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HEPATOTOXICITY STUDY OF ACUTE AND CHRONIC EXPOSURE TO DI(2-ETHYHEXYL) PHTHALATE (DEHP) IN ZEBRAFISH, *DANIO RERIO*

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ABSTRACT

Di(2-ethyhexyl) phthalate (DEHP) is the most widely known phthalate and worldwide the production and application of it is increasing over the last few years. In the present study, the impact of DEHP for acute (96 hours) and chronic (21 days) exposure to concentrations of 80, 140 and 200 µg/l on liver of zebrafish, *Danio rerio*. Hepatotoxicity studies involved evaluation of liver function enzymes such as acid phosphatase (ACP), alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate transaminase (AST) as well as the antioxidant enzyme activities namely superoxide dismutase (SOD), catalase (CAT), glutathione-s-transferase (GST), reduced glutathione (GSH), and peroxidase (POD). Abnormal levels of liver function enzymes and activity of oxidative stress enzymes were reported for both the durations of DEHP treatment. Histopathological observations included loss of hepatic cytoarchitecture, wider sinusoid spaces, hepatocytes with pyknotic nuclei, steatosis, ballooning of hepatocytes, cytoplasmic vacuolization and necrosis.

KEY WORDS : DEHP, Zebrafish, Liver function enzymes, Oxidative stress, Histopathology.

INTRODUCTION

Plasticizers are used as additives to increase the processability and give flexibility to polymers (Jamarani *et al*., 2018) and are also added to the products in cosmetic and personal care industry to enhance the staying capacity of scented products like lotions, body wash and shampoos, increase clinging properties of hair styling products, nail polish and to promote skin penetration (Walsh, 2019). Phthalates are the most commonly used plasticizers and are the active components of many food and personal care products (Koo and Lee, 2004; Frederiksen *et al*., 2007). Among other common phthalates, DEHP is the most widely known phthalate having its production and application increasing worldwide over the last few years (Li *et al*., 2022). DEHP fails to tightly bind to the polymers of plastic and hence can easily enter into the environment at the time of production, transport, storage, use and disposal. Sewage sludge and solid waste disposal sites are the primary sources of release of this phthalate into the air and water bodies (Li *et al*., 2018). It is universally considered to be an omnipresent environmental contaminant because of its wide range of applications (Koo and Lee, 2004). It has been found in many aquatic environments, including surface water, fresh water and marine ecosystems, as well as in tap water (Kim *et al*., 2002; Zhang *et al*., 2020; Hu *et al*., 2020; Zhu *et al*., 2022; Elaiyaraja *et al*., 2022).

Zebrafish, *Danio rerio*, was chosen for the present experimental research because of its numerous advantages such as small size, short life cycle, cheaper cost to maintain than rats and mice as well as easy maintenance and 70% similarity in genetic structure with humans. Zebrafish has been commonly used for toxicity studies of chemicals, pollutants and drugs in the aquatic environment (OECD, 1992; McGrath and Li, 2008; Song *et al*., 2022).

The study was carried out for acute as well as

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chronic exposure to varying concentrations of DEHP on liver biochemistry and histopathology as liver serves as a vital organ for detoxification and hence is the main target organ for many toxicants. The biochemical investigation of the activity of liver function enzymes namely ACP, ALP, ALT and AST were carried out along with antioxidant enzymes such as SOD, CAT, GST, GSH, and POD were investigated to evaluate the oxidative stress damage. Liver histopathological changes were also studied to corelate the biochemical changes with the corresponding impact on its cytoarchitecture. This study could be helpful in providing data for the ecotoxicity of DEHP to the aquatic life and its potential harm due to its increasing pollution.

MATERIALS AND METHODS

Test solution

Di(2-ethylhexyl) phthalate (DEHP) was purchased form Sigma-Aldrich, USA, Product No. 47994. The exposure concentrations of DEHP prepared were of 80, 140 and 200 µg/l.

Model animal maintenance

Zebrafish were maintained in the CCSEA registered zebrafish maintenance and breeding facility (Registration No.1936/PO/Re/S/17/CPCSEA). They were kept in 12l capacity glass tanks containing system water and acclimatized for two weeks under standard laboratory conditions, i.e. 28°C ± 1°C and photoperiod of 14:10 hours light:dark. The fish were fed twice a day with readymade dry fish food and once a day with Artemia.

Acute and Chronic toxicity exposure

One control and three experimental groups namely I, II and III each having 15 fish were maintained in separate glass tanks for the period of 96 hours exposed to DEHP of concentration 80, 140 and 200 µg/l for acute toxicity testing. Corresponding control and experimental groups were maintained for 21 days for chronic toxicity exposure to the same concentrations of DEHP.

Sample preparation for biochemical assay

Zebrafish were euthanised in ice cold water at the end of 96 hours and 21 days. Liver tissues were collected from 13 fish of each group. The pooled liver tissue was homogenized in phosphate buffer and stored at -20 ºC for determination of liver function and oxidative stress enzymes. Biochemical assays for Liver function enzymes - ALP (Kind and King, 1954), ACP (King and Armstrong, 1934), AST and ALT (Reitman and Frankel, 1957) using Erba Mannheim kit; oxidative stress enzymes - SOD (Misra and Fridovich, 1972), CAT (Aebi, 1974), GST (Habig *et al.*, 1973), GSH (Sigma-Aldrich Chemicals, USA, Catalogue Number MAK364), POD (Senthilkumar *et al.*, 2021) were performed. Spectrophotometric quantitative analysis was done using Double Beam UV-VIS Spectrophotometer LMSPUV-1200.

Statistical analysis

Results were expressed as Mean ± SD. Statistical analysis was done using One-way ANOVA followed by Bonferroni test for post hoc analysis. Differences at $P < 0.05$ were considered significant.

Histopathological studies of liver

Two fish from each of the euthanized groups of chronic duration were fixed in 10% neutral buffered formalin after cutting the head and tail region. The abdominal region from the fixed tissues were dehydrated and embedded in the paraffin wax and transverse sections of 7 µ thickness were taken using microtome (Panchal Scientific, India). The sections were stained with haematoxylin and eosin and examined under Olympus CX21i Trinocular Microscope with Magnus 5MP-HD series camera for digital imaging.

RESULTS AND DISCUSSION

Oxidative stress enzymes

In the present study both SOD and CAT activity were found to be increased after 96 hours and decreased after 21 days of DEHP exposure in all the treatment groups (Table 1). Similar results were found in zebrafish exposed to azoxystrobin where first SOD activity was increased and then decreased after prolong exposure (Han *et al.*, 2016). Li *et al.* (2022), found initial increase followed by decrease in CAT after 0.03, 0.1, and 0.3 mg/l of DEHP exposure in zebrafish. An imbalance between production and accumulation of ROS and the ability of a biological system to detoxify these ROS, leads to cell and tissue damage, known as oxidative stress (Pizzino *et al.*, 2017). After exposure to contaminants from the environment, ROS content is generally considered as a precursor biomarker that evaluates cell damage in the body (Fuzinatto *et al.*, 2015, Li *et al.*, 2022). The

failure of antioxidant defences may lead to significant oxidative damage including enzyme inactivation, protein degradation, DNA damage and lipid peroxidation (Liao *et al*., 2017). Cells make use of an antioxidant defence system based majorly on enzymes, such as SOD, CAT to protect them from cellular damage induced by ROS (Deponte, 2013). Fish can neutralize the harmful effects of ROS with the help of well-developed antioxidant defence system present in them (Zhang *et al*., 2004). Hence this sensitive antioxidant defences of fish to any ecological contamination can be used as biomarkers of aquatic ecological health (Sturve *et al*., 2008). Thus, the decreased SOD and CAT activities after chronic exposure to DEHP may indicate the failure of SOD and CAT to scavenge the free radicles formed due to prolonged exposure.

GST activity showed a gradual increase after DEHP exposure in both, acute and chronic toxicity (Table 1). The increase of GST activity in liver of treated zebrafish could be an indication of response to eliminate the excessive ROS and protect the cells from damage as also reported by Song *et al*. (2019) where an increase in GST activity after exposure to dimethyl phthalate and benzyl butyl phthalate for 7 and 28 days in earthworms (*Eisenia fetida*).

The activity of POD in the liver of zebrafish decreased after both the durations of DEHP exposure (Table 1). The decrease in glutathione peroxidase activity was found in Rohu (*Labeo rohita*) exposed to diethyl phthalate **(**DEP) (Latif *et al*., 2020). The reduction in POD levels in both acute and chronic studies could be due to its exhaustion as a result of increased production of free radicles and by the accumulation of H_2O_2 products.

GSH was found to be increased after 96 hours and decreased after 21 days of DEHP treatment (Table 1). Zebrafish exposed to chlorpyrifos (10, 30, 100, 300 mg/l) showed reduction in the GSH (Jin *et al*., 2015). The reduction in GSH after 21 days could be because of increased utilization of GSH to tackle with the increasing oxidative stress in the zebrafish due to prolonged DEHP exposure.

Liver function enzymes

The present study showed a decrease in ACP and ALP enzyme activity in zebrafish exposed to all concentrations of DEHP for both durations (Table 2). Similar results were reported by Suneetha and Veerajah (2022) in freshwater fish, *Labeo rohita* exposed to endosulfan and fenvalerate. The low enzyme activity of both these phosphatases indicates tissue damage due to DEHP exposure such as hepatic parenchymal damage and hepatocytic necrosis as observed in the histological sections (Figure 1B-D). Elevated levels were found of both aminotransferases after DEHP exposure for both durations (Table 2). Aminotransferase levels were reported to be increased after DEHP and other phthalates exposure in laboratory rats (Shi *et al*., 2020). As both AST and ALT are sensitive indicators of liver cell damage, their increased levels may indicate damage to the liver due to the accumulation and toxicity of DEHP in zebrafish.

Histopathology of liver

Zebrafish liver from control group showed normal histological structure having central vein, bile duct lined by single layer of cuboidal epithelium, cords of polygonal hepatocytes with central nucleus,

Table 1. Oxidative stress enzyme assays for acute (96 hours) and chronic exposure (21 days) of DEHP concentrations of 80, 140 and 200 µg/l as compared to the respective control groups (Activity of SOD, CAT, GST and POD expressed in µmol/min/mg of protein; activity of GSH in Nmol/mg of protein)

Enzyme	Acute exposure concentration of DEHP				Chronic exposure concentration of DEHP			
Assay	Control	80 µg/l	140 µg/l	200 µg/l	Control	80 µg/l	140 µg/l	200 µg/l
SOD	20.66 ± 0.27	23.34 ± 0.94	28.16 ± 6.58	33.16 ± 7.44*	23.44 ± 4.84	11.94 ± 0.36*	12.55 ± 0.43*	13.41 ± 0.72*
CAT	21.92 ± 0.65	24.52 ± 0.86*	25.82 ± 0.36*	29.17 ± 1.41*	20.67 ± 1.70	17.73 ± 2.89	14.38 ± 2.32*	11.43 ± 2.62*
GST	127.67 ± 2.34	131.11 ± 1.87	139.82 ± 1.35*	148.88 ± 1.52*	108.34 ± 3.55	115.95 ± 5.15	121.92 ± 4.36*	132.42 ± 3.73*
POD	309.51 ± 2.69	308.1 ± 1.95	305.69 ± 1.80	299.62 ± 4.18*	318.34 ± 3.72	295.14 ± 10.53*	275.71 ± 10.10*	255.23 ± 6.60*
GSH	30.42 ± 1.59	40.65 ± 1.85*	42.03 ± 1.91*	48.88 ± 2.09*	38.91 ± 2.33	32.28 ± 2.56	27.54 ± 3.22	22.38 ± 6.04*

Note: Values are expressed as mean ± SD; values significant at $P < 0.05$ are marked *

Table 2. Liver function enzyme assays for acute (96 hours) and chronic exposure (21 days) of DEHP concentrations of 80, 140 and 200 µg/l as compared to the respective control groups (ACP, ALP, ALT and AST expressed in IU/l)

Enzyme Assay	Acute exposure concentration of DEHP				Chronic exposure concentration of DEHP			
	Control	80 µg/l	140 µg/l	200 µg/l	Control	80 µg/l	140 µg/l	200 µg/l
ACP	10.73 ± 1.64	9.25 ± 1.69	8.46 ± 1.52	7.12 ± 2.16	10.86 ± 1.45	8.4 ± 1.51	6.52 ± 1.11*	4.1 ± 1.17*
ALP	10.84 ± 1.43	8.28 ± 2.20	6.39 ± 1.55*	4.89 ± 1.51*	13.49 ± 2.64	6.68 ± 1.71*	3.49 ± 0.89*	1.89 ± 0.54*
ALT	376.87 ± 15.99	390.62 ± 17.36	405 ± 24.49	416.87 ± 26.72	362.5 ± 21.01	395 ± 19.57	410 ± 15.81*	426.25 ± 20.15*
AST	375.06 ± 14.50	386.46 ± 10.93	394.15 ± 14.00	410.68 ± 15.19*	343.42 ± 9.73	384.01 ± 21.43	394.89 ± 29.88	411.82 ± 37.91*

Note: Values are expressed as mean ± SD; values significant at $P < 0.05$ are marked *

surrounding sinusoids (Figure 1A). DEHP treated fish exhibited alterations in liver tissues like loss of hepatic cytoarchitecture, wider sinusoid spaces, pyknotic nuclei, steatosis, ballooning of hepatocytes, cytoplasmic vacuolization and necrosis in a concentration dependent manner (Figure 1B - D). This is similar to the results of Bisai *et al*. (2022) for the study of common carp exposed to different concentrations of DEHP (10, 100 and 1000 µg/l) for 30 days. *Clarias gariepinus* exposed to DEP (50, 75, 100 and 150 µg/l) for 30 days showed hepatocytes degeneration, vacuolation and necrosis (Obiezue *et al*., 2014). Exposure of bisphenol-A led to change in the normal architecture of liver, increase lipid-like vacuolization and inflammation in sinusoids in *Ctenopharyn godonidella* (Faheem and Lone, 2018). The altered histopathology of liver demonstrates the severe adverse effects to exposure of DEHP.

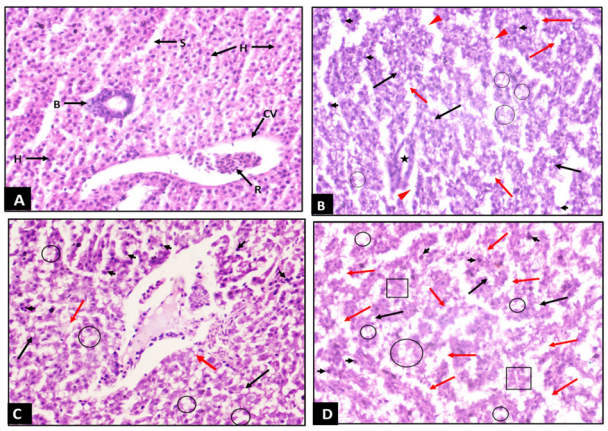

Fig. 1. Photomicrograph of T.S. of liver of zebrafish: **A** - Control group showing normal histology with cords of hepatocytes surrounding sinusoids. H- Hepatocytes with prominent nuclei, S- Sinusoids, B- Bile duct, CV- Central Vein, R- Blood cells. **B** - Experimental group of 80 µg/l of DEHP. **C** - Experimental group of 140 µg/l of DEHP. **D** - Experimental group of 200 µg/l of DEHP. Circular outline - fatty liver with areas of steatosis, Red arrows - ballooning of hepatocytes, Red triangle – wider sinusoids, Black arrow - Vacuolization, Square outline – necrosis, Black arrow head- pyknotic nuclei, Star- thickened bile duct.

CONCLUSION

This study investigated the toxic effects of DEHP on the liver of zebrafish at biochemical and histological levels. From the results of the acute and chronic toxicity studies it could be concluded that DEHP causes some major toxicity to the liver. This study provided a significant understanding of the ecotoxicity of acute and chronic exposure of DEHP in zebrafish, thereby highlighting the risk of DEHP pollution to aquatic ecosystem and eventually to human beings through the food chain. Hence, steps towards the elimination of such harmful chemicals warrant immediate and serious attention through strict regulatory measures.

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