Scope
Volume 14 Number 04 December 2024

Histopathological and Biochemical Analysis of Liver and Muscles of Fresh Water Fish*oreochromis Mossambicus* Exposed to Ossein and Dicalcium Phosphate

Dr. Remya V.K[*1], Smina. M. S[2], Jaseera M.K[2] and Chithra V.S[2]
[1]Assistant Professor and Head of the Post Graduate and Research Department of Zoology, [2]Sree Narayana College, Nattika, Thrissur, Kerala, India.

Corresponding Author: **Dr. Remya V.K**

Abstract: Gelatine, a widely used product in food, pharmaceuticals, paper production, and photography, has safe applications but poses environmental risks due to its intermediate by-products, ossein and dicalcium phosphate. Improper disposal practices by some gelatine production facilities release these pollutants into aquatic environments, creating serious ecological concerns. This study investigates the histopathological and biochemical effects of ossein and dicalcium phosphate on the liver and muscle tissues of the freshwater fish *Oreochromis mossambicus*. Ossein and dicalcium phosphate, prepared from bone powder, were introduced to the water of the experimental tanks for nine consecutive days. Key water quality parameters (salinity, dissolved oxygen, dissolved CO_2, and pH) were monitored in both groups. After exposure, liver and muscle tissues were analyzed for histopathological and biochemical changes. Histological examinations using hematoxylin and eosin staining revealed significant necrosis and disorganization of hepatocytes, while histochemical analyses indicated protein degradation. Biochemical assessments demonstrated increased glucose levels in muscle tissues, suggesting a compromised glycolytic process. Collectively, these changes resulted in abnormal behavior, homeostatic imbalances, reduced mobility, and eventual mortality in both ossein and dicalcium phosphate exposure groups. Bioaccumulation adds another layer of concern, amplifying the ecological threat. Addressing this issue requires enforceable regulations and sustainable waste treatment methods to safeguard aquatic ecosystems.

Key words: *Oreochromis mossambicus*, Gelatine, Ossein, Dicalcium phosphate, Histopathology, Biochemical analysis.

Introduction

In the modern world, the urbanization and industrialization have boosted mankind's economy through various means and ways. But at the same time, pollution of aquatic

resources has become a huge challenge and a serious threat. Due to urbanization, industrial and agricultural development, freshwater sources are dumped with different kinds of chemicals that affect the inhabiting biota. Recent years have witnessed significant attention being paid to the problems of environmental contamination by a wide variety of chemical pollutants.

Gelatin is a protein substance derived from collagen, a natural protein present in the tendons, ligaments and tissues of mammals. It is produced by the demineralization of connective tissues, bones and skins of animals usually cows and pigs. Gelatine's ability to form strong transparent gel flexible films that are easily digested, soluble in hot water and capable of forming a positive binding action have made it a valuable commodity in food processing, pharmaceuticals, photography, paper production and as a food stuff, gelatin is the basis for jellied desserts; used in the preservation of fruit and meat and to make powdered milk. It is also used to clarify beer and wine. Ossein is an intermediate product formed during the production of gelatin. Ossein is rich in collagen that is extracted from the bones of slaughtered animals. It is used for the production of gelatin. In several gelatin production factories, the waste water was discharged to nearby aquatic ecosystems. Most of the polluting activities take place in the Ossein division [1].

In order to evaluate the adverse effects of the pollutants on aquatic organism, there is a worldwide trend to complement chemical and physical parameters with biomarkers in aquatic pollution monitoring [2]. Fishes are used as excellent indicators of aquatic pollution due to their high sensitivity to environmental contaminants which may damage certain physiological and biochemical processes when contact with the organs of fishes [3]. In recent years, *Oreochromis mossambicus* has been served as a bio indicator and integrator of contaminants on various reason, Viz, wide distribution in the fresh water environment, free swimming nature, ability to respond against environmental pollution and its importance as an economic food source for human being [4]. *Oreochromis mossambicus* can survive in bad environmental conditions because of their resistance to diseases and their respiratory demands are slight, so that they can live in low oxygen environment and in high ammonia levels. Fish liver plays an important vital function in basic metabolism and it is the major organ of accumulation, biotransformation and excretion of contaminants in fish [5]. The measurement of suitable biomarkers in liver can give an idea about the health status of fish. Toxicological studies have shown that the impact of contaminants on aquatic ecosystems can be evaluated by measuring biochemical parameters in the liver of fish that respond specifically to the degree and type of contamination [6]. Also, the liver histology is used as biomarker for the environmental pollution [7] and there have been numerous reports of histopathological changes in liver of fish exposed to a wide range of organic compounds and heavy metals [2]. Aquatic hypoxia occurs when the rate of oxygen consumption by organisms during their dark cycle exceeds the rate of oxygen production or the capacity of oxygen to diffuse in from the air [8]. Fish responds to

hypoxia with a suitable means of behavioral and metabolic changes to enhance the oxygen uptake from the hypoxic environment. The Ossein released into the aquatic ecosystem makes it hypoxic environment. Hypoxia, simply the oxygen shortage that means dissolved O_2 concentration below 5-6 mg O_2/litre in fresh water. Fishes grown in such hypoxic environment trend to show considerable changes in their muscle glucose level. The biochemical analysis of muscle glucose of fishessubjected to Ossein pollution can be used as a good marker outlining the effects of hypoxia.

Dicalcium phosphate formed by the addition of calcium hydroxide to the demineralized bone mixture also imparts polluting effects to the aquatic biota. In short, Ossein and Dicalcium phosphate contribute to the hazardous polluting environment in aquatic ecosystem. The leaching of wastewater, acid and heavy metals from the solid waste has contaminated the soil and ground water.

Materials and methods

This study was conducted to analyze the effects of ossein and dicalcium phosphate on Oreochromis mossambicus, stressing changes in the liver and muscle. The duration of the study was approximately one year. *Oreochromis mossambicus*, belonging to the same age group, was collected from the Marathakkara fish farm in the Thrissur district. After collection, they were reared in a tank for acclimatization. Fish of similar sizes were separated and used for the study. First, fish were divided into two groups. The first group was labeled as the control and the other group as the experimental group. Both groups were reared in normal water. Different concentrations of ossein and dicalcium phosphate were added to the two experimental tubes for 9 consecutive days. Hydrological parameters such as salinity, dissolved O2, CO2, and pH of both the control and experiment were estimated following the standard APHA (1981). After 9 days, fish from both groups were anesthetized using chloroform. The livers of these fish were dissected and used for both histological and histochemical analyses. For biochemical analysis, fish muscles from both groups were dissected, and glucose levels were estimated using the Somogy Nelson Method. Preparation of Ossein and Dicalcium phosphate Approximately 100 g of bone powder was brought into contact with 10 ml of hydrochloric acid. The concentration was adjusted to 10 g of free HCl per litre by adding 1180 ml distilled water. The mixture was stirred thoroughly for 1–3 min. The liquid phase was then separated and the solid mass was treated with 10 ml dilute HCl and 236 ml distilled water for 2 h. (Amount of water corresponds to obtain the concentration of 50g free HCl per litre.) The resulting slurry mass was osseous. Isolation of Dicalcium phosphate Dicalcium phosphate was obtained from ossein by the addition of lime milk. Milk lime was prepared by stirring 100 mg of calcium hydroxide in 100 ml distilled water to yield lime water. Then added excess Calcium hydroxide to lime water to obtain suspended particles of Calcium hydroxide which is called milk of lime. 1000ppm, 2000ppm, 3000ppm, 4000ppm, 5000ppm, 6000ppm, 7000ppm, 8000ppm, 9000 ppm are added in 9 consecutive days to the

Histological examination

After dissecting the fish from both experiment and control, liver was removed and fixed in 10% formalin solution for 24 hours. The tissues were routinely dehydrated in an ascending series of alcohol, cleared in xylene and embedded in paraffin wax. Sections of 6µm thick were cut, processed and stained with haematoxylin and eosin. For histochemical analysis of protein Bromophenol Blue staining procedure was followed. They were examined under light microscope and photographed.

Results

The production of gelatine from crushed animal bone produces ossein and dicalcium p hosphate as intermediate products.The current study investigated the effects of ossein a nd dicalcium phosphate on the liver and muscle tissues of Oreochromis mossambicus. The contaminated aquatic environment generated considerable changes in the liver and muscle tissues of the affected fish.The hydrological conditions in the experiment group are adverse for organism survival.Salinity and dissolved oxygen levels in the experiment water sample are important pollution indicators.Whereas the control group's factors w ere advantageous for their development in captive circumstances (Table 1).

The liver histology of *Oreochromis mossambicus*

The liver cross section showed normal histology with no pathological abnormalities (Fig. 1). The fish liver is made up of two parts: parenchyma (the epithelial cells that execute the organ's primary activities) and stroma. The parenchyma comprises the numerous cells found inside the liver as well as the corresponding extracellular species. Hepatocytes account for approximately 80% of the cell population in the liver. Hepatocytes are responsible for the majority of the liver's activities. Light microscopy investigations reveal that hexagonal subdivisions of hepatic parenchyma in fish liver are indistinguishable. Histological studies of the liver of experimental fish after exposure of ossein and Dicalcium phosphate exhibited some sort of cellular degeneration (Fig. 2, 3). Necrosis and disorganization of hepatocytes is the most prominent visible change in the cross sections of treated fish liver. The clustering of hepatocytes and loosening of hepatic parenchyma also observed, so the normal cellular organization is lost. Most of the hepatocytes ruptured and cytoplasm gets disorganized. It is also observed that translocation and disorganization of nucleus occurred from the hepatocytes and they clustered in less cytoplasmic areas of liver. Migration of nucleus also observes d in some areas. The degradation of hepatic tissues observed near the blood vessel is a notable change and tissue necrosis was observed in the nearby areas. Hepatocytes are organised as tubules or cords [9, 10]. The cord like structures of hepatocytes is not often readily evident. In this configuration, hepatocytes bases are directed towards sinusoids, mostly for absorption [11, 12, and 13]. The endothelial cells that make up the walls of hepatic blood vessels are clearly distinguished by their varied colours.

Histological analysis of protein in fish liver

The hepatic cells in both the experiment (Fig. 5.7) and control (Fig. 4.6) groups were positive for the protein analysis using the Bromophenol blue technique. The positive reaction of protein to Bromophenol blue stain produces a blue hue. Bromophenol blue precipitates the protein present in the tissue and is an acidic dye that releases H+ ions, which are picked up by bipolar amino acids and converted to cations. The stain's anions react irreversibly with these cations, resulting in a rich blue color. In the control, the amino acids in the organized proteins absorb the dye, resulting in a vivid blue color. In this study, the intensity of blue hue is much lower in the experiment than in the control fish liver. In both the experiment the protein molecules get completely disorganized and the amino acids will not take up the dye. But remaining proteins take up some dye and giving light blue color. It is due to the necrosis, vaculation and rupturing of hepatocytes.

In experiments the colour production mainly contributed by some structural proteins. Other proteins are degraded. Liver has role in protein synthesis and secretion. The liver in control group has normal protein content, because the liver is normal functioning one, there were no pathological abnormalities. But in Ossein and Dicalcium phosphate treated liver protein content is very low. The histological analysis shows short term exposure of Ossein and Dicalcium phosphate can cause destruction of cells in liver, degradation of hepatocytes in close proximity to the blood vessels.

Glucose estimation in fish muscle

The effect of ossein and dicalcium phosphate on glucose content of fish muscle is given in table 2.

Table.1 The hydrological parameters of control and experiment water samples.

Parameters	**Control**	**Ossein treated sample**	**Dicalcium phosphate treated sample**
Salinity(ppt)	0.729	1.286	9.764
Dissolved oxygen(ppm)	8	4.3	4.6
Dissolved carbon dioxide(ppm)	5.06	12.8	13.7
pH	7.5	6.5	9

Table.2 Glucose content after ossein and dicalcium phosphate exposure.

Group	**MEAN±SD (mg/g tissue)**
Control	0.011±0.0089
Ossein treated sample	0.156±0.606
Dicalcium phosphate treated sample	0.198±0.322

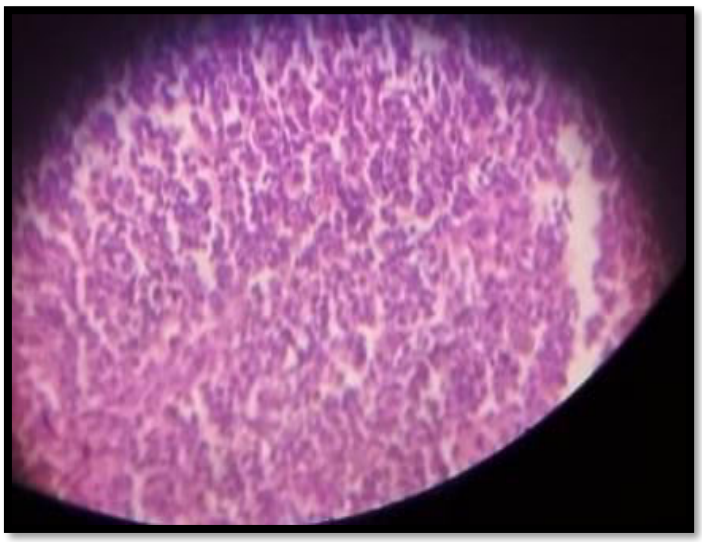

Figure 1. Liver *Oreochromis mossambicus* of control group showing liver histology

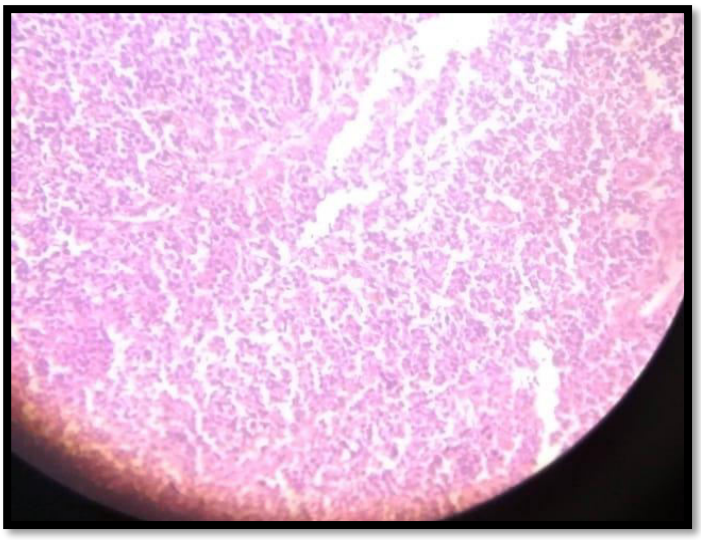

Figure 2. Liver of *Oreochromis mossambicus* of experiment group (Ossein treated) showing histopathological anomalies.

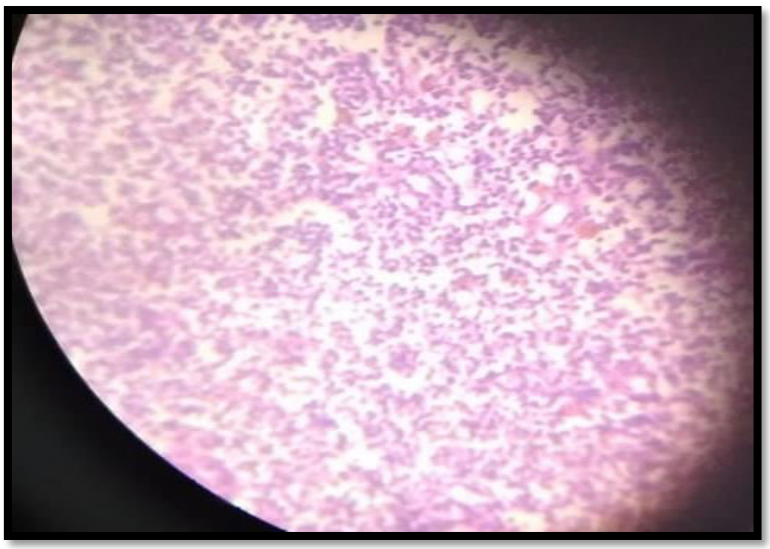

Figure 3. Liver of *Oreochromis mossambicus* of experiment group (dicalcium phosphate treated) showing histopathological anomalies.

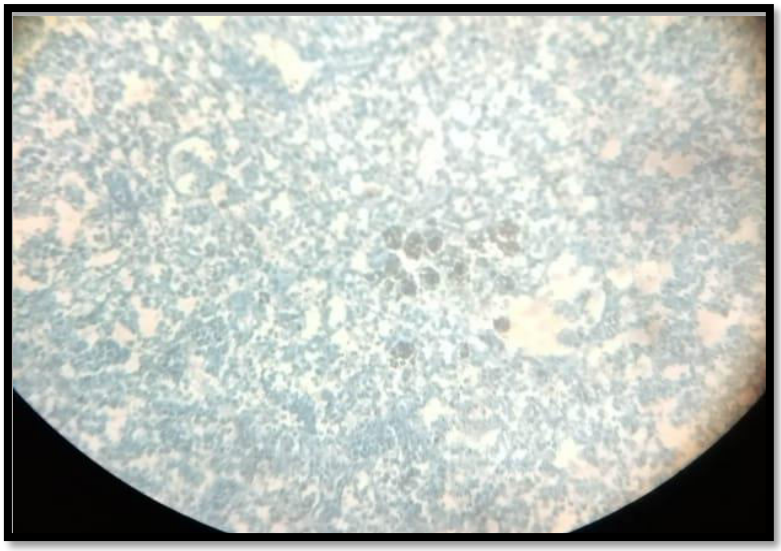

Figure 4. Histochemical analysis of protein in normal fish liver

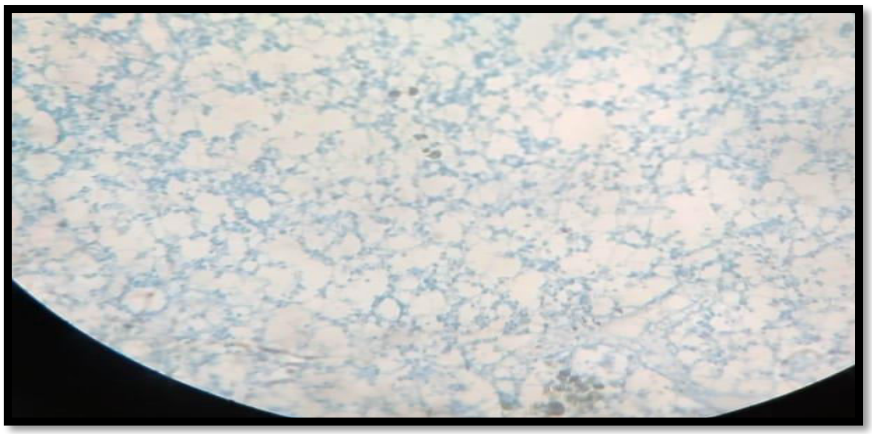

Figure 5. Histochemical analysis of protein in Ossein treated fish liver

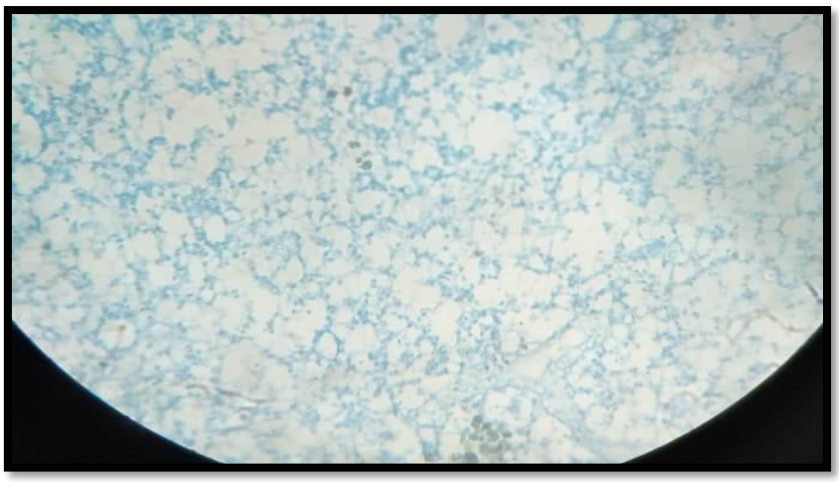

Figure 6. Histochemical analysis of protein in normal fish liver

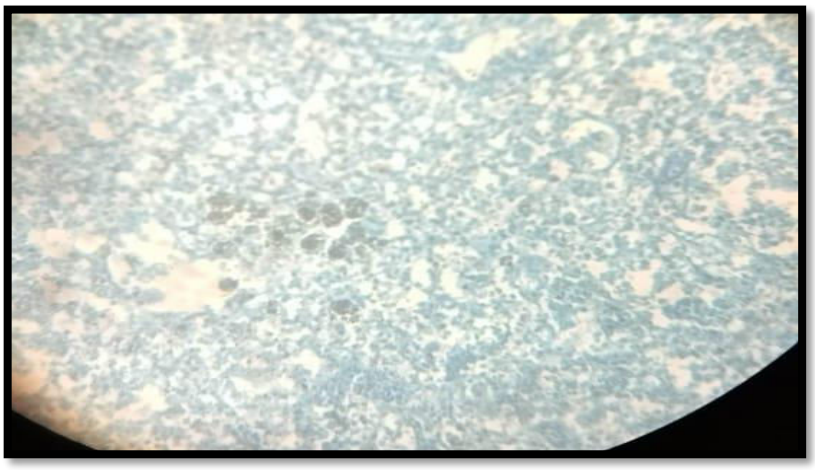

Figure 7. Histochemical analysis of protein in Dicalcium phosphate treated fish liver

Discussion

The salinity increased significantly in both treated samples, especially in the dicalcium phosphate treatment, which showed a much higher salinity (9.764 ppt) compared to both the control (0.729 ppt) and the ossein-treated sample (1.286 ppt). This rise suggests that dicalcium phosphate particularly elevates salinity, likely due to its chemical composition, which could alter the ionic balance in the water. Dissolved oxygen levels dropped significantly in both treated samples, indicating that both ossein and dicalcium phosphate has a deoxygenating effect. This reduction in DO can be stressful or even lethal for fish, as oxygen is critical for their survival and metabolic processes. The reduction in oxygen could be due to increased biological demand for oxygen in breaking down the pollutants. Both treated samples exhibited elevated CO_2 levels, with the dicalcium phosphate-treated sample showing the highest concentration. The increased CO_2 could result from the decomposition of organic materials or increased respiration due to stress. High CO_2 levels can further acidify the

water and impair oxygen exchange in fish, leading to respiratory issues. The pH levels varied considerably between the two treated samples. The ossein-treated water became more acidic (pH 6.5), which could harm fish by affecting their physiological processes. In contrast, the dicalcium phosphate-treated water became more alkaline (pH 9), indicating that it may disrupt the natural pH balance in aquatic systems. These pH shifts could be detrimental to fish health, as fish are highly sensitive to changes in water pH. The data suggests that both ossein and dicalcium phosphate significantly altered water quality, reducing dissolved oxygen, increasing dissolved CO_2, and causing notable shifts in salinity and pH. These changes could severely affect aquatic life by creating an environment that is less supportive of normal physiological functions, ultimately leading to stress, abnormal behavior, and potential mortality in fish. The findings highlight the importance of treating these waste products before release into natural water bodies to prevent ecological damage.

The control group shows the lowest concentration of glucose, indicating baseline levels in untreated fish tissues. This low concentration suggests normal physiological conditions, consistent with findings from similar studies where control groups show minimal biochemical stress indicators (Ali, 2018). Fish in the ossein-treated group exhibit a notable increase in the parameter level, with a mean of 0.156 mg/g, reflecting biochemical stress due to exposure to ossein. Previous studies show that pollutants like ossein can cause substantial biochemical alterations, disrupting normal tissue homeostasis (Kaur & Walia, 2019). The dicalcium phosphate-treated group shows an even higher concentration (0.198 mg/g) compared to both the control and ossein groups. This increase aligns with findings from studies indicating that exposure to phosphate compounds can lead to bioaccumulation and metabolic disturbances in fish, contributing to heightened biochemical stress markers in tissues (Smith, 2020). These elevated levels in the ossein and dicalcium phosphate groups suggest biochemical disruptions likely due to pollutant exposure. Both compounds seem to induce a stress response in the fish, evidenced by increased biomarker levels relative to the control. Such findings underscore the harmful effects of untreated industrial byproducts on aquatic organisms and align with broader research on pollutant-induced stress in fish (Jayanthi & Krishnamurthy, 2021).

A histopathological assay that measures the intensity of staining can be used to compare the protein content of liver cells of normal fish and fish treated with ossein and dicalcium phosphate. The histochemical study of protein in the liver offers information regarding protein breakdown. Histological alterations in the liver are often related to hepatocyte responses to toxins. These modifications have an impact on how fish liver operates normally. The current investigation found that ossein and calcium hydroxide reduced protein levels in the liver. Chlordane treatment of the freshwater fish *Labeo rohita* resulted in a considerable drop in hepatic protein content.

The exposure of liver cells to paper mill effluents resulted in a substantial reduction in total protein concentration in the cytoplasm and nucleus [15]. A reduction in hepatic protein was seen after administering n-hexane [16]. Histochemical investigation demonstrated that protein content decreased due to cellular degradation [19]. All these findings support the findings of the present study. Protein content decreased significantly in the tissue of the tannery effluent treated fish, *Cyprinus carpio*. Protein degradation was mostly caused by necrosis, degradation of the hepatic parenchyma and vacuolation of the cytoplasm [18]. A time depended reduction of protein content in the liver of *Mugil cephalus* was observed under the toxicity of carbaryl pesticide [19]. The current study reports comparable observations of protein breakdown as a result of toxicant exposure.

The histological analysis of the liver demonstrates that cellular deterioration impairs normal liver activities. Because the liver plays a key role in both the exocrine and endocrine activities. The secretions from the liver decreased, causing an abnormality in the homeostasis of the fish. As a result, the fish exhibits abnormal behavior, and the reduced movements and physical activity, leads to death. Histological investigations show that several hepatocytes in the liver of catfish exposed to a lethal dosage of fumonisin were selectively eliminated by the apoptotic process [17]. In the present study there is a drastic increase in the level of muscle glucose when exposed to ossein and dicalcium phosphate. Hypoxia causes a substantial stimulation of substrate level phosphorylation via glycolysis. When oxygen levels are low, the body switches to anaerobic metabolism, which causes end products to accumulate in the bloodstream. Endogenous glycogen serves as a primary source of fermentable fuel in fish during hypoxia exposure. Several researches found nearly identical observations to the current work. Serum glucose levels in the fish flesh rise in response to stress [20, 21, 22]. This can be attributable to a variety of causes, including a decrease in the specific activity of enzyme phosphofructokinase, lactate dehydroginase, and citrate kinase, all of which reduce glycolysis capability. Glucose concentration in fish muscle increased when fishes are exposed to 0.75, 0.06, 0.03, and 0.0 mg/l indicating an activation of anaerobic metabolism [23]. When fish are subjected to hypoxic stress, their plasma metabolites such as glucose, lactate, and fatty acids are changed [24].

Conclusion

This study investigated the effects of Ossein and Dicalcium phosphate on *Oreochromis mossambicus*. Ossein and dicalcium phosphate are intermediary compounds created during the production of gelatine from animal bone. The liver histology of the fishes exposed to ossein and dicalcium phosphate exhibits hepatocyte necrosis, clustering and cell rupturing. Histochemical study revealed the decline in protein production. Biochemical analysis of glucose in the muscle tissue shows an increase in their

concentration due to the stress they exposed. Their bioaccumulation would leads to several diseases. To ensure sustainable living and the conservation of aquatic biodiversity, reduce all forms of pollution through sustainable chemical treatment of industrial waste, the restriction of the use of persistent chemicals and the treatment of sewage in treatment plants, waste recycling, and most importantly, legislation to ensure the safe disposal of waste from factories and other enterprises.

References

1. Abdel Moneim A.M, Akahtani M. A and Elmenshway O.M (2012). Histopathological biomarkers in gills and liver of Oreochromis niloticus from polluted wetland environments, Saudi Arabia. Chemosphere 88: 1028-1035.
2. Ali, M. (2018). Biochemical stress response in fish exposed to industrial effluents. Aquatic Toxicology, 197: 73-82.
3. Almeida J. A, Novelli El, Dal PaiSilva M, Junior RA (2001). Environmental cadmium exposure and metabolic responses of the Nile Tilapia, Oreochromis niloticus. Environ pollut 114 (2): 169-175.
4. Andriana Regina Chippari-Gomes (2002). Comparative response of closely related Cichlids to graded hypoxia symposium proceedings- International congress on the biology of fish. International standard Book number ISBNI-8 94337-39-5.
5. Bansal S.K, Verma A, Gupta A. K and Dalela R.C (1979). Physiological dysfunction of the haemopoietic system in a fresh water teleost, *Labeo rohita* following chronic chlordane exposure. Part II. Alterations in certain organic components and serum electrolytes. Bull Environ Contam Toxicol 22: 674-680.
6. Barhoumi, S., Messaoudi, I., Gagnf, F and Kerkeni, A (2012). Spatial and seasonal variability of some biomarkers in *Salaria basilisca* (Pisces: Blennidae) Implication for biomonitoring in Tunisian coasts, Eco. Ind14 (1): 222-228.
7. Bedii, Cicik., Kenan, Eng (2005). The effects of Cadmium on levels of Glucose in serum and Glycogen Reserves in the liver and Muscle Tissue of *Cyprinus carpio* (L., 1758); Turk.J Vet Anim Sci (29)113-117.
8. Chowdhury, M.J., Pane, E.F., Wood, C.M (2004). Physiological effects of dietary cadmium acclimatization and water borne cadmium challenge in rainbow trout respiratory ionoregulatory and stress parameters. Comp. Biochem Physiol C Toxicol Pharmacol 139(13):163-173.
9. Figueiredo- Fernandes A, Rontainhas-Fernandes A, Rocha E and Reis, Henriques M.A (2006). Effect of paraquat on hepatic activity, liver and gonadal histology in males and females of Nile Tilapia *Oreochromis niloticus*, exposed at different temperature. Archives of Environmental Contamination and Toxicology 51(4): 626-632.
10. Goel, S.K,, Rao, G.S and Pandya, K.P (1988). Hepatotoxic effects elicited by n-hexane or n-heptane. J.Appl. Toxicol 8: 81-84.

11. Graham, J.B (1990).Ecological, evolutionary and physical factors influencing aquatic animal respiration. Am. Zool (30), 137-146.
12. Hampton, J.A., Lantz, R.C., Gold blatt, P.J., Lauren, D and Hinton, D.E (1988). Functional units in rainbow trout (*Salmo gairdneri*) liver:11 The biliary systems. Anat., Record 221:619-634.
13. Hampton, J.A., Mccuskey, P.A., Mccuskey, R and Hinton, D.E (1985). Functional units in Rainbow trout (*Salmo gairdneri*), Richardson liver: I Arrangement and histochemical properties of hepatocytes. Anat. Record213:166-175.
14. Jayanthi, M and Krishnamurthy, R (2021). Bioaccumulation and biochemical alterations in fish exposed to gelatine production wastes. Environmental Toxicology and Chemistry 40, 432-441.
15. Kaur, S and Walia, P (2019). Impact of bone- derived pollutants on the biochemical health of freshwater species. Environmental Pollution, 245, 616-623.
16. Naik, S.J.K, Devi, V.V and Piska, S (2004). Toxicity of tannery effluent on carbohydrate, protein, lipid constituents in the selected tissue of *Cyprinus Carpio* (Linnaeus). J. Aqua Biol 19 (1) :177-181.
17. Pathan, T.S., Thete, P.B., Shinde, S.E., Sonowane, D and Khillare, Y.K (2009). Histochemical changes in liver of fresh water fish, *Rasbora daniconicus*, exposed to paper mill effluent. Emir.J. Food Agric 21(2):71-78.
18. Pelgrom, S.M.G.J, Lock, R.A.C, Balm, P.H.M, Wendelaar Bonga S.E (1995). Integrated physiological response of *Oreochromis mossambicus* to sublethal copper exposure. Aquatic toxicology 32 (4): 303-320.
19. Rajene Maria Cirrascaff and Vildes Maria scusse (2008). Ultra structural and histochemical analysis of Channel cat fish (*Ictalurus punctatus*) liver treated with fumonisinB1.Brazilian archives of Biology and Technology Vol.51, No:2: 333-344.
20. Robertson, J.C and Bradley, T.M (1992). Liver Ultrastructure of juvenile Atlantic salmon (*Salmo salar*). J. morphol 211:41-54.
21. Saravanan, M., Kumar, K.P and Ramesh, M (2011). Haematological and biochemical responses of fresh water teleost fish *Cyprinus Carpio* during acute and chronic sublethal exposure to lindane. Pesticide Biochemistry and physiology 100:206-211.
22. Schar, M.I.P and Sasse, D (1985). Histochemical studies on metabolic zonation of the liver in the trout (*Salmo gairdneri*) Histochemistry 83:147-151.
23. Serafy, S.S, Abdel Hameid, N.A.H and El Daly, A.A (2009). Histological and Histochemical alterations induced by phenol exposure in *Oreochromis aureus* juveniles. Egyptian journal of Aquatic Biology and Fisheries 13(2):151-172.
24. Shivanagouda, N., Sanagouda, Bhat U.G (2013). Carbaryl Induced Changes in the Protein and Cholesterol Contents in the Liver and Muscle of Marine Benthic Fish *Mugil cephalus*. American journal of Biochemistry3(2): 29-33.
25. Shore, T.W and Jones, H.L (1989). On the structure of the vertebrate liver. J. physiol (London) 10:408 -428.

26. Smith, T. (2020). Phosphate induced metabolic disturbance in fresh water fish: A histopathological study. Journal of Fish Biology, 96, 1048-1055.
27. V.T Padmanabhan, Joseph Makkolil (2014). Contamination of the food chain by toxic metals and chemicals from a gelatine factory in Kerala, India Dol: 10. 13140 12.1. 2652.1605.
28. Val, A.L (1993). Adaptations of fishes to extreme conditions in fresh waters. In: J.E.P.W Bicudo (Ed-), the vertebrate gas transport cascade Adaptations to environment and mode of life. CRC press, Boca Raton 43-53.