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

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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.

INTRODUCTION: Phthalates, (phthalic esters or benzene dicarboxylic acid esters), are widely used in a wide variety of consumer products, formulations of pesticides, pharmaceuticals etc. 1, 2. Being non covalently bound to the polymer, they are easily released and humans are concurrently exposed through food crops, drinking water, medical devices etc. 3, 4. Zheng et al, 5 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 1 and a relationship between phthalates and testicular function 6, 7 as well as impairment of sperm quality 8 has also been reported. The epididymis plays a major role in protecting spermatozoa from ROS and secreting antioxidants into the epididymal lumen 9, 10. The spermatozoa during its maturation are under constant attack from intra- and extra-cellular ROS 11 which may result in male infertility 12. 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.
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/ Schuchardt. 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 LD50 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, lipid peroxidation (TBARS), lipid hydroperoxides (FOX), total antioxidant (water and lipid soluble) capacity, ascorbic acid and Vitamin E 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>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (gm)</th>
<th>Organ weight (gm)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td>Caput epididymides</td>
<td>Cauda epididymides</td>
</tr>
<tr>
<td>Control</td>
<td>121.52 ± 3.49</td>
<td>140.86 ± 4.96 A*</td>
<td>0.130 ± 0.009</td>
<td>0.100 ± 0.009</td>
</tr>
<tr>
<td>500 mg /kg b.wt</td>
<td>120.00 ± 4.54</td>
<td>138.69 ± 4.24 A*</td>
<td>0.125 ± 0.006</td>
<td>0.097 ± 0.007 B*</td>
</tr>
<tr>
<td>1000 mg /kg b.wt</td>
<td>120.00 ± 4.54</td>
<td>140.65 ± 4.37 A*</td>
<td>0.120 ± 0.009</td>
<td>0.094 ± 0.008 B*</td>
</tr>
<tr>
<td>1500 mg /kg b.wt</td>
<td>121.94 ± 7.13</td>
<td>142.00 ± 5.49 A*</td>
<td>0.10 ± 0.010</td>
<td>0.073 ± 0.005 B*</td>
</tr>
</tbody>
</table>

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)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Organ</th>
<th>Control</th>
<th>Experimental Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>500mg/kg b.wt.</td>
</tr>
<tr>
<td>Total Protein (mg /gm)</td>
<td>Caput</td>
<td>26.03± 1.49</td>
<td>25.08± 2.58</td>
</tr>
<tr>
<td></td>
<td>Cauda</td>
<td>28.18± 0.23</td>
<td>20.29± 0.44*</td>
</tr>
<tr>
<td>Lipid Peroxidation (µ mole MDA/mg protein)</td>
<td>Caput</td>
<td>0.392± 0.005</td>
<td>0.554± 0.008*</td>
</tr>
<tr>
<td></td>
<td>Cauda</td>
<td>0.51 ± 0.005</td>
<td>0.75± 0.010*</td>
</tr>
<tr>
<td>Lipid Hydroperoxides (µ mole hydroperoxides / mg protein)</td>
<td>Caput</td>
<td>2.95 ± 0.144</td>
<td>3.25± 0.03*</td>
</tr>
<tr>
<td></td>
<td>Cauda</td>
<td>2.69± 0.02</td>
<td>3.91 ± 0.04*</td>
</tr>
<tr>
<td>Total Antioxidant Capacity (a) Water Soluble (µg ascorbic acid/ mg protein)</td>
<td>Caput</td>
<td>0.132± 0.005</td>
<td>0.123± 0.003</td>
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<tr>
<td></td>
<td>Cauda</td>
<td>0.161± 0.007</td>
<td>0.204± 0.001*</td>
</tr>
<tr>
<td>(b) Lipid Soluble (µg α-tocopherol / mg protein)</td>
<td>Caput</td>
<td>1.59 ± 0.02</td>
<td>1.524± 0.01</td>
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<tr>
<td></td>
<td>Cauda</td>
<td>0.913± 0.04</td>
<td>0.855±0.08</td>
</tr>
</tbody>
</table>

Multiple comparison of means were performed

* Significant P <0.05

Incapable 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)

<table>
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<tr>
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<th>Organ</th>
<th>Control</th>
<th>Experimental Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>500mg/kg b.wt.</td>
</tr>
<tr>
<td>Ascorbic Acid (µg Ascorbic acid/ gm tissue)</td>
<td>Caput</td>
<td>91.27 ± 1.24</td>
<td>109.47 ± 2.52*</td>
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<tr>
<td></td>
<td>Cauda</td>
<td>78.79 ± 2.61</td>
<td>87.34 ± 0.77</td>
</tr>
<tr>
<td>Vitamin E (µg α- tocopherol / gm tissue)</td>
<td>Caput</td>
<td>6601.18 ± 174.00</td>
<td>4638.17 ± 76.98*</td>
</tr>
<tr>
<td></td>
<td>Cauda</td>
<td>6999.11 ± 180.85</td>
<td>5286.10 ± 48.89*</td>
</tr>
</tbody>
</table>

Multiple comparison of means were performed

* Significant P <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. 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. 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 6.

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 2.

Lipid peroxidation (LPO) is a free radical mediated process that involves the formation and propagation of lipid radicals, the uptake of O₂ and the rearrangement of the double and unsaturated lipids resulting in a variety of degraded products that eventually causes destruction of membrane lipids 22. 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 23. Fukuoka et al. 24 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 radicals. 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 25. 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 26.

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 27 and regenerating active tocopherol 28. It has been shown to react directly with superoxide 29, hydroxyl radicals 30 and singlet oxygen 31. 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 32. 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 apecies. 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 33 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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