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Effect on histological and sperm kinetics in DBP exposed Wistar rats

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Abstract: *Di-n-butyl phthalate (DBP) is a ubiquitous environmental pollutant, extensively used as a softener for polyvinyl chloride resins. A study was conducted to evaluate its effect on reproductive function of Wistar rats. DBP was given orally at a dose of 500, 1000 and 1500 mg kg^{-1} body weight for 7 days. Evaluating histological and fertility parameters assessed reproductive function. Significant reduction in seminiferous tubule diameter, Leydig cell nuclear diameter (except at dose 500 mg), number of primary spermatocytes, secondary spermatocytes and spermatids were observed. Caudal sperm density and viability reduced significantly. Decrease in serum testosterone was also observed. Evidence indicates that DBP exposure causes dose dependent testicular toxicity and has the potential to induce adverse effect.*

Key words: *DBP, Testis, Sperm density, Sperm viability, Testosterone*
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Introduction

Phthalates, produced millions of tons annually worldwide, are used widely in consumer and industrial products. Being ubiquitous environmental contaminant it is reported in food, air and water (ASTDR, 2001) and reproductive-age of women (CDC, 2003). Several environmental contaminants have been found to act as endocrine disruptors (Haffor and Abou-Tarkoush, 2004; Kumar *et al*., 2004). Reduced viability of F_1 egg ropes in *Chironomus riparius* exposed to diethyl phthalate was also reported (Kim and Lee, 2004). Antiandrogenic effect was reported in rodents on exposure to di-n-butyl phthalate (DBP), butylbenzyl phthalate (BB_ZP) and diethylhexyl phthalate (DEHP) during gestational, lactation and pubertal age (Gray *et al.,* 1999, 2000; Foster *et al.,* 2001; Parks *et al*., 2000). Severe damage to the reproductive system of mature F1 male rats after 250 mg kg^{-1} DBP administration (Zhang *et al.,* 2004), Leydig cell aggregation on fetal exposure to DBP (Mahood *et al*., 2005) was observed. Sertoli cells in DBP-exposed fetal testes had retracted apical processes, altered organization of vimentin cytoskeleton and abnormal cell-cell contacts with gonocytes (Kleynenova *et al.,* 2005). Duty *et al.* (2005) reported that association between environmental levels of phthalates and altered reproductive hormone levels in men did not change in the expected pattern. In the present study an attempt has been made to study the effect of DBP on testes, caudal sperm parameters and serum testosterone profile in Wistar rats.

Materials and Methods

Colony bred male Wistar rats (120-122 g) were housed in polypropylene cages with stainless steel grills, maintained in a well-ventilated animal room (12 hr light : 12 hr dark) in animal house facility, Department of Zoology, University of Rajasthan and provided standard rat feed (Aashirwad Ltd., Chandigarh) and tap water *ad libitum*.

Forty male animals were randomly divided into 4 groups of 10 each: Control, Group 2, Group 3 and Group 4. Dibutyl phthalate (DBP,CAS 84-74-2) [99%, M = 278.35 g/mol, density 1.047 (at 20°C) MERCK - Schuchardt] was dissolved in soybean oil. Wistar rats of group 2, 3 and 4 were given DBP (500, 1000 and 1500 mg/kg^{-1} b.wt) orally for a period of 7 days. Control group animals received an equal volume of vehicle (soybean oil) for 7 days. The doses were selected below LD_{50} values, which ranged from 8-20 g kg^{-1} body weight.

Animals were autopsied twenty four hours after the last dose. Testis and epididymis were excised and weighed.

(a) Histological analysis: Testes were fixed in Bouin's fixative, cut at 5 μm, processed and stained with Erhlich haematoxylin and alcoholic eosin (Humanson, 1972).
Morphometric measurement (Seminiferous tubule diameter and Leydig cell nuclear diameter) and cell population (preleptotene spermatocytes, pachytene spermatocytes, secondary spermatocytes, round spermatids) count (corrected by using Abercrombie's formula, 1946) was done.

(b) Sperm analysis: Sperm density and sperm viability were assessed in cauda epididymis by the method given in Sigma bulletin (1998).

(c) Hormonal assay: Serum testosterone was determined by radioimmunoassay (RIA) using Coat-A-Count kit (Diagnostic Production Corporation, USA).

Statistical analysis: Between groups comparison of Wistar control rats and rats treated with different doses of di-n-butyl phthalate was done using analysis of variance (ANOVA) and when significant treatment effects were detected paired 'F' test was used to identify specific differences between treatments (Arora *et al*., 2007). Data were expressed in mean ± SE and $p < 0.05$ was considered significant.

Table - 1: Morphometric measurement of seminiferous tubular diameter (μm) and Leydig cell nuclear diameter (μm) in testes of DBP treated Wistar rats (mean ± SE)

	Control (C)	500 mg kg^{-1} body weight (E_1)	1000 mg kg^{-1} body weight (E_2)	1500 mg kg^{-1} body weight (E_3)	ANOVA
Seminiferous tubular diameter	250.04 ± 0.05	238.66 ± 0.30	204.48 ± 1.06	192.50 ± 0.31	F_{obs} = 2181.6842 $F_{crit\ 0.05}$ = 3.49
Leydig cell nuclear diameter	5.54 ± 0.03	5.345 ± 0.01	3.212 ± 0.03	1.875 ± 0.11	F_{obs} =794.2423 $F_{crit\ 0.05}$ =3.49

Table - 2: Testicular cells of DBP treated Wistar rats (mean ± SE)

	Preleptotene spermatocytes	Pachytene spermatocytes	Secondary spermatocytes	Round spermatids
Control (C)	22.4 ± 0.18	28.94 ± 1.84	54.81 ± 0.7	32.77 ± 1.4
500 mg kg^{-1} b.wt. (E_1)	19.93 ± 0.5	20.11 ± 0.37	43.94 ± 0.29	21.36 ± 0.41
1000 mg kg^{-1} b.wt.(E_2)	15.96 ± 0.61	17.66 ± 0.8	26.18 ± 0.54	14.41 ± 0.81
1500 mg kg^{-1} b.wt.(E_3)	8.94 ± 0.13	11.48 ± 0.71	13.11 ± 0.91	- - -
ANOVA	F_{obs} = 0.0945 $F_{crit\ 0.05}$ = 2.86	F_{obs} = 0.045 $F_{crit\ 0.05}$=2.86	F_{obs} = 0.2049 $F_{rit\ 0.05}$= 2.86	F_{obs} = 0.1195 $F_{crit\ 0.05}$= 2.88

Table - 3: Caudal sperm parameters of DBP treated Wistar rats (mean ± SE)

	Control (C)	500 mg kg^{-1} body weight (E_1)	1000 mg kg^{-1} body weight (E_2)	1500 mg kg^{-1} body weight (E_3)	ANOVA
Sperm density (million ml^{-1})	82.48 ± 1.36	54.32 ± 1.81	22.03 ± 0.98	10.46 ± 0.53	F_{obs} =2033.465 $F_{crit\ 0.05}$= 3.49
Sperm viability (%)	68.95 ± 1.49	20.02 ± 1.20	15.3 ± 0.48	7.9 ± 0.21	F_{obs} =981.864 $F_{crit\ 0.05}$= 3.16

Table - 4: Serum testosterone (ng ml^{-1}) of DBP treated Wistar rats (mean ± SE)

	Control (C)	500 mg kg^{-1} body weight (E_1)	1000 mg kg^{-1} body weight (E_2)	1500 mg kg^{-1} body weight (E_3)	ANOVA
Serum testosterone	2.54 ± 0.41	2.23 ± 0.18	1.48 ± 0.04	0.80 ± 0.02	F_{obs} =0.717 $F_{crit\ 0.05}$=3.49

Table - 5: Paired 'F' test analysis after ANOVA

	Seminiferous tubule diameter	Leydig cell nuclear diameter	Preleptotene spermatocytes	Pachytene spermatocytes	Secondary spermatocytes	Round spermatids	Sperm density	Sperm viability	Serum testosterone
Control compared E_1	S	S	-	-	-	-	S	S	-
Control compared E_2	S	S	-	-	-	-	S	S	-
Control compared E_3	S	S	-	-	-	-	S	S	-
E_1 compared E_2	S	S	-	-	-	-	S	S	-
E_1 compared E_3	S	S	-	-	-	-	S	S	-
E_2 compared E_3	S	S	-	-	-	-	S	S	-

Where p < 0.05 = Significant (S)

Results and Discussion

Di-n-butyl phthalate caused significant decrease in seminiferous tubular diameter and Leydig cell nuclear diameter indicating impairment of Leydig cell function. The atrophic state of Leydig cell could be due to altered LH secretion affecting the testosterone synthesis. Phthalate mediated reproductive toxicity (Akingbemi *et al.,* 2001) in the developing testis is thought to occur by a complex interaction between Sertoli, Leydig and germ cells (Mylchreest *et al.,* 2002). Reports exist that phthalate esters disrupts testosterone biosynthesis (Shultz *et al.,* 2001; Barlow *et al.,* 2003) and alters Leydig cell differentiation (Huguchi *et al.,* 2003) and Sertoli cells (Fischer *et al.,* 2003; Bockelheide, 1993). Peroxisome proliferator- activated receptor α (PPAR-α) mediates phthalate induced toxicity (Ward *et al.,* 1998). However, PPAR-γ may play a role in reproductive toxicity as it has been found in human testes, ovary, placenta and embryo. Gazouli *et al.* (2002) reported antiandrogenic effect of phthalates is mediated by PPAR-α dependent inhibition of Leydig cell PBR gene expression. Antispermatogenic effect was evident in the present study by the reduced number of spermatogenic cells. Phthalate monoester toxicity has also been found to be associated with an increased rate of apoptosis of germ cells (Richburg and Bockelheide, 1996), which may be partially responsible for loss of spermatogenic cells in the testes. Differentiation of spermatogenic cells under the control of testosterone is probably mediated by Sertoli cells (Courot and Kilgour, 1984). The significant reduction in the number of secondary spermatocytes and spermatids is indicative of non-availability of ABP from Sertoli cells (Hess *et al.,* 1988). Spermatocytes and spermatids are highly sensitive to androgen concentration. Decrease in testosterone level influences spermatocyte and spermatid stage as is evident in the present study.

Disruption of the testicular homeostatic mechanism influences considerably the epididymal contents. Statistical analysis by ANOVA revealed that reduction in caudal sperm density and viability in the present study is significantly influenced by different dose levels, which may be due to decreased androgen level. Reduced sperm count (Duty *et al*., 2003, 2004; NTP-CERHR 2000; Wine *et al.,* 1997) sperm motility (Duty *et al.,* 2003) severe reproductive damage to testis, epididymis and epididymal sperm parameters (Zhang *et al.*, 2004) after exposure to environmental contaminants has been reported. The reduced number and motility of sperms may be responsible for antifertility effects of DBP. The current study indicates dose response relationship between phthalates and testes and sperm parameters.

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