Sub Acute Nephrotoxic Effect of Diethyl Phthalate on Rat

Hetal Roy*, Zarana Patel, Pooja Chauhan, Suresh Balakrishnan

Divisional of Toxicology, Zoology Department, Faculty of Science, The M. S. University of Baroda, Vadodara, India-390 002

Abstract
Diethyl phthalate has many industrial uses, as a solvent and vehicle for fragrance and cosmetic ingredients. This study was designed to examine the diethyl phthalate induced toxicity on rat kidney. SD rats were treated orally with 420 and 840 mg/Kg body weight/day for 14 days. Activities of antioxidant enzymes and histopathology of kidney were analyzed at the end of the study period. Diethyl phthalate treatment was found to induce oxidative stress in rat kidney, as evidenced by significant decreases in SOD, GPx and catalase activities and GSH level, along with marked increase in TBARS levels. The effect of DEHP was more pronounced in 840 mg/Kg body weight/day treated rats. Vacuolar degeneration and tubular destruction were the common observed histopathological change in treated kidney. Present findings emphasized the potential role of diethyl phthalate induced renal damage by depleting the oxidant stressor activity in rat.

1. Introduction
Di(2-ethylhexyl) phthalate (DEHP) has broad spectrum of uses as a pharmaceutical formulations, primarily as a plasticizer in enteric-coatings of solid oral drug products to maintain flexibility, softeners of plastics, solvents in perfumes, and additives to nail polish, as well as in lubricants and insect repellents (Edgar et al., 2001). There are several significant routes of exposure for human to phthalates, including inhalation (Fromme et al., 2002; Adibi et al., 2003), ingestion of food contaminated with phthalates or medication containing phthalates coatings (Hauser et al. 2004) and dermal absorption (Koch et al., 2002). Therefore, human is routinely exposed by DEHP. Anderson et al. (2001) observed accumulation of DEHP metabolites and its excretion through urine of human. With reference to above finding, it can hypothesize that continuous intake of DEHP may develop renal damage.

Oxidative stress is a term used to describe various deleterious processes resulting from an imbalance between the excessive formation of reactive oxygen species ROS and/or reactive nitrogen species RNS and limited antioxidant defenses. Whilst small fluctuations in the steady-state concentration of these oxidants may actually play a role in intracellular signaling (Droge, 2002), uncontrolled increases in the steady-state concentrations of these oxidants can lead to free radical mediated chain reactions which
indiscriminately target various biomolecules like proteins, DNA (Stadtman and Levine, 2000; Turrens, 2003). It results in massive cell damage inducing cellular mutations, tissue breakdown and immune compromise (Valko et al., 2007).

1.1. Objective of Research
Our aim was to understand DEHP induced sub acute nephrotoxicity in rats. The objective of this study was to evaluate DEHP induced oxidative stress in renal tissue. To observe ROS induced histopathological alteration of kidney due to DEHP oral exposure was one of the parameter of current study design.

1.2. Justification of Research
DEHP is commonly used plasticizer, even in household item and so human is routinely exposed by target test compound. After long period of time DEHP moved out from the plastic material and human is easily encounter by it. Due to such exposure of xenobiotic compound ROS level is increased which is harmful for living organisms. Oxidative stress is frequently associated with pathogenesis of renal failure and their complications. Present investigation is intended to provide an overview of oxidative stress induced histopathological alteration by the Diethyl Phthalate in rat kidney after 14 days of sub acute exposure.

2. Material and Methods

2.1. Experimental Protocol
Healthy male SD albino rats (Rattus norvegicus) (wt: 250±30 gm; age: 7–8 weeks) were grouped into four i.e. Control (CN), Vehicle control (VC) and Treated (Low dose (LD) and High dose (HD)) groups. Rats were given food and water ad libitum. Protocol was approved by Departmental ethical committee according to CPCSEA. DEHP was given orally at the dose of 420 and 840 mg/Kg body weight/ day in 5% DMSO for 14 days of duration and 5% DMSO is given to vehicle control rats. The animals were sacrificed on the 15th day after overnight fasting. Kidney was removed and washed in PBS.

2.2. Stress Marker parameters
Kidney tissue was homogenized in PBS, centrifuged and supernatant was separated. MDA was determined by the method of Buege and Aust (1978) based on the principle of thiobarbituric acid (TBA) reacts with MDA and forms red color. The activity of superoxide dismutase (SOD) was assessed by method described by Marklund and Marklund (1974). Catalase activity was by the method described by Sinha (1972). Dichromate in acetic acid is reduced to chromic acetate, when heated in presence of hydrogen peroxide with the formation of perchromic acid as an unstable intermediate. The chromic acetate formed is measured at 590nm.

The method described by Rotruck et al. (1973) was adopted for estimation of glutathione peroxidase (GPx) activity which was measured by following the increase in absorbance at 412nm using 5,5'- dithio-bis-2- nitrobenzoic acid (DTNB) as a substrate and the enzyme activity is expressed in terms of µg of GSH consumed/ mg tissue. The reduced glutathione level was determined by the method of Beutler et al. (1963). This method was based on the development of yellow colour when thiol reagent, DTNB reacts with GSH present in tissue sample forming 5-thio nitrobenzoic acid (TNB) and GS-TNB, which can be measured at 412nm using UV/VIS Perkin–Elmer spectrophotometer.

2.3. Histopathological examinations
The kidney samples were fixed in 10% buffered formalin, processed through routine histological preparation and stained by haematoxylin–eosin (H&E). Mounted slides were examined and photographed under a light microscope (Leica DM2500).

2.4. Statistical analysis
Data generated from the experiment were subjected to statistical analysis and presented as mean and standard error around mean. The statistical significance of the differences between the mean values of control and experimental groups was evaluated through one-way analysis of variance (ANOVA) followed by Bonferroni’s post-hoc test. Statistical analysis performed using GraphPad prism (version 6) software.

3. Results
The administration of toxic dose of DEHP caused a significant increase in LPO activity, as measured by the increase in TBARS level with reference to that of control group. Sub acute oral intoxication of
Table 1: Effect of DHEP on the activities of LPO, antioxidant enzyme and non enzymatic antioxidant after subacute oral exposure in rat kidney

<table>
<thead>
<tr>
<th>Group</th>
<th>LPO (nmol MDA/min/mg tissue)</th>
<th>Catalase (µmole H₂O₂ liberate/minute / mg protein)</th>
<th>SOD (%inhibition/min/mg tissue)</th>
<th>GPx (mM of GSH consumed/ mg tissue)</th>
<th>GSH (µg/ gm tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>21.9±1.2*b</td>
<td>22.5±0.8</td>
<td>15.7±0.6</td>
<td>28.6±0.7</td>
<td>4.77±0.6</td>
</tr>
<tr>
<td>VC</td>
<td>22.4±1.4</td>
<td>23.9±0.8</td>
<td>15.6±0.8</td>
<td>29.3±1.2</td>
<td>4.9±0.7</td>
</tr>
<tr>
<td>LD</td>
<td>25.6±1.9*</td>
<td>14.3±0.9**</td>
<td>14.1±0.95</td>
<td>25.9±1.3*</td>
<td>3.8±0.7</td>
</tr>
<tr>
<td>HD</td>
<td>32.5±2.6**</td>
<td>9.5±1.1****</td>
<td>13.7±1.2*</td>
<td>23.8±1.6**</td>
<td>3.1±0.9**</td>
</tr>
</tbody>
</table>

*bValues are expressed as Mean±SE; n=5 for each group; * p ≤ 0.05; ** p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001

DEHP significantly increased the formation of TBARS in low dose group (P≤0.05) and high dose group (P≤0.001) of rats (Table 1). Increase level of TBARS in treated kidney indicating a marked production of oxidative stress.

Activity of antioxidant enzyme was measured which is the important parameter to analyzed the status of reactive oxygen species. Table 1 illustrates that the administration of DEHP depleted the activity of SOD, catalase, GPx and GSH in kidney homogenate of rat. The level of SOD activity was lowered down significantly in high dose (p≤0.05) and also observed decreased in low dose but not significant, compare to that of reference group. There was no observed change in vehicle control rats as compare to reference group. Activity of catalase also decreased at the significant level of p≤0.0001 (57.7% less than the control group) in 840mg/Kg body weight dose and p≤0.001 (36.45% less than the control group) in 420mg/Kg body weight dose group of rats.

GSH Level was decreased significantly as compare to that of untreated rats. Value was found highly significant in both the treatment groups. Increased activity of GSH was assessed in LD group (18.42% higher) compare to that of HD group of animal. Significantly depleted level of GSH was found in high dose treatment of DEHP in comparison with control as well as vehicle control. Observed LD values of GSH was lowered but not found statistically significant in comparison with untreated rats. GPx activity was also observed low in treatment group. Value of HD observed significantly higher (P≤0.001) as compare to experiment control rats.

Microscopically, examined section of untreated kidney tissue was observed with normal histoarchitectural structure. Kidney of control rat show intact Bowman’s capsule, distal tubules with intact cuboidal cell and proximal tubules with

![Figure 1](image1.png)

Figure 1: (A) Control rat kidney with renal corpuscle (20X), (B) Renal tubules with cuboidal epithelium and intact glomerulus (40X). G- Glomerulus, RT- Renal tubule

![Figure 2](image2.png)

Figure 2: (A) Kidney of DEHP treated rat with glomeruli degeneration. (B) Renal tubular necrosis with colloid filled cavity (GN- Glomerular necrosis)

showed well vascularize glomeruli with intact podocyte. In DEHP treated group of rat, degenerative lesion was observed in kidney. Exposure of DEHP resulted in glomeruli degeneration. Experimental treated kidney section show tubular epithelial vacuolization and renal tubular necrosis with colloid filled cavity (Figure 2A & 2B).
Figure 3: (A) DEHP treated kidney showing vacuolar degeneration of renal tubule (Green arrow), (B) Focal glomerulonephritis (*) and pyknotic nuclei of renal tubules (Green arrow).

Figure 4: Spongiform renal tubular necrosis (A–10X, B–40X)

Treated kidney showed increased vacuolar degeneration of renal tubule, Focal glomerulonephritis and pyknotic nuclei of renal tubules (Figure 3A & 3B). Spongiform of renal necrosis was prominent feature of HD group of kidney after 14 days of oral treatment (Figure 4A & 4B).

4. Discussion

Diethyl phthalate induced oxidative Stress is caused by an imbalance of Pro-oxidant and antioxidants in the system, Which raises the physiological level of ROS, including free Oxygen species and Peroxides and leads to oxidative DNA, Lipid and Protein damage (Valko et al., 2007). Antioxidant enzymes such as SOD, GPX, LPO and CAT compose the most important intracellular antioxidant defense system to prevent cellular damage caused oxidative stress. Any disruption of this defense system will cause accumulation of ROS and lead to oxidative damage (Agarwal et al., 2008). In the present study; it was found that DEHP induces oxidative stress by increasing thiobarbituric acid reactive substances (TBARS) in rat kidney homogenates. Lipid peroxidation is a major harmful consequence of reactive oxygen species (ROS) formation (Seo et al., 2004). Increased lipid peroxidation could lead to severe cell organelle damage leading to impairment in the various metabolic functions of the cell (Pereira and Rao 2006). The higher level of TBARS in SD rats and marine culture fish was observed by Pereira and Rao (2006) and Kang et al. (2010) respectively. Determination of malondialdehyde (MDA) by TBARS is used as an index of the extent of lipid peroxidation. In the present study, elevated MDA level is due to the oxidative damage of protein by elevated oxygen free radicals (Schafer and Buettner 2001). SOD activity in treated rats was observed decreased in present study. Result is coincides with many researchers finding (Erkekoglu et al., 2010; Huang et al., 2015; Mohammad et al., 2015).

Decreased level of catalase activity in the treated group of DEHP was observed, as compared to control. The decrease in catalase activity was reported in other studies (Emiko et al., 2002; Umamaheswari and Senthilnathan, 2014; Zhang, 2015). Catalase deficiency makes kidney more susceptible to oxidant tissue injury and renal fibrosis in mice (Mizuho et al., 2001). Reduced GSH level was observed in experimental treatment group. The decreased level of GSH was reported by other findings due to Diethyl Phthalate intoxication (Emiko et al., 2002; Kang et al., 2010). Significant decline in renal GPx activity was observed in this study coincides with Umamaheswari and Senthilnathan (2014) who have noticed decreased GPx activity in Tilapia fish.

In the present study, it was observed that administration of DEHP result in marked nephrotoxicity. Marked effect was observed due to continuous 14 days of exposure. The changes in kidney parenchyma involved both glomerular and tubular elements. Focal necrosis of renal tubules in the form of vacuolation was observed. Treated dose produced widespread massive damage in renal tubules and glomerular atrophy. Elif (2006) reported tubular damage and tubular obstruction due to DEHP exposure which also affect glomerular filtration rate. In the study of Ikekale et al. (2011), the exposure of fish to DEHP increased vacuolization in the renal tissue and degeneration of glomeruli.
Conclusion

In this study we have assayed the alteration in the stress marker enzyme activities of the kidney of rat, after exposure to two concentration of DEHP. The observation registered in this experiment, reveal significant decline in enzyme activity of SOD, CAT, GSH, GPx, in DEHP treated renal tissues. Resultantly, DEHP is caused histo-architectural changes in kidney and leads histophalogical lesion.

Research Highlights

Diethyl Phthalate oral exposure is potent to generate reactive oxygen species in kidney tissue after 14 days of treatment. Elevated level of ROS disturbs cell antioxidant system and increased LPO activity in treatment group of DEHP. Increased MDA level resulted into histopathological alteration of Kidney tissue due to DEHP toxicity.

Limitation

Present study is only for 14 days of DEHP oral exposure; one can precede this study for sub chronic or more which will elaborate better hazards effect on human health. After sub acute study, it is clear that DEHP has potential to develop nephrotoxicity. Biochemical parameter in like, Creatinine level, Creatine kinase, NAG activity and urine analysis study with correlation of present data will advocate better on nephrotoxic potential of diethyl phthalate.

References


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