



**STUDY OF THE ENZYME AND HISTOPATHOLOGICAL EFFECTS OF CHROMIUM
III CHLORIDE IN THE FRESH WATER FISH *CIRRHINUS MRIGALA***

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ABSTRACT

Metals occur naturally in the earth's crust, and their contents in the environment can vary between different regions resulting in spatial variations of back ground concentrations. With the advent of rapid industrialization, urbanization and other developmental activities, most of the water sources are becoming contaminated and causing deleterious effects on aquatic bio-systems. The aim of the present work is to study the enzyme and histopathological effects of Chromium III chloride in the fresh water fish *Cirrhinus mrigala*. The LC₅₀ at 96 hours was determined by the Probit Analysis Method. The experiment was designed to expose the fish to different sub chronic doses of Chromium III chloride. One trough served as control and four experimental troughs were maintained. Each trough was provided with 34mg/l, 36mg/l, 38mg/l and 40mg/l of chromium III chloride. Each trough contained ten fishes and the experiment was conducted in triplicate. The duration of the experiment was for 30 days. Enzymes were estimated after 30 days and the histopathology of the liver and muscle was studied. A significant decrease was observed in the levels of Alkaline phosphatase, Acid phosphatase, Lipase and Amylase. Histopathology studies showed degeneration in the liver and muscle. Most of the heavy metals are present in the edible portion of the fish. Humans may also be affected by eating fish and this can cause a few health problems.

KEY WORDS: Chromium III chloride, Probit analysis, alkaline phosphatase, acid phosphatase, Lipase, Amylase, histopathology, LC 50.

INTRODUCTION

Metals occur naturally in the earth's crust, and their contents in the environment can vary between different regions resulting in spatial variations of back ground concentrations. "Heavy metal" is a general communal term assigned to the collection of metals and metalloids having atomic density greater than 4000 kg/m³ and specific gravity greater than five (Garbarino *et al.*, 1995). They are the natural components of earth crust. Most of the heavy metals other than cadmium, mercury and lead are essential micro nutrients needed for living beings at lower concentration, but at higher concentration they can lead to severe poisoning.

With the advent of rapid industrialization, urbanization and other developmental activities, most of the water sources are becoming contaminated and causing deleterious effects on aquatic bio-systems (Salukn *et al.*, 1982; Al-Attar, 2005). Such heavy metals are of special concern because of their diversified effect on aquatic flora and fauna even at small concentrations by combining with cell organelles, macromolecules and metabolites (Berman and Lal, 1994; Vinodhini and

Narayanan, 2009). According to Forstner and Prosi (1979) the harmful effects of heavy metals are due to incomplete biological degradation.

Chromium naturally occurs in the earth's crust. Chromium is released into the environment from natural and anthropogenic sources. The largest release is from industrial discharges. Metal processing, tannery facilities, chromate production, stainless steel welding, and ferrochrome and chrome pigment production make use of chromium. Chromium is a non-biodegradable, persistent type of heavy metal and its compounds are known to have toxic potentials.

Chromium gains entry into the body through the lungs, gastrointestinal tract and to a lesser extent through skin. Inhalation is the most important route for occupational exposure, whereas non-occupational exposure occurs via ingestion of chromium-containing food and water. Chromium is very toxic by dermal and inhalation routes and causes lung cancer, nasal irritation, nasal ulcer, hypersensitivity reactions and contact dermatitis. Chromium is required by the human body to promote the

action of insulin for the utilization of sugars, proteins and fats.

Heavy metal accumulation can also be caused by the food source, possibly leading to bio-magnification, the augmentation of toxins up the food chain (Per-Arne *et al.*, 1997). Fish is at the higher level of the food chain and incorporates large quantities of metals and the accumulation depends upon the intake and elimination from the body (Nishijo *et al.*, 2004).

Abedi *et al.*, (2013) observed the enzymatic activities in common carp *Cyprinus carpio* influenced by sublethal concentrations of cadmium, lead, chromium. This study was to assess the effects of heavy metals agents on two biochemical enzyme activities, Alanine aminotransferase, Aspartate aminotransferase as well as Alkaline phosphatase and amylase.

Bioaccumulation and histopathological changes induced by toxicity of Mercury (HgCl₂) in Tilapia Fish *Oreochromis niloticus* was studied (Mohammed *et al.*, 2016; Chavan and Mulae, 2014). Effect of lead nitrate on the histopathology of the gills, liver and kidney of the fresh water fish, *Crihinnus mirgala* (Nimmy and Joseph, 2018), showed degeneration. The gills exposed to sublethal concentration of chromium showed mild histological changes. Fusion of gill lamellae, hypertrophy and degeneration of epithelium were predominant after 30 days. The aim of the present work is to study the enzyme and histopathological effects of Chromium III chloride in the fresh water fish *Cirrhinus mrigala*.

MATERIALS AND METHODS

Experimental design

The fish were acclimatized to laboratory conditions for 15 days before starting the experiment and normal fish feed were provided at 9am every day. The fishes were divided into 5 equal groups consisting of 10. Each group was transferred separately to plastic troughs. They were exposed to Lead nitrate III chloride for 96 hours with concentrations of 310mg/l, 320mg/l, 330mg/l and 340mg/l. Mortality was recorded once in every 24 hours. The LC₅₀ at 96 hours was determined by the Probit Analysis Method (Finney, 1971).

To determine the sub lethal concentration of chromium III chloride, $\frac{1}{10}^{\text{th}}$ of the concentration of LC₅₀ value for 96 hours, was taken. The experiment was designed to expose the fish to different sub chronic doses of Chromium III chloride. One trough served as control and four experimental troughs were maintained. Each trough was provided with 34mg/l, 36mg/l, 38mg/l and 40mg/l of chromium III chloride. Each trough contained ten fishes and the experiment was conducted in triplicate. The duration of the experiment was for 30 days.

Collection of fish serum

Blood samples were collected in eppendorf tubes from the gills of experimental fishes. The blood samples were incubated at room temperature for coagulation and the serum was collected. The collected serum was subjected to centrifugation for 15 minutes at 3000 rpm. The harvested serum was then stored at -4°C for assaying blood serum enzymes.

Estimation of enzymes

Acid phosphatase

Principle

α -naphthylphosphate is hydrolyzed by serum acid phosphatase to α -naphthol and inorganic phosphate. The rate of hydrolysis is proportional to the enzyme activity present. The α -naphthol produced is coupled with Fast Red TR to produce a colored complex which absorbs light at 405 nm. The reaction can be quantified photometrically because the coupling reaction is instantaneous (Hillman *et al.*, 1971).

Calculation

One international unit is defined as the amount of enzyme that catalyzes the transformation of one micromole of substrate per minute under defined conditions.

$$\frac{\Delta A/\text{Min} \times 106 \times 1.1}{12.9 \times 10^3 \times 1.0 \times 0.1} = \text{u/L} = \Delta A/\text{Min} \times 853$$

Alkaline phosphatase

Principle

The liberated phenol is measured colorimetrically in the presence of 4-aminophenazone and potassium ferricyanide (Belfield and Goldberg, 1971).

Calculation

$$\text{Enzyme activity (IU/L)} = \frac{\text{Abs}_{\text{SAMPLE}}}{\text{Abs}_{\text{STANDARD}}} \times 75$$

Amylase

Principle

α -Amylase hydrolyzes the 2-chloro-4-nitrophenyl- α -D-maltotrioside (CNPG3) to release 2-chloro-4-nitrophenol (CPNP) and form 2-chloro-4-nitrophenyl- α -D-maltoside (CNPG2), maltotriose, and glucose. The rate of formation of the 2-chloro-4-nitrophenol can be detected spectrophotometrically at 404 nm to give a direct measurement of α -amylase activity in the sample (Kennedy *et al.*, 1999).

Calculation

$$\frac{\Delta \text{Abs}/\text{min} \times \text{TV} \times 1000}{\text{MMA} \times \text{SV} \times \text{LP}} = \text{U/L } \alpha\text{-amylase in sample}$$

TV	-	Total assay volume (ml)
MMA	-	Millimolar absorptivity of 2 chloro-p-nitrophenol
SV	-	Sample volume (ml)
LP	-	Light path

Lipase**Principle**

Serum triglycerides are hydrolyzed to glycerol and free fatty acids by lipase. In the presence of ATP and glycerol kinase (GK), the glycerol is converted to glycerol-1-phosphate. The glycerol-1-phosphate is then oxidized by glycerol phosphate oxidase (GPO) to yield hydrogen peroxide. The condensation of hydrogen peroxide with 4-chlorophenol and 4-aminophenazone (4-AA) in the presence of peroxidase (POD) produces a red colored quinonimine dye which absorbs at, or near 500nm (Roy *et al.*, 1971).

Calculation

Triglycerides results are expressed as mg/dl or mmol/L.

$$\text{Triglycerides} = \frac{\text{Abs sample}}{\text{Abs standard}} \times \text{Conc. Standard}$$

Histopathological study of tissues

Liver and muscle tissue excised from fishes of the control and experimental groups were fixed with 10% formalin solution. After proper dehydration by graded alcohols, paraffin blocks were prepared and 4-5 μm thick ribbons were cut in a rotator microtome and were stained with Eosin and Haematoxylin. The histopathological changes observed were photographed.

Statistical analysis

Student 't' test, one way ANOVA and DMRT was done for all the parameters of the study using IRRISTAT version 3/93 software.

RESULTS AND DISCUSSION**Lc₅₀**

50% mortality was observed at a concentration of 340 mg/l of Chromium III chloride. The log concentration using probit analysis (Finney, 1953) is 2.53.

ENZYME PROFILE**Alkaline phosphatase**

The value of alkaline phosphatase in the control is 112 ± 3.00 . A notable decrease in alkaline phosphatase has been observed in 34mg/l, 36mg/l, 38mg/l, 40mg/l chromium III chloride treated fishes (110 ± 3.00 , 77.30 ± 3.00 , 102.17 ± 7.64 , 63.40 ± 3.00) respectively. The one way ANOVA for alkaline phosphatase in all the treated samples is significant at 1% level. DMRT for control and experimental is significant at 5% level (112.80^a , 110.40^b , 77.30^d , 102.16^c and 63.40^e).

Acid phosphatase

The value of acid phosphatase in the control is 4.00 ± 3.00 . A notable increase has been found in 36mg/l chromium III chloride treated fish (6.20 ± 3.00). Decrease in acid phosphatase has been observed in 34mg/l, 38mg/l, 40mg/l chromium III chloride treated fishes (2.10 ± 1.00 , 1.40 ± 1.00 , 1.00 ± 1.00). The one way ANOVA for acid phosphatase in all the treated samples

is significant at 1% level. DMRT for control and experimental is significant at 5% level. (4.00^b , 2.10^c , 6.20^a , 1.40^d , 1.00^e).

Amylase

The mean value of the control is 34.00mg/l. There is a significant decrease in all the experimental groups namely 34mg/l, 36mg/l, 38mg/l, 40mg/l of the chromium III chloride treated fishes. The values are 15.00 ± 5.00 ($P < 0.01$), 10.10 ± 3.00 ($P < 0.01$), 5.57 ± 4.00 ($P < 0.01$) and 3.00 ± 2.00 ($P < 0.01$). The one way ANOVA for the levels of amylase in the control and experimental is significant at 1% level. The mean comparison by DMRT is significant for all the groups at 5% level (34.00^a , 15.00^b , 10.00^c , 9.00^d and 3.00^e).

Lipase

The control value for lipase is 20.00 ± 2.00 . There is a significant decrease in all the experimental groups namely 34mg/l, 36mg/l, 38mg/l, 40mg/l of the chromium III chloride treated fishes. Decrease in lipase has been observed in (15.40 , NS), (10.10 , $P < 0.05$), (5.57 , $P < 0.01$), (4.40 , $P < 0.01$). The one way ANOVA for the levels of lipase in the control and experimental is significant at 1% level. The mean comparison by DMRT is significant for all the groups at 5% level (34.00^a , 15.00^b , 10.00^c , 9.00^d , 3.00^e).

HISTOPATHOLOGY

The histopathological examination of muscle and liver at the end of 30 days treatment of chromium III chloride at concentrations of 34mg/l, 36mg/l, 38mg/l and 40mg/l of Chromium III chloride is compared with that of control.

Muscle

The control fish shows normal appearing striated skeletal muscle bundles. In the 34mg/l treated fish, the muscle shows minimal lymphocytic infiltrate in between muscle fibres. The 36mg/l treated fish shows normal appearing muscle fibres with minimal lymphocytic infiltrate in between the muscle fibers. The 38mg/l treated fish shows necrosis of skeletal muscle fibres. The 40mg/l treated fish shows degeneration of skeletal muscle fibres.

Liver

The histology of liver tissue in the control group showed normal hepatocytes with lymphocytic infiltrate in the portal triad. The 34mg/l treated fish showed foamy degeneration of hepatocytes. The 36mg/l treated fish showed congestion of sinusoids and foamy degeneration of hepatocytes. The 38mg/l and 40mg/l treated fish showed foamy degeneration of hepatocytes.

Table 1: Percentage (%) mortality in *Cirrhinus mrigala* treated with different Concentrations of Chromium III chloride.

S.NO	NO. OF FISHES	TOXICANT CONCENTRATION mg/l	MORTALITY IN ANIMALS TREATED WITH CrCl ₃	
			96 hours	%
1	1	310	1	10%
2	10	320	1	10%
3	10	330	3	30%
4	10	340	5	50%

Table 2: LC₅₀ value of chromium III chloride and the 95% confidence limit in *Cirrhinus mrigala*.

LC ₅₀ (LOG CONCENTRATION)	95% CONFIDENCE		PROBIT EQUATION	CHI-SQUARE
	LOWER LIMIT	UPPER LIMIT		
2.53	4.72	5.15	Y= -85.94 + 35.90 X	4.56

Table 3: Enzyme analysis of *Cirrhinus mrigala* after 30 days of exposure to Chromium III chloride.

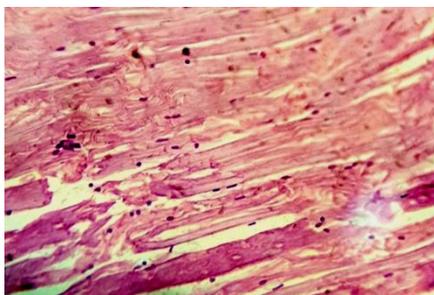
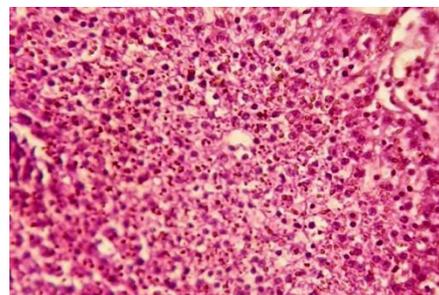
Analysis	Control	Treatment with Chromium III chloride			
		A 34mg/l	B 36mg/l	C 38mg/l	D 40mg/l
Alkaline phosphatase	112.80±3.00 ^{NS}	110±3.00 ^{NS}	77.30±3.00 ^{**}	102.17±7.64 ^{NS}	64.40±3.00 ^{**}
Acid phosphatase	4.00±1.00 ^{NS}	2.10±1.00 ^{NS}	6.20±3.00 ^{NS}	1.40±1.00 ^{NS}	1.00±1.00 ^{NS}
Amylase	34.00±3.00 ^{**}	15.00±5.00 ^{**}	10.00±3.00 ^{**}	9.00±4.00 ^{**}	3.00±2.00 ^{**}
Lipase	20.00±2.00 ^{NS}	15.40±3.00 ^{NS}	10.10±4.00 [*]	5.57±2.52 ^{**}	4.40±2.00 ^{**}

Values are mean ± standard deviation of the samples in each group; **-significant at p<0.01; *-significant at p<0.05; NS- not significant

Table 4: One way ANOVA for the enzyme analysis of the serum in the freshwater fish *Cirrhinus mrigala* treated with various concentrations of chromium III chloride.

Analysis	Df	SS	MS	F	PROB	CV%
Alkaline phosphatase	4	5703.75	1425.93	319.23	0.000 ^{**}	2.27
Acid phosphatase	4	55.77	13.94	11.62	0.002 ^{**}	37.26
Amylase	4	522.62	130.65	182.31	0.000 ^{**}	7.63
Lipase	4	1688.40	422.10	324.69	0.000 ^{**}	8.03

df- degrees of freedom; SS- Sum of Squares; MS- Mean Square; F- F test; P- Probability; CV- Coefficient of Variation; **- significant at p<0.01 level; *- significant at p<0.05.

**Fig: 1 Muscle of control fish (HE x 400)****Fig: 2 Liver of control fish (HE x400)**

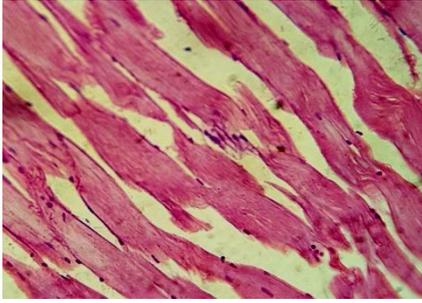


Fig 3: Muscle of fish treated with 34mg/l of Chromium III chloride (HEx400)

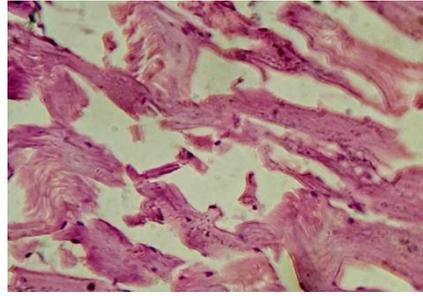


Fig 4: Muscle of fish treated with 36mg/l of Chromium III chloride (HEx400)

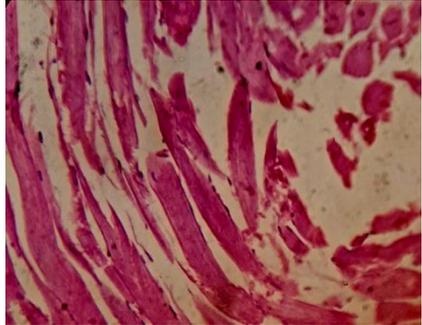


Fig 5: Muscle of fish treated 38with mg/l of Chromium III chloride (HEx400)

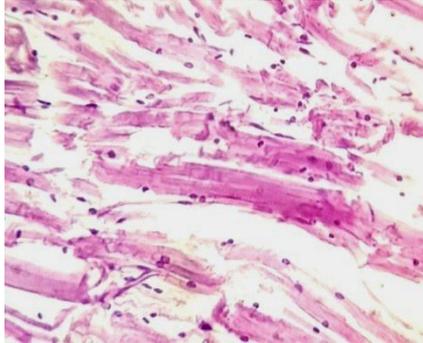


Fig 6: Muscle of fish treated 40with mg/l of Chromium III chloride (HEx400)

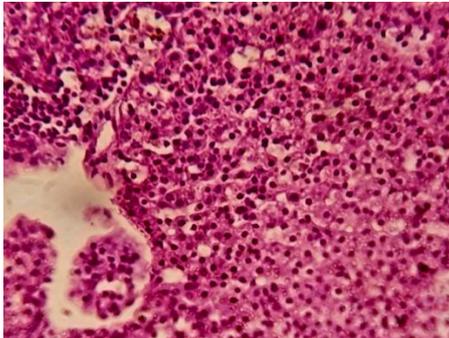


Fig 7: Liver of fish treated with 34mg/l of Chromium III chloride (HEx400)

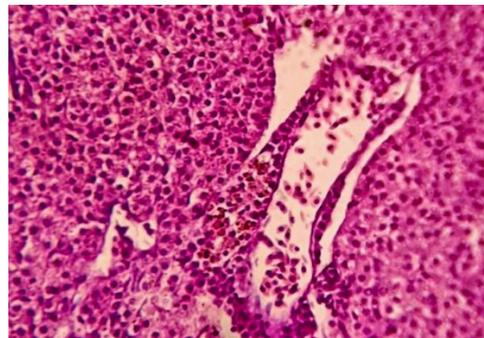


Fig 8: Liver of fish treated 36with mg/l of Chromium III chloride (HEx400)

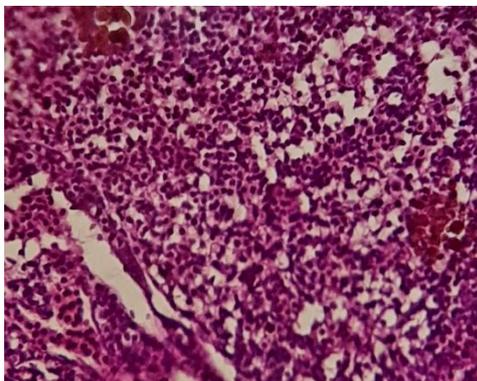


Fig 9: Liver of fish treated with 38mg/l of Chromium III chloride (HEx400)

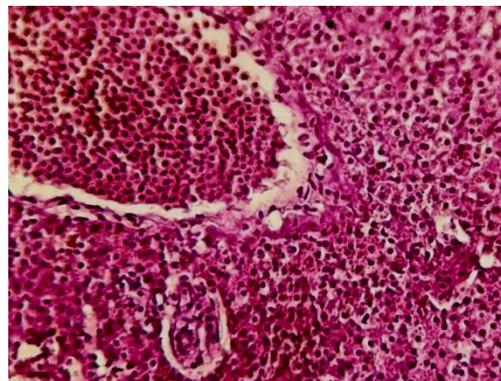


Fig 10: Liver of fish treated with 40mg/l of Chromium III chloride (HEx400)

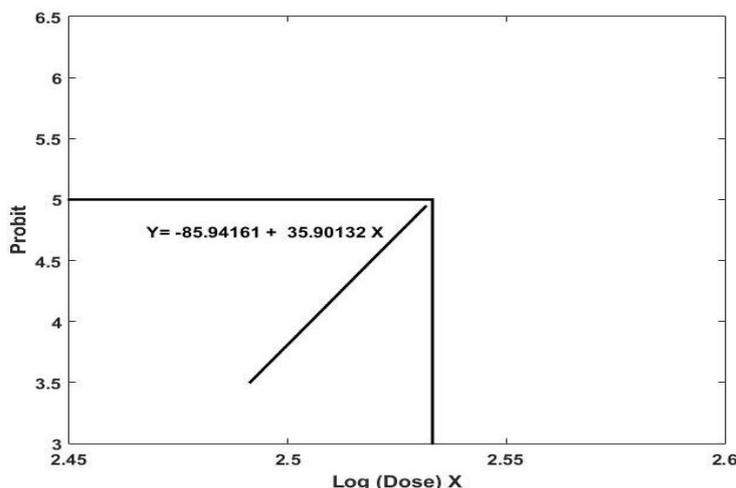


Fig 11: Regression graph showing LC₅₀ for fishes treated with different concentrations of Chromium III chloride.

LC 50

Chromium compounds cause excess mucous secretion, damage in the gill respiratory epithelium and the fish may die with symptoms of suffocation (Benoit, 1976). Sublethal effects of chromium in fish were directly related to the inhibition of various metabolic processes (Nath and Kumar, 1987). The distinction in acute toxicity may be due to alteration on water quality and trial species. Fishes that are very sensitive to the toxicity of one material may be less or even not sensitive to the toxicity of another metal in the ecosystem (Hedayat *et al.*, 2012).

ENZYME PROFILE

In toxicological studies, changes in concentration of enzyme activities often directly reflect cell and organ damage in specific organs (Casillas *et al.*, 1983).

ALKALINE AND ACID PHOSPHATASE

Alkaline phosphatase (ALP) is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, such as, nucleotides, proteins and alkaloids. It is a stress marker enzyme most effective in an alkaline environment that catalyzes the hydrolysis of phosphorous compounds and the transfer of phosphoryl groups to an acceptor molecule. The rate of catalytic activity of the enzyme is inversely proportional to the concentration of inorganic phosphate in the ambient environment (Dyhrman and Palanik, 1999).

This enzyme could serve as a good indicator of intoxication because of its sensitivity to metallic salts (Boge *et al.*, 1992). It is a lysosomal, hydrolytic enzyme with an acid pH optimum. It takes part in the dissolution of dead cells and as such serves as a good indicator of stress condition in the biological system (Gupta *et al.*, 1983; Verma *et al.*, 1984).

Abnormal levels of ALP in blood most often indicate a problem with liver, gallbladder, or bones. However, they may also indicate malnutrition, kidney cancer tumors,

intestinal issues, pancreas problems, or a serious infection. Liver is one of the main sources of ALP, but some is also made in the bones, intestines, pancreas, and kidneys.

AMYLASE AND LIPASE

Sultana and Lomte, (1997) reported decrease in amylase activity in *L. marginalis* and explained that mercury chloride and copper chloride are potent inhibitors of amylase activity. Mukke and Chinte, (2012) studied the enzyme activity in the hepatopancreas of freshwater crabs, *Barytelphusa guerini*, and observed a significant decrease in lipase activity after the exposure HgCl₂ and CuSO₄. The continuous decrease was seen in lipase activity after acute exposure of HgCl₂ and CuSO₄.

A low amylase level in blood may indicate permanent damage to the amylase producing cells in the pancreas. Decreased levels can also be due to kidney disease and toxemia. Significantly low lipase level can reveal permanent damage to pancreatic cells that produce lipase.

HISTOPATHOLOGY LIVER AND MUSCLE

Long term exposure to hexavalent chromium exhibit several alterations in behavior, physiology, cytology, histology and morphology. Decrease in antibody production and lymphocyte count, reduction in spleen weight (Arunkumar, 2006).

High doses of heavy metals cause visible external lesions such as discoloration and necrosis on the liver (Cavas *et al.*, 2005). *Oreochromis mossambicus* exposed to Cadmium showed liver alterations in the form of hyalinisation, hepatocyte vacuolation, cellular swelling and congestion of blood vessel (Dyk *et al.*, 2007). Epithelial swelling of the renal tubules and mitochondrial and endoplasmic reticulum swelling (cloudy swelling) were observed in kidney of *Dicentrarchus labrax* exposed to cadmium.

Histological examination of liver of fish treated with mercury showed loss of cellular architecture. Fabio *et al.*, (2015) showed Melano macrophage Centers in the liver and spleen. These structures were formed by macrophage aggregates, which presented pigmented material varying from pink to red and light brown to dark brown. Moreover, different levels of fibroses were observed in the liver. Liver of the fishes are sensitive to environmental contaminants because many contaminants tend to accumulate in the liver at much higher levels than in the other organs (Heath, 1995).

Sherine *et al.*, (2017) observed that liver treated with KBr showed congestion of sinusoid and diffuse lymphocytic infiltrate in the parenchyma with bile stasis, congestion, bile stasis, loss of hepatocytes and architecture, central vein dilatation and ballooning degeneration, focal dilatation of bile ducts and widening of sinusoids, bile duct proliferation and diffuse mononuclear cell infiltrate in the entire parenchyma.

Saad *et al.*, (2012) observed that fish inhabiting polluted water displayed epithelial lesions in muscle tissue that would most probably be invaded by micro-organisms which might cause severe epidermal pathology, resulting in degeneration of muscle bundles. Study on market carp corroborated by the findings of Abbas and Ali (2007), noted several histological variations such as destruction and vacuolation in the muscle cells of *Oreochromis* species, following exposure to chromium.

Fish exposed to heavy metals may reflect histological alterations where the muscles would reflect degeneration in its muscle bundles with certain focal areas of necrosis (Kaoud and El-Dahshan, 2010). Sangeetha and Deeparani (2017) observed that the exposure to sublethal concentration of CdNPs, produced marked thickening and separation of muscle bundles, haemolysis, necrosis, lesions with reduced compactness and pronounced intramuscular oedema with minor dystrophic changes.

CONCLUSION

The study concludes that the exposure of *Cirrhinus mrigala* to sublethal concentrations of chromium III chloride has significantly altered the physiological responses. The toxic responses are reflected by the enzymatic and histological changes. Most of the heavy metals are present in the edible portion of the fish. Humans may also be affected by eating fish and this can cause health problems.

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