Int. J. Environ. Res., 6(3):769-778, Summer 2012
ISSN: 1735-6865

Toxic Effects of Zinc Cyanide on Some Protein Metabolites in Fresh water fish, *Cirrhinus mrigala* (Hamilton)

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Received 20 June 2011; Revised 12 Feb. 2012; Accepted 22 Feb. 2012

ABSTRACT: Effect of zinc cyanide on protein and certain metabolites content and some enzymes activities was evaluated in liver, muscle and gill tissues of the freshwater fish, *Cirrhinus mrigala,* during exposure and following cessation of exposure. Fish exhibited significant alterations in the biochemical parameters in all tissues studied. Levels of total protein decreased in all tissues, where as free amino acids (FAA) and protease activities significantly increased ($P<0.05$). Similarly decrease was observed in the ammonia level with increased urea and glutamine levels at all exposure periods. The enzymes involved in the protein metabolism altered under the zinc cyanide intoxication. Increase in the protease and aminotransferases revealed amplified transamination processes. Significant increase of phosphatases indicated increased rate of phosphorylation and transport of molecules across the cell membrane. Withdrawal study also exhibited significant recovery in all above biochemical parameters, in all tissue after the 7th day post recovery treatment. Present study exhibited negative effects of zinc cyanide on protein metabolism. Fish with low protein content were not fit for human consumption. Patterns of effects on intermediary metabolism suggest avenues to determine the mechanisms by which such effects occur.

Key words: Protein metabolism, Enzyme activity, Zinc cyanide, *Cirrhinus mrigala*

INTRODUCTION

The aquatic environment is continuously being contaminated with toxic chemicals from industrial, agricultural and domestic activities (Ghaderi *et al.,* 2012; Mhadhbi *et al.,* 2012; Clemente *et al.,* 2012; Divis *et al.,* 2012; Ashraf *et al.,* 2012, Okuku and Peter, 2012; Ekmekyapar *et al.,* 2012). Cyanides are one of the major classes of toxic substances used on a large scale in the mining, electroplating, printed circuit board manufacturing, steel and chemical industries. Consequently, these industries, discharge large quantities of cyanide-containing liquid waste (Way, 1984). Often, such wastes also contain significant amounts of heavy metals, viz., copper, nickel, zinc, silver and iron (Mudder and Whitlock, 1984). Cyanide readily combines with most major and trace metals, property that makes it useful in extracting metals from ores (Patil and Paknikar, 2000). And because cyanide is carbon based compound, it reacts readily with other carbon-based matter, including living organisms. Owing to the highly reactive nature of cyanide ions, metal complexes of variable stability and toxicity are readily formed. Since the formation of metal cyanide complexes does not eliminate the toxicity of cyanide, these must be removed from wastewaters prior to their discharge in the environment (Patil and Paknikar, 2000).

Cyanide toxicity resulted primarily from inhibition of cytochrome oxidase, the terminal enzyme of the mitochondrial electron transport chain (Way, 1984), resulting in histoxic hypoxia and impairment of energy production. In natural waters, cyanide may affect fish populations by direct lethal or through sublethal toxic effects due to the large pollution of industrial wastes. The extent of cyanide toxicity in fish depends mainly on the rate of its detoxification in vivo (Mudder and Whitlock, 1984). Fish occupy a prominent position in the field of toxicology; in studies concerning both human and ecological health. Sublethal concentrations that do not cause death over the short term but do harm the individual, thus making it expend resources to survive in a state of altered equilibrium. Much of the toxicological interest in cyanide has been focused on its rapid lethal action; however, the most widespread problems arising from cyanide are from chronic/ sub chronic exposures (Mathangi and Namasivayam, 2000). Toxicity of cyanides to various aquatic organisms has been reviewed (Eisler, 1991; USEPA, 1984). Cyanide exposure is known to produce

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a variety of biochemical changes in animals (Way, 1984). Although it has been reported that cyanide induced some histopathological derangements in fish liver (Dixon and Leduc, 1981), the data obtained were not correlated with tissue and serum enzyme measurements. However, there are no reports on the effect of metal cyanides on the various aspects of the physiology and biochemistry of the fish. Exposure to these toxicants may cause stress in fish without necessarily leading to death. Stress response is characterized by biochemical and physiological changes which may be manifest in both acute and chronic toxicity tests (Alabaster *et al.,* 1983). The disruption of biochemical and physiological integrity is assessable by the changes in the enzyme activities in functional organs (van der Oost *et al.,* 2000). In addition to inhibition of cytochrome oxidase, biological alterations caused in fish due to the toxic stress by cyanides can be sensitively recognized as a measure of metabolism, since the metabolism integrates elements such as enzyme activity and modulation, substrate pools and physiological response.

Protein is the most important and abundant biochemical constituent present in the animal body. Understanding the metabolism of protein becomes necessary in the light changes that take place in its profile during cyanide intoxication (Okafor *et al.,* 2008). Both the protein degradation and synthesis are sensitive over a wide range of conditions and show changes to a variety of physical and chemical modulators. The physiological and biochemical alterations observed in an animal under any physiological stress can be correlated with the structural and functional changes of cellular proteins. Proteins occupy a unique position in the metabolism of cell because of the proteinaceous nature of all the enzymes which mediate at various metabolic pathways (Harper, 1979). Changes in enzymes profile are important pollution indices. It has been reported that alteration in the enzymatic activities of the fish treated with cyanide is due to increased permeability of the cell, as well as direct effect of cyanides on the tissues (Alabaster *et al.,* 1983). Transamination is one of the principal pathways for the synthesis and deamination of amino acids, enabling carbohydrate and protein metabolism during fluctuating energy demands of the fish under various adaptive conditions (Gabriel *et al.,* 2009). Maintenance of internal homeostasis through biochemical processes in the Krebs's cycle may be reflected in varying levels of the enzymes occasioned by cellular damage in the functional organs (Heath, 1991). Both aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) are stimulated are stimulated when disease process affects cell integrity and ALT is a more liver-specific enzyme (Gabriel *et al.,* 2009). The present study was carried with an aim to investigate the sublethal effects of zinc cyanide on biochemical parameters in functional tissue of *Cirrhinus mrigala*. The fish *C. mrigala* was selected for the present study because it is used as an important culture fish in India and contributes to 81.3% of inland catch along with the other major carps.

MATERIALS & METHODS

Healthy freshwater fish, *C. mrigala* (6±0.5 cm, 3±0.2 g) were collected from the State Fisheries Department, Bhadra reservoir project, Shimoga, Karnataka, and used as the test animal. These fish were kept in cement tanks (30 × 30 × 20 cm) for 15 days. The food was provided in the form of rice bran and groundnut oil cake (3:1). Dechlorinated tap water was used for acclimatization as well as for experimentation. The physico-chemical parameters of water was estimated according to APHA (2005) were as follows: temperature 25 ± 1 ºC, pH 7.2 ± 0.2 at 25ºC, dissolved oxygen 6.3 ± 0.8 mg/L, total hardness 23.4 ± 3.4 mg as $CaCO_3$/L, phosphate 0.39 ± 0.002 µg/L, salinity 0.01ppm, specific gravity 1.001 and conductivity less than 10 µS/cm. Zinc cyanide (99%) was procured from Loba chemicals Mumbai (India). To determine the 96 h LC_{50} value, fish were exposed to different concentrations of zinc cyanide. Each concentration was repeated three times with a parallel controls. The recorded mortality data were subjected to probit analysis (Finney, 1971) and the 96 h LC_{50} value of zinc cyanide to *C. mrigala* was found to be 0.343 mg/L.

A total of sixty fish were exposed to sublethal concentration of zinc cyanide for a period of 15 days, these served as exposed group. This level of exposure is one-third (0.114 mg/L) the value of 96 h LC_{50} (0.343 mg/L). After 15 days of exposure period, 15 fish from exposed group were transferred into cyanide-free water for 7 days (post-recovery period) in order to study the withdrawal effect. A group of 15 fish were kept in toxicant free water as controls. The toxicant, recovery and the control water was changed every 24 hrs. Sampling of fish from exposed, and control group were done after 5, 10 and 15 days. Five observations were made for each metabolite or enzyme and means and standard deviations were calculated. One way analysis of variance was employed to compare the differences between control and exposure group and also between control and recovery groups. In all the cases, differences were considered statistically significant at $p<0.05$ (Daniel, 1987).

After completion of treatment, fish were removed from test chamber, washed with clean water and sacrificed by a sharp blow on the head. Liver, muscle

and gill tissues were dissected and used for biochemical analysis. Metabolites examined were total protein, FAA, glutamine, urea and ammonia. Enzymes were protease, alanine aminotransaminase (E.C.2.6.1.2) (ALT), aspartate aminotransaminase (E.C. 2.6.1.1) (AST), alkaline phosphatase (ALP; E.C. 3.1.3.1), acid phosphatase (AcP; E.C. 3.1.3.2).

Total protein was estimated by the method of Lowry et al. (1951), using bovine serum albumin as standard and protein content was expressed as mg / g wet wt of tissue. FAA was estimated by using the method of Moore and Stein (1954) and values are expressed as mg of amino acid nitrogen released / g wet.wt of tissue. Urea was estimated according to Natelson (1971) and Glutamine by using the method of acid hydrolysis described by Colowick and Kaplon (1967) and the values were expressed as µm / g wet wt of tissue. Ammonia content was estimated with the Nesslers reagent as described by Bergmeyer (1965) the values were expressed as µm / g wet wt of tissue. Protease levels were estimated using the method of Davis and Smith (1955) and values were expressed as µm of amino acid nitrogen released / mg of protein/ hour. The enzymes ALT and AST were estimated by the method of Rietman and Frankel (1957) as described by Bergmeyer (1965). The enzyme activity was expressed as µmol of pyruvate formed/mg protein/h. Acid and Alkaline phosphatase were estimated by following Kind and King (1954). The enzyme assays were made after preliminary standardization regarding linearly with respect to time of incubation of enzyme concentration. In all the above cases 5% homogenate was used for the estimation.

RESULTS & DISCUSSION

Exposure to sublethal concentration of zinc cyanide caused significant alterations in the biochemical parameters of the fish *C. mrigala*. In general, the edible freshwater fish constitute one of the major sources of nutritious food for humans. The biochemical changes induced by zinc cyanide stress is due to disturbed metabolism manifested as inhibition of enzymes, retardation of growth, damage and dysfunction of the tissues. The total protein content decreased significantly in all the tissues, the maximum decrease was observed in muscle (-45.46%) on 15th day followed by liver (-39.96%) and gills (-35.13%) during zinc cyanide treatment (Fig.1). Recovery in the amount of protein was noted after 7 days in toxicant free water. Proteins are the constituents of the cell membrane, have a major role in the interactions between intra and extra cellular media and as enzyme, proteins participate in the intricately balanced sub cellular functions (Soundararajan and Veeraiyan, 2010). In fish, proteins are one of the main energy sources which play an important role in the maintenance of blood glucose. Further, under the stress conditions, the proteins consumed by the fish are not stored in the body tissues and hence the treated fish meet their extra energy requirements from body proteins which are mobilized to reduce glucose, which is made available for fish by the process of gluconeogenesis (Virk and Sharma, 2003). The decrease in protein content throughout the exposure, and gradual recovery, are similar to fish, *C. mrigala* exposed to free cyanide (Prashanth and Neelagund, 2007). The altered mobility and low content of proteins in muscles reflect a change in the rate of synthesis and degradation of protein, lowered working capacity under the impact of stress (Arai, 1974).

The FAA pool was increased significantly in the tissues of the fish during exposure to Zinc cyanide (Fig 1). Maximum increase was observed on the 10th of exposure period. Among the tissues, gills showed maximum increase in the FAA (76.78%), followed by liver (71.79%) and muscle (65.07%). Elevated FAA levels observed in the present study might be due to increased energy production by supplying them as keto acids into TCA cycle through aminotransferases to contribute energy needs during toxic stress (Sambasiva Rao, 1999). The hike in the FAA level has functional relevance for meeting energy demands and is involved in osmoregulation as well (Prashanth and Neelagund, 2007). The enhanced FAA may be due to depletion of reserved glycogen, so the fish can try to yield metabolic energy by gluconeogenesis process (Naveed *et al.*, 2010). Similar findings were observed by Vijuen and Steyn (2003) in various animals during different toxic conditions. The lowering of proteins and elevation of FAA are apparently inter-related and are indicative of metabolic utilization driving a possible source of energy to meet the energy demand under stress (Prashanth and Neelagund, 2007).

Protease is an enzyme that breaks the peptide bond to produce amino acids and other simpler peptides. In comparison to the control, zinc cyanide intoxication induced the highest protease activity after 15 days of exposure (Fig. 2). The increased protease activity in liver (54.72%), gill (52.47%) and muscle (49.07%) tissues was clearly reflected the breakdown of proteins. Under proteolysis, enhanced breakdown dominates over synthesis. While in the case of anabolic process, increased synthesis dominates the protein breakdown (Harper, 1979). Enhanced protease activity and decreased protein

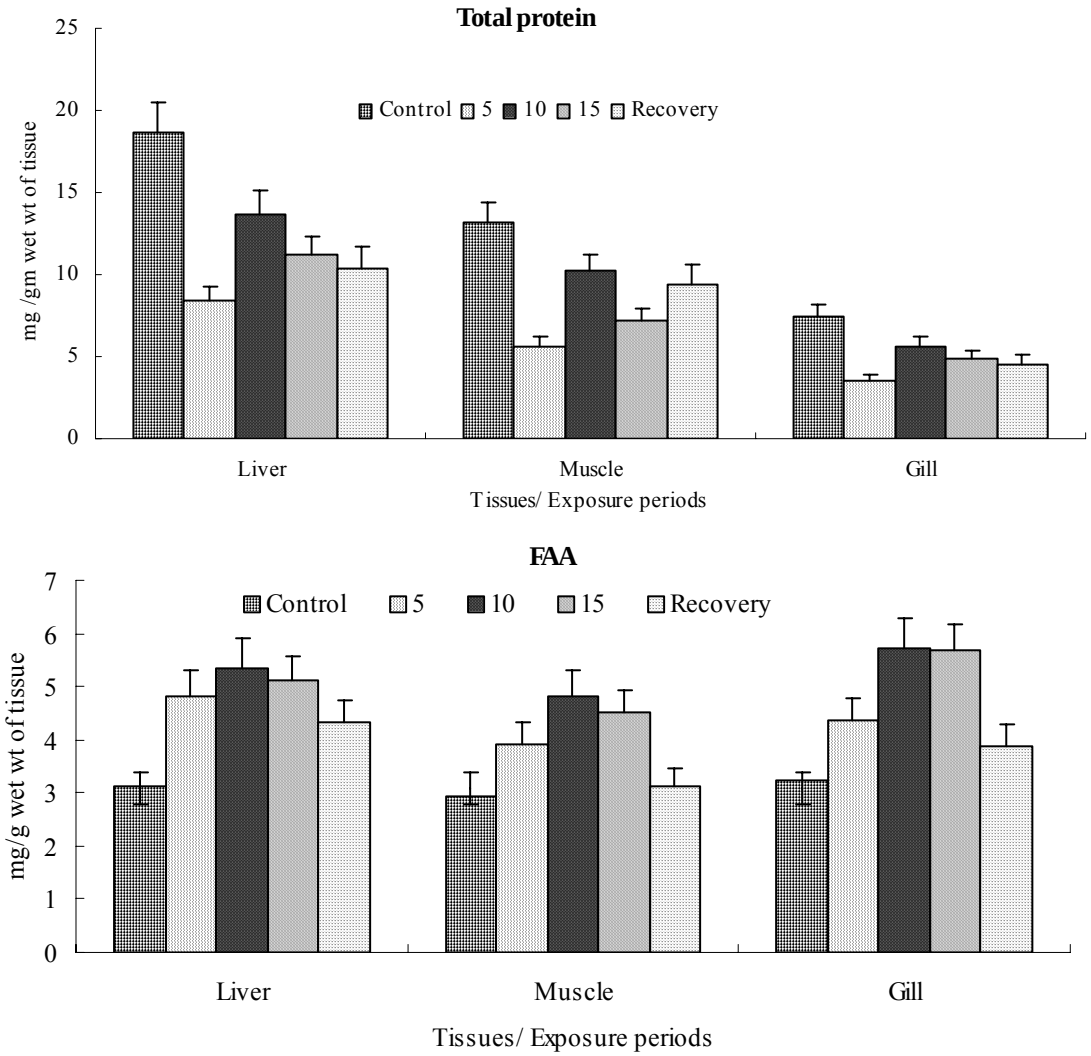

Fig. 1. Changes in total proteins, free amino acids (FAA) of *C. mrigala* after sublethal exposure to zinc cyanide. (All values are means ± SD five individual determination are statistically different at $p \leq 0.05$)

level has resulted in a marked elevation in the FAA content in all the tissue and at all time intervals (Fig. 3). Proteolytic enzymes participate in the break down of protein molecules into amino acids and these amino acids are in turn oxidized to furnish energy for body function (Saravanan *et al.*, 2010). Induction of proteolysis as a result of elevated protease activity reflecting in the decrease of the protein levels of different tissues of *C. mrigala* exposed to cypermethrin has been documented by Prashanth (2006).

Ammonia level exhibited decreasing trend in all the tissues (Fig. 2). Maximum decrease was observed in the liver (-51.94%) tissue followed by gill (-45.03%) and muscle (-40.43%). Decrease could be due to conversion of ammonia into citrulline and glutamine (Campbell, 1997). Some fish can reduce the rate of ammonia production from amino acid catabolism to slow down the build up of ammonia internally (Ip and Chew, 2010). Ammonia cannot be stored for longer period of time in the body as it leads to endogenous ammonotoxicity. In the present study decreased ammonia content in the tissues might be an indication of protein anabolism under cyanide intoxication (Prashanth and Neelagund, 2007). This decline in the ammonia content suggests that the ammonia might have been converted into urea and glutamine, which are non toxic compounds.

Urea content increased in all the tissues. Maximum increase was observed in the gill (70.21%) followed by liver (64.31%) and muscle (54.78%) (Fig.3). This increase in urea contents in all the tissues might be related to the pronounced increase of transaminases in response to continuous exposure to the toxicant. The presence of urea in extra hepatic tissues might be due to the vascular mobilization and translocation in the tissues. Although urea and

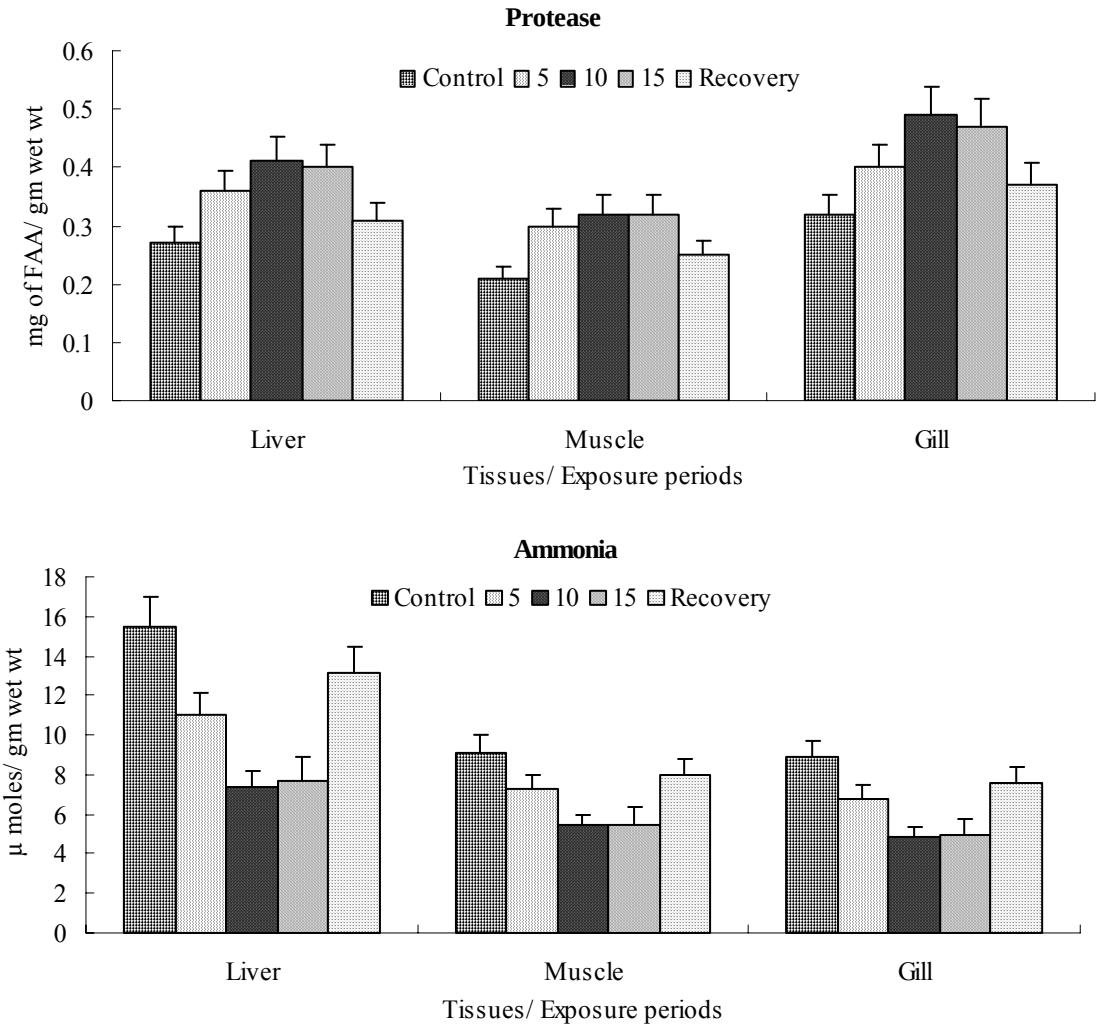

Fig. 2. Changes in Protease and ammonia of *C. mrigala* after sublethal exposure to zinc cyanide. (All values are means ± SD five individual determination are statistically different at $p \leq 0.05$)

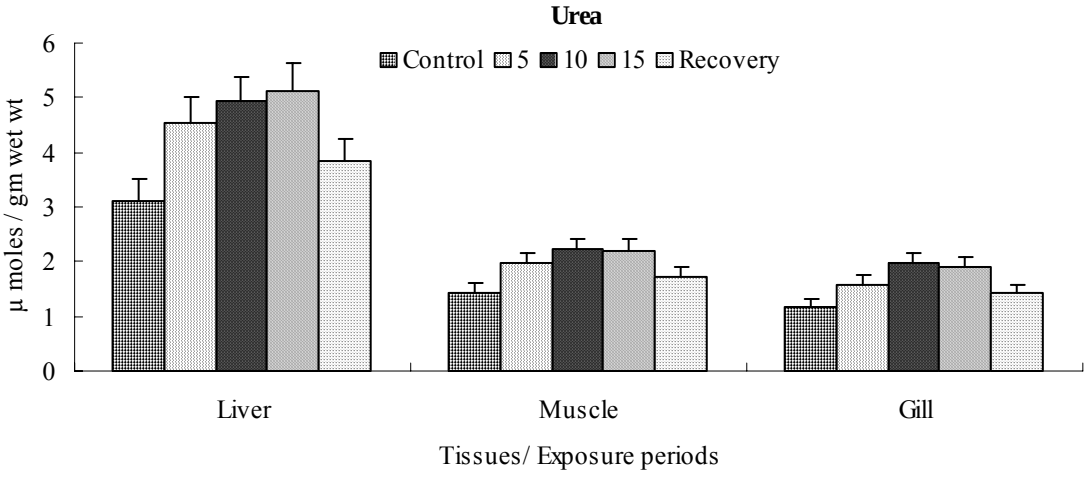

Fig. 3. Changes in total Urea and Glutamine of *C. mrigala* after sublethal exposure to zinc cyanide. (All values are means ± SD five individual determination are statistically different at $p \leq 0.05$)(Continues)

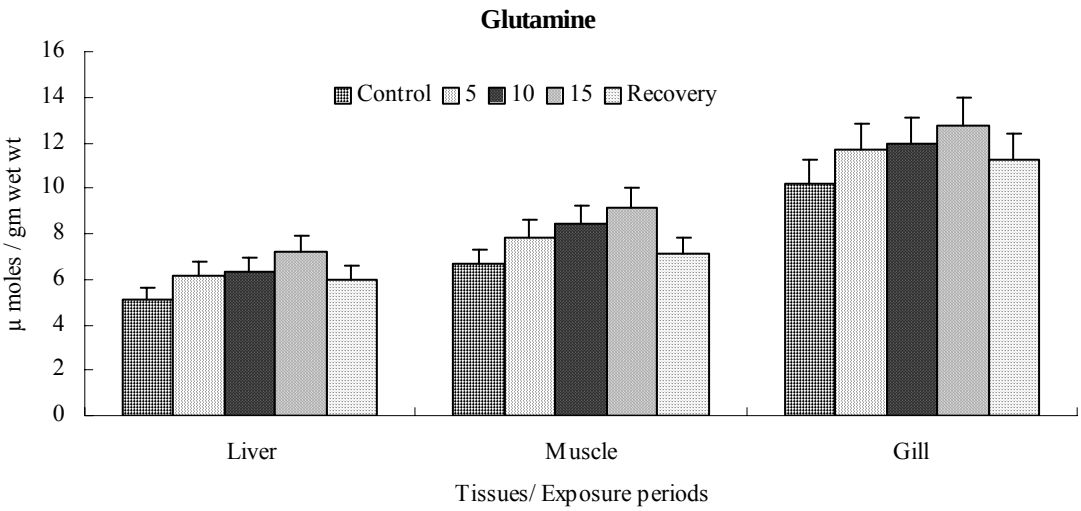

Fig. 3. Changes in total Urea and Glutamine of *C. mrigala* after sublethal exposure to zinc cyanide. (All values are means ± SD five individual determination are statistically different at $p \leq 0.05$)

glutamine were retained for the purpose of osmoregulation instead of being excreted under stress condition (Sambasiva Rao, 1999).

Glutamine is the major amino acid found in the circulatory system. Its role is to carry ammonia to and from various tissues but principally from peripheral tissues to the kidney, where the amide nitrogen is hydrolyzed by the enzyme glutaminase, this process regenerates glutamate and free ammonium ion, which is excreted in the urine (Campbell, 1997). In the present study zinc cyanide exposure produced progressive increase in the glutamine content and the increase was in the following order: liver (40.61%)> muscle (37.10%)>gill (24.52%) (Fig.3). Increased glutamine content might be due to triggering of the operation of detoxification of ammonia, chiefly by way of formation of less toxic nitrogenous substances, namely urea and glutamine (Krebs, 1980). Increase might also be due to the stimulation of glutamate synthetase activity under sublethal exposure to zinc cyanide. Increased Urea and Glutamine levels in the tissue might be due to activation of urea and TCA cycle respectively.

The activity levels of Alanine and aspartate aminotransferase enzyme exhibited transient increasing trend (Fig. 4). Both the activity was enhanced in all the tissues. Maximum increase in the ALT was observed in liver (42%), gill (40.65%) and muscle (34.81%). Similarly, AST also exhibited similar trend in the liver (50.62%), muscle (49.52%) and gills (40.93%). Similar findings were also observed by Naveed et al. (2010) in *Channa punctatus*. Increased activities of both aminotransferases indicated amplified transamination processes. An increase in transamination occurs due to amino acid input into the TCA cycle in order to cope with the energy crisis during toxicant-based stress (Venkateswara Rao, 2006). This elevation in the ALT and AST indicating the increased turnover of FAA and glutamate formation during cyanide stress in fish. The higher levels of FAA observed in the present investigation (Fig.1) support this assumption. Since the transaminases are considered as an index of gluconeogenesis, stimulation of its activity in the present study suggests increased mobilization of FAA into tissue gluconeogenesis, as reported during treatments with free cyanide (Prashanth and Neelagund, 2007). Increased FAA coupled with stimulation of transaminases also suggests increase in feeding of keto-acids into the TCA cycle due to greater availability of amino acids and participation for greater needs of energy synthesis during zinc cyanide exposure.

Phosphatase is an important constituent of many biological processes involving genetic transduction because it can regulate the proteins to which they are attached (Ogundele *et al.*, 2010). The Alkaline and Acid phosphatases were enhanced during toxic exposure period and under stress condition. AcP serves as a biochemical marker for lysosomal activity while ALP indicates membrane transport and integrity in the neuronal architecture. AcP exhibited progressive increase in its activity in all the tissues at all the exposure periods (Fig 5). Maximum increase was noticed in the muscle tissue (47.88%) followed by liver (42.49%) and gill (41.83%). Withdrawal experiment showed significant recovery in the AcP activity in all the tissues. ALP also exhibited similar trend (Fig. 5).

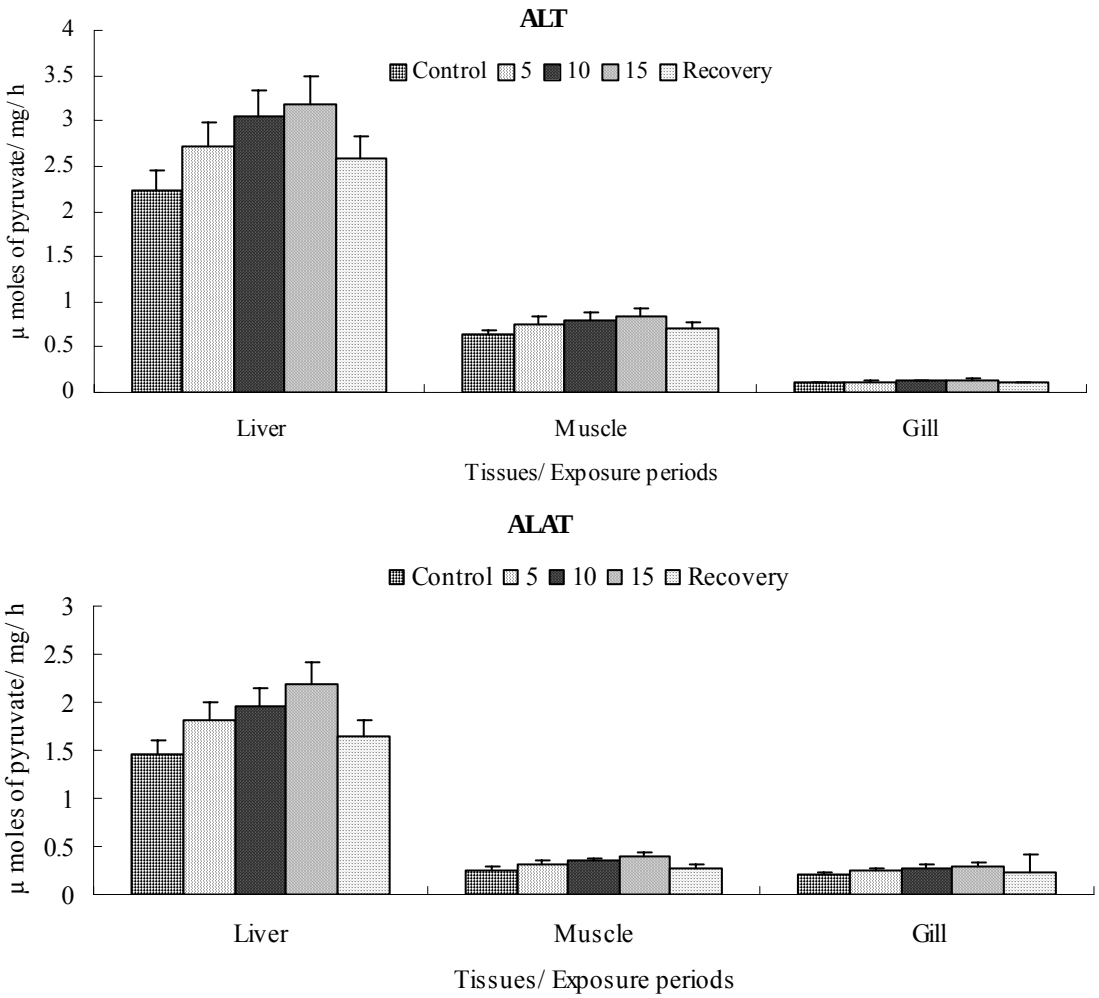

Fig. 4. Changes in ALT and ALAT activity of *C. mrigala* after sublethal exposure to zinc cyanide. (All values are means ± SD five individual determination are statistically different at $p \leq 0.05$)

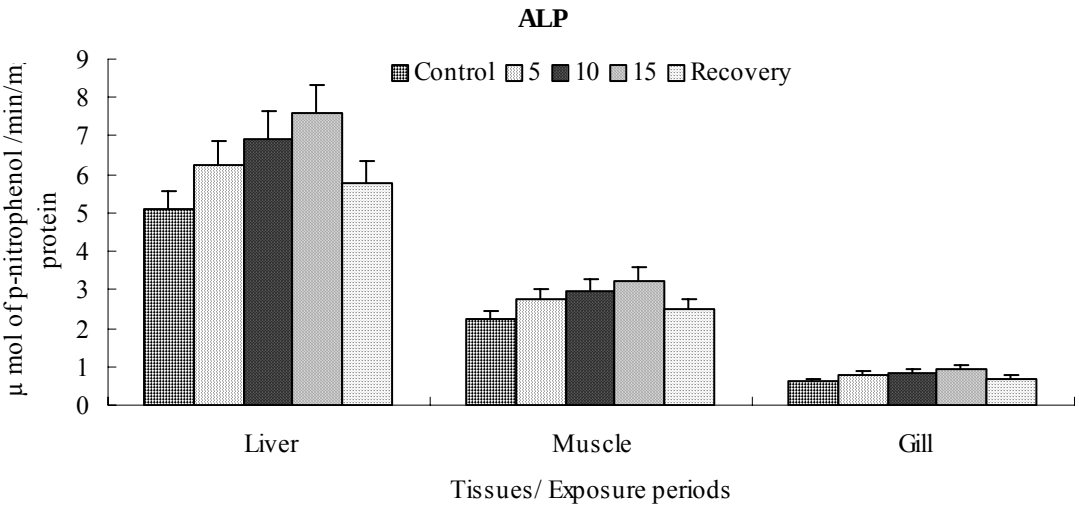

Fig. 5. Changes in ALP and ALP of *C. mrigala* after sublethal exposure to zinc cyanide. (All values are means ± SD five individual determination are statistically different at $p \leq 0.05$)

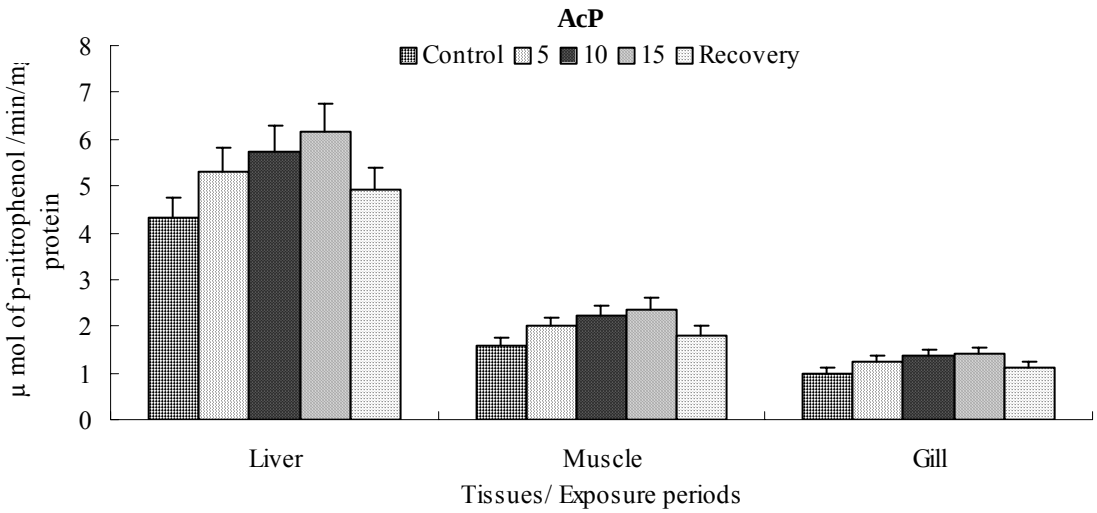

Fig. 5. Changes in ALP and ALP of *C. mrigala* after sublethal exposure to zinc cyanide. (All values are means ± SD five individual determination are statistically different at $p \leq 0.05$)(Continues)

Maximum increase was observed in the liver (49.25%), followed by gill (48.74%) and muscle (45.97%). The elevated levels of phosphatases may indicate the increase in the rate of phosphorylation and transport of molecules across the cell membrane. Since, cyanide has anti-phosphatase activity, so reduction in protein level may be due to the inhibition of alkaline phosphatase activity, as it plays an important role in protein synthesis (Pilo *et al.,* 1972). Significant increase phosphatase activity might be due to cellular damage. Similar observations were made by Naveed et al. (2010) in the fish *C. baturachus*, exposed to endosulfan and kelthan and attributed the observed changes as an indicator of hepatic tissue damage and dysfunction. As such, the fish can utilize stored proteins to overcome the toxic stress. Under toxic stress, the levels of key enzymes involved in proteins metabolism were changed.

CONCLUSION

Results of the present study show alteration in protein metabolism of the freshwater fish *C. mrigala* under sublethal exposure to zinc cyanide. The parameters recovered to near to control levels in most cases by 7 days post recover period, which is indicative of the reversibility of zinc cyanide effects within reasonable concentration limits. The recovery in all activities after cyanide-withdrawal from medium suggests that fish were apparently relieved from the biochemical effects of zinc cyanide stress within 7 days. Faster recovery may also be because of metabolic compensation. The fish seems to develop mechanisms to reduce metabolic impairment of extended period of exposure. Since the concentration of zinc cyanide (sublethal) was continuously present throughout, the decline or elevation only up to a particular length of time indicates the initial susceptibility of the fish to the metal cyanide. The trend of recovery may be attributed metabolic adjustments as a compensator measure.

ACKNOWLEDGEMENT

Authors are thankful to the University Grant Commission (UGC), New Delhi for the financial support as Major Research Project (F.No.34-459/2008 SR). We thank Kuvempu University for providing necessary infrastructural facilities to carry out the experiments. The help and support of Fishery Department BR Project, Shimoga, are gratefully acknowledged.

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