Cadmium ion induced changes in the protein catabolism of *Oreochromis mossambicus*

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Abstract- The euryhaline fish *Oreochromis mossambicus* was exposed to three sub-lethal concentrations of Cadmium ion, for 7 days to evaluate the role of protein catabolism in fulfilling the immediate energy needs of fishes under Cadmium ion induced stress. The levels of tissue protein, free amino acid, plasma ammonia and the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and glutamate dehydrogenase (GDH) were estimated in some of the vital tissues like gills, liver, kidney, muscle and blood of *O. mossambicus*. The rates of ammonia excretion, oxygen consumption and ammonia quotient (AQ) were also estimated. The significant ($P< 0.05$) decrease in the levels of proteins concomitant with remarkable increase in the level of free amino acids, ALT, AST and GDH activities in these vital tissues of fish species elucidated the protein catabolism as one of the main mechanism of meeting out the immediate energy demand of the fishes in condition of cadmium exposure. The AQ in treated fish increased significantly ($P< 0.05$), which indicate a marked increase in the catabolism of proteins during cadmium ions induced stress.

Index Terms- Aminotransferase, Cadmium, Catabolism, Glutamate dehydrogenase, *Oreochromis mossambicus.*

I. INTRODUCTION

Heavy metals are one of the important environmental pollutant. The contamination of aquatic environments by heavy metals has become a global problem. Many estuarine and coastal aquatic environments have been sinks for industrial and agricultural effluents. Therefore, the aquatic organisms are at high risk of health. Out of the several heavy metals in the industrial waste streams, Cadmium is often used in environmental studies because it is a non-essential metal [1] and a non-degradable cumulative pollutant. It is highly toxic, widely distributed in the environment and can adversely affect organisms at relatively low concentrations [2]. Tobacco smoke is one of the most common sources of cadmium [3]. Cadmium is widely used in steel industry alloys, in batteries and in pigments used in paints, inks, plastic, and enamels [4].

Cadmium is well known for its toxic effect on aquatic organisms [5]. In fish, the heavy metals have adverse effect on growth and reproduction and cause osmoregulatory stress [6,7,8,9,10]. Cadmium-exposed fish may show skeletal deformities, alterations in several enzymatic systems, including those involved in neurotransmission, transepithelial transport and intermediate metabolism, variation of mixed function oxidase activities, abnormal swimming, changes in individual and social behaviour, and metabolic disorders [11, 12]. More recently, it has been shown that sub lethal Cadmium also causes important changes in the swimming activity of *C. carpio* in captivity [13,14]. The alterations in the metabolic rate, the excretion of ions (e.g., ammonium), respiration, food consumption, and growth rates are important among the biochemical and physiological effects by the exposure to heavy metals [15, 16, 17]. Teleost fishes use protein as the main source of energy for their metabolic processes. Amino acids provide 14 –85% of the energy requirements of teleost fish [18]. Because proteins are a major source in the metabolism of teleost fishes and heavy metals may be involved in the normal working of these molecules, it is important to study the changes in protein metabolism after metal exposure in detail.

The objective of the present study was to determine levels of total protein, free amino acids, and plasma ammonia and to investigate the response of AST, ALT, and GDH activities in tissues of *Oreochromis mossambicus* (Peters) exposed to sub lethal concentrations of cadmium ion for 7 days. In order to assess the changes caused by cadmium ion on protein catabolism, ammonia quotient, ammonia excretion and Oxygen consumption were also investigated in the study.

II. RESEARCH ELABORATIONS

Animals and Experimental Exposure

Oreochromis mossambicus (15±8 g) commonly known as Tilapia obtained from the culture ponds of Kerala University of Fisheries and Ocean Studies, Puduvyppu were acclimatized to laboratory conditions, for about one month before experiments, in 5000L tanks where a continuous gentle flow of dechlorinated tap water was maintained. The physicochemical parameters of water were estimated daily [19]. The tap water had dissolved oxygen content of 7.8 ppm, pH 7.0 ± 0.32, temperature 26 ± 3^0C, salinity 0 ppt and hardness below detectable amounts and they were fed on a commercial diet *ad libitum*.

Sublethal toxicity studies

The acclimatized fishes were sorted in to batches of six each for sub lethal toxicity studies. The bioassays were conducted in 60L tubs containing dechlorinated tap water. The *Oreochromis*

mossambicus were exposed to a concentration of 0.92, 1.84 and 3.06 mg L^{-1} cadmium ions, (equivalent to 1/10, 1/5 and 1/3 of 96 h LC50 values obtained in acute bioassays) for 7 days by addition of calculated amounts of Cd^{++} from a 1M $CdCl_2$ stock prepared in deionised water. To maintain the quality of water as well as to prevent the degradation of cadmium chloride the water as well as the toxin were replenished at every 24 h intervals along with proper provision of aeration. The fishes were fed during the experiment once in a day. The feeding was stopped 24 h prior to their sacrifice. Suitable controls were maintained to nullify any other effect that likely to affect the fish.

Sampling of Tissues

Blood was drawn from the common cardinal vein in 1ml syringe. The blood was mixed with an appropriate amount of anticoagulant like ethylene diamine tetra acetic acid (EDTA). This preparation should be mixed immediately and thoroughly to avoid clotting. The solution was then centrifuged for 5-10 minutes at 2,000 rpm. The supernatant fluid was then separated and assayed.

Another set of treated fishes were sacrificed by pithing (by damaging the brain and severing the spinal cord between the head and trunk region using a sharp needle), dissected and different organs were surgically removed and the tissues viz. gills, liver, kidney and muscle were removed from its body. The tissues were wiped thoroughly using blotting paper to remove blood and other body fluids, washed in ice-cold 0.33M sucrose, and again blotted dry. Cell free homogenates (5% homogenates of gill, liver, muscle and 1% homogenates of kidney tissues), in 0.33M cold sucrose solution were prepared using Kemi Model-KHH1 homogenizer with Teflon coated pestle followed by centrifugation at 1000g for 15minutes in a refrigerated centrifuge (REMI-C24) for carrying out biochemical assays or stored at -20^0C until further use. The homogenates were purified further as per the requirement of different methods. The spectrophotometric estimations were carried out by using Hitachi-2900 UV-Visible spectrophotometer with quartz cuvettes against the suitable blank.

Methods used for biochemical analysis

a. Estimation of protein

Protein concentrations of the sample were quantified according to Lowry et al. [20] using bovine serum albumin as standard. The soluble proteins were purified by precipitation with equal volume of 10% TCA and pellet obtained after centrifugation at 1000 rpm for 15 minutes were dissolved in 1ml of 0.1N NaOH.

b. Estimation of Free Amino Acids (Ninhydrin Positive Substances)

Total free amino acids also known as Ninhydrin positive substances were estimated by Ninhydrin method using leucine as standard [21].

c. Assay of activities of transaminases (ALT and AST)

The activities of alanine aminotransferase (ALT) (EC 2.6.1.2) and aspartate aminotransferase ((AST) (EC 2.6.1.1)) were estimated according to the method of Mohun and Cook [22]. Briefly, 0.5 ml of substrate either for ALT (0.1 M phosphate buffer, pH 7.4; 0.2 M DL- alanine; 2mM 2-oxoglutarate) or AST ((0.1 M phosphate buffer, pH 7.4; 1.0 M aspartic acid; 2mM 2-oxoglutarate) was taken and incubated in water bath at 37 ^{o}C for 3 min. Thereafter, the substrate was mixed with 0.2 ml of enzyme solution (tissue homogenate). The reaction mixture was incubated at 37 ^{o}C for 60 min (for ALT) and 30 min (for AST) with intermittent shaking. The reaction was stopped by the addition of 1 ml DNPH and was further incubated for 20 min. The intensity of the colour developed by the addition of NaOH (10 ml, 0.4 N) was monitored calorimetrically at 540 nm against the distilled water blank. The activities of ALT and AST were calculated in terms of µ moles of pyruvate liberated / min / mg protein.

d. Assay of Glutamate dehydrogenase (GDH) (EC 1.4.1.2)

For Glutamate dehydrogenase assay[23], the reaction mixture consisted of 2.1 ml phosphate buffer, 0.2 ml enzyme source, 0.1 ml NADH, 0.2ml Ammonium acetate, 0.2 ml EDTA and 0.1 ml Triton X-100. The above mixture was equilibrated at room temperature for 10 minutes. Started the reaction by adding 0.1 ml of 2-oxoglutarate, and the rate of change of extinction at 340 nm with time were noted (ε NADH-6.3 × 10^3 litres mol^{-1} cm^{-1}). The enzyme activity was calculated as micromoles of NADH oxidized / minute / mg protein.

e. Estimation of plasma ammonia

Ammonia in the serum sample was estimated using the method of Boltz and Howel [24]. One ml of deporteinized plasma was taken for the assay. The optical density was read at 625 nm against a blank. A set of standard ammonia solutions were also treated similarly. The values were expressed as micromole/L.

f. Determination of rate of Ammonia excretion by Oreochromis mossambicus

Ammonia in the sample was estimated using the method of Boltz and Howel [24]. Two litre water containing 0.92 mg/L, 1.84mg/L and 3.06 mg/L cadmium chloride each was taken in separate tanks. A tank containing tap water served as the control. One *O. mossambicus* with known weight was introduced to each tank. Immediately after the exposure, 1 ml of water sample was taken from each tank to determine the initial ammonia content in water. Samples were taken from each tank after a period of 1 hour and the final ammonia content in water was assayed. A set of standard ammonia solutions were also treated similarly.

g. Determination of rate of Oxygen consumption by Oreochromis mossambicus

Two-litre water containing 0.92 mg/l, 1.84mg/l and 3.06 mg/l cadmium chloride each was taken in separate tanks. A tank containing tap water served as the control. Each *O. mossambicus* with known weight were introduced in to the tanks. An even layer of liquid paraffin was poured over the water in the tanks to prevent further dissolution of atmospheric oxygen in to it. Immediately siphon water from each tank to the DO bottles taking all precautions to reduce contact of water with air to a minimum. Samples were taken again from each tank after one hour. The oxygen concentration in the sample was estimated using the Winkler's method.The experiment was repeated until concordant values were obtained.

Statistical Analysis

The statistical analysis was carried out using the software SPSS 13.0 package. One- way ANOVA followed by Tukey's test was carried out for the comparison between different

concentrations in each tissue and for determining the significant difference between different concentrations of toxin in plasma ammonia levels, ammonia excretion rates and oxygen consumption rates of the fish. Significance level (P-value) was set at 0.05 in all tests.

III. RESULTS

Cadmium ion induced alteration in the levels of Tissue protein

The results shown in Fig 1 demonstrate significant decrease in the levels of total protein in all studied organs of the fish in response to the treatment of cadmium ions compared to control group.

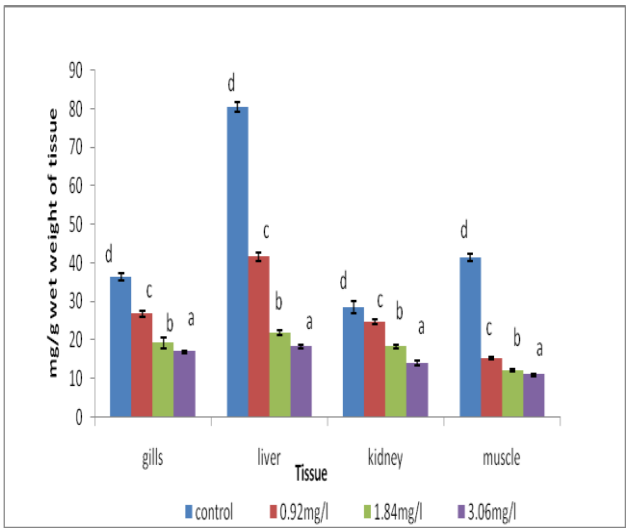

Figure 1: Protein content in the various tissues exposed to different concentrations of Cadmium chloride. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA)

Alterations in the levels of free amino acid on cadmium treatment

The levels of free amino acid were found to exhibit high degree of alteration in all studied organs on cadmium treatment (Fig 2). The levels of free amino acids were observed to show significant increase in all concentrations of cadmium ion. Maximum increase in free amino acids was observed in liver followed by kidney, muscle and gills

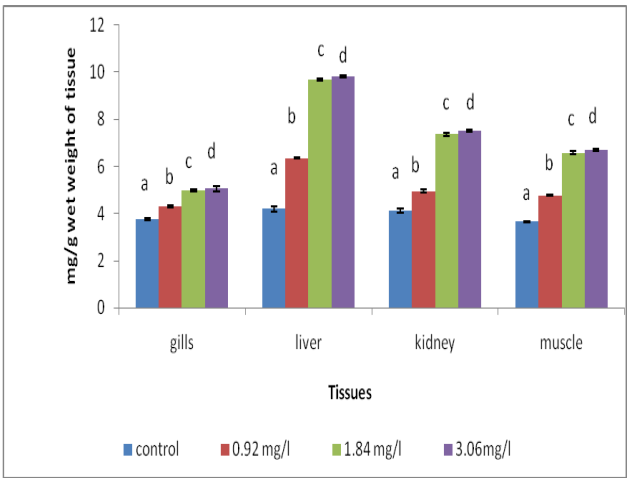

Figure 2: Free Amino acid content in the various tissues exposed to different concentrations of Cadmium chloride. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA)

Cadmium ion induced changes in transamination and oxidative deamination.

The results illustrated in Fig 3 show significant increase in the activity of alanine amino transferase (ALT) in gills, liver, kidney and muscle of the fish on treatment with cadmium ions. The maximum increase was seen in kidney followed by liver.

The data showed in Fig 4 display remarkable enhancement in the activity of aspartate aminotransferase (AST) in gills, liver, kidney and muscle of both fish species in response to cadmium. The maximum increase was observed in liver followed by kidney.

The results depicted in Fig 5 demonstrate that cadmium ions were able to cause notable variations in the activity of glutamate dehydrogenase (GDH) in liver, muscle and kidney of the fish). A statistically significant increase in Glutamate dehydrogenase (P<0.05) was observed in liver, kidney and muscle of the treated groups compared to control. No significant variation was observed in gills of the treated groups compared to control.

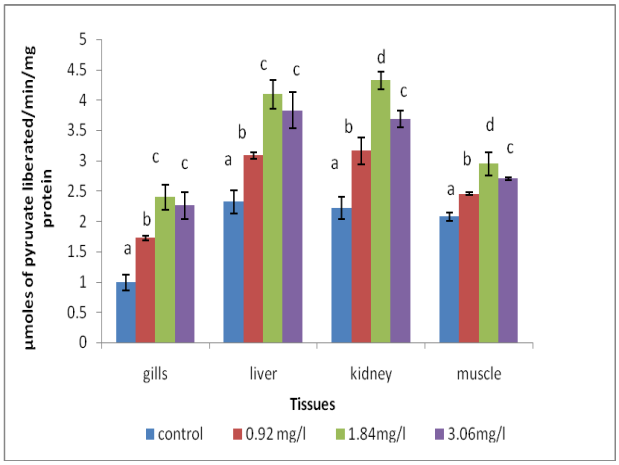

Figure 3: Effect of different concentrations of Cadmium chloride on the ALT activity of various tissues of *O.*

mossambicus. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA).

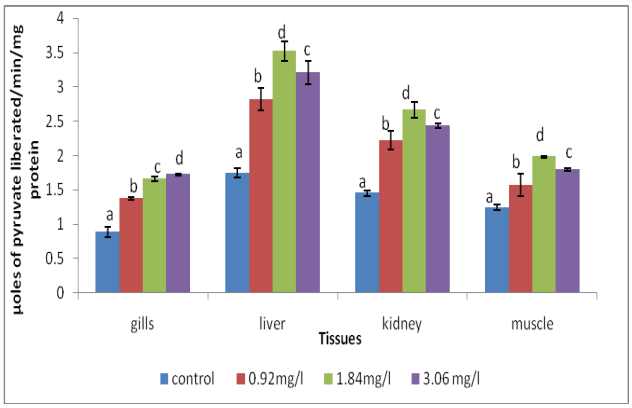

Figure 4: AST activity in the various tissues exposed to different concentrations of Cadmium chloride. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA).

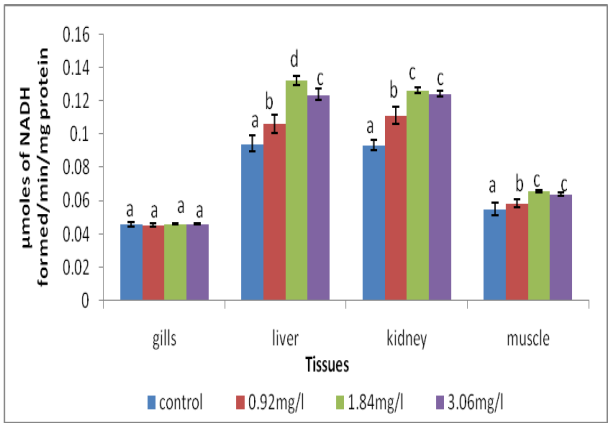

Figure 5: GDH activity in the various tissues exposed to different concentrations of Cadmium chloride. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA)

Effect of cadmium ions on the levels of plasma ammonia

The results of effect of cadmium ions on the level of plasma ammonia in fish are presented in Fig 6. One-way ANOVA followed by Tukey's test showed that there was significant (P<0.05) increase in the plasma ammonia in all the treated groups compared to control.

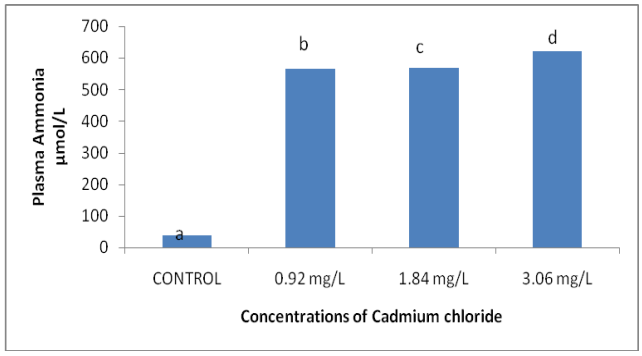

Figure 6: Levels of Ammonia in the plasma of O. *mossambicus* exposed to different concentrations of Cadmium chloride. Each bar diagram represents mean ± S.D. On each bar, values with different lower case letters vary significantly (P<0.05) (One-way ANOVA).

Effect of cadmium ions on the rates of ammonia excretion

O. mossambicus exposed to varying sub lethal concentration of Cadmium chloride exhibited no significant (P<0.05) variations in the rates of excretion of ammonia compared to control (Fig 7). One-way ANOVA followed by Tukey's test has been carried out to ascertain the statement

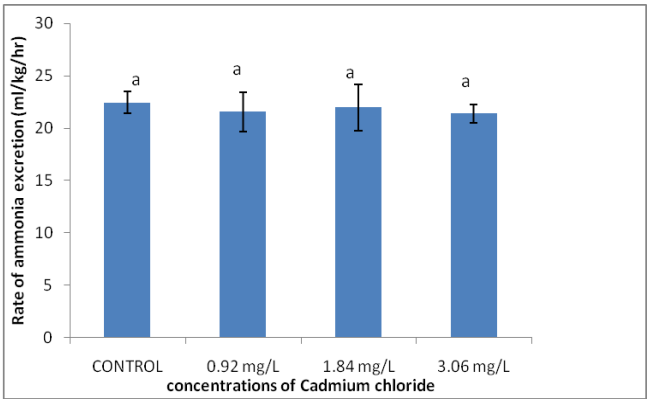

Figure 7: Rates of ammonia excretion by *Oreochromis mossambicus* exposed to different concentrations of Cadmium chloride. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA)

Effect of cadmium ions on the rates of oxygen consumption

One-way ANOVA followed by Tukey's test showed that there was significant decrease (p<0.05) in the rate of Oxygen consumption of fish treated with cadmium chloride compared to control group (Fig 8)

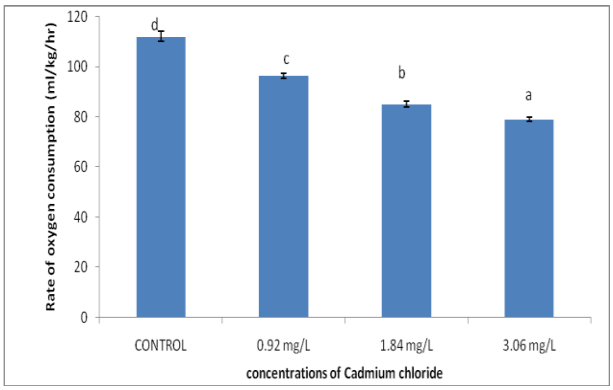

Figure 8: Rates of oxygen consumption by *Oreochromis mossambicus* exposed to different concentrations of Cadmium chloride. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA)

Effect of cadmium ions on the ammonia quotient

O. mossambicus exposed to varying sub lethal concentration of Cadmium chloride exhibited significant (P<0.05) increase in the Ammonia quotient (Fig 9). One-way ANOVA followed by Tukey's test has been carried out to ascertain the statement

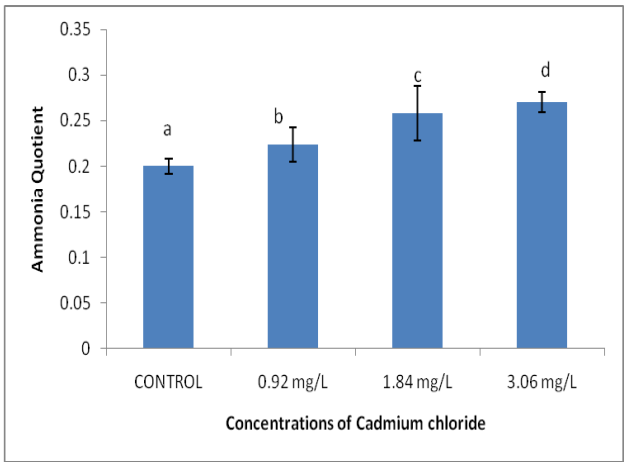

Figure 9: The Ammonia quotient of *Oreochromis mossambicus* exposed to different concentrations of Cadmium chloride. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA).

IV. Discussion

The present study demonstrated that the fish *Oreochromis mossambicus* exposed to sub-lethal concentrations of cadmium ion (0.92 mg/l, 1.84 mg/l, and 3.06 mg/l) for 7 days displayed a significant decrease (p<0.05) in the level of protein in the gills, liver, kidney and muscle than the control. Proteins in an animal are being constantly degraded and re synthesized from the free amino acid pool in tissue. A dynamic steady state always prevails between these two opposite processes of protein catabolism and anabolism. During stress conditions, the balance between anabolism and catabolism will be impaired. The metabolism move towards more catabolic state and the tissue protein may undergo proteolysis. Reduction in the protein content in the tissues suggests its increased degradation in to amino acids. The increased free amino acid pool [25] can be used for ATP production by transamination reactions or gluconeogenic pathway. Tissue protein content has been suggested as an indicator of xenobiotic-induced stress in aquatic organisms [26]. Meena Kumari et al. [27] observed a decrease in protein level in *Labeo rohita* treated with copper. A decrease in the protein content was also found in the hepatopancreas of edible crab *Scylla serrata* exposed to cadmium and the gills, liver, kidney, muscle, and intestine of the common carp exposed to mercury [28, 29].

Significant increases in the level of free amino acids were observed when *Oreochromis mossambicus* exposed to cadmium ions. Among the organs studied liver showed highest increase because it is the major site of amino acid catabolism. The decline in total protein content and the simultaneous increase in free amino acid in the tissues studied indicate the activation of protein catabolism to counteract the cadmium chloride induced toxic stress. The free amino acids are mobilized in order to cope with the extra energy demands under stress conditions [30]. The increased protein breakdown is a functional response to deal with the extra energy requirements to cope with Cadmium stress [29]. De Smet and Blust [30] also observed similar increase in free amino acids in common carp *Cyprinus carpio* exposed to cadmium.

The aminotransferases are known to play an important role in the utilization of amino acids for the oxidation and/or for gluconeogenesis [31]. while GDH, a mitochondrial enzyme, catalyzes the oxidative deamination of glutamate generating α-ketoglutarate, an important intermediate of the TCA cycle. Significant increase (p<0.05) in the activities of alanine aminotransferase and aspartate aminotransferase were observed in the tissues of *O. mossambicus* exposed to cadmium ion for 7 days. The alteration in aminotransferase activities indicates changes in energy metabolism in response to an enhanced energy demand to compensate the stress situation. De Smet and Blust [30] indicated that elevated activities of AST and ALT in liver, kidney of *Cyprinus carpio* following Cd exposure. The increased activities of the two major aminotransferases AST and ALT in fish may thereby enhance transamination for the channelling of free amino acids into the TCA cycle and/or to favour gluconeogenesis [32]. In the present study, an increased activity of GDH observed in the liver, kidney and muscle tissues may be because of an increase in glutamate oxidation, resulting in increase in ammonia production and α- ketoglutarate formation at the expense of NAD. The increase in the activity of GDH was found to be most prevalent in liver and kidney indicated significant role of these organs in the deamination. Similarly, an enhancement in the activity of GDH due to carbofuran intoxication was observed in liver and muscle tissues of *C batrachus* [33]. Kumar et al. [34] observed significant increase in the activity of GDH in *Channa punctatus* and *Clarias batrachus* on treatment with cypermethrin. The activity of gill GDH is

lower than the other tissues of fish [35,36,37,38]. The significant increase in the activities of alanine and aspartate aminotransferases and GDH could be due to incorporation of keto acids into the TCA cycle.

Fish have a remarkable capacity to use proteins as an energy source [18], and ammonia is the major end product of nitrogen metabolism. A significant elevation of plasma ammonia was observed in fish exposed to sub lethal concentrations of Cadmium ion for 7 days. The reason for this seems to be a combined effect of elevated, stress induced, ammonia production and an unchanged excretion despite an elevated plasma-to-water gradient [39,40,41,42]. An increase in ammonia production as a result of metal induced stress together with an impaired ability to excrete ammonia across the gill is the typical response to metal exposure in freshwater fish and leads to elevated plasma ammonia levels [42]. Increased ammonia production can arise from a general corticosteroid-mediated stress response that includes increased protein catabolism and gluconeogenesis [43]. No significant change in ammonia excretion was observed compared to control in *O.mossambicus* treated with Cadmium ion. The reason for this seems to be an impaired ability to excrete ammonia across the gill is the typical response to metal exposure [42]. De Boeck et al. [44] observed similar results when treated with copper in the common carp, *Cyprinus carpio*.

Oxygen consumption of aquatic animals is a very sensitive physiological process and therefore, alteration in the respiratory activity is considered as an indicator of stress of animals exposed to heavy metals. A significant decrease in the oxygen consumption was observed when *Oreochromis mossambicus* exposed to sub-lethal concentrations of cadmium ions. The decrease in oxygen consumption may be due to intimate contact with water contaminated with cadmium, which decreases the oxygen diffusing capacity of the gills. Metals may induce various disturbances in fish gills. Excessive secretion and coagulation of mucus impair gas exchange across the secondary lamellae epithelium [45, 46]. Most of the nitrogenous end products of freshwater fish originate from protein catabolism, with ammonia as the principal end product, the contribution of protein catabolism to the total energy production of the fish can be assessed by determination of the ammonia quotient (AQ = mole to mole ratio of ammonia excreted to oxygen consumed.[44,47,48,49,18]. A significant increase in the ammonia quotient was observed in *O.mossambicus* when exposed to cadmium chloride for 7 days. This was supported by De Boeck et al. [44] who also observed similar increase in AQ in common carp when treated with copper. Thus, although oxygen consumption is reduced by cadmium chloride exposure, protein catabolism appears to remain constant, or is at least less affected, and becomes relatively more important. The catabolism of proteins might be more than the measured quantity using AQ because significant accumulation of ammonia occurred in the plasma due to impaired excretion. The rate of protein breakdown is acute as evident in this study.

In conclusion, the present study illustrates the impact of Cadmium chloride on the catabolism of proteins and amino acids, in *Oreochromis mossambicus*. Proteins are known to play dominant role in accomplishing the immediate energy demand in recovering from the stress. The cadmium chloride toxicity in the fish *Oreochromis mossambicus* enhances the catabolism of proteins to handle the extra energy demand. The elevation in free amino acid content, ALT, AST, GDH and plasma ammonia along with a reduction in total protein content of tissues indicate a boost in protein catabolism. The AQ in treated fish increased significantly, which indicate a marked increase in the catabolism of proteins during cadmium ion induced stress.

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