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ORIGINAL ARTICLE

EFFECT OF INDIVIDUAL AND CO-EXPOSURE OF COPPER AND CADMIUM ON PHOSPHATASE ACTIVITY IN FRESHWATER FISH, *Cyprinus carpio*.

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ABSTRACT

The present study is aimed to investigate the alterations of acid and alkaline phosphatase activities in gill, liver and kidney tissue of freshwater fish *Cyprinus carpio* exposed to the sublethal concentrations of 1/3rd and 1/8th of 96 hr LC_{50} range of individual and binary mixture of Cu and Cd for short term (120 hrs) and long term (90 day) experimentation respectively. The observed enzyme activities in control fish were most pronounced in liver followed by kidney and gill. The single and binary exposure of Cu and Cd showed a marked inhibition of both the ACP and ALP activity in all the tissues of treated fish under short and long term exposure. Further, the inhibition of ACP and ALP activity in all the tissues of metal treated groups were increased with increasing of exposure time. In addition, among the single and co-exposure of Cu and Cd, the ACP and ALP activity in various tissues of *C. carpio* showed the maximum of inhibition in fish exposed to the mixture of Cu and Cd than in single exposure which might be due to the synergistic interactions of Cu and Cd.

Key Words: Copper, Cadmium, Cu-Cd mixture, *Cyprinis carpio*, ACP, ALP

1.INTRODUCTION

The discharge of potentially toxic trace metals into the environment has become a global problem. As a result of industrial activities and technological development, the amount of heavy metal ions discharged into streams and rivers by industrial and municipal waste-water have been increasing. In addition to natural sources of heavy metals (i.e. geologic weathering, volcanic activity, and animal excretion), there are several anthropogenic sources like mining and metallurgical industry, agriculture (use of fertilizers and pesticides), waste leaching, and battery production, fossil fuel burning, paint and chlor-alkali industries (Forstner and Whitman, 1979). The toxic metals are held to be the most dangerous, since continuous exposure of aquatic organisms to their low concentration may result in bioaccumulation and transfer to man through food web (Karadede *et al.*, 2004). Heavy metal constitutes serious types of pollution in freshwater and being stable compounds; they are not readily removed by oxidation and affect the animal. Heavy metals have a unique property of accumulation over a period of time, along a food chain and a very high level can be accumulated in an organism from very low level concentration in water and sediments (Bose *et al.*, 1994).

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Copper is a trace element which is essential to the function of specific proteins enzymes. However, at high concentrations, it may be toxic to organisms. The increasing industrial activities and the use of $CuSO_4$ as a fungicide in agricultural practices as well as in the control of algae and pathogens in fish culture ponds have increased the copper concentrations in aquatic systems. Cadmium is one of the most toxic heavy metals and its environmental concentration is increasing due to industrial and agro-chemical usages and anthropogenic activities. This is very dangerous pollutant which affects animals, arriving through the food chain (ATSDR, 2001).

Chemicals and heavy metals may significantly damage certain physiological and biochemical processes when they enter the organs of fishes (Jen and Hon, 2003). Biochemical approach has been advocated an early warning of potentially damaging changes in stressed fish. Elagamy and Uner (1999) stated enzyme activities are considered as sensitive biochemical indicators of imminent hazardous effects in fish and have been used as important parameters in the testing of water. Phosphatase is a hydrolytic enzyme, leading to the release of ortho-phosphate from phosphorus compound based on the optimum pH which are classified into acid phosphatase (ACP, optimum pH $\leq$ 6.0) and alkaline phosphatase (ALP, optimum pH $\geq$ 8.0) (Jansson *et al.*, 1988; Hongxia *et al.*, 2012). Acid phosphatase is a lysosomal, hydrolytic enzyme in nature which frees attached phosphate

groups from other molecules during digestion (Verma *et al*., 1984) and found in endoplasmic reticulum (De Duve *et al*., 1955). It helps in the dissolution of the cell after its death (Chetna and Aditi, 2011). Alkaline phosphatase is a polyfunctional enzyme present in the plasma membrane of all cells. It is majorly found in the hepatocytes at the bile pole pinocytic vesicle and golgi complex (Roy *et al*., 2012). This enzyme could serve as a good indicator of liver intoxication because of its sensitivity to metallic salts (Boge *et al*., 1992).

Majority of works have showed that the changes of acid and alkaline phosphatase activity in fishes are due to individual metal exposure. Variation of acid phosphatase and alkaline phosphatase activities have shown in the fish *Cyprinus carpio* to cadmium (De la Tore *et al*., 2000) and copper (Karan *et al*., 1998); *Catla catla* to arsenic (Lavanya *et al*., 2011) and *Oreochromis niloticus* to zinc (Younis *et al*., 2012). However, very little information is available on the comparison of single and combined metal effects with the phosphatase activity in animal system. Therefore, the present investigation was carried out to analyse the effects of single and co-exposure of Cu and Cd in the activity of acid and alkaline phsophatase in different tissues of fish *C. carpio* under short and long-term exposure.

2.MATERIALS AND METHODS

Experimental Condition

Healthy adult fish of *Cyprinus carpio* with body length 20±2 cm and body weight 200±10 g were selected for experiments. The fish were collected from the Kamaraj fish farm at cholatharam, 30 km away from Annamalai University and were transported to laboratory and acclimatized for two weeks before exposure. The tap water used for the experiments had a pH value of 7.2 to 7.6, dissolved oxygen 7 to 7.5 mg/L, total hardness of 240 to 260 mg/L and alkalinity of 165-168 mg/L. Supplemental aeration was provided to maintain dissolved oxygen levels and the temperature was kept at 26±1^{0}C. Experimental fish were fed daily with commercial fish pellet once a day during acclimation. Under laboratory conditions, water was renewed two or three times in a week.

Exposure Chemical

The copper chloride ($CuCl_2.2H_2O$) and cadmium chloride ($CdCl_2.H_2O$) manufactured by Merck Specialities Private Limited, Mumbai was used for the present study. The 96 hr LC_{50} concentration of Copper, Cadmium and Cu plus Cd was 38.36 mg/L, 92.23 mg/L and 23.90 mg/L respectively for the experiment fish by using the probit analysis method (Finney, 1971).

Experimental Design

Fishes were divided into eight equal groups each comprising of 50 fishes. Each group was kept in separate plastic tanks. The first group was kept as control for short term and the fishes were maintained in water containing normal water without any treatment. The experimental fishes were exposed to one-third of 96 hr LC_{50} concentration of Cu (12.78 mg/L) (Group 2), Cd (30.74 mg/L) (Group 3) and Cu+Cd (7.96 mg/L) (Group 4), for short term (interval of 24, 72 and 120 hours) experiment. The fifth group was kept as control for long term exposure. The one-eighth of 96 hr LC_{50} concentration of Cu (4.79 mg/L) (Group 6), Cd (11.53 mg/L) (Group 7) and Cu+Cd (2.98 mg/L) (Group 8) were used for long term experiments (90 day). The experiments were carried out using a static-renewal method.

Estimation of Enzyme (ACP and ALP) Activity

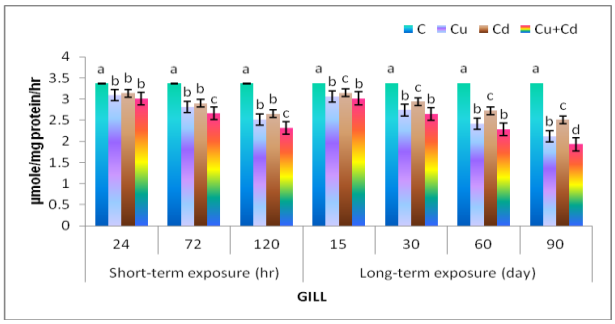
The experimental fish were sacrificed after short and long term exposure periods and the tissues of gill, liver and kidney were removed, weighed before homogenization. The weighed tissues were homogenized using 10 ml. chilled distilled water and centrifuged at 3,000 rpm for 10 minutes. The supernatant was then used for enzymatic studies. Acid Phosphatase (ACP) and Alkaline Phosphatase (ALP) were analyzed according to the methods of Tennis Wood *et al*., (1976). The optical density was measured at 415 nm (ACP) and 400 nm (ALP) using a UV-VIS Spectrophotometer. The values are expressed in µmole/mg protein/hour.

Statistical analysis

Experimental data are presented as Mean ± Standard Deviation (Mean ± SD). Statistical analysis was implemented using SPSS (10.0 version) statistical package. One-way ANOVA was used to compare variables among the different groups.

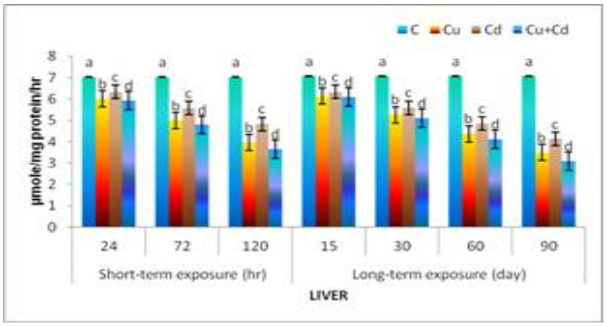
3.RESULTS

Fig. 1. Changes in ACP activities in gill of *C. carpio* exposed to Cu (12.78 and 4.79 mg/L), Cd (30.74 and 11.53 mg/L) and Cu+Cd (7.96 and 2.98 mg/L) under short and long term exposure



Values are Mean±S.D., Sample Size (N) = 6.;Different letter designations denotes significant at 5% level among the exposure groups within the same time of interval; C = Control

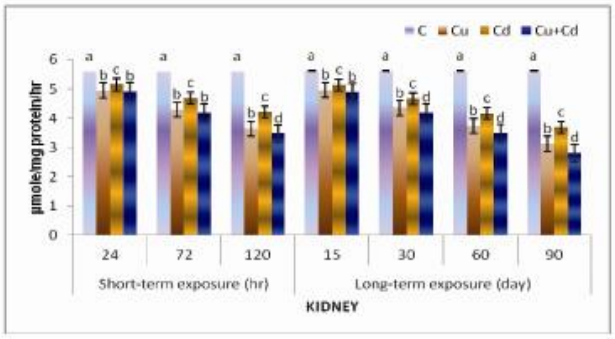
Fig. 2. Changes in ACP activities in liver of *C. carpio* exposed to Cu (12.78 and 4.79 mg/L), Cd (30.74 and 11.53 mg/L) and Cu+Cd (7.96 and 2.98 mg/L) under short and long term exposure



Values are Mean±S.D., Sample Size (N) = 6.;Different letter designations denotes significant at 5% level among the exposure groups within the same time of interval; C = Control

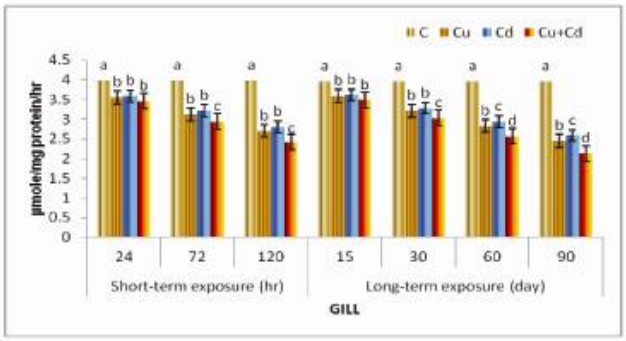
Figure 1 to 6 represents the changes of acid and alkaline phosphatase activities in the tissues of gill, liver and kidney of fish *C. carpio* exposed to individual and combined mixture of Cu and Cd for short and long term exposure at an interval of 24, 72 and 120 hr and 15, 30, 60 and 90 day, respectively. The activity of ACP in the tissues of fish exposed to the single and combined mixture of Cu and Cd showed a marked inhibition after the short-term and long-term exposure. The rate of inhibition was higher in the liver followed by kidney and gill.

Fig. 3. Changes in ACP activities in kidney of *C. carpio* exposed to Cu (12.78 and 4.79 mg/L), Cd (30.74 and 11.53 mg/L) and Cu+Cd (7.96 and 2.98 mg/L) under short and long term exposure



Values are Mean±S.D., Sample Size (N) = 6.;Different letter designations denotes significant at 5% level among the exposure groups within the same time of interval; C = Control

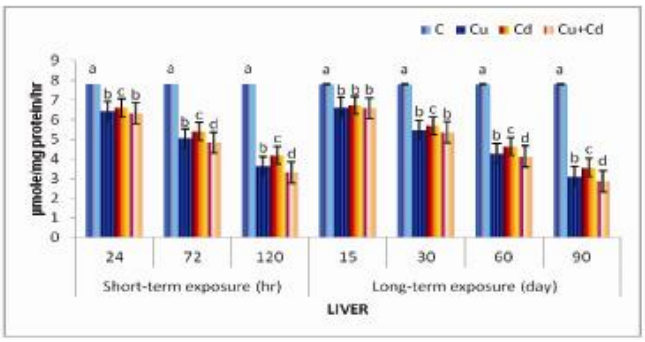
Fig. 4. Changes in ALP activities in gill of *C. carpio* exposed to Cu (12.78 and 4.79 mg/L), Cd (30.74 and 11.53 mg/L) and Cu+Cd (7.96 and 2.98 mg/L) under short and long term exposure



Values are Mean±S.D., Sample Size (N) = 6.;Different letter designations denotes significant at 5% level among the exposure groups within the same time of interval; C = Control

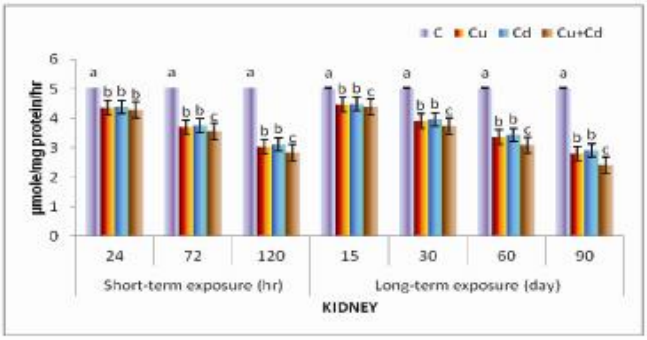
The maximum inhibition of ACP activity in liver of fish exposed to Cu, Cd and Cu plus Cd shows 43.81% and 50.28%, 31.77% for short term (120 hr) and 41.58% and 48.37% and 56.21% for long term (90 day) experimentation over the control groups. Next to the liver, the decreased level of ACP activity was found in kidney followed by gill tissue in both short and long-term exposure. Similar to ACP activity, there was maximum significant reduction of ALP activity in liver tissue followed by kidney and gill of *C. carpio* exposed to Cu, Cd and Cu plus Cd mixture under short and long term exposure. Further, in fishes exposed to copper, cadmium and copper+cadmium under short and long term experimentation, the inhibition of ACP and ALP activity in all tissues was more remarkable in long term exposure than in short term exposure. In addition, among the single and co-exposure of Cu and Cd, the maximum inhibition in both ACP and ALP activity was observed in Cu plus Cd mixture in both short and long term exposure, which might be due to the synergistic effect of copper and cadmium in fish *C. carpio*.

Fig. 5. Changes in ALP activities in liver of *C. carpio* exposed to Cu (12.78 and 4.79 mg/L), Cd (30.74 and 11.53 mg/L) and Cu+Cd (7.96 and 2.98 mg/L) under short and long term exposure



Values are Mean±S.D., Sample Size (N) = 6.;Different letter designations denotes significant at 5% level among the exposure groups within the same time of interval; C = Control

Fig. 6. Changes in ALP activities in kidney of *C. carpio* exposed to Cu (12.78 and 4.79 mg/L), Cd (30.74 and 11.53 mg/L) and Cu+Cd (7.96 and 2.98 mg/L) under short and long term exposure



Values are Mean±S.D., Sample Size (N) = 6.;Different letter designations denotes significant at 5% level among the exposure groups within the same time of interval; C = Control

4.DISCUSSION

Heavy metals are known for their strong action on biological tissues (Meenakumari *et al*., 2010). Metal ions once absorbed into the body are capable of reacting with a variety of active binding sites and then disrupting the normal physiology of an organism which may lead to the death of the organisms. The toxic effect of heavy metals on enzyme system depends on the capacity of toxicants to react with ligands, which is essential for the normal functioning of enzymes. Thus enzyme bioassay can provide diagnostic tool to assess a change or damage caused to organism due to administration of heavy metals (Harper *et al*., 1978).

The present results showed that ACP and ALP activity decreased significantly in different tissue of *C. carpio* exposed to single and combined mixture of Cu and Cd in both short and long-term exposure. This inhibited level of tissue enzymes may be attributed to tissue damage (because of toxicant) that lead to leakage from tissue into blood and / or actual inhibition of tissue enzymes. The decrease of ACP and ALP activity in tissue due to Cu (or) Cd toxicity was similar to that obtained by Shalaby *et al.* (2000) who have recorded that a significant reduction in ACP and ALP in liver and kidney of fish *C. carpio* after toxication with Cd. Further, the decreases in phosphatase activity in the current

study were dependent on the time of exposure, exposure concentration of test metals (Cu, Cd, Cu plus Cd) and their cumulative accumulation at cellular level in the test fish. The correlations between accumulation of heavy metals and abnormal enzyme activities in different tissues have also been reported in the freshwater fish *Channa punctatus* exposed to cadmium (Dubale and Shah, 1981). The present results support the report of Shwetha *et al*. (2012) who have observed a decrease in ACP and ALP activities in liver, gill and muscle which might be due to the uncoupling of phosphorylation by heavy metals. Dalela *et al*. (1980), Magar and Afsar (2013) are also on the opinion that uncoupling of oxidative phosphorylation has been the main reason for inhibition of acid phosphatase.

Exposure of the fish to Cu and Cd, both individual and combined, the inhibition of ACP was higher in the liver followed by kidney and gill tissue in both short and long-term experimentation. ACP acts as marker enzyme for the detection of lysosomes in cell fractions and can be altered by the presence of xenobiotics (Magar and Asfar, 2013). Aruljothi (2013) has reported that the accumulated arsenic in hepatocytes are passed to the lysosome and destruct the cell membrane which in turn leads to hepatic damage and releases the acid and alkaline phosphatase. Similarly, the decreased lysosomal membrane stability was observed by Regoli *et al*., (1998) on exposure to copper. James and Soni (1994) observed the inhibition of acid phosphatase activity in different tissues of mice exposed to mercury chloride. Further, they have stated that this enzyme might diffuse into the cell by treatment of toxicants and get utilized for the digestion of cellular organelles, which are responsible for its secretion, resulting in the decrease of ACP activity.

The results of the present study are in agreement with those of Borah *et al*. (1996), who have suggested that the declined level of acid phosphatase activity in *Heteropneustes fossilis* exposed to rogor might be due to the severe histopathological changes in tissues. Further, Karuppasamy (2000) have suggested that the inhibition of ACP activity in metal exposed fish might be due to increased necrosis in the tissues like hepatocytes, gills and brain.

Similarly, Hongxia *et al*. (2012) have also demonstrated the inhibition of ACP activity in the liver, kidney, gill and spleen of Cu exposed fish. Sastry and Subhadra (1985) reported that a significant reduction in gill and hepatic ACP level of *Heteropneustes fossilis* after Cd treatment for 15, 30 and 60 days. De Smet *et al*. (2001) noted a declined level of ACP activity in the gill, liver and kidney of Cd exposed *C. carpio*. Moreover, Gill *et al.*, (1991) and (1992) reported that the hepatic, branchial and renal ACP activities were decreased in rosy barb (*Barbus conchonius*, *Puntius conchonius*) toxicated with Cd as well as copper. However, it is important to observe that the differences in tissue AKP in response to metals are indicative of the possible use of AKP as biomarkers for aquatic ecotoxicology as already established in the literature (Jiraungkoorskul *et al*., 2003).

ALP is a non-specific enzyme in the tissue especially liver that plays an important role in dephosphorylation of organic compounds. In higher animals, this enzyme is involved in bone formation and in membrane transport (Molina *et al.*, 2005). Furthermore, alkaline phosphatase (ALP) is involved in the synthesis of nuclear protein, nucleic acid and phospholipids (Das *et al*., 2004). These enzymes are associated with transmembrane transport mechanism, ion transport, maintenance of ionic strength in the organ (Moog, 1946; Aruljothi, 2013). Seth *et al*. (1969) and Sarita and Shrivastava (2011) have reported that ALP is important for permeability process at the membrane sites. Fluidity of the membrane is responsible for the functioning of the membrane bound enzymes and various other transports (Geetha *et al*., 1990).

The present study showed that ALP activity was reduced in all organs of *C.* carpio exposed to Cu, Cd and Cu plus Cd during the periods of short and long-term experimentation. This decrease of ALP activity in the present study was much coincides with the findings of Ahmed *et al*. (1997), who have studied the effect of copper and chromium on oxygen consumption and phosphatase activity in *S. serrata*. Further, they have reported that there was a decrease in alkaline phosphatase activity in muscle, hepatopancreas and haemolymph. Intoxication with cadmium caused significant retardation of ALP activity in the liver and kidney of cat fish, *Heteropneustes fossilis* (Sastry and Subhadra, 1985). Sreenivasan *et al.,* (2011) reported that the impact of cypermethrin on the variations in the alkaline phosphatase (ALP) in the gills of *S. hydrodroma*. Sarasus and Andal (2005) also found a decrease level of ALP in liver and gill in *Hypophthalmichthys molitrix* and *Catla catla* after exposure to distillery effluent for 30 days. Kamble *et al*. (2011) also found reduced level of ALP activity in liver, muscle and kidney of *Bariliusburna* when exposed to dimecron. Sastry and Gupta (1978) have stated that the lead nitrate (6.8 mg L^{-1} for 125 days) inhibits alkaline phosphatase activities in the liver of *Channa punctatus*.

The decreased activity of ALP in all the tissues, particularly in liver could be due to the consequence changes in the permeability of plasma membrane in addition to changes in the balance between synthesis and degradation of enzyme protein thus lowering the enzyme activity (Thenmozhi *et al*., 2011). Sunmonu *et al*. (2009) have also observed significant decrease of ALP in liver under anthracene toxicity and it is possibly due to leakage of the enzyme from cytosol across the damaged plasma membrane into the general blood circulation or decreased enzyme synthesis on account of organ dysfunction. Accumulation of toxicants in the liver would alter its function (Premdas and Anderson, 1963) and this could be true in the present study where the metals Cu and Cd have caused destruction in the liver thereby, reducing the synthesis of enzyme protein.

The findings of Ramesh and Gracelyn (2013) reported that the decreases in the ALP activity as an indicator of degenerative changes in the membrane transport system. The decreasing trend of ALP activity under single and binary exposure of copper and cadmium stress observed in *C. carpio* could be also correlated to the histopathological changes and functional impairments. This finding is in good agreement with Shwetha *et al*. (2012) who suggested that the decreased level of ALP activity in zinc cyanide exposed fish liver, muscle and gills were due to necrosis and dysfunction of organs.

Results of the present study also indicate that there is another possibility for the decrease in ALP activity in all tissues might be due to the inhibition of this enzyme on protein directly or indirectly by copper and cadmium intoxication. Karuppasamy (2000) stated severe inhibition of ALP activity found in the liver, gill and brain by phenylmercuric acetate could be due to the interaction of chemical with the cofactors and regulators of the enzyme. Further, he stated that the observed decreases of ALP enzyme activity probably would facilitate the increased activity of phosphorylation enzyme in the tissues of fish and cause subsequent break down of glycogen for energy release during toxic stress.

According to Shaikila *et al*. (1993) severe acidosis may be the cause for inhibition of alkaline phosphatase activity in intoxicated liver, which inturn could be adaptive for the fish to meet the energy demand by the anaerobic breakdown of glycogen. The above observation is in accordance with that of the present study where reduced level of total carbohydrate content have been observed in different tissues under short and long-term exposure to the metals of Cu, Cd and Cu plus Cd mixture coinciding with the decreased level of ALP activity.

5.CONCLUSION

In the present study, the exposure of common carp under heavy metal stress caused considerable alterations in enzyme activities in *C. carpio.* This enzymatic changes in *C. carpio* exposed to various levels of Cu, Cd and their mixture revealed their effects on intermediary metabolism of *C. carpio.* Further, the present study pointed out that enzymatic responses in fish exposed to contamination of metals in environment can be used as indicator for both the tissue damage and biomarkers of pollution.

6.ACKNOWLEDGEMENTS

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