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Effect of Di-(2-Ethylhexyl) Phthalate on Certain Haematological and Histological Parameters of Common Carp *(Cyprinus carpio)*

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Abstract

Di-(2-Ethyl-Hexyl) Phthalate (DEHP) is a plasticizer commonly found in aquatic environment due to wide use of plastics for various purposes. In this study, common carp (*Cyprinus carpio*) (length 14.2 ± 0.82 cm and weight 32.8 ± 0.66 g) were exposed to DEHP at three different concentration levels i.e., 10, 100 and 1000 µg l^{-1} water for 30 d to find out its effect on certain haematological and histological parameters. Haematological studies revealed significant ($p<0.05$) decrease in erythrocyte counts, while leukocyte counts were significantly increased in treated group with increasing concentration of DEHP. Further, treatment of DEHP in concentration dependent manner showed deformities in erythrocytes whereas leukocytes were unaffected. The total plasma protein level was significantly ($p<0.05$) decreased in lower to higher concentration of the exposure. Histological studies showed gill and liver tissues were affected with impaired histoarchitecture in a dose dependent manner.

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1. Introduction

Di-(2-Ethyl-Hexyl) Phthalate (DEHP), a plasticizer commonly used in the formulations of polyvinylchloride and other kind of plastics. Approximately 8.4 million tonnes of plasticizers are produced annually in the world of which DEHP is the most widely used phthalate compound and produced at more than 2 million tonnes[1]. Over the years, concerns have increased due to DEHP mediated toxicity on different animals. This compound is not chemically bound to plastics and frequently leaches out into the surrounding environment[2-3] and therefore responsible for contaminating those which are in close contact[4]. It can be absorbed with biological fluids and redistributed into different host tissues increasing the risk of health hazards like developmental, reproductive and respiratory anomalies[5]. It is absorbed from the lungs, gut and through the skin[6] and its toxic effect on reproductive and hepatic cells is well known in several animals[7-8].

DEHP exerts adverse effects on liver such as increased liver weight, elevated liver enzyme levels and histological changes in higher vertebrates[9-11]. Studies have shown that phthalate compounds have endocrine disrupting properties and have adverse reproductive effects in mammals. It effects on male reproductive health such as reduced testosterone level and reduced sperm count[12], anti-androgenic and estrogenic effects in mammalian male including human[13-14]. The effect of DEHP is well studied in various aquatic animals. Literatures indicate that short-term and long-term exposure of phthalate including DEHP had pronounced effect on various haematological and histological parameters of fish like *Pseudobagrus fulvidraco*[15], *Oreochromis mossambicus*[16] and *Clarias gariepinus*[17-18]. Further, marked histopathological changes in various tissues including gill and liver due to phthalate-based compounds were recorded in different fish species[19-21].

In India, information of DEHP toxicity in different fish species is meagre. Some reports exist on the contamination of water bodies with this compound. Earlier, Selvaraj *et al.*[22] found 514 ng l^{-1} DEHP in the water samples of Kaveri River, India whereas in river sediments it was 2 to 1438 ng g^{-1} dry weight. However, Srivastava *et al.*[23] reported 31.61 ng g^{-1} DEHP in sediments of Gomti river, India, which were well above the USEPA sediment guideline value. No information is available on toxicity of DEHP in any of the cultured fish species in India till date except Revathy and Chitra[21], who had studied histopathological changes in *O. mossambicus* after acute exposure of DEHP. However, *Cyprinus carpio* is one of the major cultured species throughout the world and is popular in Asian countries including India[24]. Looking into the DEHP contamination to be very common in aquatic environments due to widespread applications of plastics, this study was carried out to find out the effects of different concentrations of DEHP on certain haematological parameters and histoarchitecture of gill and liver tissue of common carp, *C. carpio*.

2. Materials and Methods

2.1 Experimental Fish

For this experiment, the juvenile of common carps (*C. carpio*), average length of 14.2 ± 0.82 cm and average weight of 32.8 ± 0.66 g were collected from local fish dealer and acclimatized in 3000 l capacity of cemented cistern at the outdoor facility of Department of Fishery Sciences, Vidyasagar University, Midnapore, West Bengal, India for 15 d. After acclimatization, they were segregated into various groups and the experiment was conducted under 12 h light and 12 h dark photoperiod with aerated condition.

2.2 Chemicals Used

A technical grade of DEHP (purity 99%) used in all experiments was obtained from SRL Laboratory Ltd. (Mumbai, India). Stock solutions were made by dissolving DEHP in acetone (100 g l^{-1}) and kept at 4 °C until further use. Serial dilutions were made as per requirement from the stock solution to obtain the exposure concentrations. All other chemicals used in this study were analytical grade.

2.3 Experimental Design

The experimental fishes were divided in four different groups to study the effect of DEHP on common carps. 12 glass tanks of 80 l capacity were used (3 per each group) and in each tank 10 numbers of fish were kept. Fishes of different experimental group were exposed to acetone dissolved DEHP supplemented through water at

three different concentrations. The three different DEHP treated groups were lower concentration of 10 µg l^{-1} water, medium concentration of 100 µg l^{-1} water and a higher concentration of 1000 µg l^{-1} water. The control group was treated with 0.001% acetone alone. The experiment was conducted for 30 d and during this period, commercial carp feed (CP Aquaculture Pvt. Ltd., India) was fed twice daily at the rate of 5% body weight. Complete water replenishment was done from each experimental tank at a week interval and different doses of DEHP were made from stock solution and exposed to fishes. During the experimental trial, water quality parameters were measured as per the methods given in APHA[25]. The water temperature was 32 °C to 34 °C, while the pH, dissolved oxygen and the alkalinity of the water were 7.2 ± 0.2, 5.8 ± 0.4 mg l^{-1} and 82 ± 1.2 mg l^{-1}, respectively. During the study period, behavioural changes and mortality if any were observed in the experimental groups.

2.4 Haematological Study

Blood samples were collected from the caudal peduncle of the fish of all groups after 30 d of experimental period. Blood smear was made on clean grease free glass slide and stained with Gimesa stain to observe the toxic effect of DEHP treatment on the morphological features of blood cells under microscope (Carl Zeiss, Axio-Cam ERc5s).

Similarly, the total erythrocyte counts and total leukocyte counts were done by using Neubauer haemocytometer[26]. The total plasma protein level of fishes from control and DEHP exposed group were estimated as per Lowry method[27].

2.5 Histological Study

After the exposure period, fishes from all groups were sacrificed under tricaine methanesulfonate (MS-222) anaesthesia followed by a big blow on head. Tissues samples like gill and liver were collected for histological studies. Tissues were first fixed in Bouin's fluid for 24 h and then processed with paraffin blocks and sectioned at 5 µm. The sections were stained by hematoxylin and eosin stain (H & E stain) and examined under microscope (Carl Zeiss, Axio-Cam ERc5s).

2.6 Statistical Analysis

All the data are presented as mean ± Standard Error of the mean (SE). The differences between control and different exposure groups for erythrocyte counts, leukocyte counts and plasma protein levels were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test performed with SPSS statistical software package (version 23.0). The significant level (p) was set at 0.05.

3. Results

3.1 Mortality and Behavioural Changes

During the experimental period, no mortality was recorded in any of the experimental group. Fish in control group were active with well synchronized movements. They mostly settled to the base of the experimental tanks, sometimes came to the surface of the water and actively responded to the slight disturbance. However, the DEHP treated fishes showed erratic swimming, loss of equilibrium, restlessness, lack of feeding and gulping at surface etc.

3.2 Haematological Changes

Haematological studies indicated marked effect of DEHP on the blood parameters of common carps in a dose dependent manner. Morphologically, the erythrocytes were found to be oval shaped with prominent cell membranes and nucleus in control group. But shape of erythrocytes in the DEHP treated fishes turned into triangular and more deformed erythrocytes were observed with increasing concentration of DEHP (Figure 1). On the contrary, no morphological deformities or abnormalities could be demonstrated in the leukocytes even at higher concentration of DEHP. The study on the blood cells counts of the exposed fishes showed that the total erythrocyte counts were significantly ($p<0.05$) decreased (Figure 2). The erythrocyte count was least ($1.38 \pm 0.01 \times 10^6$ µl^{-1}) in 1000 µg l^{-1} DEHP treated group, while it was $2.26 \pm 0.02 \times 10^6$ µl^{-1} in control group. On the contrary, total leukocyte counts were significantly ($p<0.05$) increased with increasing concentration of DEHP compared to control group (Figure 3). In control group, the total leukocyte count was $22.4 \pm 0.6 \times 10^3$ µl^{-1},

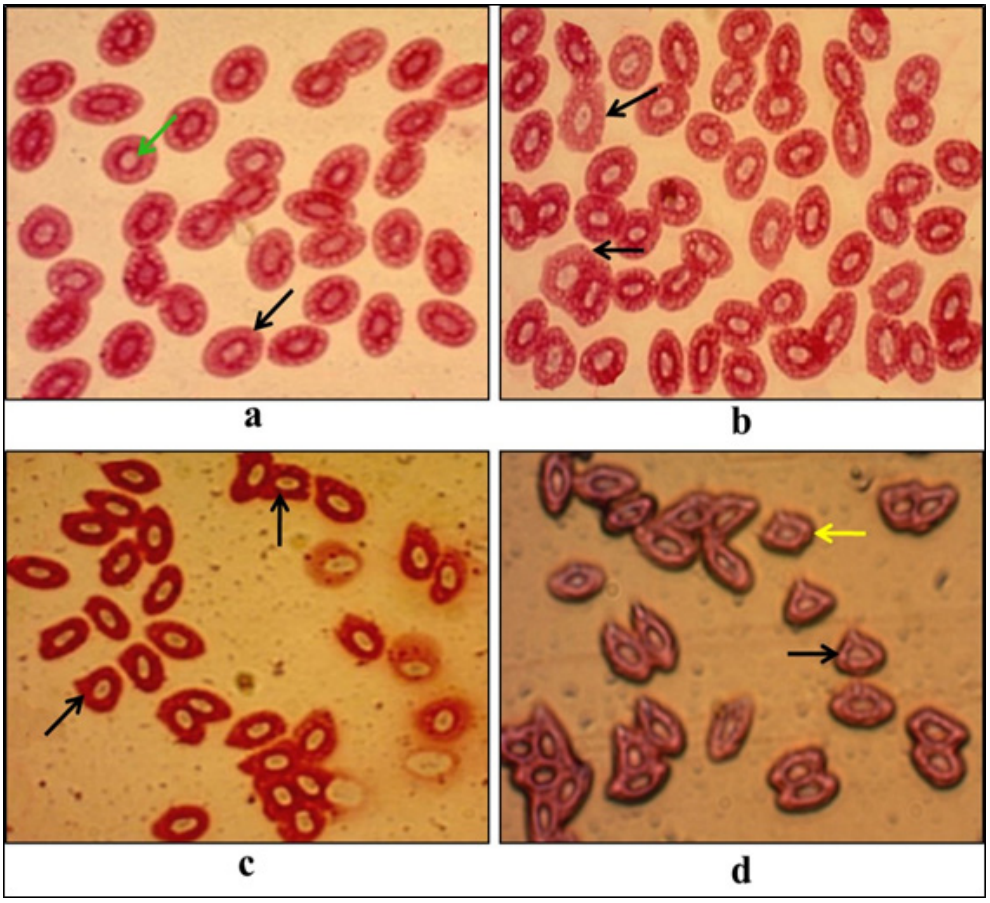


Figure 1. Photomicrograph of erythrocytes of *C. carpio* (Gimesa stain, 1000X). **1a** (control group) oval shaped erythrocytes with prominent cell membranes (black arrow) and nucleus (green arrow), **1b** (10 µg l^{-1} DEHP treated group) beginning of deformation in erythrocytes (black arrow), **1c** (100 µg l^{-1} DEHP treated group) irregular shaped erythrocytes (black arrow), and **1d** (1000 µg l^{-1} DEHP treated group) triangular (black arrow) and highly deformed (yellow arrow) erythrocytes.

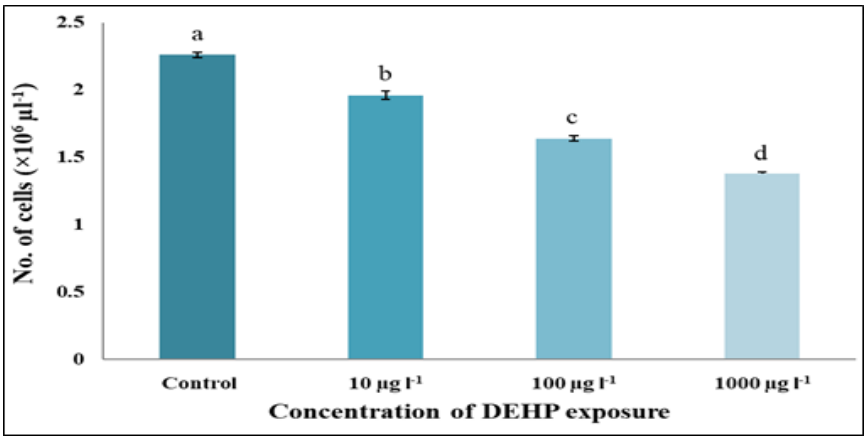


Figure 2. Erythrocyte counts (×10^6 μl^{-1}) of control and different DEHP treated group. All data are expressed as mean ± SE (n = 6) of 3 replicate tanks and values with different superscripts are significantly different at 0.05 level.

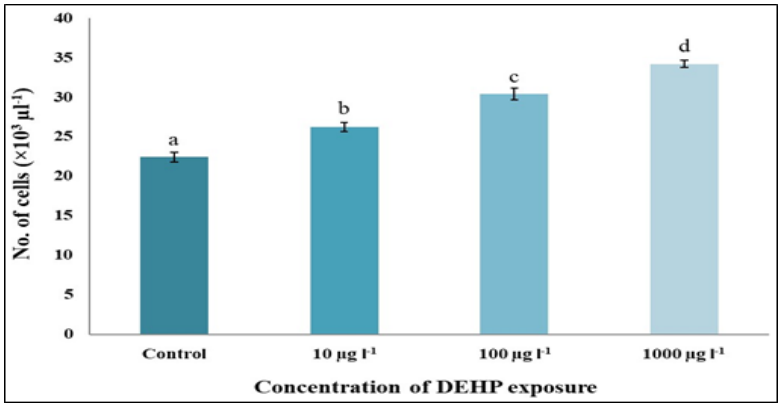


Figure 3. Total leukocyte counts ($\times 10^3$ μl^{-1}) of control and different DEHP treated group. All data are expressed as mean ± SE (n = 6) of 3 replicate tanks and values with different superscripts are significantly different at 0.05 level.

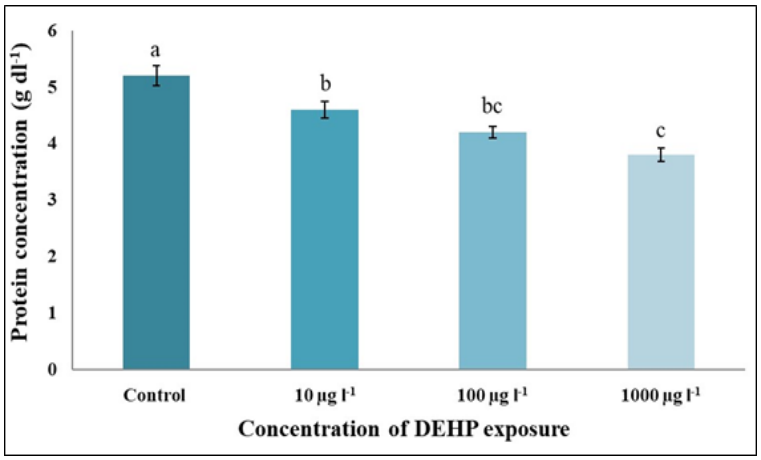


Figure 4. Total plasma protein concentration levels (g dl^{-1}) of control and different DEHP treated group. All data are expressed as mean ± SE (n = 6) of 3 replicate tanks and values with different superscripts are significantly different at 0.05 level.

while in 1000 µg l^{-1} treated group the value was found to be 34.2 ± 0.48 × 10^3 μl^{-1}. Similarly, the total plasma protein concentration levels were also significantly ($p<0.05$) decreased with increasing concentration of DEHP as compared to control group (Figure 4). The plasma protein content in the control group was 5.2 ± 0.18 g dl^{-1} however decreased protein content (3.8 ± 0.12 g dl^{-1}) was recorded in 1000 µg l^{-1} DEHP treated group.

3.3 Histological Changes

The gill tissue of control fish showed normal architecture of gill filament, primary and secondary gill lamellae (Figure 5a). However, gill tissues in each group of DEHP treated fishes showed marked changes in a concentration dependent manner. In the lower concentration of DEHP (10 µg l^{-1}) treated fish, damaged gill epithelium, fusion

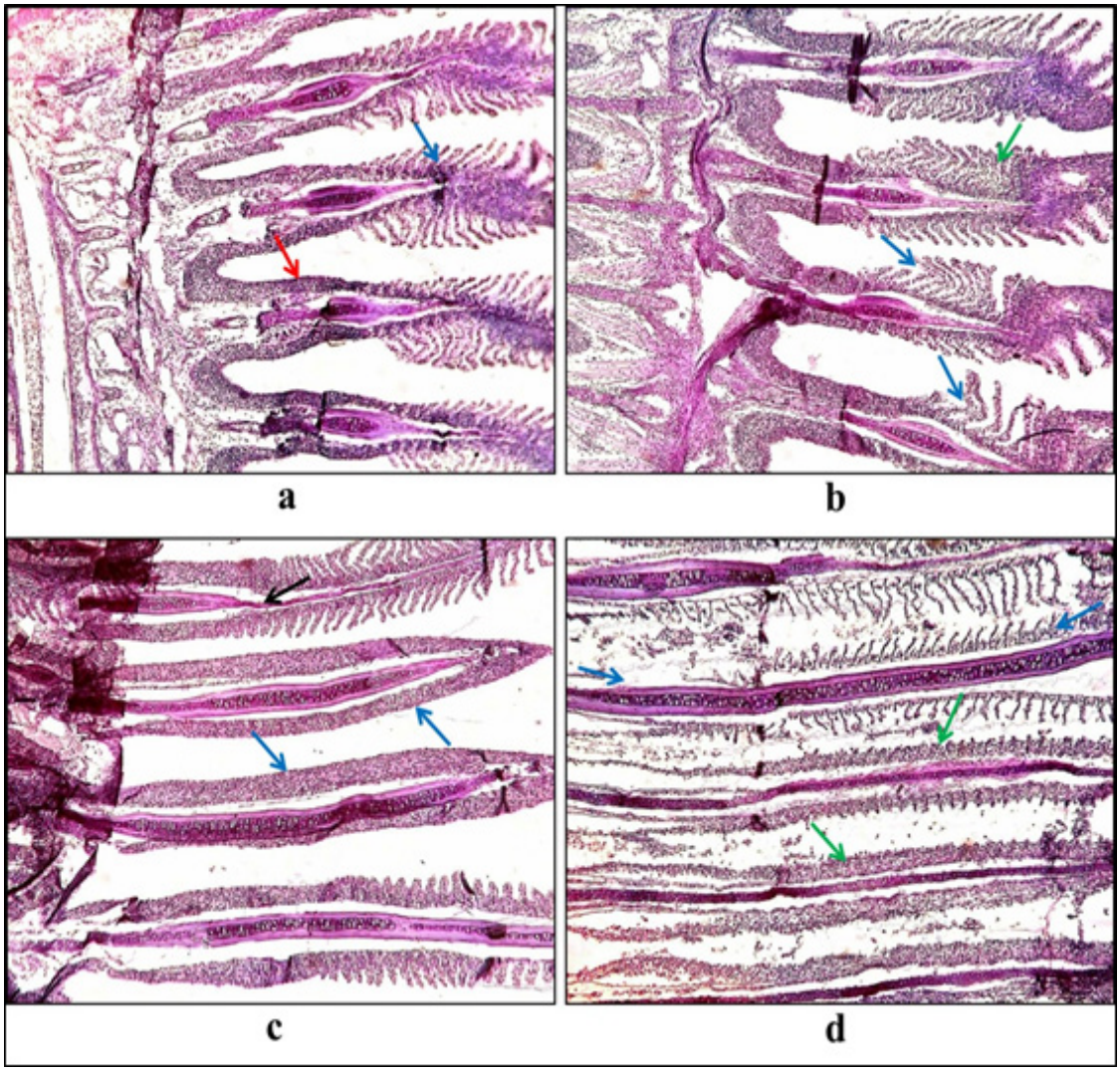


Figure 5. Photomicrograph of gills of *C. carpio* (H & E stain, 100X). **5a** (control group) normal structure of secondary lamellae (blue arrow) and primary lamellae (red arrow), **5b** (10 µg l^{-1} DEHP treated group) curling of secondary lamellae (blue arrow) and fusion of secondary lamellae (green arrow), **5c** (100 µg l^{-1} DEHP treated group) absence of secondary lamellae (blue arrow) and erythrocyte infiltration (black arrow), and **5d** (1000 µg l^{-1} DEHP treated group) hypertrophy (blue arrow) and necrosis in primary lamellae (green arrow).

of secondary lamellae and curling in secondary lamellae were observed (Figure 5b). The group treated with medium concentration (100 µg l^{-1}) of DEHP showed loss of gill arches and secondary lamellae and massive erythrocyte infiltrations (Figure 5c). Group treated with higher concentration (1000 µg l^{-1}) of DEHP showed necrosis in primary lamellae, hypertrophy and enlarged primary lamellae of gill (Figure 5d).

The liver of control fish showed normal organization of the hepatic cells and blood capillaries (Figure 6a). Degenerated hepatocytes with elongated nucleus were observed in liver tissues in the group treated with lower concentration of DEHP (Figure 6b). Hepatocytes showed vacuolization, necrosis and elongated nucleus in the group treated with medium concentration of DEHP (Figure 6c). However, in group treated with

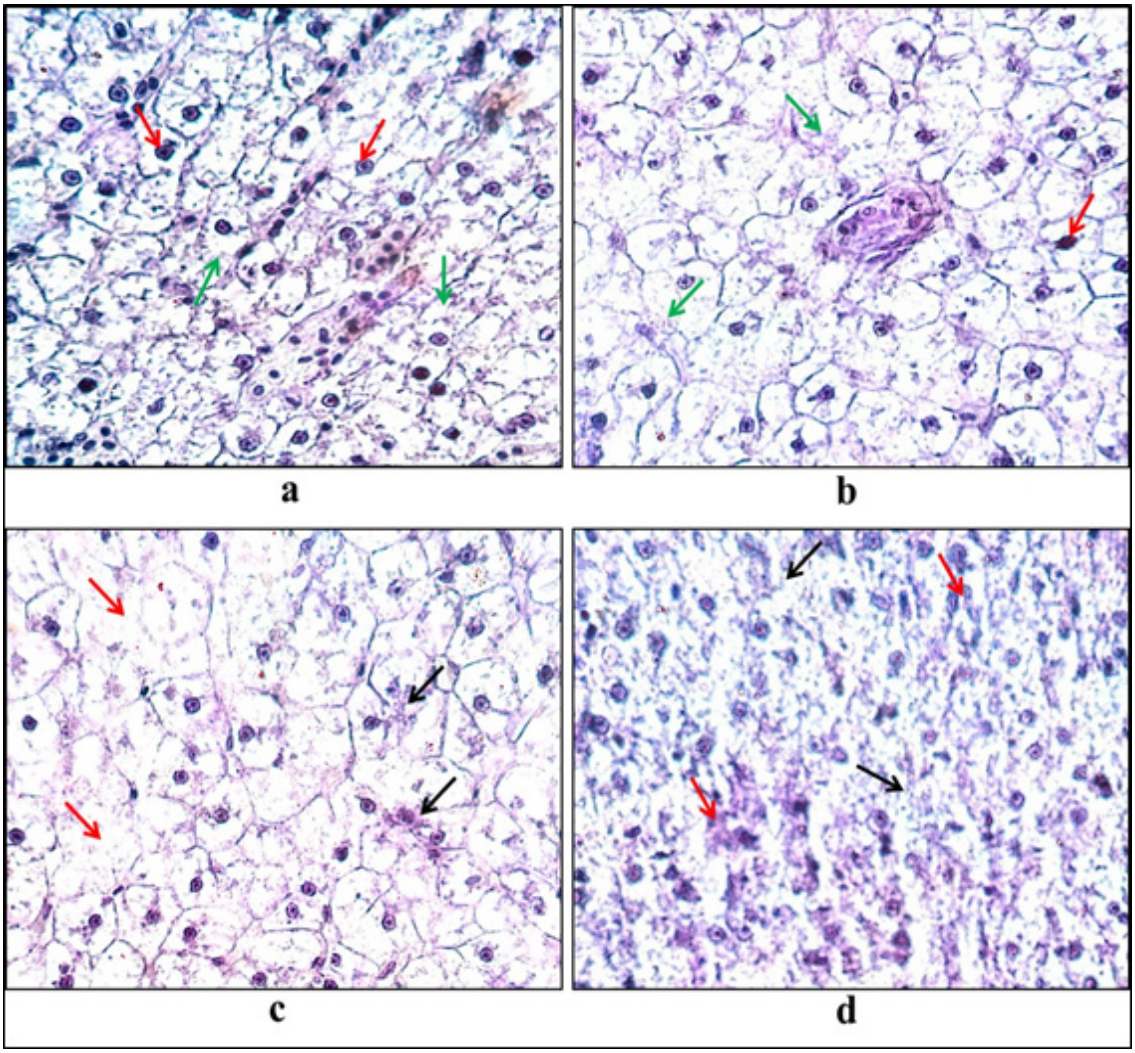


Figure 6. Photomicrograph of liver of *C. carpio* (H & E stain, 1000X). **6a** (control group) normal structure of hepatocyte (green arrow) and the nucleus (red arrow), **6b** (10 µg l^{-1} DEHP treated group) elongated nucleus (red arrow) and degenerated hepatocyte (green arrow), **6c** (100 µg l^{-1} DEHP treated group) vacuolization (red arrow) and necrosis (black arrow), and **6d** (1000 µg l^{-1} DEHP treated group) enucleated hepatocyte (black arrow) and complete necrosis (red arrow).

higher concentration of DEHP (1000 µg l^{-1}), enucleated hepatocytes with complete necrosis were observed (Figure 6d).

4. Discussion

DEHP is one of the most abundantly produced phthalate products and are released widely into the environment. DEHP that reaches to aquatic environment may cause adverse effects on food chains of aquatic organisms. In the present study, toxicity tests on the bottom feeding freshwater fish, *C. carpio*, was done as a preliminary step for the assessment of the environmental risks of DEHP on aquatic systems. Any changes in fish behaviour are considered as one of the sensitive biomarkers to evaluate the exposure to the toxicant[28,29]. Behaviour of the fishes was monitored continuously throughout the study. Herein, fishes in the DEHP

treated groups showed erratic swimming, restlessness, lack of feeding and loss of equilibrium. Similar result was found when *O. mossambicus* was exposed to 0.59 mg l^{-1} and 1.18 mg l^{-1} of Di-Butyl Phthalate (DBP) for 56 d[20].

Haematological parameters are extremely useful tool to study the toxicological impact on animal tissues[30,31]. In this study, the total erythrocyte counts were significantly decreased and leukocyte counts were significantly increased in order of increasing concentration of DEHP. In any hemopoietic organism, decreased erythrocyte count can be an indicator of anaemia which leads to inhibition of erythropoiesis[32]. The results of our study are corroborated with the findings of Obiezue *et al.*[17]. They have demonstrated decreased erythrocyte count with increased leukocyte count in *C. gariepinus* after the application of DEP at the rate of 50 to 150 µg l^{-1} for 30 d. Significant decrease in erythrocyte count was reported in fish like *P. fulvidraco*, induced by intraperitoneal injection of DEHP at the dose of 300 to 1000 mg kg^{-1} body weights of fish for 3 d[15]. In our study, decreased erythrocyte counts as well as morphological deformities indicate that erythrocytes were affected or destroyed after the DEHP exposure. However, increased in leukocyte counts could be attributed to a stimulation of the immune system in response to stress caused by DEHP exposure. Among the biochemical parameters, plasma protein level varied significantly and thus extensively used to assess the impacts or stress stimulated by environmental contaminants[33-35]. In this study, the total plasma protein concentration levels were significantly decreased in a concentration dependent manner. Similar result was observed in *P. fulvidraco* when induced with DEHP of 1000 mg kg^{-1} body weights for 3 d[15]. Yuan et al.[36] also found significant increase in the total serum protein content with increasing concentration of DEHP exposure (0.1 mg l^{-1} and 0.5 mg l^{-1}) in *P. fulvidraco* after 56 d experimental trial.

Histological study is a noteworthy and relevant parameter to understand the changes that occurred in the structural organization of organs due to exposure to toxicants[37]. In the present study, fishes in lower and medium concentration of DEHP treated group (10 and 100 µg l^{-1}) showed damage of gill epithelium, degeneration of gill arches and curling with absence of secondary lamellae. The effects were more pronounced with hypertrophy, necrosis in primary lamellae and enlarged primary lamellae at higher concentration of DEHP (1000 µg l^{-1}) treated group. Similar results have been reported in *O. mossambicus* with several damages in gill structure including upliftment and blebbing of lamellar epithelium, erythrocyte infiltration, aneurysm, curling and loss of secondary lamellae, enlarged primary lamellae and complete destruction of gill lamellae after exposure to varying concentration of DEHP (10 to 60 mg l^{-1}) for 96 h[21]. Similarly, Zeid and Khalil[20] found congestion of lamellar blood capillaries and haemorrhage, focal epithelial proliferations, fusion of gill filaments and leukocytic infiltrations in *O. mossambicus* exposed with DBP at 0.59 mg l^{-1} and 1.18 mg l^{-1} for 56 d. Further, *C. gariepinus* exposed to DEP (50 to 150 µg l^{-1}) for 30 d showed prominent oedema of the lamellar epithelia, loss of lamellar epithelia, mild oedema and raising of the filament[17]. It is therefore clear that exposure to DEHP could result in disorganization of gill structure as noticed by damages in gill epithelium, hypertrophy, absence and fusion of secondary lamellae.

Liver plays a primary role in the metabolism and excretion of xenobiotic compounds with morphological alternations[38]. In the present study, DEHP treated fishes exhibited alterations in liver tissues like degenerated cytoplasm, cytoplasmic vacuolization, elongated nucleus, a few regions with enucleated hepatocytes and complete necrosis in a concentration dependent manner. Similar types of result were also reported in the liver tissue of *C. gariepinus* exposed to DEP (50 to 150 µg l^{-1}) for 30 d[17] as well as in *C. striatus* exposed to 40 mg l^{-1} DEP for 21 d[18]. However, *O. mossambicus* exposed to DBP at 0.59 mg l^{-1} and 1.18 mg l^{-1} for 56 d showed vacuolations in the hepatocytes, hydropic degeneration with pyknotic nuclei, necrosis in portal areas, lymphocytes infiltrations, haemorrhage and lymphocytes infiltrations in liver tissue[20]. Exposure of high concentration DEHP (60 mg l^{-1}) for 96 h in *O. mossambicus* showed vacuolization, erythrocyte infiltration, elongated or absence of hepatic nucleus, necrosis and bile stagnation in organ like liver[21]. Yuan *et al.*[36] revealed that exposure of 0.1 and 0.5 mg l^{-1} of DEHP for 56 d exerts its toxic effects in *P. fulvidraco* by interfering with hepatic metabolism. In this study, the development of degenerated hepatocyte, vacuolization and necrosis in DEHP treated *C. carpio* could be due to possible accumulation of DEHP in liver tissue.

5. Conclusion

In the present study, DEHP exhibited toxic effect with changes in haematological as well as histological parameters in fish like *C. carpio*. Even at low concentration (10 µg l^{-1}) treatment, DEHP caused changes in blood parameters as well as altered the histoarchitecture of gill and liver tissue. However, further study is necessary to find out the target organ as well as deposition of DEHP in various tissues of fish.

6. Acknowledgements

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7. Conflict of Interest

Conflict of interest among authors is none.

8. References

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