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In vivo changes in the activity of (gill, liver and muscle) ATPases from *Catla catla* as a response of copper cyanide intoxication

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ABSTRACT

The present study was conducted to assess the effect of complex metal cyanide (copper cyanide) on Na^+K^+, Mg^{2+} and Ca^{2+}-ATPase activity in different tissues of Indian major carp, Catla catla. Exposure of fish to two sublethal concentrations (0.253 and 0.152 mg/L) (selected on the basis of 1/3rd and 1/5th of 96 h LC_{50}) of copper cyanide for different time intervals (5, 10 and 15 d) revealed significant inhibition of enzyme activity in gill, liver and muscle tissues. Inhibition was both dose and time dependent and reversible at post recovery period. Activity of Mg^{2+}-ATPase was inhibited more than that of Na^+K^+ and Ca^{2+}-ATPase. Harmful effect of cyanide complex was reversible in 7 day post recovery period in all the tissues studied. The present study suggests that this complex cyanide alters the membrane permeability of ATPases, resulting in the breakdown of the active transport mechanism. In fact, the impairment of activity of enzymes which carry out key physiological role could cause alterations in physiology of whole organisms.

Keywords: ATPase, Metal cyanide, Inhibition, Tissues

INTRODUCTION

There has been a great deal of physiological work on the effects of cyanides in aquatic fauna. In aquatic environment, presence of cyanides in lower concentrations was found to alter the structure and function of aquatic organisms and this is more probable than their mortality [1]. Copper cyanide is an important compound for electroplating of copper and also used as a catalyst in polymerizations, and as insecticide, fungicide, and biocide in marine paints. The compound is useful reagent in organic synthesis [2]. It is ranked as one of the hazardous compounds to ecosystems and human health [3]. Although metal cyanide complexes are much less toxic than free cyanide; their dissociation releases free cyanide as well as the metal cation which can also be toxic. Even in the neutral pH range of most surface water, copper cyanide complex can dissociate sufficiently to be environmentally harmful if in high enough concentrations [4]. Stability of copper cyanide depends upon the pH of water and therefore, the potential environmental impacts and interactions (i.e. their acute or chronic effects, attenuation and re-release) can vary [5].

Even though the cyanide compounds tend to undergo fairly rapid degradation in the environment, however the organism may be exposed to sublethal concentrations for extended periods. Aquatic organisms, particularly fish, are

highly sensitive to cyanide and its toxicity to fish has been reported [6]. Sublethal effects of cyanide in fish were directly related to the inhibition of various metabolic processes [1, 4, 7-8]. Ion transport was found to be depressed by toxicants in several important osmoregulatory systems of teleost, leading to structural damage to various organs like gills, muscle and liver [9].

Adenosine triphosphatases (ATPase) is a group of enzymes that play an important role in intracellular functions and are considered to be a sensitive indicator of toxicity [10]. They hydrolyze adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and inorganic phosphate (Pi). In this process, the energy released becomes available for cation transport. ATPase, in various ion dependent forms, is a membrane-bound enzyme and responsible for the transport of ions through the membrane and thus regulates, among others, cellular volume, osmotic pressure and membrane permeability. These enzymes, especially sodium, potassium activated ATPase (Na^+K^+-ATPase), play a central role in whole body osmoregulation [8], in that they provide energy for the active transport of Na^+ and K^+ across the cell membrane also affect the transepithelial movements of cations in gills [11]. Detection of ATPase inhibition could prove to be an important index for tolerable levels of a large group of environmental contaminants [12]. Inhibition of ATPase by xenobiotics may produce adverse effects in the organism. It disrupts ATP utilization within the synaptic area and alters the energy metabolism of the nerve terminated by secondarily altering the activities of other enzymes for which ATP or ADP may have allosteric effects. Xenobiotics can also alter ATPase activity by disrupting energy-producing metabolic pathways or interacting directly with the enzyme [11].

Fish are able to uptake and retain different xenobiotics dissolved in water via active or passive processes. They can be used to detect and document pollutants released into their environment. The interest in understanding the physiological mechanisms associated with fish responding to environmental stresses has been growing [13]. Studying the biological responses to environmental chemicals through the use of biomarkers provides means to understand environmental levels of pollutants in biological terms, and more importantly, it can be used for the assessment of environmental quality in specific situations. Therefore present investigation was carried out to assess the toxic potential of complex cyanide on tissue membrane ATPase activity.

MATERIALS AND METHODS

Chemicals

Copper cyanide (97% pure) was procured from Loba chemicals limited, Mumbai, India. Other chemicals used in the study were purchased from Merck (India) limited, Mumbai.

Collection and Maintenance of fish

Indian major carp, *C. catla*, weighing 1.5±0.2 g with average size of 2±0.5 cms were collected from the Division of fish breeding, State Fisheries Department, Bhadra Reservoir Project, Shimoga, Karnataka, India Reservoir. Fish were maintained in glass aquaria under laboratory conditions for a minimum period of two weeks before experimentally used. Commercial fish food pellets (Nova. Aq. Pvt. Ltd, not less that 3% of the body weight) was provided as food, and Water was exchanged every 24 h to minimize contamination from metabolic wastes.

Experimental design

Experimental tanks were supplied with 20 L of dechlorinated tap water (temperature 27±1 ºC, pH 7.4±0.2, dissolved oxygen 6.8±0.5 mg/L, total hardness 23.4±3.4 mg as $CaCO_3$/L,). Sublethal concentrations viz., 0.253 and 0.152 mg/L were based on the 1/3rd and 1/5th of 96 h LC_{50} of copper cyanide to the fish *C. catla*. A total of 60 fishes were selected and were divided into three groups of 20 each. Fish in group I were kept in cyanide free tap water served as control. Group II and III received 0.253 and 0.152 mg/L of the toxicant. Fishes were exposed to both sublethal concentrations for three different time intervals (5, 10, and 15 d). After the completion of each exposure (5, 10 and 15 d) and recovery (7 d) period, five fish each were sacrificed and gill, muscle and liver tissues were quickly dissected out, cleaned in cold saline, blotted dry and used for the enzyme studies.

Enzyme assay

A 5% homogenate (w/v) was prepared for each tissue sample using 0.25 M sucrose prepared in 0.3 mM Tris–HCl buffer in a glass homogenizer at 4 °C. Homogenates were centrifuged at 1000 ×g at 0±4 °C for 20 min. Supernatants obtained were used for enzyme assay. Activity of Mg^{2+} Ca^{2+} and Na^+K^+-ATPase was assayed spectrophotometrically [14], with slight modification. Standard reaction mixture of 2ml for total ATPase activity contained of Tris–HCl buffer, 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, and 0.3 ml of enzyme extract. Mg^{2+}-ATPase activity was

measured in the presence of 1 mM ouabain in the above reaction mixture which is a specific inhibitor of Na^+K^+-ATPase activity. Where as for Ca^{2+}-ATPase the reaction mixture contained 5 mM of $CaCl_2$ instead of 100 mM NaCl, 20 mM KCl. After pre-incubation for 5 min at 37 °C, reaction was initiated by adding ATP (100 mM) to the reaction mixture followed by incubation for exactly 30 min at given temperature. Reaction was stopped by adding 2 ml of 10% ice-cold TCA (w/v) and centrifuged at 1000g for 10 min. Inorganic phosphate produced in the supernatant was estimated by the method of [15].

Statistical analysis

Arithmetic mean (±SD) of the ATPase activity obtained from the five replicate samples was used in the present study. Statistical evaluation between the cyanide treated and corresponding control group was analyzed by analysis of variance (ANOVA). Significant differences among the means was considered at a level of $p<0.05$.

RESULTS

Marked changes were noticed in the activity of enzymes in gills, muscle and liver. Activities of Na^+K^+, Mg^{2+} and Ca^{2+}-ATPase were inhibited on exposure of fish to both the sublethal concentrations of metal cyanide in all three tissues studied (gill, muscle and liver) (Table 1, 2 and 3). Rate of inhibition of Mg^{2+}-ATPase was higher than Na^+/K^+ and Ca^{2+}-ATPase in all tissues and the inhibition was tissue specific. The order of inhibition in the activity of Mg^{2+}-ATPase in the tissue was gill>liver>muscle, Na^+K^+-ATPase liver>muscle>gill and Ca^{2+}-ATPase liver>gill>muscle respectively. Marked inhibition was recorded in the higher concentration of cyanide exposure. Gill tissue showed maximum inhibition 52.31%, followed by liver (43.61%) and muscle (39.33%) at higher concentration. However after 7 day of post recovery, the activity levels of all the ATPases came to near control values, indicating negative effect.

Table 1: Effect of sublethal concentrations of copper cyanide on Mg^{2+}-ATPase levels (µmol Pi liberated/mg protein/h) in different tissues of *C. catla.*

	Sublethal 1/3rd (0.253 mg/L)					Sublethal 1/5th (0.152 mg/L)			
	Control	5	10	15	Recovery	5	10	15	Recovery
Gill	10.3179 [a]	8.6121 [e]	7.2173 [g]	4.9210 [i]	9.1263 [c]	8.9174 [d]	7.9611 [e]	6.4686 [h]	9.7274 [b]
% Change		-16.53	-30.05	-52.31	-11.55	-13.57	-22.84	-37.31	-5.72
SD	0.105	0.138	0.505	0.786	0.238	0.353	0.289	0.420	0.289
Muscle	3.2195 [a]	2.6643 [e]	2.2333 [h]	2.0404 [i]	2.9382 [c]	2.9131 [d]	2.5970 [f]	2.3971 [g]	3.1604 [b]
% Change		-17.24	-30.63	-36.62	-8.74	-9.52	-19.34	-25.54	-1.84
SD	0.276	0.337	0.003	0.479	0.453	0.596	0.211	0.114	0.197
Liver	4.1361 [a]	3.2681 [f]	3.3127 [e]	2.5227 [i]	3.8017 [c]	3.6115 [d]	2.9039 [h]	3.1966 [g]	3.9072 [b]
% Change		-20.99	-19.91	-39.01	-8.09	-12.68	-29.79	-22.72	-5.53
SD	0.216	0.075	0.277	0.153	0.172	0.256	0.275	0.197	0.289

Data are means ±SD (n = 5) for an organ in a row followed by the same letter are significantly different (p < 0.05) from each other according to Duncan's multiple range test.

Table 2: Effect of sublethal concentrations of copper cyanide on Na^+K^+-ATPase levels (µmol Pi liberated/mg protein/h) in different tissues of C. catla

	Sublethal 1/3rd (0.253 mg/L)					Sublethal 1/5th (0.152 mg/L)			
	Control	5	10	15	Recovery	5	10	15	Recovery
Gill	4.6080 [a]	3.8358 [d]	2.9138 [i]	3.2759 [g]	4.1987 [c]	3.7208 [e]	3.2635 [h]	3.6256 [f]	4.4673 [b]
% Change		-16.76	-36.77	-28.91	-8.88	-19.25	-29.18	-21.32	-3.05
SD	0.177	0.425	0.373	0.109	0.504	0.164	0.115	0.425	0.160
Muscle	2.6854 [a]	2.3271[e]	2.0714 [g]	1.6293 [i]	2.3895 [d]	2.4072 [c]	2.1605 [f]	1.8236 [h]	2.5983 [b]
% Change		-13.34	-22.87	-39.33	-11.02	-10.36	-19.55	-32.09	-3.24
SD	0.164	0.176	0.459	0.292	0.357	0.249	0.158	0.504	0.131
Liver	6.3921 [a]	5.2471 [e]	4.7762 [g]	3.6044 [i]	5.9213 [c]	5.5689 [d]	5.1161 [f]	4.6316 [h]	6.1355 [b]
% Change		-17.91	-25.28	-43.61	-7.37	-12.88	-19.96	-27.54	-4.01
SD	0.158	0.824	0.160	0.467	0.214	0.150	0.230	0.292	0.206

Data are means ±SD (n = 5) for an organ in a row followed by the same letter are significantly different (p < 0.05) from each other according to Duncan's multiple range test.

Table 3: Effect of sublethal concentrations of copper cyanide on Ca^{2+}-ATPase levels (µmol Pi liberated/mg protein/h) in different tissues of *C. catla*

	Sublethal 1/3rd (0.253 mg/L)					Sublethal 1/5th (0.152 mg/L)			
	Control	5	10	15	Recovery	5	10	15	Recovery
Gill	7.6147 [a]	6.2523 [e]	5.0796 [i]	5.3053 [h]	6.6851 [d]	6.7439 [c]	5.3616 [g]	5.8638 [f]	7.0606 [b]
% Change		-17.89	-33.29	-30.33	-12.21	-11.44	-29.59	-22.99	-7.28
SD	0.160	0.206	0.026	0.187	0.150	0.050	0.177	0.476	0.533
Muscle	4.1351 [a]	3.7393 [d]	3.6392 [e]	2.9165 [i]	3.1642 [h]	3.8550 [c]	3.3550 [f]	3.2619 [g]	3.8948 [b]
% Change		-9.57	-11.99	-29.47	-23.48	-6.77	-18.87	-21.12	-5.81
SD	0.099	0.361	0.124	0.403	0.520	0.215	0.578	0.451	0.588
Liver	5.7962 [a]	4.6816 [d]	3.3095 [i]	4.3437 [f]	4.7265 [c]	4.4241 [e]	4.2657 [g]	3.9128 [h]	4.9778 [b]
% Change		-19.23	-42.90	-25.06	-18.45	-23.67	-26.41	-32.49	-14.12
SD	0.478	0.066	0.203	0.077	0.048	0.308	0.539	0.029	0.164

Data are means ±SD (n = 5) for an organ in a row followed by the same letter are significantly different ($p < 0.05$) from each other according to Duncan's multiple range test.

DISCUSSION

Physiological manifestations in an organism treated with a toxicant are resultants of alterations which have taken place at cellular and sub-cellular levels. Single group of biomolecules primarily responsible for such alterations at sub-cellular level are enzymes whose activity is most important for various biochemical reactions to sustain life [11]. Enzymes are highly sensitive to changes in the environment, and are used as models to study mode of action of toxicant at sub-cellular level. ATPases are key enzymes of energy metabolism and had been demonstrated to be one of the targets of cyanide [16]. These enzymes hydrolyzes the terminal pyrophosphate bond of ATP to provide the energy for ion-pump to drive the membrane transport of mono- and divalent-ions (viz., Na^+, Mg^{2+} and Ca^{2+}). Na^+K^+-ATPase are primarily responsible to maintain equilibrium of Na^+ and K^+ ions by controlling active transport across membrane. However, Mg^{2+}-ATPase is mainly concerned with maintaining cellular balance of Mg^{2+} ions which are needed for many important biological functions including protein biosynthesis and cell growth [13]. Ca^{2+}-ATPase maintains low intracellular Ca^{2+} ions than the Ca^{2+} concentration of extra cellular medium [10].

Cyanide is known to inhibit cytochrome oxidase at the active site and this blocks the terminal event in electron transport. This single site of action is responsible for the rapid and often fatal toxic effects of cyanide [4]. As a result, oxidative phosphorylation is compromised and an increased demand on glycolysis [1, 17]. This result in ATP depletion and the effects include: interference with membrane integrity, ion pumps, and protein synthesis, loss of cell function and perhaps cell death [18].It may be noted that fall in ATPase activity can enhance release of neurotransmitter in rat [19], and thus can affect in a similar way as fish cytochrome.

In the present study, inhibition in the activity of ATPases increased at higher concentration of toxicant reaching a plateau with maximum inhibition exceeding 50% of normal level (Table 1, 2 and 3). Maximum inhibition in the enzymes was found 10th d of exposure in both the concentration and was found reversible in post-recovery period, reaching near to control values in all the tissues. Time required for recovering enzyme activities was much longer in comparison to inhibition. Inhibition in the ATPase affected neural integration, ionic balance and availability of metabolic energy for various physiological functions [20]. At sub-lethal concentrations recovery of damages took place slowly and gradually resulting into recovery of affected fish. Out of three tissues studied, ATPase activity of gill revealed comparatively faster inhibition indicating a tissue specific sensitivity to toxicant [12]. In aquatic organism, gills having larger surface area of exposure are found to be the major source for toxicant uptake [9].

Activity of Mg^{2+}-ATPase was inhibited more than that of Na^+/K^+ and Ca^{2+}-ATPase. ATPase being an integrated enzyme of mitochondria, any damage to the mitochondria ultimately alters its activities which would interfere with conversion of oxidative energy to phosphorylated energy [21]. Thus it can be inferred that mitochondrial ATPase, is the target site for the action of cyanides. Although Na^+/K^+-ATPase and Ca^{2+} ATPase as well were suggested to be the target sites, but the supporting evidence to that effect is meager [17]. Mg^{2+} -ATPase does not transfer Mg^{2+} across the gill epithelium, which is essential to the integrity of the cellular membrane, intercellular element and to the stabilization of branchial permeability [18]. Damages in the membrane architecture may be the reason for the enzyme inhibition during the sublethal; treatment with copper cyanide. Another possible reason may be the non availability of the substrate like ATP molecule, which resulted in the inhibition of ATPases. Observations made by Begum, [12] and Prashanth and David [9] supports the present study.

Lipid peroxidation in vivo has been identified as one of the basic deteriorative reactions in cellular mechanisms of the cyanide induced oxidative stress in fresh water fishes [22]. Free radical generated during the catalytic cycle of cyanide would have induced the peroxidation process in membrane lipids. The observed results indicate that peroxidation occurring in the damaged tissues, which brings about a change in the structure and inactivates a number of membrane bound enzymes and protein receptor, which finally disrupts the membrane integrity [18]. Significant decrease in the Na^+K^+-ATPase activity of *C. catla* after exposure to copper cyanide mainly refers to the changes in the membrane lipid content, which in-turn have been shown to influence the inhibition in Na^+K^+-ATPase activity. Similar observations were made by Balambigai and Aruna [23] in the fish, *Cyprinus carpio* exposed to copper sulphate. Some studies have shown that xenobiotics modulate intracellular Ca^{2+} concentration by acting on the endoplasmic reticulum Ca^{2+}-ATPases [24]. Furthermore the Ca^{2+}pump may act synergistically with the Na^+/Ca^{2+} exchanger [25]. Decreased Ca^{2+}ATPase due to the activity of cyanide may lead to high internal Ca^{2+}level [16]. Increase in intracellular accumulation of Ca^{2+} ions there by increases the release of neuro-transmitter from the synaptic vesicles by exocytosis [19], which may inhibit Na^+K^+-ATPase.

CONCLUSION

The marked loss in the activities of membrane-bound ATPases may also be due to the loss of protein-SH, because of increased lipid peroxidative damage of cell membranes. The present study confirms that copper cyanide induced alterations in the level of membrane-bound ATPase activity in freshwater teleost fish (*C. catla*). The present findings warrant future studies to explore ATPase as possible biomarkers of cyanide related incidents in ecotoxicology. The major findings of the present experiment validate the ATPase activity can be taken as meaningful index of cellular activity and forms a useful toxicological tool.

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