a reduction in GnRH secretion, causing the suppression of circulating level of LH and consequently testosterone concentration [36]. *In vitro* studies have demonstrated that phthalates can have a direct effect on adult Leydig cell function [24]. These results coincided with previous studies which showed decreased serum testosterone level and an impairment of steroid hormone metabolism after DBP exposure [37].

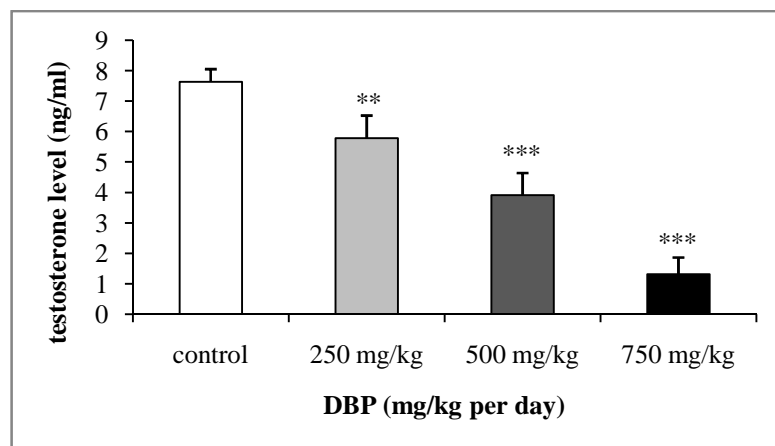
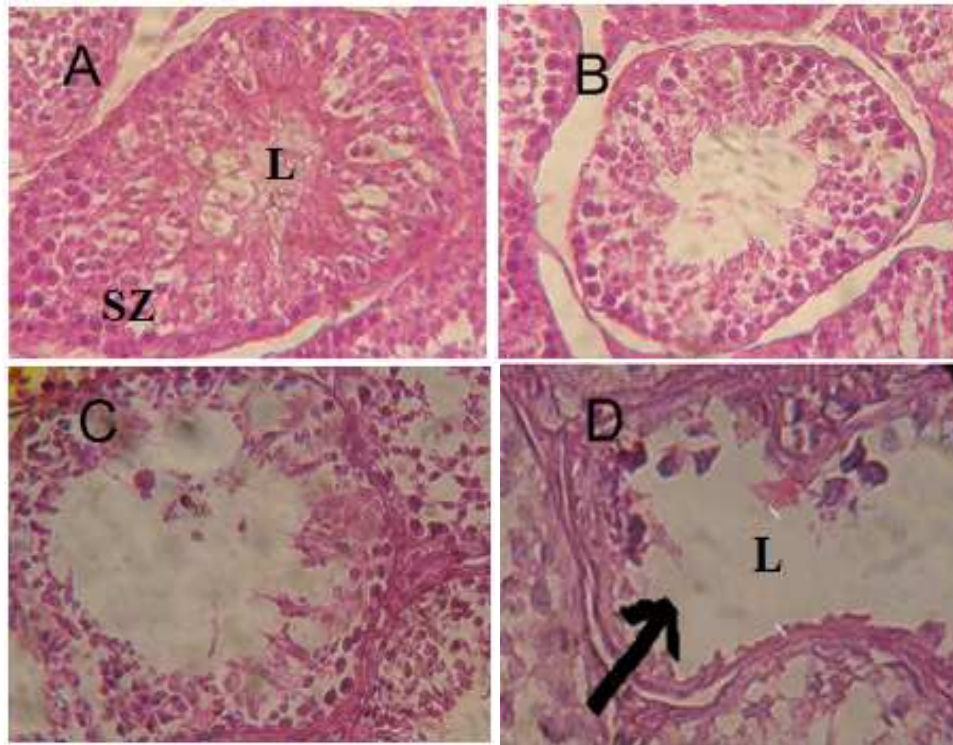


Figure 2: Testosterone levels (ng/ml) after administration of DBP for 30 days is shown

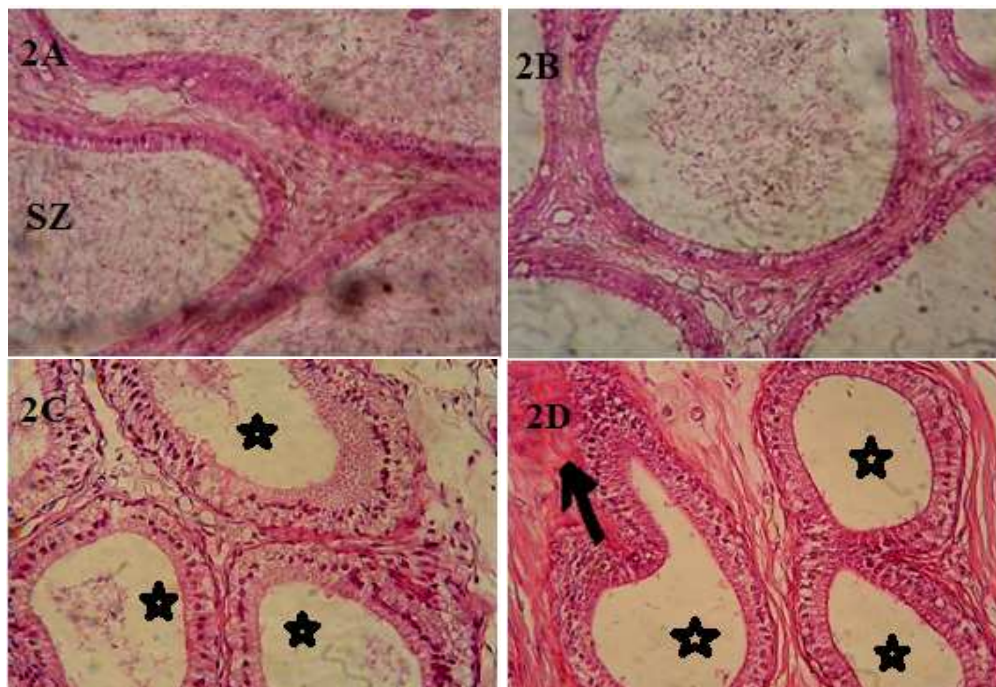
Results are expressed as the means ±S.E.M. (n=6). Statistically significant differences were determined by one-way ANOVA (\*\*P≤0.01; \*\*\*P≤0.001 versus control)

Histopathologically, DBP-treated of male rabbits for 30 days imparted adverse effects on the seminiferous epithelium and epididymal morphology. The arrangements of cells in rabbits' seminiferous epithelium exposed to DBP were irregular and the inter-cellular connections were not compact (Figure 3). Result indicated that this treatment could disturb the junctions between Sertoli cells and germ cells. Testicular atrophy may be explain by the depletion of zinc after DBP exposure [7] and the alteration of Vimentin cytoskeleton organization [38], or membrane alteration in Sertoli cells leading to sloughing of spermatogenic cells [38]. It is known that Vimentin is an important Sertoli cell cytoskeleton component, and it plays an important role in positioning the Sertoli cell nucleus and anchoring spermatogenic cells to the seminiferous epithelium [39]. Damaged Vimentin filaments were associated with seminiferous epithelium disintegration, which was reversed during the recovery of spermatogenesis after the critical conditions subsided [17]. Epididymis is the important portion of male genital organs, which plays a crucial role in the storage and maturation of spermatozoa [40, 41]. In the present study, the dose-dependent epididymal toxicity induced by DBP was shown to be associated with regressive epididymal histological structure (Figure 4).



**Figure 3: Histological changes of rabbit's testis in the different treatment groups**

The photomicrographs were taken at X 400 magnification by hematoxylin and eosine staining. (A) Control rabbits showed a compact and regular arrangement of germ cells in the seminiferous tubules. (B) Vacuolization of Sertoli cells in rabbits treated with DBP at 250 mg/kg per day. (C) Seminiferous tubules in rats treated with DBP at 500 mg/kg per day showed an irregular arrangement of germ cells and absence of spermatocytes. (D) Rabbits treated with 750 mg/kg per day of DBP showing marked a reduction in diameter and degeneration of the seminiferous tubules (ST) with necrosis of spermatocytes, spermatids and defoliation of many spermatocytes into lumen (L) of the ST (dark arrow)



**Figure 4: Histological changes of the rabbit's epididymis in the different treatment groups**

The photomicrographs were taken at X 400 magnification by hematoxylin and eosine staining. 2A. Epididymis of the control group. 2B. Epididymis in rabbits of 250 mg/kg di-n-butyl phthalate (DBP) exposure group showed no obvious morphologic changes. 2C. Epididymis in rabbits of 500 mg/kg DBP exposure group showing atrophy of epididymal tubules, the Lumina were oligozoospermic (star). 2D. Epididymis in rabbits of 750 mg/kg DBP exposure group showing the interstitial vascular was hyperemia (arrow); the Lumina were oligozoospermic (star).

In summary, these results demonstrated that exposure to DBP resulted in adverse effects on the reproductive system of male adult rabbits. It has been found that DBP could disturb the secretion of testosterone, induce the production, motility, vitality and morphology of sperm and causes histological damage in reproductive organs. Further investigation is needed to explore the complex mechanism of action of this chemical in male rabbits.

#### Acknowledgment

The authors would like to thank Dr LechkhebYousria for her scientific help of this manuscript. Thanks are also given to Prof Taher Ali for statistical assistance.

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