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Effect of Exposure to Sublethal Concentrations of Zinc Cyanide on Tissue ATPase Activity in the Fresh Water Fish, *Cirrhinus mrigala* (Ham)

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Abstract: The present study was conducted to evaluate the effects of a Zinc cyanide ($Zn(CN)_2$) on ATPase (Na^+-K^+-, Mg^{2+}, Ca^{2+}) activity of freshwater fish, *Cirrhinus mrigala* (Ham). Fish were exposed to two sublethal concentrations (0.114 and 0.068 mg/L) and the effects were studied in liver, muscle and gill tissues at the end of 5, 10 and 15 days of exposure. Cyanide intoxication resulted marked changes in ATPases, represented by significant decrease in the activities. Maximum inhibition was observed at higher concentration. Order of inhibition in the activity was Na^+-K^+-> Ca^{2+} > Mg^{2+} ATPase. These results imply an immediate adaptive response to the stress, demonstrating alterations in the ATPase activities in the tissues of cyanide treated fish. Present study clearly proposes that ATPase enzymes can be used as biomarkers of exposure to aquatic organisms under cyanide intoxication.

Key words: Cyanide, ATPases, Major carp, Response, Alteration

Introduction

Adenosine triphosphatases (ATPases) are complex set of enzyme systems found in invertebrates and vertebrates (Carfagna *et al.* 1996). These enzymes play a central role in physiological functions of a cell as energy transducers by coupling the chemical reactions (Takao 1985). In cells, whatever mechanisms are used to maintain viability under anoxia, ATP- reducing and ATP-utilizing reactions must be curtailed in concert so that transmembrane ionic gradients are maintained (Cotou *et al.* 2001, Begum 2011). When this balance is broken, cells inevitably become sensitive to anoxia, a process which eventually leads to inhibition of ion transport with loss of trans-membrane ion gradients and anoxic depolarization. However, if cells adapt to the new situation, a new steady state will be achieved and this include a general reduction of metabolic processes and probably a changed allocation of energy utilization (Pablo 1996). Membrane localization is the key to the physiological function of ATPases which are coupled with pumping of cations across the membranes from one intracellular component to another.

ATPases have requirement for Na^+-K^+, $Mg^{2+}$and Ca^{2+} ions for their activity and involve in the cleavage of ATP to ADP/AMP and inorganic phosphate with liberation of energy (Begum 2011). In addition to its fundamental importance to ion transport, ATPase activity could be used as an indicator of physiological changes. Osmotic regulations in freshwater fishes are intimately bound to control ionic concentration as

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well as cell and body volume (Kumosani 2004). These membrane enzymes which carry out ion transport with parallel energy production are well characterized and variation in their activity can be used to measure the toxic impact of chemicals (Cotou *et al.* 2001).

Stress response is characterized by biochemical and physiological changes in both acute and chronic toxicity tests (Tiwari, Singh, 2004). The disruption of biochemical and physiological integrity is assessable by the changes in the enzyme activities in functional organs (De La Torre *et al.* 2000). Knowledge of sublethal effects of toxic compounds on enzymatic activities is very important to delineate the health status of fish and to provide a future understanding of ecological impacts (Singh, Singh 2002). Enzymatic activities are useful 'markers' of physiological damage to organs (De La Torre *et al.* 2000, Adamu, Iloba 2008), thus are needed to be assayed in the test fish.

Aquatic vertebrates live in an intimate contact with the environment making them susceptible to pollutants. Among them, fish are the major target for the action of toxicants. Fish are largely being used for assessment of the quality of aquatic environment and as such can serve as bioindicators of environmental pollution (Prashanth, Neelagund 2007). Primary site of action of cyanide is presumed to be the central nervous system (CNS) (Okolie, Audu 2004). Cyanide inhibits the mitochondrial enzyme cytochrome oxidase in the respiratory electron transport chain of the mitochondria, impairing both oxidative metabolism and the associated process of oxidative phosphorylation (daya *et al.* 2000). Additionally, a number of other enzymatic processes are also inhibited which exacerbate the cyanide toxicity (Prashanth, Neelagund 2007). Cyanide, a metabolic inhibitor which prevents resynthesis of adenosine triphosphate (ATP) in the axon, expected to reduce the efflux of ions to a very low value (Unnisa, Devaraj 2007). The reduction of ATP has been suggested to cause disturbances in cellular metabolism, leading to histotoxic hypoxia in the fish (Begum 2011). Investigations into the effects of cyanides on fish have a diagnostic significance in evaluating the adverse effects to human health. Most studies on the effects of environmental pollutants are confined to reporting physiological changes after or either exposure to toxicants. Very little attention has been paid to study the effects of these toxicants on enzymatic parameters of freshwater fish. Therefore, the primary aim of this study was to evaluate the toxic effect of $Zn(CN)_2$ on the ATPase activities of commercially valuable freshwater fish *C. mrigala.*

Materials and methods

Test fish

Indian major carp, *C. mrigala*, were obtained from local fisheries department (BRP). Healthy fingerlings (5±0.5 cm, 2±0.2 g) were selected and acclimatized to laboratory conditions for one week before the start of the experiments. Dechlorinated tap water was used for the bioassay experiments. Water was renewed every day and a 12-12 h photoperiod was maintained during acclimatization and test periods. The physico-chemical characteristics of the water was analyzed following the methods mentioned in APHA (2005) and are found as follows, temperature 25±1 ºC, pH 7.2±0.2 at 25 ºC, dissolved oxygen 6.3±0.8 mg/L, carbon dioxide 6.3±0.4 mg/L, total hardness 23.4±3.4 mg as $CaCO_3$/L, phosphate 0.39±0.002 µg/L, salinity 0.01 ppm, specific gravity 1.001 and conductivity less than 10 µS/cm. Fish were fed regularly with commercial fish food pellets during acclimatization and test periods. No mortality was observed during the study period.

Test chemical and Experimental protocol

Zinc cyanide (97% purity) was procured from Loba chemicals Pvt. Ltd, Mumbai, India and the stock solutions was prepared by dissolving $Zn(CN)_2$ in double distilled water in standard volumetric flask. The working concentrations were prepared from this standard stock and required quantity of $Zn(CN)_2$ was drawn directly from this working solution using micropipette. Sublethal test concentration used (0.114 mg/L and 0.068 mg/L) was based on the 1/3rd and 1/5th of 96h-LC_{50} value (0.343 mg/L) for *C. mrigala* previously determined by Shwetha, Hosetti (2009). All other chemicals used were of analytical grade obtained from Merck (Mumbai). The period of exposure was a major factor that has to be considered to determine the toxic effects of $Zn(CN)_2$ on the fish. Hence, the present study was designed to study the sublethal effects on 5th, 10th and 15th days of exposure.

Fish were sacrificed to collect tissues samples at the end of each exposure periods for both the con-

centrations. Each tissue sample was crushed with the mortar and pestle and the extraction was prepared by 0.25M sucrose buffer pH 7.4 (1:10 W/V) and then centrifuged. Five percent of the tissues homogenate was used for each assay. All the process carried out at 5 ºC and the supernatant was used to determine the enzyme activities. Levels of Na^{+}-K^{+}-ATPase, Mg^{+2}-ATPase and Ca^{2+}-ATPase activities were estimated quantitatively in the liver, muscle and gill tissue of the fingerlings exposed to $Zn(CN)_2$ by determination of inorganic phosphate liberated during the hydrolysis of the substrate adenosine triphosphate at 37 ºC (Post, Sen, 1967). One unit of enzyme activity was defined as that amount which catalyzed hydrolysis of one micro mole (µM) of inorganic phosphate from ATP per minute under given assay conditions. Concentration of protein was estimated by following Lowry *et al.* (1951) using BSA as standard. Mean values and standard deviations were calculated for each test group. These results were compared to determine toxic effects by one way analysis of variance (ANOVA) and Duncan's significant difference test was used for mean separation. The statistical analysis was performed and the significance level was set at $p<0.05$ and $p<0.01$.

Results and Discussion

After 15 days of exposure to both sublethal concentrations liver, muscle and gill tissues of the test fish exhibited significant alterations in ATPase activities probably due to stress from the toxicant concentration and duration of exposure. ATPases, the membrane bound enzymes, any damage to cellular organelles due to toxicants would certainly results in decreased activity levels and same has been observed in freshwater fish, *C. mrigala* treated with $Zn(CN)_2$. Dose and time dependent inhibition of ATPase activity was observed in the present study (Table 1, 2 and 3). Though, stress is an energy demanding process and the animal mobilizes energy substrates to cope with stress metabolically (Singh, Singh 2002). It appears that enzymatic changes possibly will modify the transport across the plasma membrane. The membrane bound enzyme ATPase is involved in the uptake of certain neurotransmitters and also in active ion transports (Bhattacharya 2000).

Sublethal concentrations of cyanide had a significant ($p<0.05$) impact on the liver, muscle and gill ATPases levels of *C. mrigala*. Inhibition of ATPase activities could be expected to have metabolic or ionic effect in fishes in relation to osmoregulation (Parvez 2006). Observed changes in the levels of ATPases in *C. mrigala* could be attributed to pathological changes in tissues such as liver and gills which are involved in the exchange of ions between the fish and the surrounding water and to the reduction of Na^{+}-K^{+}-ATPase activity, which plays a central role in whole body ion regulation under toxicant exposure (Begum 2011). Previous studies (De Boeck *et al.* 2001, Monteiro *et al.* 2005) reported that the osmoregulatory disturbances associated with an increased epithelial permeability and inhibition of active ion uptake, subsequently to the decline of ATPase activity and a decrease in the number of active chloride cells.

Na^{+}-K^{+}-ATPase, are located in the cell membrane has been found to be involved in the active transport of Na^{+} and K^{+} across the cell membrane (Unnisa, Devaraj, 2007). Sodium and potassium are essential for the activity of many enzymes and have been implicated in the transport of ATP which participates in several metabolic processes. In the present study Na^{+}-K^{+}-ATPase activity in tissue decreased significantly in liver, muscle, and gills of both sublethal exposure and the decrease was duration-dependent. Maximum inhibition was in the group that was given the 0.114 mg/L $Zn(CN)_2$. Gill tissues exhibited maximum inhibition (40.78 and 32.72%), followed by liver (40.51 and 27.24%) and muscle (38.41 and 27.30%) compared to control (Table. 1). The inhibition of Na^{+}-K^{+}-ATPase in gills probably disturbs Na^{+}, K^{+} pump, resulting in erratic entry of Na^{+} into the cell along the concentration gradient and the water molecule follows along the osmotic gradient. This process may cause swelling of the cell and finally membrane ruptures (Oruc *et al.* 2002). The inhibition of this enzyme by cyanide thus prevents the buildup of high ion concentrations in the extracellular spaces resulting in obstruction of the movement of internal destructive extra ions towards the external medium via the leakage junctions (Begum 2011).

Mg^{2+}-ATPase has a unique role in energy synthesis through oxidative phosphorylation in mitochondria and presumed to be present in all types of cells (Siraj Mohiyuddin *et al.* 2010). It is responsible for trans-epithelial regulation of Mg^{2+} ions, which are essential to the integrity of the cellular membrane, intracellular cements and the stabilization of branchial permeability (Parvez *et al.* 2006). In most

Table 1. Na^+/K^+-ATPase activity (µmol Pi liberated/mg protein/h) in the tissues of *C. mrigala* fingerlings exposed to sublethal concentrations of Zinc cyanide.

Exposure periods in days							
		Sublethal 1/3rd (0.106 mg/L)			Sublethal 1/5th (0.064 mg/L)		
	Control	5	10	15	5	10	15
Liver	10.0319	8.9797**	7.9305**	5.9676**	9.2391**	7.8741**	7.2988**
% Change		-10.49	-20.95	-40.51	-7.9	-21.51	-27.24
SD	0.174	0.166	0.15	0.137	0.112	0.126	0.17
Muscle	7.8211	6.8701*	5.4036**	4.8170*	7.2086*	6.5543*	5.6856**
% Change		-12.16	-30.91	-38.41	-7.83	-16.2	-27.3
SD	0.141	0.147	0.132	0.123	0.158	0.113	0.112
Gills	12.5919	11.1569*	7.8065**	7.4567*	11.5179**	9.2391*	8.4720*
% Change		-11.4	-38	-40.78	-8.53	-26.63	-32.72
SD	0.213	0.237	0.229	0.211	0.125	0.153	0.166

Values are expressed mean±SD (n=5) and the values are significant at $p<0.05$ (**) and $p<0.01$(*)

Table 2. Mg^{2+}ATPase activity (µmol Pi liberated/mg protein/h) in the tissues of *C. mrigala* fingerlings exposed to sublethal concentrations of Zinc cyanide.

Exposure periods in days							
		Sublethal 1/3rd (0.106 mg/L)			Sublethal 1/5th (0.064 mg/L)		
	Control	5	10	15	5	10	15
Liver	2.1675	2.0193*	1.7260**	1.3650*	2.0870**	1.8726*	1.5004**
% Change		-6.84	-20.37	-37.02	-3.71	-13.6	-30.78
SD	0.096	0.089	0.099	0.054	0.102	0.091	0.06
Muscle	1.1743	1.0277**	0.9138**	0.77952*	1.0615*	0.9803**	0.8370**
% Change		-12.48	-22.18	-33.62	-9.6	-16.52	-28.72
SD	0.008	0.009	0.005	0.006	0.007	0.01	0.007
Gills	7.4898	6.7460*	5.9902*	5.2682**	6.9491*	6.4189*	5.5390*
% Change		-9.93	-20.02	-29.66	-7.22	-14.3	-26.05
SD	0.151	0.137	0.146	0.123	0.109	0.133	0.114

Values are expressed mean±SD (n=5) and the values are significant at $p<0.05$ (**) and $p<0.01$(*)

Table 3. Ca^{2+}ATPase activity (µmol Pi liberated/mg protein/h) in the tissues of *C. mrigala* fingerlings exposed to sublethal concentrations of Zinc cyanide.

Exposure periods in days							
		Sublethal 1/3rd (0.106 mg/L)			Sublethal 1/5th (0.064 mg/L)		
	Control	5	10	15	5	10	15
Liver	4.6166	3.8468*	3.3730**	2.8315*	3.9822*	3.4971**	3.1925*
% Change		-16.67	-26.94	-38.67	-13.74	-24.25	-30.85
SD	0.094	0.103	0.08	0.076	0.083	0.061	0.074
Muscle	3.4065	3.0934**	2.8182*	2.4314*	3.1724**	2.9470*	2.6748**
% Change		-9.19	-17.27	-28.62	-6.87	-13.49	-21.48
SD	0.103	0.097	0.089	0.091	0.09	0.055	0.059
Gills	7.0975	6.1030**	5.4826*	4.4560*	6.3625*	5.6743*	4.7493*
% Change		-14.01	-22.75	-37.22	-10.36	-20.05	-33.08
SD	0.132	0.12	0.125	0.105	0.1	0.108	0.104

Values are expressed mean ± SD (n=5) and the values are significant at $p<0.05$ (**) and $p<0.01$(*)

cases Mg^{2+}-ATPase is taken as an index of general ATPase activity because of its abundant distribution and dual localization in mitochondria and cytosol (Lehninger, Albert 1988). In the present study, the Mg^{2+}-ATPase activity showed progressive inhibition in liver, muscle and gill tissue. Significant inhibition was detected in Mg^{2+}-ATPase activity (Table 2), ranging from liver (37.02 and 30.78%) > muscle (33.62 and 28.72%) > gills (29.66 and 26.05%) at 1/3rd and 1/5th sublethal concentration respectively. Maximum inhibition of enzyme activity was observed in the hepatic tissue. The inhibition of Mg^{2+} ion-dependent ATPase in fish, *C. mrigala* probably might have caused a blockage in the transport of ions across the membrane and reduced synthesis of ATP production (Unnisa, Devaraj 2007). Further the decrease in Mg^{2+}-ATPase activity might be due to low operation of oxidative pathway, resulting in decreased formation of free energy and altered cellular energy metabolism (Daya *et al.* 2000). Mg^{2+}-ATPase is a phospholipids dependent enzyme (Cutkomp *et al.* 1982) and alterations in the chemical and physical characters of phospholipids would therefore alter the enzyme activity. This possibility was also supported by Begum (2011) and Cutkomp *et al.* (1982).

Table 3 represents Ca^{2+}-ATPases in the tissues of *C. mrigala* exposed to $Zn(CN)_2$. Significant inhibition was observed in all the tissues ranging from, liver (38.67%) > gills (37.22%) > muscle (28.62%) in 1/3rd and at 1/5th gills (33.08%) > liver (30.85%) > muscle (21.48%) respectively. The inhibition of Ca^{2+}-ATPase activity may be due to the inhibition of oxidative phosphorylation (Tiwari *et al.* 2002), and degradation products of lipid peroxidation on the enzyme molecule (Ardelt *et al.* 1994, Daya *et al.* 2000). Ca^{2+} in the membrane assists the cross-linking of skeletal proteins and binds to anionic sites in the lipid bilayer and alters membrane fluidity (Curry 1992). Inhibition of Na^{+}-K^{+}-ATPase activity would be expected to cause a reduction of Ca^{2+} transport out of the cell by means of the Na^{+}/Ca^{2+}exchanger or even Ca^{2+} influx (Gmaj, Murer 1988) and expected to elevate Na^{+} concentration and reduce K^{+} levels. Decrease in Ca^{2+}-ATPases has been reported by several workers (Colls, Murphy 1992, Okolie, Audu 2004). Unnisa, Devaraj (2007) observed that cyanide, specifically inhibits the activity of Ca^{2+}-ATPase in fish. Similarly, Odunuga, Adenuga (1997) observed that cyanide, specifically inhibits the activity of the brain microsomal Ca^{2+}-ATPase in rats.

In the present study, it was noticed that increase in the duration of exposure of cyanide caused decrease in the activity of ATPase enzymes in the liver, muscle and gill tissues. The inhibition disrupted ATP utilization within the synaptic area and alter the energy metabolism of the nerve terminated secondarily altering the activities of other enzymes for which ATP or ADP may be allosteric effects (Unnisa, Devaraj 2007). Thus, ATPases are very sensitive to chemical interaction and can be used as reliable biomarker for the mechanistic toxicity studies of toxicants. It has been found that increasing duration of exposure of cyanide caused decreased activity of ATPases in the tissues especially in liver and muscle. This could be due to cyanide induced effect on cell membrane because of their strong affinity for interaction with membrane lipids causing inhibition of membrane bound ATPase enzymes activity by affecting enzyme complex (Babu *et al.* 1990, Pala *et al.* 1991). The present data could be very useful in cyanide induced risk assessment. It has been observed that these noxious chemicals are more toxic to fish in comparison with other higher animals including man.

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