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Hematological and histopathological effects of xenoestrogen (Bisphenol A) on liver and kidney functions of Mozambique tilapia fish (*Oreochromis mossambicus*)

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Abstract
Xenoestrogens are chemical compounds which include phytoestrogens, polychlorinated biphenyls, bisphenol A (BPA) and phthalates, which have very serious diverse effects on fish health. The present study was designed to determine the toxicological influences of xenoestrogen BPA exposure on biochemistry and histopathology of Mozambique tilapia (*Oreochromis mossambicus*). The fish was exposed to sublethal concentration of BPA in aquariums for 28 days. After the completion of the trial, blood samples were taken and stored in EDTA tubes. The liver and kidney of the fish were removed and preserved in 4% formalin. After dehydration, 5 µm thick sections were cut and stained with hematoxylin. Biochemical tests such as alanine transferase (ALT), aspartate transferase (AST), total protein, blood glucose, blood creatinine, and complete blood count (CBC) were analyzed. Histological examination of kidney and liver of the control group revealed normal architecture, while the liver of the treated group showed vacuolation in hepatocytes, aggregation of lymphocytes, necrosis, increased hemorrhages, erythrocyte infiltration, and hepatocellular necrosis, while the kidney showed degeneration of glomerulus and renal tubule. Presence of melanomacrophages and hypertrophy in tubular epithelial cells increased the space in between glomerulus and enlarged Bowman's capsule. ALT, AST, plasma glucose, WBCs, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and plasma creatinine and protein values increased significantly as compared to those of the control group, while RBCs, hematocrit test (HCT) and MCH were decreased significantly.

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Introduction

Aquaculture is growing rapidly in Pakistan and there is an enormous potential for development of the sector. Fish is a lean source of protein, vitamins and minerals, such as selenium, vitamin B12, niacin, phosphorus and potassium. It is a source of cheap and precious animal protein for human population (Makori et al., 2017). Thus, fish industry makes a major role to the economy of Pakistan as an earner of foreign exchange. Due to the cheap cost of tilapia farming, it has been known as the "aquatic chicken" of the trade. The estimated annual value of the farmed tilapia production is 1.8 billion US$. Tilapias have become the second most common aquaculture species and have been introduced into at least 90 countries on all continents except Antarctica (Fitzsimmons, 2001).

Liver is the vital organ for the metabolism and detoxification of xenobiotics, including BPA; due to

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this the liver is largely visible to BPA effects and could be vulnerable to lower doses than other organs. Liver also plays its vital role in metabolic activities. Liver is located in the cranial region of abdominal cavity, ventral to the swim bladder. The kidneys of fish play a vital role in excretion and regulation of the water balance in maintaining a fish's internal environment and osmosis (Mahboob et al., 2020; Nkwuda et al., 2020). Within freshwater fish, the kidney is responsible for preventing excess solute loss as the fish contain more salts within their blood than the water located around their bodies. Due to this concentration gradient, water will naturally diffuse into the fish through osmosis causing large amounts of water to build-up inside. Therefore, the kidneys in this type of fish increase the amount of water that passes out in their urine and actively reabsorb the salts that would pass out as well to maintain that balance (Tavares-Dias, 2022). This, therefore, results in the production and excretion of large amounts of dilute urine. This urine is so dilute that it is almost completely composed of water. The functional and structural unit nephrons consist of renal corpuscles which include Bowman's capsules, glomerulus and renal tubules.

Freshwater adulteration with heavy metals and chemicals has been described to be one of the most thought-provoking issues in most of countries (Hezbullah et al., 2016). Approximately, 70,000 anthropogenic chemicals are released into the aquatic ecosystems (Metzler and Erica, 2001). It has been widely reported that waste from agricultural and industrial processes containing highly toxic chemicals like pesticides that lead to pollution of aquatic environments including rivers, ponds and lakes (Langston, 1990; Choppala et al., 2013; Aslam et al., 2022). The accretion and persistence of insecticides and pesticides in the aquatic environment pose a threat to biological life, as witnessed by the chronic and acute poisoning of fish and other aquatic organisms. Environmental pollutants can cause change in the biochemical parameters on organisms like fish and can react in aquatic environment synergistically (Moore and Waring, 2001).

Exposure to endocrine-disrupting chemicals (EDCs) is presently observed as one of the most serious anthropogenic fears to biodiversity and ecosystems. Bisphenol A (BPA) is found in polycarbonate plastics and epoxy resins (Sharma and Chadha, 2021; Afzal et al., 2022). Major uses of BPA include glazing and sheeting of electrical and electronic goods, electronic storage media and household equipment, including bottles, utensils and containers. BPA enters into the water bodies through manufacturing plants and effluent discharge. BPA is additionally free throughout transport process, and degradation of plastic and PVC pipes additionally end in BPA unharnessed (Flint et al., 2012; Afzal et al., 2022). Thus, the present investigation was undertaken to assess that up to what extent BPA could adversely affect the functions of liver and kidneys in Mozambique tilapia fish.

Materials and Methods

Live samples of *Oreochromis mossambicus* irrespective of sex with specific weight and length were collected from the ponds of Fish Hatchery, Manawan, Lahore, Pakistan. All fish were of equal weight 95-100 g and equal length 30-32 cm. The collected samples were brought to the research laboratory alive in plastic bags filled with water and oxygen. Live fish was transferred to aquaria and all aquaria were maintained at similar environmental conditions.

Preparation of BPA dose

BPA was purchased from Sigma (USA) Chemicals. Three different levels of 0.5 mg/L, 01 mg/L and 1.5 mg/L were prepared. Fish was divided into four groups, 3 treatment groups were labeled as group I, group II and group III, respectively, while group IV was control. Before starting the test, all experimental aquaria were cleaned and filled with dechlorinated tap water. Physical and chemical parameters like pH at 7.4–7.8, temperature at 28–30 ℃, dissolved oxygen 5.86–10.53 ppm was maintained throughout the experiment. Fish was fed with a commercial feed containing 30% protein.

Biochemical and histological analysis

After the completion of the trial, fish were removed from the aquarium by a fry net and anesthetized immediately using chloroform. Blood samples from each group were taken by cardiac puncture with the help of syringes and were stored in EDTA tubes for biochemical and hematological tests. Tissue samples of liver and kidney from each group were stored in 4% formalin.

From the preserved blood, different biochemical tests like total protein content, plasma glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood creatinine and CBC were analyzed. The preserved tissues were fixed, dehydrated, embedded, paraffined in wax and microtomy was done. Sections of 2-3 µm were mounted on slides with Meyer's albumin-stained hematoxylin. The stained sections were observed under a binocular research microscope of lens 400X attached with a CCD camera. Appropriate sections and portions of the tissues were photographed using the MICAPS 3.7

image analyzer software.

Results

Histopathology of control group

Histological examination of liver of the control group showed normal hepatic architecture. The components of liver such as lobules were also normal. The hepatocytes were normal in appearance having central nuclei, arranged in a group around the central vein, and there was no tissue alteration. There were no pathological abnormalities, no inflammation, and hydropic degenerations (**Figure 1**). Histological examination of the control kidney also showed normal renal capsule nephrons and tubules having slightly spherical glomerulus with proper Bowman's capsules. No renal tissue inflammation, necrosis, fibrosis, atypia or malignant were seen (**Figure 2**).

Treated Group I

The histological examination of the liver of the treated group I showed no gross hydrophobic degeneration of hepatocytes. However, in some areas vacuolation in hepatocytes was evident with adjacent aggregates of lymphocytes, erythrocytes infiltration into blood sinusoids, and slightly increased hemorrhage's necrosis atypia or malignancy (**Figure 3**). Kidneys of group I showed focal renal tubular degeneration and presence of monocular cells infiltration in intra-tubular areas. The increase of space in between the glomerulus and enlarged Bowman's capsule revealed no renal tissue necrosis, fibrosis, atypia or malignance (**Figure 4**).

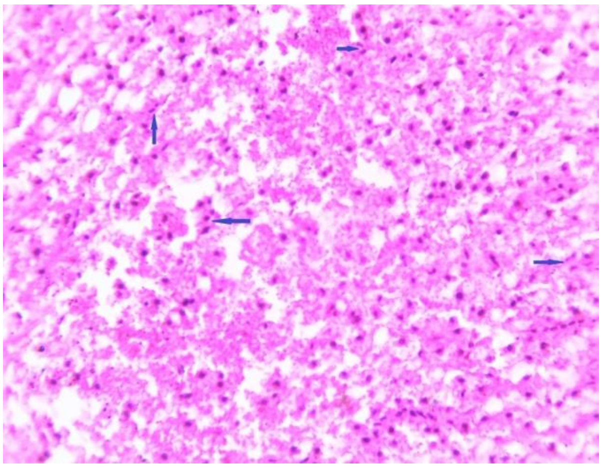

Figure 1. Histological examination of the control fish liver reveals normal hepatic architecture

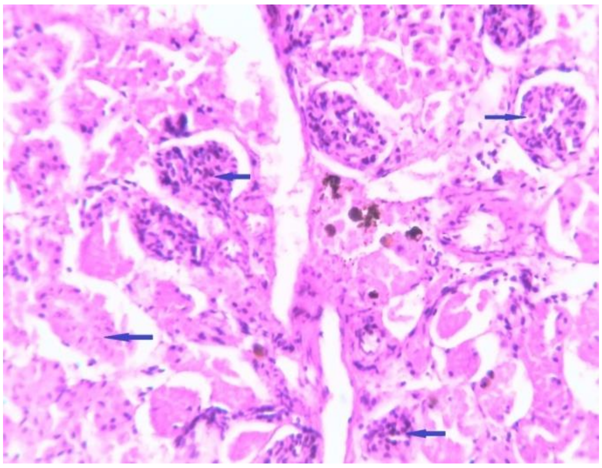

Figure 2. Histological examination of the control fish kidney reveals normal renal capsule, nephrons and tubules

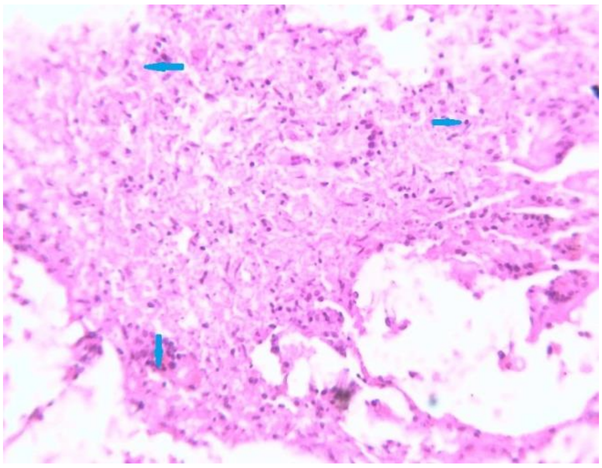

Figure 3. Histological examination of the liver treated with 0.5 mg/L BPA reveals vacuolation in hepatocytes, erythrocyte infiltration into blood sinusoids and slightly increased hemorrhage

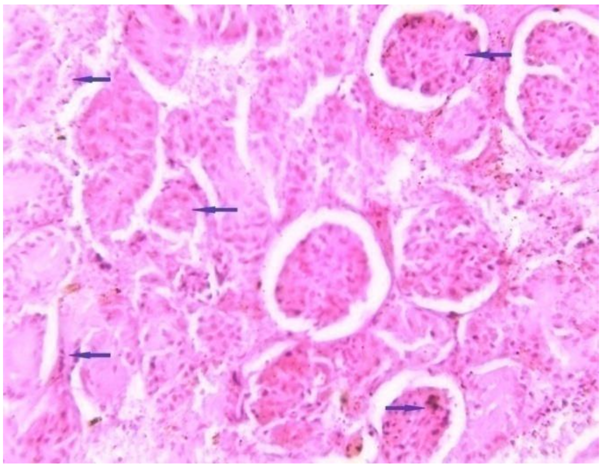

Figure 4. Histological examination of the 0.5 mg/L BPA. The kidney reveals focal renal tubular degeneration and presence of mononuclear cells infiltration in the intratubular presence of melanomacrophages

Treated Group II

The liver of group II fish showed initiation of degeneration of hepatocytes and nuclei. There was increased vacuolation in hepatocytes, erythrocyte infiltration into blood sinusoids and increased hemorrhage. Hepatocyte necrosis was evident, and no atypia or malignancy seen (**Figure 5**). The kidney of Group II showed initiation of focal renal tubular degeneration and presence of monocular cells infiltration in intratubular areas, presence of space in between the glomerulus and the enlarged Bowman's capsule. No renal tissue necrosis, fibrosis atypia or malignancy were seen (**Figure 6**).

Treated Group III

Group III fish liver showed degeneration of hepatic cells, moderate increase in number of vacuoles and pleomorphic nuclei. Erythrocyte infiltration into the blood sinusoids and increased hemorrhages' hepatocyte necrosis was observed, but no atypia or malignancy was seen (**Figure 7**). The histological examination of Group III kidney revealed degeneration of glomerulus and renal tubule. Presence of melanomacrophages and hypertrophy in tubular epithelial cells were also evidenced. Increased space between the glomerulus and enlarged Bowman's capsule was seen. No renal tissue necrosis, fibrosis, atypia or malignancy were observed (**Figure 8**).

Biochemical parameters

Administration of sublethal concentration of BPA caused a significant decrease in both liver and kidney proteins in all experimental groups, while plasma glucose levels increased as compared to those of the control group. AST and ALT values showed a gradual and a significant increase in all BPA treated groups as shown in **Figures 9-10**. The increased activity of serum enzymes was due to damaged hepatocytes and increased cell permeability, leading to a leakage of enzymes from damaged hepatocytes into serum. Plasma glucose and creatinine values were increased in a dose-dependent manner in the experimental groups (**Table 1**).

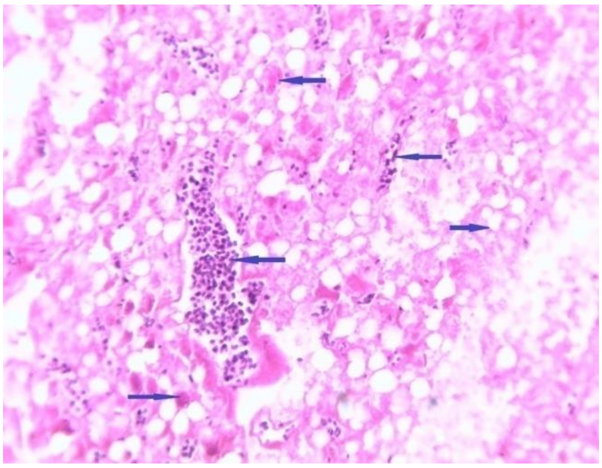

Figure 5. Histological examination of liver treated with 1 mg/L BPA

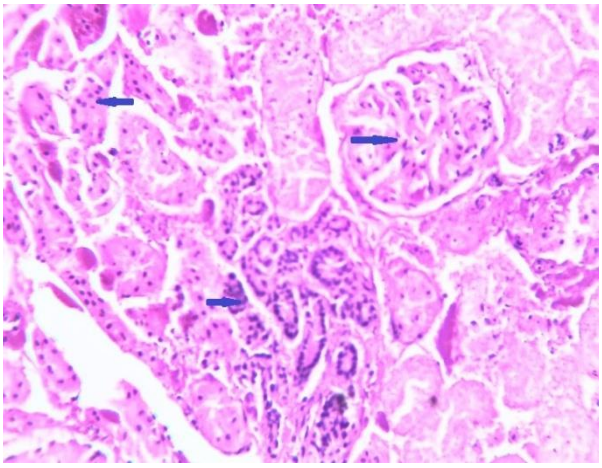

Figure 6. Histological examination of kidney treated with 1 mg/L BPA

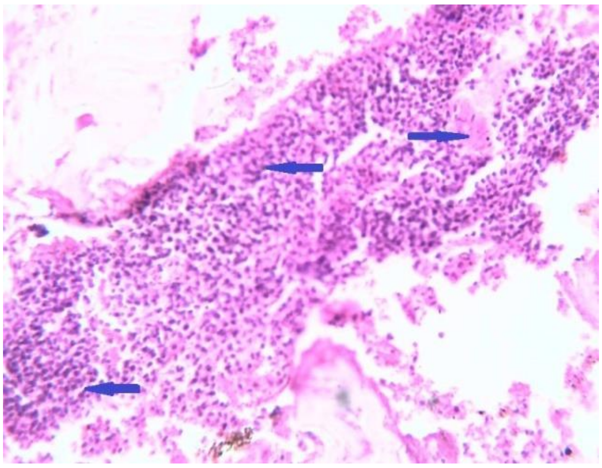

Figure 7. Histological examination of liver treated with 1.5 mg/L BPA

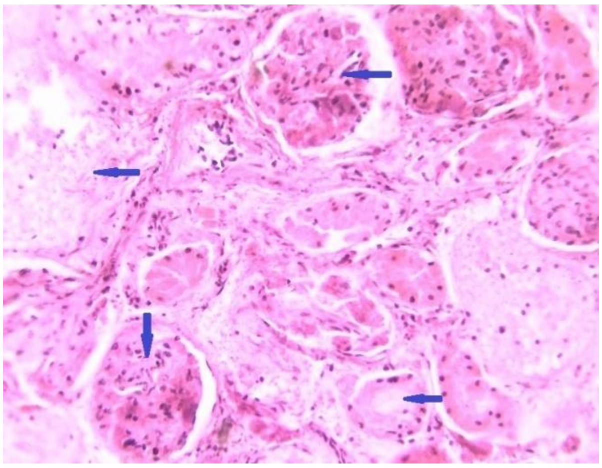

Figure 8. Histological examination of kidney treated with 1.5 mg/L BPA

Table 1. Changes in the biochemical parameters of liver and kidney in *O. mosambicus* treated with sublethal concentrations of BPA

Group	Fish organ	Total protein (mg/mL)	ALT (SGPT) (IU/L)	AST (SGOT) (IU/L)	Plasma glucose (mg/dL)	Plasma creatinine (mg/dL)
Control (0 mg/L)	Liver	37.8	19.8 ± 0.5	40.5 ± 0.9	30.5 ± 1.1	1.01 ± 1.7
	Kidney	6.9	21.6 ± 1.9	24.1 ± 1.7		
Group I (0.5 mg/L)	Liver	43.7	40.9 ± 1.7	141.5 ± 1.6	41.6 ± 1.6	1.9 ± 1.5
	Kidney	7.5	30.7 ± 2.3	38.6 ± 0.5		
Group II (1.0 mg/L)	Liver	52.3	55.5 ± 2.7	146.2 ± 0.6	52.5 ± 0.8	2.3 ± 1.2
	Kidney	9.7	45.6 ± 2.3	46.2 ± 1.7		
Group III (1.5 mg/L)	Liver	60.2	81.8 ± 1.9	154.2 ± 0.8	61.5 ± 1.9	3.1 ± 2.1
	Kidney	15.3	61.5 ± 2.6	53.7 ± 0.6		

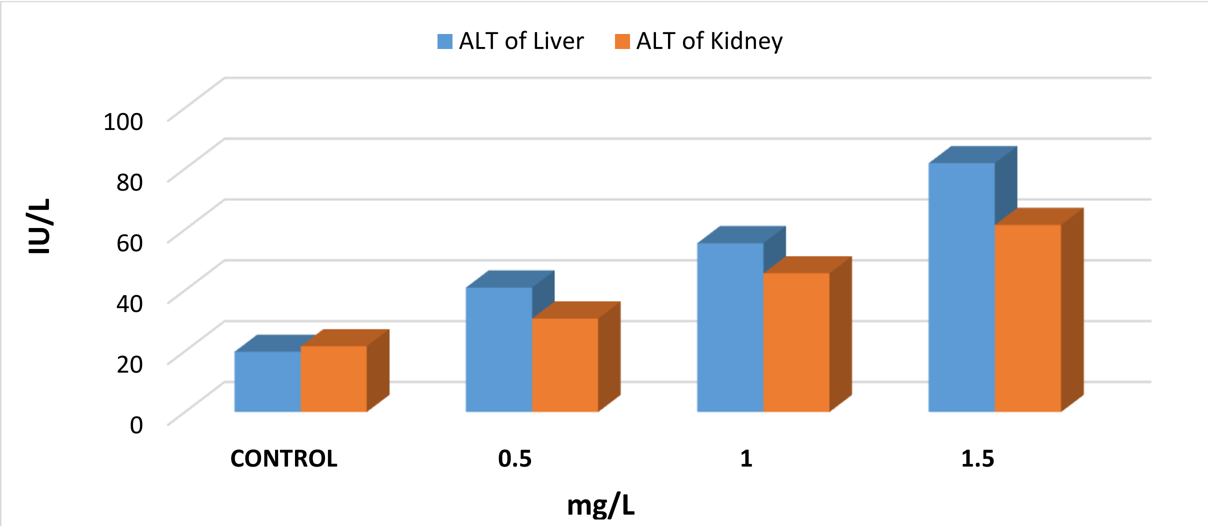

Figure 9. ALT of liver and kidney of the fish exposed to sublethal concentrations of BPA

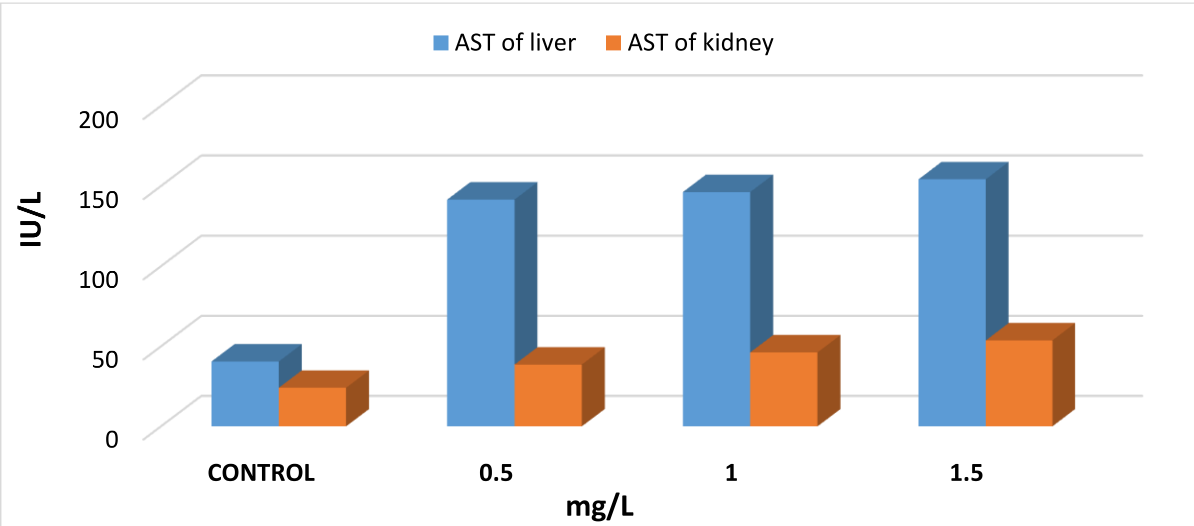

Figure 10. AST of liver and kidney exposed to the sublethal concentrations of BPA

Hematology analysis

Exposure of sublethal concentrations of BPA showed a significant decrease in hemoglobin and total RBC values in the experimental groups. There was an increase in WBC with respect to that of the control group. A consistent decrease in HCT (PVC) values from the control to the experimental groups was recorded. MCV and MCH values also gradually decreased with increase in the level of BPA. The MCHC values increased with increase supplementation of BPA. Mean hematological parameters of *Oreochromis mosambicus* after exposure to different concentrations of BPA is presented in **Table 2**.

Table 2. Mean hematological parameters of *Oreochromis mosambicus* exposed to sublethal concentrations (0.5, 01 and 1.5 mg/L) of BPA

Group	Hemoglobin (g/L)	WBC (x 10^3/µL)	RBC (x 10^6/µL)	HCT (L/L)	MCV (µm^3)	MCH (pg)	MCHC (g/dL)
Control	7.2 ± 1.9	15.4 ± 0.6	1.4 ± 2.1	21.6 ± 1.7	154.2 ± 0.8	51.4	41.2
Group I (0.5 mg/L)	6.3 ± 1.6	16.5 ± 0.7	1.3 ± 1.8	19 ± 0.9	145 ± 0.7	48.4	47.0
Group II (1 mg/L)	5.8 ± 1.1	16.7 ± 0.5	1.1 ± 2.2	17 ± 1.2	158 ± 0.5	46.7	51.1
Group III (1.5 mg/L)	5.4 ± 1.7	17.3 ± 0.3	0.9 ± 2.5	16 ± 1.6	150 ± 0.6	20.0	49.4

MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, and MCHC = mean corpuscular hemoglobin concentration.

Discussion

Toxic potential of a toxicant is directly related with severity of damage it causes in organisms. Histopathological biomarkers approach is a great source of assessment of environmental risk and genotoxicity evaluation in fish and water bodies (Rana et al*.,* 2017). The contamination of fresh water with a wide range of contaminants has become a great concern over the last few years. Once a toxicant enters in the body of an organism, different biochemical and physiological responses occur which may be adaptive or may lead to toxicity. The biochemical processes are the most sensitive indicators of the pollutant damage.

However, in our study the rate of histopathological changes was more in fish when exposed to higher concentration of BPA. Fish liver is the vital organ for detoxification of BPA. The liver plays a key role in degradation and detoxification of pollutants. Therefore, the alterations in liver of aquatic fauna are a mirror of aquatic pollution of their habitat (Moon et al., 2012). The liver is mostly associated with the toxification and biotransformation processes due to function, position and blood supply (Van der Oost et al., 2003). Numerous histological changes were reported in liver of fish due to exposure to xenoestrogens. Liver histology is highly delicate and is a precise way to analyze the effects of any pollutant on fish. In the present study, *Oreochromis mossambicus* exposed to different concentrations of BPA showed clear histological changes in liver, increased vacuolation in hepatocytes, dilated blood vessels, sinusoids, necrosis, nuclear pyknosis, vacuolation, increased infiltration of melanomacrophages into hepatopancreas, loss of contact between hepatocytes and pancerocytes, degeneration of hepatic cells, moderate increase in number of vacuoles and pleomorphic nuclei. Erythrocyte infiltration into the blood sinusoids and increased hemorrhages' hepatocytes necrosis were also evident with no atypia or malignancy. Similar results were shown by Faheem et al (2016) in BPA-induced histopathological changes *Catla catla* and *O. mossambicus* (Deepa et al., 2017)

Fish kidneys are major hematopoietic and osmoregulatory organs. In the present study, kidney of the control group showed a normal architecture with no abnormalities. Increased concentration of BPA on the treated group caused abnormalities with focal renal tubular degeneration of glomerulus and renal tubules, glomerulus expansion, aggregation of melanomacrophages, nuclear hypertrophy, and blood congestion. Similar results were shown in *Catla catla* kidney when treated with different concentrations of BPA (Faheem et al*.,* 2016).

In the current study, ALT activity after exposure of the fish to sublethal concentrations of BPA under laboratory conditions was significantly increased in the tissues when compared to the control values. The results showed that ALT activity was higher in liver than that kidney which seems to be tissue specific. The increased levels of ALT in liver and kidney of the treated groups indicate a significant tissue damage which may have been due to interruption in normal physiological and biochemical processes. In the high dose BPA group, the AST and ALT levels were nearly double that of the control group. Similar results were shown in which the AST activity was also increased significantly when compared to control value in both liver and kidney stinging catfish *Heteropnestes fossilis* (Pal and Reddy, 2018).

AST (aspartate aminotransferase) is a specific enzyme of liver but also found in skeleton muscles and kidney. It helps in glycogenesis from amino acids in association with ALT. In the present study, the AST activity in the experimental treated groups was also increased significantly when compared to that in the control group. This increased activity was an alarming signal of liver tissue damage leading to a discharge of enzymes into the blood stream. Another cause of increase level of these enzymes may be due to hepatic cells injury or enlarged synthesis of enzymes (Rastiannasab et al., 2016).

In the present research, protein levels increased consistently in both liver and kidney with increase in the levels of BPA. High levels of total protein can mean that either albumin or globulin is high, i.e., high levels of albumin mean an organism is dehydrated and high globulin levels identify blood diseases such as multiple myeloma or autoimmune diseases such as lupus, kidney disease, or liver disease (Younis et al., 2013). Under stress condition fish mobilizes proteins as an energy source through the oxidation of amino acids (Jia et al., 2017). Decrease in tissue protein content in fish reveals the toxic stress in the conversion of tissue protein into soluble protein reaching in the blood for its usage. This decrease in plasma protein could be due to change in protein and free amino acids metabolism and their synthesis in liver. The increase in total protein content in liver and kidney of fish indicates accumulation of this biochemical in the vital tissues under BPA stress (Akram et al., 2021). In long-term exposure to BPA, much of the energy must have been used up by the fish to compensate the stress, resulting in depletion of proteins (Martyniuk et al., 2020). The increased values of total protein particularly in liver may have been enhanced rate of protein synthesis under BPA-induced stress (Guru and Arockiaraj, 2022).

Glucose is the vital source of energy in all vertebrates and is a susceptible indicator of environmental stress in fish. Fish blood contains normally 40-90 mg/dl of glucose (Patriche, 2009). In the present study, increased levels of glucose in all three BPA-treated groups were recorded compared to the control. The hepatic cells have many important functions such as secretion of bile, detoxification and synthesis of several components of blood plasma, storage of glycogen and release of glucose in the blood (Kumar et al., 2016). The results suggest that reduction in glycogen is probably due to its more rapid breakdown (glycogenolysis) which releases glucose into the circulatory system to meet the increased energy requirements under stress condition (Kamalam et al., 2017). It is a consistent indicator of environmental stress and is believed as a secondary response induced by toxicant like BPA (Firat et al., 2011). Under stressful circumstances, an individual stores energy reserves in the form of glycogen in the muscles and liver that in turn increases glucose concentration in plasma (Bartonkova et al., 2017).

In the current study, serum creatinine levels were increased significantly in *O. mossambicus* administered with sublethal concentrations of BPA compared with those in the control group. Serum creatinine and uric acid are vital indicators of renal health. They are also considered as potential biomarkers for muscle and purine metabolism (Hamed and Tawwab, 2017). Increased creatinine level indicates weakened kidney function (Kulkarni and Pruthviraj, 2016). The increased levels of serum creatinine may be due to renal dysfunction, reduced renal blood flow due to stress, diabetes acromegaly, damage of tissue or dietary or poor excretion, increased synthesis, or reduced degradation activity of BPA (Amin and Hashem, 2012). The present results confirm that BPA treated fish suffered with glomerular dysfunction as blood levels of creatinine depend largely on glomerular function.

In the present study, PCV value decreased noticeably with rising toxicity of BPA at 28 days of exposure time. Due to a significant reduction in RBC, PCV decreased. The decrease in hematological variables (PCV, Hb, and RBC) of the exposed fish may have been due to hemolysis and shrinkage of RBC leading to important decrease in hematocrit value which results in fish anemia (Burgos-Aceves et al., 2019). The increase rate of the breakdown of RBC or decrease rate of structure of RBCs might also be responsible for decrease in RBC count. Increase in WBCs count occurred as a pathological response since these WBCs play a vital role during invasion by stimulating the hemopoietic tissues and the immune system by producing antibodies and chemical substances working as defense against infection. WBCs are key cells in the immune system, because of their core defensive function. The WBCs reacts immediately to the change in standards due to toxicants. During toxic exposure period, the WBC counts were enhanced. It indicates that fish can develop a defensive mechanism to overcome the toxic stress.

Conclusion

The biochemical parameters like total proteins, ALT and AST in the different tissue of fish provided vital information about the quality of the environment. The changes in the tissues brought about alterations in the histological organization of vital organs of fish due to the exposure of BPA. The toxic chemical in body cells of aquatic organism altered the biochemical components which were indicators of the harmful effect of pollutants. Thus, alterations in biochemical parameters of toxic chemical -treated fish were concluded as important tool for the assessment of water quality in the field of environmental toxicology.

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For studies involving Human/Animal subjects, a formal ethical approval has been sought by authors from ethical committee/review board of their parent institutions according to their internal guidelines.

Handling of bio-hazardous materials
The author(s) certify that all experimental materials were handled with care during collection and experimental procedures. After completion of experiment, all materials were properly discorded to minimize any types of bio-contamination(s).

Availability of primary data and materials
As per editorial policy, experimental materials, primary data or software codes are not submitted to the publisher. These are available with corresponding author and/or with other author(s) as declared by the corresponding author of this manuscript.

Author's consent
All authors contributed in designing and execution of the experiment. All contributors have critically read this manuscript and agree for publishing in IJAEB.

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