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Effect of EDTA on Reduction of Copper Toxicity in *Oreochromis mossambicus* (Peters)

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The indiscriminate discharge of industrial effluents, raw sewage wastes and other waste pollute most of the environments and affect survival and physiological activities of target organisms. Metals and pesticides, in particular have a tendency to accumulate and undergo food chain magnification (Vinikour *et al.* 1980). They could also cause catastrophic diseases like Minamata and Itai-Itai. Some of these organisms, like fish, are consumed by human beings. Hence, reduction of toxic elements in aquatic environments by acceptable methods is needed. Unfortunately in India neither Government bodies nor industries take effective measures to control the level of toxicants in the environment. Synthetic compounds like ethylene diamine tetraacetic acid (EDTA) are known to be effective chelating agents of heavy metals. Studies (Lewis *et al.* 1972; Lawrence et *al.* 1981; Licop 1988) reveal that the introduction of Na-EDTA in metal polluted water enhances the survival chances of marine organisms. However, there is paucity of information on the correlation between the introduction of chelating agents in metal polluted water and the reduction of metal toxicity. There is also not much information on the optimum dosage of the chelating agent that is required to reduce metal toxicity. The present work was designed to study the effect of the chelating agent EDTA on the reduction of copper toxicity in the freshwater cichlid fish *Oreochromis mossambicus*.

MATERIALS AND METHODS

Freshwater fish, *Oreochromis mossambicus* were collected from a local pond and held for 30 days in laboratory conditions (DO: 4.27 ± 0.6 ml/l; temperature: 29.1 ± 0.6°C; pH: 7.7 ± 0.06; salinity: 0.13 ± 0.003 ppt and hardness ($CaCO_3$): 90 ± 3.8 ppm). During acclimatization, water was changed daily and fish were fed *ad libitum* with pelletized diet containing 35% protein. Acclimated fish (11.3 ± 0.7g) were exposed to different concentrations of copper (0, 3.0, 3.5, 4.0, 4.25, 4.5, 4.75 and 5.0 ppm) and mortality was observed for 96 hr. A static renewable bioassay method was adopted for the determination of 96 hr median lethal concentration (Sprague 1973); probit analysis was followed for the calculation of 96 hr LC_{50} (Litchfield and Wilcoxon 1949). Control group was maintained in metal-free water. The 96 hr LC_{50} value of copper for *O. mossambicus* was 4.27 ppm and its 95% confidence limits were 3.72 (lower limit) and 4.90 (upper limit). Stock solution of copper was prepared by dissolving 3.93g of analar grade (Merck) $CuSO_4 7 H_2O$ in 11 of distilled water and then diluted with freshwater to obtain the desired concentration (4.27 ppm) for the present study.

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Active and healthy fish (11.3 ± 0.7 g) were chosen from the acclimation tank and starved for 24 hr prior to the commencement of the experiment. The fish were divided into 6 groups of 20 individuals each and they were exposed for 28 days as follows:

Table 1. Experimental groups and their notation

S.No.	Groups	Notation
1	Control (Metal-free water)	C
2	Copper (4.27 ppm) alone	Cu
3	Copper (4.27 ppm) + 0.125g EDTA/l	CuEDTA1
4	Copper (4.27 ppm) + 0.25g EDTA/l	CuEDTA2
5	Copper (4.27 ppm) + 0.50g EDTA/l	CuEDTA3
6	Copper (4.27 ppm) + 1.0g EDTA/l	CuEDTA4

(For ease of reference, hereafter control and experimental groups will be referred by their notation). The chosen levels of chelating agent EDTA were added to the medium along with copper (see Table 1) on day 0, to assess the effect of EDTA on the reduction of metal uptake and improvement of haematological parameters in *O. mossambicus*. The medium was mixed well after the addition of copper and EDTA and then test individuals were introduced. The experiment was conducted in epoxy coated cement tanks (105 l capacity) containing 80 l of test medium. 35% protein feed was offered as diet to test animals in a feeding tray once in a day at 0800 hr and uneaten food was removed after 2 hr of feeding. Faecal matter was randomly collected by using feeding trays and dried in a hot air oven at 60°C to estimate the copper content. The medium was not changed during the experiment and all tanks were aerated for 14 hr/day. The hydrobiological parameters of DO, temperature, pH, salinity and the hardness of exposures were not much varied and they averaged to 3.57 ± 0.5 ml/l, 29.2 ± 0.5°C, 7.8 ± 0.4, 0.14 ± 0.02 ppt and 95 ± 4.5 ppm respectively.

Three fish were removed from each experimental group (except Cu group) on days 0, 3, 7, 14 and 28 and blood collected and analysed for routine haematological parameters. There was 40% mortality in Cu group and therefore only 2 fish were sacrificed. Blood was collected in a watch glass containing the required amount of 6% EDTA as an anticoagulant from 3 experimental fish at a time by cutting the caudal peduncle using a sharp knife. Haematological parameters were estimated according to routine clinical method (Wintrobe 1978). Blood cells (RBC and TLC) were counted by using an improved Neubauer counting chamber. Haemoglobinometer was used to determine the haemoglobin content of blood. Oxygen carrying capacity of blood was calculated by multiplying the haemoglobin content with 1.25, oxygen combining power of Hb/g (Johansen 1970). Two-way ANOVA was applied to determine the significance of interaction between EDTA, copper level and exposure period on blood cells. Analysis of covariance (Snedecor 1961) was applied to test the time-and dose-dependent significance of EDTA levels on copper and exposure period.

Copper content in liver, muscle, gill, faeces and water were estimated at the end of the experiment on day 28. Three replicates of samples (except water) were digested with a mixture of concentrated nitric acid and perchloric acid in the ratio 1:2 until the formation of a white residue at 100°C in a water bath. The cooled residue was dissolved completely by adding 1 N HCl and made up to 25 ml with distilled water (FAO 1975). The copper concentration in water was estimated following the method of APHA

(1993). The solution was filtered through cotton wool and the filterate was subjected to metal analysis in atomic absorption spectrophotometry (GBC 906 AA model). The instrument was calibrated using standards prepared from copper sulphate.

RESULTS AND DISCUSSION

Animals exposed to median lethal concentration of copper showed significant (ANOVA: $P < 0.05$) decrease in RBC (Red Blood Corpuscles) count, Hb (Haemoglobin) content, Ht (Haematocrit) value and oxygen carrying capacity of blood. TLC (Total Leucocyte Count) and ESR (Erythrocyte Sedimentation Rate) showed the opposite trends (Table 2). Blood parameters were improved in fish exposed to copper with different levels of EDTA. For instance, the RBC count of fish held in metal free water was 1.97×10^6 m m^{-3} and it significantly (ANOVA: $P < 0.05$) declined to 0.72, 0.56, 0.49 and 0.44 x 10^6 m m^{-3} in fish exposed to copper alone on days 3, 7, 14 and 28 respectively; however, the RBC count improved to 0.94, 1.33, 1.34 and 1.37×10^6 m m^{-3} in fish belonging to the group CuEDTA2. There was two fold increase in RBC count in fish belonging to group CuEDTA2 as compared with fish exposed to copper alone. However, the improvement in RBC count due to the addition of EDTA was neither time-nor dose-dependent (Covariance: $P > 0.05$) response (See Table 2). Similar trends were obtained for Hb, Ht and oxygen carrying capacity of blood. The significant reduction in RBC, Hb, Ht values in *O. mossambicus* exposed to copper alone resulted in anaemia. This might be due to the inhibition of erythropoiesis coupled with enhanced rate of destruction of erythrocytes in haemopoietic tissue (Gardner and Yevich 1970) and haemodilution (Lauren and McDonald 1985). An increase in ESR and MCV values suggests that the anaemia was of the macrocytic type. Oxygen carrying capacity of blood also declined in *O. mossambicus,* perhaps due to the significant reduction in RBC count and Hb content (Table 2). This could affect tissue respiration (James and Sampath 1995). An increase in TLC was mainly due to an increase in the population of basophil, neutrophil and lymphocytes.

The present study reveals that EDTA when added to the copper media, significantly ($P < 0.05$) reduced the copper level in water and metal uptake in tissues as compared to animals exposed to copper alone. The concentration of copper in water in group Cu alone was 2.661 mg/l and it significantly ($P < 0.05$) declined to 1.087, 0.862 and 0.739 mg/l in groups CuEDTA1, CuEDTA2 and CuEDTA3 respectively. The uptake of copper m liver tissue of animals exposed to copper alone was 2.1 mg/g wet tissue and it significantly ($P < 0.01$) declined to 0.948, 0.423 and 0.379 mg/g wet tissue in animals belonging to groups CuEDTA1, CuEDTA2 and CuEDTA3 respectively (Table 3). Similar trends were observed in muscle and gill also. This suggests that EDTA can bound with Cu^{2+} ions in test media and produce a stable ion (Cu^{2+}) exchanged EDTA complex, leaving a minimum number of free ions in water and thus reducing the chance for metal uptake by tissues (Table 3). Besides, the EDTA added groups eliminated more amount of copper from the body through faeces. Copper elimination through faeces in fish exposed to copper alone was 0.188 mg/g dry matter and it significantly ($P < 0.01$) enhanced to 2.06, 5.44 and 7.45 mg/g dry matter respectively in fish belonging to groups CuEDTAl, CuEDTA2 and CuEDTA3. The formation of ion exchanged EDTA complex (not estimated due to lack of facilities) in water and elimination of more amount of copper through faeces, evidently reduced the metal burden in tissues and thereby improved the haematological parameters of fish exposed to copper containing EDTA. The addition of chelating agent, zeolite (sodium aluminosilicate) to sublethal levels of

Table 2. Effect of median lethal concentration of copper and addition of chelating agent EDTA on haematological parameters of *O. mossambicus*. Each value is the mean ($\overline{X}$±SD) of three estimations.

Exposures	Exposure period (Days)					Covariance
	0	3	7	14	28	
Red Blood Corpuscles (x 10^6 mm^{-3})						F value
C	1.97 ± 0.06	1.89 ± 0	2.07 ± 0.02	2.10 ± 0.27	2.15 ± 0.07	
Cu	1.97 ± 0.06	0.72 ± 0.07	0.56 ± 0.07	0.49 ± 0.06	0.44 ± 0.06	
CuEDTA1	1.97 ± 0.06	0.90 ± 0.05	1.01 ± 0.06	1.18 ± 0.13	1.21 ± 0.08	0.011
CuEDTA2	1.97 ± 0.06	0.94 ± 0.03	1.33 ± 0.28	1.34 ± 0.07	1.37 ± 0.15	0.007
CuEDTA3	1.97 ± 0.06	0.95 ± 0.05	1.37 ± 0.14	1.41 ± 0.15	1.43 ± 0.21	
CuEDTA4	1.97 ± 0.06	0.91 ± 0.06	1.02 ± 0.08	All fish dead		
Total Leucocyte Count (x 10^3 mm^{-3})						
C	43.80 ± 2.15	43.40 ± 1.06	42.90 ± 2.60	43.80 ± 2.35	44.60 ± 3.07	
Cu	43.80 ± 2.15	97.50 ± 2.12	100.15 ± 3.32	113.00 ± 6.71	120.00 ± 7.84	
CuEDTA1	43.80 ± 2.15	63.40 ± 2.71	68.60 ± 2.50	64.30 ± 5.14	60.45 ± 4.00	0.105
CuEDTA2	43.80 ± 2.15	57.75 ± 3.35	61.00 ± 2.07	56.07 ± 3.07	53.01 ± 5.10	0.154
CuEDTA3	43.80 ± 2.15	59.18 ± 3.22	58.59 ± 4.14	53.01 ± 2.06	48.50 ± 3.51	
CuEDTA4	43.80 ± 2.15	61.08 ± 1.91	68.10 ± 3.01	All fish dead		
Haemoglobin (g%)						
C	6.55 ± 0.07	6.65 ± 0.14	6.85 ± 0.07	6.84 ± 0	6.85 ± 0.14	
Cu	6.55 ± 0.07	2.80 ± 0	2.40 ± 0.19	3.30 ± 0.07	3.20 ± 0	
CuEDTA1	6.55 ± 0.07	4.00 ± 0.14	4.30 ± 0.07	4.50 ± 0.09	6.50 ± 0.09	0.204
CuEDTA2	6.55 ± 0.07	4.35 ± 0.07	4.60 ± 0.14	5.16 ± 0.07	5.60 ± 0.07	0.123
CuEDTA3	6.55 ± 0.07	4.45 ± 0.07	4.90 ± 0.07	5.75 ± 0.07	6.20 ± 0.14	
CuEDTA4	6.55 ± 0.07	4.39 ± 0	4.61 ± 0	All fish dead		
Oxygen Carrying Capacity of Blood (mlo_2 g^{-1} Hb)						
C	7.74 ± 0.08	7.80 ± 0.17	6.69 ± 0.08	7.68 ± 0	7.80 ± 0.14	
Cu	7.74 ± 0.08	3.36 ± 0	2.88 ± 0.23	3.96 ± 0.08	3.84 ± 0	
CuEDTA1	7.74 ± 0.08	4.80 ± 0.17	5.16 ± 0.08	7.80 ± 0.08	7.80 ± 0.11	
CuEDTA2	7.74 ± 0.08	5.22 ± 0.08	5.52 ± 0.17	7.96 ± 0.08	7.92 ± 0.08	
CuEDTA3	7.74 ± 0.08	5.34 ± 0.08	5.88 ± 0.08	8.10 ± 0.08	8.04 ± 0.17	
CuEDTA4	7.74 ± 0.08	5.16 ± 0	5.53 ± 0	All fish dead		
Haematocrit (%)						
C	40.68 ± 3.11	41.74 ± 2.46	40.00 ± 3.00	39.75 ± 2.35	38.96 ± 2.84	
Cu	40.68 ± 3.11	11.82 ± 1.57	15.00 ± 1.36	12.50 ± 1.50	10.00 ± 1.07	
CuEDTA1	40.68 ± 3.11	18.41 ± 2.38	25.00 ± 2.20	29.00 ± 3.00	30.00 ± 2.14	0.413
CuEDTA2	40.68 ± 3.11	17.24 ± 1.08	28.00 ± 2.00	36.00 ± 2.07	40.00 ± 3.81	0.007
CuEDTA3	40.68 ± 3.11	23.00 ± 0.24	35.90 ± 0.37	37.50 ± 2.50	45.00 ± 2.80	
CuEDTA4	40.68 ± 3.11	17.97 ± 1.09	31.40 ± 1.59	All fish dead		
Erythrocyte Sedimentation Rate (mm/hr)						
C	2.35 ± 0.07	2.45 ± 0.07	2.42 ± 0.04	2.50 ± 0.06	2.46 ± 0.13	
Cu	2.35 ± 0.07	2.96 ± 0.14	3.47 ± 0.06	4.75 ± 0.07	6.89 ± 0.35	
CuEDTA1	2.35 ± 0.07	2.55 ± 0.07	2.98 ± 0.09	3.26 ± 0.09	3.60 ± 0.16	
CuEDTA2	2.35 ± 0.07	2.53 ± 0.13	2.88 ± 0.14	2.30 ± 0.09	2.70 ± 0.09	
CuEDTA3	2.35 ± 0.07	2.50 ± 0.09	2.75 ± 0.06	2.55 ± 0.07	2.19 ± 0.14	
CuEDTA4	2.35 ± 0.07	2.52 ± 0.14	2.81 ± 0.09	All fish dead		
Mean Corpuscular Volume (F/l)						
C	203.05 ± 6.57	216.93 ± 7.24	193.24 ± 7.68	189.29 ± 3.27	181.21 ± 9.07	
Cu	203.05 ± 6.57	164.17 ± 2.88	267.86 ± 9.00	224.09 ± 8.79	227.00 ± 2.01	
CuEDTA1	203.05 ± 6.57	204.56 ± 4.72	247.52 ± 3.54	330.51 ± 7.58	247.93 ± 3.81	
CuEDTA2	203.05 ± 6.57	183.25 ± 4.17	210.52 ± 8.31	205.28 ± 0.85	291.97 ± 8.19	
CuEDTA3	203.05 ± 6.57	242.10 ± 6.90	154.81 ± 7.91	180.41 ± 2.59	314.69 ± 2.98	

Analysis of covariance: All F values are statistically insignificant

Table 3. Effect of median lethal level of copper and the role of EDTA on copper distribution in tissues, faecal matter and water. Each value is the mean ($\overline{X}$±SD) of three observations.

Exposures	Tissue copper uptake (mg Cu /g wet tissue)			Faeces (mg Cu /g dry matter)	Water (mg Cu /l)
	Gill	Liver	Muscle		
C	0.038 ± 0.001	0.055 ± 0.004	0.076 ± 0.005	0.005 ± 0	0.007 ± 0
Cu	1.197 ± 0.081	2.104 ± 0.206	1.078 ± 0.063	0.188 ± 0.064	2.661 ± 0.123
CuEDTA1	0.413 ± 0.014	0.948 ± 0.064	0.667 ± 0.021	2.060 ± 0.142	1.087 ± 0.074
CuEDTA2	0.294 ± 0.053	0.423 ± 0.035	0.293 ± 0.043	5.440 ± 0.343	0.862 ± 0.043
CuEDTA3	0.267 ± 0.081	0.379 ± 0.053	0.117 ± 0.007	7.450 ± 0.516	0.739 ± 0.022

cadmium, significantly reduced the retention of cadmium in body tissues and this indirectly improved the growth of catfish *Heteropneustes fossilis* (*James et al. 1997*). Muramota (1980) found that metal chelating compounds NTA (Nitrilo triacetic acid) and EDTA reduced metal toxicity in fish by preventing accumulation of cadmium in tissues which supports the present findings. He also states that Cd-EDTA complex was quickly excreted through urine since it was not reabsorbed in fish kidney (Babiker and Rankin 1975). Planas-Bohne and Lehman (1983) found low level of cadmium in tissues due to increased excretion of metals through faeces and urine when rats were administered cadmium intravenously along with EDTA. The role of chelating compounds in reducing metal toxicity in plant was studied by Srinivas (1993) who found that chelated metal was less toxic than that of their ionic forms. Free metal ions caused severe deleterious effects on aquatic organisms than complexed forms (Nor 1987; Gerhardt 1993).

Elimination of copper through faeces and reduction of copper level both in water and tissues were maximum in 0.5g EDTA/l added to copper exposure, and hence it is considered an optimum dose. Further, addition of EDTA beyond the optimum dose (0.5g/l) caused mortality of fish after the 7th day (See Table 2). Perhaps the chelating agent removed not only the accumulated copper but also all the trace elements like copper which already existed in the body, which are physiologically important. Davey *et al.* (1973) reports that an over dose of chelating agent caused the deleterious effects on development, survival and growth in crustacean larvae. Liao *et al.* (1983) added 200 g EDTA to 20-40 ton larval rearing medium to encourage penaeid larval development and survival. Moreover, application of EDTA in shrimp hatcheris is a routine practice to remove ammonia in more advanced countries in the West (Simon 1981; Mock 1982; Fox 1983). Based on the present study, it is recommended that an optimum dosage of 0.5g EDTA/l can effectively remove copper from contaminated water and improve the physiological functions/activities of fish.

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