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## EFFECT OF Di-n-BUTYL PHTHALATE ON THE EPIDIDYMAL ANTIOXIDANT SYSTEM OF WISTAR RATS

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### Keywords:

Di-n-butyl phthalate, lipid peroxidation, lipid hydroperoxides, total antioxidant capacity, ascorbic acid, Vitamin E.

**ABSTRACT:** The study evaluates the effect of di-n-butyl phthalate (DBP), a ubiquitous environmental contaminant, on epididymal antioxidant system of Wistar rats. Adult Wistar rats were orally administered DBP (500, 1000 and 1500 mg/ kg b.wt) for 7 days. Body weight increased significantly while caput and cauda epididymal weight decreased. Significant reduction in total protein concentration, lipid soluble antioxidant capacity and vitamin E levels were observed. Water soluble antioxidant activity decreased in caput epididymis at all dose level while it increased significantly in cauda epididymis. A dose dependent increase in lipid peroxidation, lipid hydroperoxides and ascorbic acid levels has been observed. The results strongly suggests that enhanced lipid peroxidation and lipid hydroperoxides in caput and cauda epididymis is indicative of disruption of membrane fluidity and integrity which may partly be responsible for alteration in total antioxidant capacity and primary defense system-ascorbic acid and vitamin E. In fact, it is an imbalance between the generation and neutralization of reactive oxygen species which leads to epididymal oxidative stress which may be partly responsible for affecting fertility.

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**INTRODUCTION:** Phthalates, (phthalic esters or benzene dicarboxylic acid esters), are widely used in a wide variety of consumer products, formulations of pesticides, pharmaceuticals etc. <sup>1, 2</sup>. Being non covalently bound to the polymer, they are easily released and humans are concurrently exposed through food crops, drinking water, medical devices etc. <sup>3, 4</sup>. Zheng et al, <sup>5</sup> reported that DBP accumulation in the biological system through dermal route was less as compared to oral route with maximum DBP being metabolized within 2 to 3 days.

With variable routes of entry into the human body it can have an effect on reproductive and development process. Environmental contaminants have been reported to affect both male and female fertility<sup>1</sup> and a relationship between phthalates and testicular function <sup>6, 7</sup> as well as impairment of sperm quality <sup>8</sup> has also been reported. The epididymis plays a major role in protecting spermatozoa from ROS and secreting antioxidants into the epididymal lumen <sup>9, 10</sup>. The spermatozoa during its maturation are under constant attack from intra- and extra-cellular ROS <sup>11</sup> which may result in male infertility<sup>12</sup>. The present study was undertaken to evaluate the effect of di-n-butyl phthalate on total protein, lipid peroxidation and lipid hydroperoxides, total (water and lipid soluble) antioxidant capacities and non- enzymatic antioxidants - ascorbic acid and Vitamin E.

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**MATERIALS AND METHODS:**

**Chemicals and reagents:** Di (n-butyl) phthalate (DBP) [99%; M = 278.35 g/mol; density 1.047 (at 20°C)] was obtained from MERCK/ Schudardt. All the chemicals were procured either from Hi-Media or Sigma Chemicals Co.

**Animals and experiment:** Male Wistar rats (120-122 g) were housed in polypropylene cages with stainless steel grills, maintained in a well ventilated animal room (12h: 12h:: light: dark) and provided standard feed (Aashirwad Ltd., Chandigarh) and tap water *ad libitum*.

**Experimental design:** Forty male animals were randomly divided into 4 groups of 10 each: group 1 (control), groups 2, 3 and 4. DBP (dissolved in soyabean oil) was administered by gavage to groups 2, 3 and 4 at a dose level of 500, 1000 and 1500 mg/kg b.wt. respectively for 7 days. Group 1(control) were administered equal amount of soyabean oil. All the groups were fed on standard feed and water *ad libitum*. The doses were selected below LD<sub>50</sub> values, which ranged from 8-20g / kg b.wt. (<http://www.inchem.org/>). The experiments were approved by Departmental Animal Ethical Committee. Animals were sacrificed twenty-four hours after the last dose under light ether anesthesia. Organs viz. caput and cauda epididymides were excised, trimmed off of

extraneous tissues, weighed and stored at -70°C until analysis.

**Biochemical analysis:** The homogenate was prepared in Remi homogenizer and centrifuged at 2000 rpm (R8C Laboratory REMI Centrifuge) or 10,000 rpm (Sigma refrigerated high speed laboratory centrifuge) so as to remove the cell debris. Supernatant was used for biochemical assays. Total protein<sup>13</sup>, lipid peroxidation (TBARS)<sup>14</sup>, lipid hydroperoxides (FOX)<sup>15</sup>, total antioxidant (water and lipid soluble) capacity<sup>16</sup>, ascorbic acid<sup>17</sup> and Vitamin E<sup>18</sup> were assayed. Absorbance was measured on Carl-Zeiss spectrophotometer, Germany and GBC 911 UV-vis spectrophotometer, Australia.

**Statistical analysis:** Data were analyzed by Sigma Stat 3.5 Software (Cranes Software International Ltd, Bangalore, India) using one-way ANOVA and if the difference was significant then Duncan's post hoc test was carried out. Data were expressed as mean ± SEM. Differences were considered significant at P<0.05.

**RESULTS:** Significant change in body weight was observed when initial body weight (control and experimental groups) was compared with their final weight. (**Table 1**). Non-significant decrease in caput epididymis was recorded while a significant increase was observed in cauda (P<0.05) (**Table 1**).

**TABLE 1: BODY WEIGHT (gm) AND ORGAN WEIGHT (gm). (Mean ± SEM)**

Groups	Body weight		Organ weight	
	Initial	Final	Caput epididymides	Cauda epididymides
Control	121.52 ± 3.49	140.86 ± 4.96 A*	0.130 ± 0.009	0.100 ± 0.009
500 mg /kg b.wt	120.00 ± 4.54	138.69 ± 4.24 A*	0.125 ± 0.006	0.097 ± 0.007 B*
1000 mg /kg b.wt	120.00 ± 4.54	140.65 ± 4.37 A*	0.120 ± 0.009	0.094 ± 0.008 B*
1500 mg /kg b.wt	121.94 ± 7.13	142.00 ± 5.49 A*	0.10 ± 0.010	0.073 ± 0.005 B*

A – Initial body weight Vs Final body weight

B – Control group Vs treatment group

\* Significant P <0.05

Total protein decreased in caput and cauda epididymis. Lipid peroxidation (TBARS) and lipid hydroperoxides (FOX) increased significantly in caput and cauda epididymides. Water soluble antioxidant capacity decreased in caput while a

significant increase was observed in cauda. Lipid soluble antioxidant capacity decreased in both caput and cauda epididymis although the decrease was significant in caput epididymis after 1000 mg /kg b.wt and 1500 mg /kg b.wt. (**Table 2**).

**TABLE 2: EFFECT OF Di-n-BUTYL PHTHALATE ON CAPUT AND CAUDA EPIDIDYMIDES OF WISTAR RATS. (Mean ± SEM)**

Parameters	Organ	Control	Experimental Groups		
			500mg/kg b.wt.	1000 mg/kg b.wt.	1500 mg/kg b.wt.
Total Protein (mg /gm)	Caput	26.03± 1.49	25.08± 2.58	23.84± 0.85	21.48± 0.82
	Cauda	28.18± 0.23	20.29± 0.44*	19.12± 0.97*	18.14 ± 0.49*
Lipid Peroxidation μ mole MDA/mg protein)	Caput	0.392± 0.005	0.554± 0.008*	0.616 ± 0.018*	0.877 ± 0.005*
	Cauda	0.51 ± 0.005	0.75± 0.010*	0.838 ± 0.032*	0.897±0.017*
Lipid Hydroperoxides μ mole hydroperoxides / mg protein)	Caput	2.95 ± 0.144	3.25± 0.03*	4.05± 0.10*	4.82±0.03*
	Cauda	2.69± 0.02	3.91 ± 0.04*	4.119± 0.002*	5.35±0.067*
Total Antioxidant Capacity (a) Water Soluble μg ascorbic acid/ mg protein)	Caput	0.132± 0.005	0.123± 0.003	0.119 ± 0.002	0.109±0.003**
	Cauda	0.161± 0.007	0.204± 0.001*	0.203± 0.003*	0.215±0.002*
(b) Lipid Soluble μg α-tocopherol / mg protein)	Caput	1.59 ± 0.02	1.524± 0.01	1.501± 0.98*	1.36±0.04*
	Cauda	0.913 ± 0.04	0.855± 0.08	0.724 ± 0.072	0.668±0.014

Multiple comparison of means were performed

\* Significant P &lt;0.05

Increase in ascorbic acid level was evident while vitamin E concentration decreased significantly at

all dose levels in caput and cauda epididymis. (Table 3).

**TABLE 3: EFFECT OF Di-n-BUTYL PHTHALATE ON CAPUT AND CAUDA EPIDIDYMIDES OF WISTAR RATS. (Mean ± SEM)**

Parameters	Organ	Control	Experimental Groups		
			500mg/kg b.wt.	1000mg/kg b.wt.	1500 mg/kg b.wt
Ascorbic Acid (μg Ascorbic acid/ gm tissue)	Caput	91.27 ± 1.24	109.47 ± 2.52*	192.15 ± 0.62*	219.85 ± 4.72*
	Cauda	78.79 ± 2.61	87.34 ± 0.77	117.85 ± 4.42*	199.87± 3.93*
Vitamin E (μg α- tocopherol / gm tissue)	Caput	6601.18 ± 174.00	4638.17 ± 76.98*	2696.49 ± 84.28*	1661.79 ± 28.34*
	Cauda	6999.11 ± 180.85	5286.10 ± 48.89*	3790.76 ± 37.54*	1459.41 ± 34.64*

Multiple comparison of means were performed

\* Significant P &lt;0.05

**DISCUSSIONS:** There is no substantial human evidence for intake of DBP via oral route yet it may occur via drinking water (stored in plastic containers), food (packed in plastic bags), medical devices (plastic tubings) etc<sup>19, 20</sup>. The spermatozoa during its transit in the epididymis are at a risk of lipid peroxidation as free radicals are generated by themselves and the epididymal cells<sup>21</sup>. DBP exposed groups revealed a diminution in the total

protein in caput and cauda epididymis. Epididymal expression of proteins seems to be altered by the redox status of the cell.

High levels of unsaturated fatty acids (PUFA's), especially decosahexanoic acids which constitute 50% PUFA's (human sperm), are known to contribute to membrane fluidity and flexibility and are precursors of prostaglandin and leukotrienes-

important factors for sperm motility and inflammatory process. Decrease in caudal sperm density and viability provides some evidence that phthalate disrupts testosterone level<sup>6</sup>.

Present investigation revealed that DBP initiated lipid peroxidation promoting the formation of malondialdehyde as well as formation of lipid hydroperoxides due to generation of oxidative stress which effects not only the essential biomolecules of the cell but also causes loss of membrane integrity and change in membrane permeability which in turn would have an impact on fertilizing potential of spermatozoa. Similar findings were also reported<sup>2</sup>.

Lipid peroxidation (LPO) is a free radical mediated process that involves the formation and propagation of lipid radicals, the uptake of O<sub>2</sub> and the rearrangement of the double and unsaturated lipids resulting in a variety of degraded products that eventually causes destruction of membrane lipids<sup>22</sup>. This could probably lead to oxidation of lipids in the basal region of the epididymal cell and of PUFA's in the sperm plasma membrane. Two step percoll gradient study revealed association of sperm mid-piece abnormalities with malondialdehyde generation<sup>23</sup>. Fukuoka et al.<sup>24</sup> demonstrated increased oxidative damage of proteins, lipids and DNA after DBP exposure. Soyabean oil (a source of phytoestrogen) has been used in the present study as vehicle. The amount of oil was 0.1 ml/day and no adverse effects were observed in control animals. Thus, a synergistic effect for increased lipid peroxidation is ruled out.

Of the total antioxidant capacity, the water-soluble fraction appears to be a major contributor as compared to the lipid soluble antioxidant capacity. The study revealed decreased water and lipid-soluble antioxidant capacities although an increase was observed in cauda water soluble activity. The free radicals generated in the aqueous phase appear to be significant. Water-soluble antioxidants cannot suppress oxidation of lipids generated within the lipophilic domain. The decline in caput water soluble activity reflects it being consumed during the process. However, an increase in cauda probably accounts for the water soluble antioxidants being stimulated on account of free radical being generated which is proportional to the

increased lipid peroxidation. The lipid soluble antioxidants suppress the oxidation as it proceeds in this domain and is consumed as it scavenges the lipid peroxy. This explains the decline in caput and cauda lipid antioxidant capacity.

Ascorbic acid and Vitamin E acts as potent scavengers of free radicals in the biological system<sup>25</sup>. And plays an essential role in the maintenance of membrane integrity providing protection against oxidative damage. Although low molar ratio of Vitamin E is present in the biological membranes as against phospholipids which are prone to damage due to oxidative stress but still it acts as a first line of defense against lipid peroxidation of PUFA in the membrane system<sup>26</sup>.

Vitamin C has been shown to be more efficient than other plasma components although it acts synergistically with tocopherols at blocking the initiation of lipid peroxidation by trapping peroxy radicals in the aqueous phase before it can react with lipids in the plasma membrane<sup>27</sup> and regenerating active tocopherol<sup>28</sup>. It has been shown to react directly with superoxide<sup>29</sup>, hydroxyl radicals<sup>30</sup> and singlet oxygen<sup>31</sup>. It is generally regarded as a protective agent that nullifies free radicals by donating a single electron followed by a proton to yield a chemically reduced non-radical product and ascorbyl radicals.

The ascorbyl radical dismutates to ascorbate and dehydroascorbic acid. Glutathione may regulate Vitamin C<sup>32</sup>. Its cooperative action with Vitamin E protects against microsomal peroxidation. An imbalance between ascorbate and Vitamin E level as observed in the present study would account for increased generation of lipid peroxides and reactive oxygen species. Since DBP is lipid soluble hence would cause damage (lipid peroxidation and lipid hydroperoxides) inside the membrane systems which would lead to marked depletion in vitamin E levels. Synergisms between vitamins C and E have amply been documented<sup>33</sup> as decline in vitamin E causes ascorbate to become prooxidant.

**CONCLUSIONS:** The results from the present study strongly suggests that increased lipoperoxidation and lipid hydroperoxides concentrations in caput and cauda epididymis were indicative of disruption of membrane fluidity and

integrity which may partly be responsible for alteration in other biochemical parameters. In fact, it is an imbalance between the generation and neutralization of ROS which leads to epididymal oxidative stress which may affect fertility.

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## REFERENCES:

- Heudorf U, Mersch-Sundermann V and Angerer J: Phthalates: Toxicology and exposure. *Int J Hyg Environ Health* 2007; 210: 623-634.
- Zhou D, Wang H, and Zhang J: Di-n-butyl phthalate (DBP) exposure induces oxidative stress in epididymis of adult rats. *Toxicol Indust Health* 2011; 27: 65-71.
- Zhang YH, Zheng LX and Chen BH: Phthalate exposure and human semen quality in Shanghai: A cross-sectional study. *Biomed Environ Sci* 2006; 1: 205-209.
- Wittassek M, Heger W, Koch HM, Becker K, Angerer J and Kolossa-Gehring M: Daily intake of di(2-ethylhexyl)phthalate (DEHP) by German children—a comparison of two estimation models based on urinary DEHP metabolite levels. *Int J Hyg Environ Health* 2007; 210: 35-42.
- Zheng Q, Wei C, Wu Y, Li Ke, Ding S, Yuan J, Yang Xu and Chen M: Approach to distribution and accumulation of dibutyl phthalate in rats. *Food Chem Toxicol* 2013; 18-27.
- Nair N, Bedwal S, Kumari D, Bedwal S and Bedwal RS: Effect on histological and sperm kinetics parameters in DBP exposed Wistar rats. *J Env Biol* 2008; 29: 769-772.
- Scarano WR, Toledo FC, Guerra MT, Pinheiro PF, Domeniconi RF, Felisbino SL, Campos SG, Taboga SR and Kempinas WG: Functional and morphological reproduction aspects in male rats exposed to DBP *in utero* and during lactation. *Toxicol Environ Health* 2010; 73: 972-984.
- Hauser R, Meeker JD, Singh NP, Silva MJ, Ryan L, Duty S and Calafat AM: DNA damage in human sperm is related to urinary levels of phthalate monoester and oxidative metabolite. *Human Reprod* 2007; 22: 688-695.
- Chabory E, Damon C, Lenoir A, Kauselmann G, Kern H, Zevnik B, Garrel C, Saez F, Cadet R, Henry-Berger J, Schoor M, Gottwald U, Habenicht U, Drevet JR and Vernet P: Epididymis seleno-independent glutathione peroxidase 5 maintains sperm DNA integrity in mice. *J Clin Invest* 2009; 119: 2074-2085.
- Liu X and Li JY: Update of the studies on epididymal oxidative stress. *Zhonghua Nan Ke Xue* 2009; 15: 161-164.
- Dacheux JL, Gatti JL and Dacheux F: Contribution of epididymal secretory proteins for spermatozoa maturation. *Microscopy Res Tech* 2003; 61: 7-17.
- Aitken RJ and Koppers AJ: Apoptosis and DNA damage in human spermatozoa. *Asian J Androl* 2011; 13: 36-42.
- Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 1976; 72: 248-254.
- Okhawa H, Oishi N and Yagi K: Assay for lipid peroxides in animal tissues by thiobarbituric acid reactions. *Anal Biochem* 1979; 95: 351-358.
- Jiang ZY, James VH and Wolff SP: Ferrous ion oxidation in the presence of xylenol orange for the detection of lipid hydroperoxides in low density lipoprotein. *Anal Biochem* 1992; 202: 384 - 389.
- Prieto P, Pineda M and Aguilar M: Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. *Anal Biochem* 1999; 269: 337 - 341.
- Roe JM and Keuthers CA: Detection of ascorbic acid in whole blood and urine through the 2, 4 - DNPH derivative of dehydroascorbic acid. *J Biol Chem* 1943; 147: 399 - 407.
- Desai ID: Vitamin E methods for animal tissues. *Methods Enzymol* 1984; 105: 138-143.
- Cao XL: Phthalate esters in foods: Sources, occurrence and analytical methods. *Comp Rev Food Sci Food Saf* 2010; 9:21-43.
- Fierens T, Servaes K, Van holderbeke M, Geerts L, De Henauw S, Sioenland Vanermen G: Phthalates in food –Part 1: Analysis of food products and packaging materials sold on Belgian market. *Food Chem Toxicol* 2012; 50(7):2575-2583.
- Cacuzza M, Sikka SC, Athaydei KS and Agarwal A: Clinical relevance of oxidative stress and sperm chromatin damage in male infertility damage: An evidence based analysis. *Int Braz J Urol* 2007; 33: 603-621.
- Dattani JJ, Moid N, Highland HN, George LB and Desai RR: Ameliorative effect of curcumin on hepatotoxicity induced by chloroquine phosphate. *Environ Toxicol Pharmacol* 2010; 30: 103-109.
- Aitken RJ, Harkiss D and Buckingham DW: Analysis of lipid peroxidation mechanisms in human spermatozoa. *Mol Reprod Dev* 1993; 35(3): 302-315.
- Fukuoka M, Zhou T, Tanaka A, Ikemoto I and Machida T: Mechanism of testicular atrophy induced by di-n-butyl phthalate in rats. Part2. The effects of some testicular enzymes. *J Appl Toxicol* 1990; 10: 285-293.
- Ambali SF, Ayo JO, Ojo SA and Esievo AN: Coadministration of vitamin C and E ameliorates chronic chlorpyrifos-induced erythrocyte osmotic fragility in Wistar rats. *Aus J Basic App Sci* 2010; 4: 1015-1021.
- Singh V, Kharb S, Ghalaut PS and Gupta S: Serum vitamin E in chronic myeloid leukaemia. *J Ass Physicians* 2000; 48: 201-203.
- Craven PA, Derubertis FR, Kagan VA, Melhem M and Studer RK: Effects of supplementation with Vitamin C or E on albuminuria, glomerular TGF-  $\beta$ , and glomerular size in diabetes. *J Am Society Nephrol* 1997; 8: 1405-1414.
- Niki E: Action of ascorbic acid as a scavenger of active and stable oxygen radicals<sup>1,2</sup>. *Am J Clin Nutr* 1991; 54: 1119S- 1124S.
- Hemila H, Roberts P and Wikström M: Activated polymorphonuclear leucocytes consume vitamin C. *FEBS Lett* 1985; 178: 25-30.
- Bielski BH: Chemistry of ascorbic acid radicals. *Ascorbic acid: chemistry, metabolism, and uses. Adv Chemical Series* 1982; 200: 81-100.
- Bodannes RS and Chan PC: Ascorbic acid as a scavenger of singlet oxygen. *FEBS Lett* 1979; 105:195-196.
- Sevanian A, Davies KJ and Hochstein P: Serum urate as an antioxidant for ascorbic acid. *Am J Clin Nutr* 1991; 54: 1129S- 1134S.
- Durak D, Uzun FG, Kalender S, Ogutcu A, Uzunhisarcikli M and Kalender Y: Malathion induced oxidative stress in human erythrocytes and the protective effect of vitamins C and E *in vitro*. *Environ Toxicol* 2009; 24: 235-242.

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