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Impact of Copper and Its EDTA Complex on the Glutathione-Dependent Antioxidant System in Freshwater Fish (*Carassius auratus*)

H. Liu,[1,2] J. F. Zhang,[1] H. Shen,[1] X. R. Wang,[1] W. M. Wang[2]

[1] State Key Laboratory of Pollution Control and Resources Reuse, School of Environment, Nanjing University, Nanjing, 210093, People's Republic of China
[2] Department of Agricultural Engineering, Hohai University, Nanjing 210098, People's Republic of China

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Copper is an essential trace element required for maintaining cellular function and is an integral part of a number of copper-containing enzymes. However, high intracellular copper levels can be toxic. Excessive copper can lead to alterations in the intracellular protein machinery either directly via denaturation of enzymes and or indirectly via generation of reactive oxygen species (ROS) (Pourahmad and O'Brien, 2000; Droge, 2002). Aquatic organisms are protected against ROS by antioxidant enzymes and low molecular weight scavengers (Winston and Di Giulio, 1991; Peters and Livingstone, 1996). Antioxidant systems have been studied for some years in fish and bivalves experimentally exposed to chemicals or collected from polluted areas (Di Giulio et al., 1989; Winston and Di Giulio, 1991; Stegeman et al., 1992; Lemaire and Livingstone, 1993).

Glutathione (GSH), the most abundant cellular thiol, is involved in metabolic and transport processes and in the protection of cells against the toxic effects (Meister and Anderson, 1983). If potentially toxic H_2O_2 is present in a tissue, GSH is oxidised by glutathione peroxidase (GPx) to glutathione disulfide (GSSG). GSSG is reduced back to GSH by glutathione reductase (GR). When toxic xenobiotics are not conjugated to GSH, they can combine covalently, e.g., with DNA, RNA or cell proteins and thus cause serious cell damage. Several studies have shown that glutathione homeostasis can be used as a biomarker in a variety of fish species being exposed to different xenobiotics (Otto and Moon, 1996; Hasspieler et al., 1994). Studies on aquatic animals, particularly on marine species, have demonstrated that the antioxidant systems could be used to develop relevant indices in explaining the sensitivity of some aquatic species to xenobiotics (Wenning et al., 1988; Livingstone et al., 1990; Lemaire and Livingstone, 1993). However, few reports have been published concerning the toxicological effects of copper and its organic ligands on laboratory aquatic organisms.

The aim of this study is to investigate the long-term effects of copper (Cu^{2+}) and its EDTA complex on the glutathione-dependent antioxidant system. Levels of

Correspondence to: X. R. Wang

GR, GSH and oxidized glutathione (GSSG) were measured in the liver of freshwater fish (*Carassius auratus*).

MATERIALS AND METHODS

In this research, experimental reagents of analytical or ultra-pure grade from home or abroad (Amresco) were used. The copper ion stock solution with a concentration of 1000mg/L was prepared by dissolving copper sulfate in 10.0ml (1:1) HNO_3, then adding distilled water to 1.0L. $Na_2EDTA{\cdot}2H_2O$ was used in preparation for Cu-EDTA complex. EDTA complex of copper in test solutions were calculated by using MINTEQA2 (Brown and Allison 1990) with Cu-EDTA making up over 99% of the total copper species.

The study organism, the freshwater fish, *Carassius auratus*, was obtained from a local aquaculture base (Lukou, Nanjing). The fish had average body length and weight of about 7.10 cm and 5.32 g. The water temperature was maintained at 16.0±1.0 °C under a 12 h light/dark photoperiod and fish were acclimated for at least 2 weeks before experimentation with the total mortality of fish near zero. Air flow was continuous and artificial dry food was provided once daily.

Eleven groups were exposed to copper ion and Cu-EDTA solutions at the concentrations of 0.0025, 0.005, 0.01, 0.05 and 0.25mg/L with one group used for the control. Fish were randomly selected into the aquaria with the rate of fish/water 2.84g/L. During the experiment, water pH was kept 7.0±0.1 and water hardness about 100 mg/L $CaCO_3$.

After 40-d exposure, eight freshwater fish were taken from each group for parallel samples. Fish samples were weighed, dissected and their livers were dissected for weight determination after rinsing in physiological salt water. The liver of each fish was analyzed separately. About 0.10 g of liver tissue was homogenized after addition 1.0 mL of 10.0 mM Tris buffer (pH 7.5) for detection of enzyme activities. The extracts were centrifuged at 1×10^4 rpm for 10 minutes at 4 °C and preserved at -85 °C for analysis.

The determination of protein in fish liver was carried out by the method of Bradford (1976). The absorbance was measured by a UV-1600 spectrophotometer. Bovine serum albumen (BSA, Sigma) was used as the protein standard.

Glutathione reductase (GR) activity was determined according to Bergmeyer (1963). The final assay mixture (3.0 mL) contained tissue supernatant, 0.1mmol/L phosphate buffer, 1mmol/L Na_2EDTA, 1mmol/L GSSG and 0.2mmol/L NADPH-Na_4. The absorbance was determined at 340 nm.

Determination of GSH was performed by the method of Hissin and Hilf (1976). Final assay mixture (2.0 mL) contained 100 μL of the diluted tissue supernatant,

1.8 mL of phosphate-EDTA buffer, and 100 μL the OPT (O-Phthalaldehyde) solution, containing 100 μg of OPT. Fluorescence at 420 nm was determined with activation at 350 nm.

Oxidized glutathione levels were determined after the formation of an adduct between the reduced glutathione contained in the biological sample and N-ethyl-maleimide (Hissin and Hilf, 1976). The final assay mixture contained 100 μL of the diluted tissue supernatant, 2.8 mL of NaOH, and 100 μL the OPT (O-Phthalaldehyde) solution, containing 100 μg of OPT. Fluorescence at 420 nm was determined with activation at 350 nm.

Data were expressed as Mean±SD and analyzed using the program SPSS 10.0 for windows. One-way analysis of variance (ANOVA) and Tukey test were used to test differences between groups. The difference was considered significant if the corresponding *P*-value was less than 0.05.

RESULTS AND DISCUSSION

After 40-day exposure, the glutathione systems responded differently to copper ion and Cu-EDTA. Glutathione reductase (GR) has a crucial role in cellular antioxidant protection because of its ability to generate reduced glutathione. It is shown in Figure 1, hepatic GR activity decreased significantly with the increasing concentrations of Cu^{2+} and Cu-EDTA, and there was no significant change in GR activity in response to 0.0025 and 0.005 mg/L of Cu^{2+} addition (Table 1). For 0.01 mg/L of Cu^{2+} exposure and higher, GR activity decreased markedly, and it was much lower under 0.25 mg/L Cu^{2+} than 0.01 mg/L Cu^{2+} stress (Table 1). GR activities were significantly inhibited for 0.005 mg/L Cu-EDTA and higher, and it was much higher under 0.25 mg/L Cu-EDTA than 0.005 and 0.01 mg/L Cu-EDTA. The changes of GR activity among other groups didn't show significant differences (Figure 1 and Table 1). Our observations may result from the contribution of organic complexes in enhancing toxicity of copper ranging between 0.005 and 0.01 mg/L. Similarly, the bioavailability of trace metals was poorly correlated to the bioaccumulation and toxicity in Daphnia in the presence of humic acid (Winner and Gauss 1986).

Compared to the controls, GSSG content increased markedly under 0.0025, 0.01 and 0.05 mg/L of Cu^{2+} exposure (Table 1), but it was inhibited at 0.25 mg/L Cu^{2+} stress (Figure 2). GSSG contents significantly increased for 0.01 mg/L Cu-EDTA and higher, and it reached the greatest value at the highest exposure (Figure 2), with an activation rate at 44% ($P<0.01$) (Table 1). It was shown that the increase of GSSG content was alleviated with the addition of EDTA when exposure concentration is less than 0.25 mg/L. These results provided an evidence for the role of EDTA in the detoxification of metals. In glutathione antioxidant defense systems, GSSG is reduced back to GSH by glutathione reductase. The decrease of glutathione reductase activity (Figure 1) may cause the accumulation of GSSG.

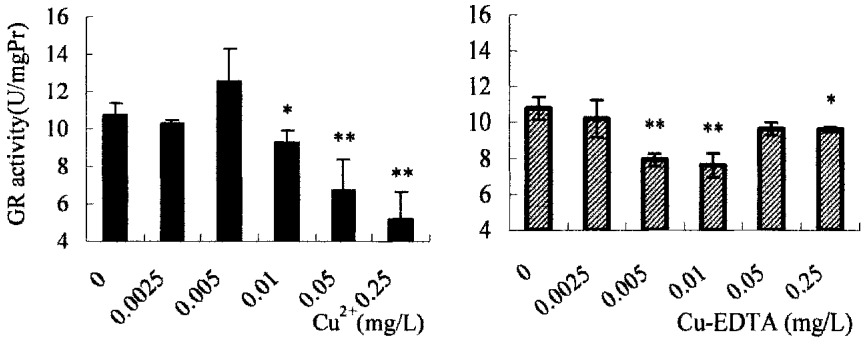

Figure 1. The effects of Cu^{2+} and Cu-EDTA on GR activity in the liver of *Carassius auratus*. *: $P < 0.05$; **: $P < 0.01$.

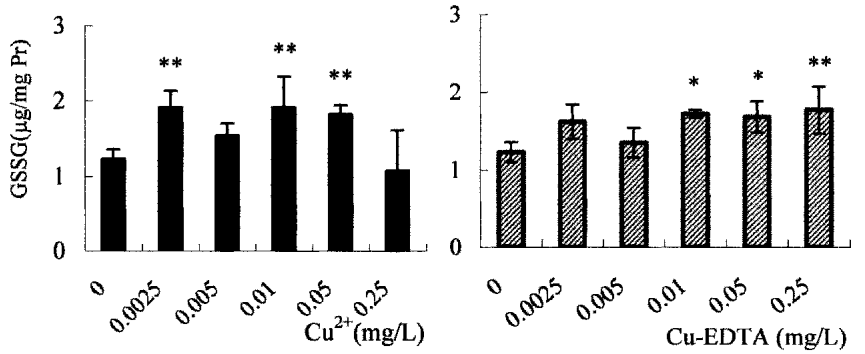

Figure 2. The effects of Cu^{2+} and Cu-EDTA on GSSG content in the liver of *Carassius auratus*. *: $P < 0.05$; **: $P < 0.01$.

Changes of GSH content in the liver of *Carassius auratus* were shown in Figure 3. Hepatic GSH contents were elevated significantly for 0.0025 mg/L of Cu^{2+} exposure and higher ($P<0.01$), and it increased 167% with 0.01 mg/L of Cu^{2+} exposure compared to the controls ($P<0.01$) (Figure 3 and Table 1). The increase of GSH contents under Cu-EDTA exposure was lower as compared to the same concentration of Cu^{2+} exposure, and it reached the greatest value at 0.005 mg/L of Cu-EDTA with an activation rate at 105% ($P<0.01$) (Figure 3 and Table 1). GSH contents can be increased due to an adaptive mechanism to slight oxidative stress through an increase in GSH synthesis. The GSSG accumulation in freshwater fish (Figure 2.) may induce the biotransformation of GSSG to GSH.

Hepatic GSH/GSSG ratio was calculated by dividing GSH content by GSSG content at exposure to Cu^{2+} and Cu-EDTA (Figure 4). The changes of GSH/GSSG ratio with 0.0025~0.005 mg/L of Cu^{2+} and Cu-EDTA treatments were similar, and increased significantly at 0.005 mg/L of Cu^{2+} and Cu-EDTA exposure, with activation rate at 72% and 98% compared to the controls ($P<0.01$)

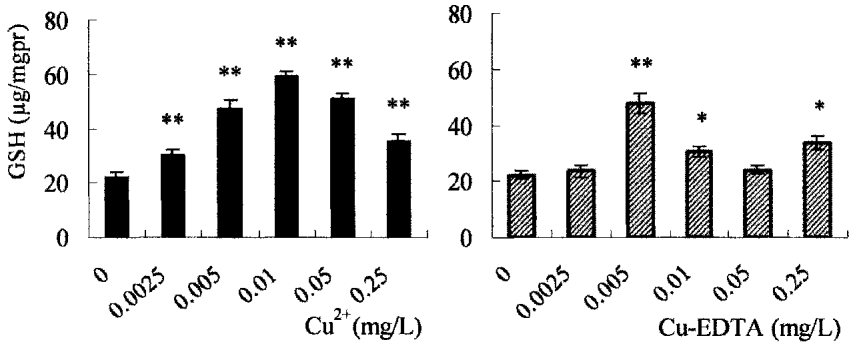

Figure 3. The effects of Cu^{2+} and Cu-EDTA on GSH content in the liver of *Carassius auratus*. *: $P < 0.05$; **: $P < 0.01$.

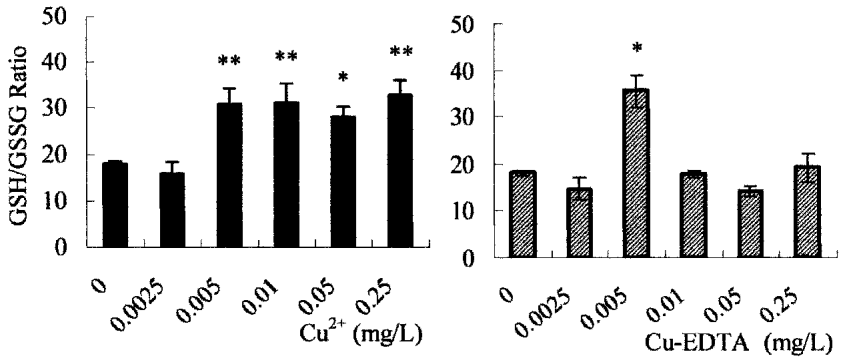

Figure 4. The effects of Cu^{2+} and Cu-EDTA on GSH/GSSG ratio in the liver of *Carassius auratus*. *: $P < 0.05$; **: $P < 0.01$.

(Table 1), respectively. In contrast, the GSH/GSSG ratio with 0.01~0.25 mg/L of Cu^{2+} treatment was higher than the same concentration of Cu-EDTA treatment. This suggests that the glutathione detoxification system was induced in the liver of fish exposed to a copper ion concentration over 0.005 mg/L.

In general, the effects of copper ion and Cu-EDTA on GR, GSH and GSSG of fish liver were different from each other, and it depends not only on the species of copper, but also on the types of enzymes. An eventually significant inhibition of GR, slight induce of GSSG contents and eventually significant induce of GSH contents were observed in fish liver at exposure to copper solutions. GSH can be served as the most sensitive parameter of the glutathione system for copper pollution. The present study shows that EDTA complexes may either enhance or reduce toxicity of copper on GR activity, GSSG and GSH contents, which may be due to the different complexation ability of copper ions at different concentrations. We suggest further study of this phenomenon.

Table 1. Results of Tukey analysis for multiple comparisons in response to GR activity, GSSG content, GSH content and GSH/GSSG ratio with Cu^{2+} or Cu-EDTA exposure to show the levels of significance (P).

C (mg/L)	Source of variation							
	Cu^{2+}				Cu-EDTA			
	GR	GSSG	GSH	GSH /GSSG	GR	GSSG	GSH	GSH /GSSG
0	ABab	Bbc	De	BCb	Aa	Bc	Cc	Bb
0.0025	ABab	Aa	Cd	Cb	Aab	ABabc	Cc	Bb
0.005	Aa	ABab	Bb	Aa	Bc	ABbc	Aa	Aa
0.01	BCb	Aa	Aa	Aa	Bc	ABab	Bb	Bb
0.05	CDc	Aa	Bb	ABa	Aab	ABab	Cc	Bb
0.25	Dc	Bc	Cc	Aa	Ab	Aa	Bb	Bb

The different upper case letters in each column of the table mean very significantly different (P<0.01) and the different lower case ones mean significantly different (P <0.05) by Tukey analysis.

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