Changes of superoxide dismutase and catalase activities in crucian carp（*Carassius auratus*）exposed to copper and recovery response

Hongxia Jiang[1] . Hongmei Yang[2]. Xianghui Kong[1]* . Shuping Wang[1]. Huiyun Guo[1]

[1]College of Fisheries, Henan Normal University, Xinxiang, Henan 453007, China
[2]Editorial Board of Journal of Zhengzhou University, Zhengzhou, Henan 450001, China
xhkong@htu.cn

Abstract: In this study, the effects of antioxidant enzyme parameters in the freshwater fish *Carassius auratus* to water-borne copper (Cu^{2+}) exposure were assessed, and the reversibility of enzyme activities post-copper exposure were studied. The fish were exposed to different concentrations (0.05, 0.1, 0.2, 0.5 and 1.0 mg/L) of copper for 96 h, and then the 1.0 mg/L exposure group was transferred to the clean water (control water without the introduced copper) and exampled after 1, 5, 10, 15, and 30d to assess the recovery profile. Responses of the activities of superoxide dismutase (SOD) and catalase (CAT) in kidney, gill, spleen and brain to copper exposure and the recovery profile were investigated. As shown in the results, the changes of the two antioxidant enzymes activities in different organs of *C. auratus* exposed to copper are different, SOD and CAT activities in gill and spleen increased at low copper concentrations and decreased at high copper concentrations. SOD and CAT activities in brain all decreased at various copper concentrations, while SOD activity in kiney increased at all copper concentrations. After removing 1.0 mg/L copper exposure, all the antioxidant enzyme activities recovered to the normal levels within 30 days, and SOD and CAT activities in gill normalized in the fastest speed, while the both enzyme activities in kindey normalized in the slowest speed. SOD and CAT activities in brain of *C. auratus* are more sensitive to copper exposure, which can be used as sensitive biomarkers to assess copper contamination in aquatic ecology.
[Jiang HX, Lei MY, Kong XH, Wang SP, Guo HY. **Changes of superoxide dismutase and catalase activities in crucian carp（*Carassius auratus*）exposed to copper and recovery response**. *Life Sci J* 2013;10(1):3281-3288] (ISSN: 109 7-8135). http://www.lifesciencesite.com. 414

Keywords: Copper exposure; antioxidant enzyme; recovery; *Carassius auratus*

1. Introduction

Copper is an essential micronutrient for growth, metabolism and enzyme activities of various organisms, while it becomes toxic and has a potential hazard to aquatic organisms when its concentration increases above certain natural levels. (Handy, 2003). For example, copper can result directly in impairment of protein function, peroxidation of lipids, damage to DNA and organelles, and depletion of ATP (Stohs and Bagchi, 1995; WHO, 1998). However, in recent years, environmental concentration of copper has been increasing in aquatic environments as a result of anthropogenic activities such as mining and smelting, agricultural and industrial emissions and municipal wastes. Copper pollution is particularly serious in aquaculture environments, because $CuSO_4$ is often used as algaecide, fungicide and bactericide to control the growth of phytoplankton and fish disease in aquaculture. The toxicity of copper to fish has been studied extensively by many workers in a wide range of biochemical, physiological, and behavioral effects in a number of fish species (Shariff et al.2001; Cerqueira and Fernandes 2002; Kolok et al.2002; Oliveira et al.2004). Moreover, the attention has also been focused on changes of enzymatic activities induced by copper, which showed the utility of enzymatic activity changes in biomonitoring program as an early warning indicator reflecting fish health status and copper pollution level (Antognelli et al.2003; Carvalho and Fernandes 2008; Atli et al.2006).

Some recent studies suggest that copper exhibits redox potential and generates reactive oxygen species (ROS) such as hydrogen peroxide, superoxide radicals, hydroxyl radicals resulting in oxidative stress (Mates 2000; Vutukuru et al., 2006;). While fish have developed several protective mechanisms to remove ROS before the detrimental effects occur in cell. Antioxidant enzymes are radical scavenging enzymes, which form the first line of defense against free radicals in organisms. The oxygen-scavenging antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GR). These enzymes can protect cellular membrane against the damage of ROS. SOD and CAT are two main enzymes in antioxidant systems, they play an indispensable role in scavenging radical under copper stress. SOD catalyzes the reduction of superoxide radical into hydrogen peroxide, which is eliminated by CAT into oxygen and water. (Di Giulio et al.,1995; Halliwell and Gutteridge, 2001). Thus, the two antioxidant enzymes contribute to the maintenance of a relatively low ROS level in cells (Hidalgo et al., 2002).

Although SOD and CAT have been demonstrated to be effective indicators for environmental stress in a variety of fish species (Atli et al.,2006; Vutukuru et al., 2006; Yi et al.,2007; Eyckmans et al.,2011). However, few work has been performed to compare the effectiveness of antioxidant enzymes in different organs for monitoring copper pollution in water using crucian carp. Crucian carp (*C.auratus*), an omnivorous fish, is widely distributed in freshwater in China. It is readily available throughout the year and easy to culture. Moreover, it can tolerate the highly polluted and eutrophic water. These properties make it well suited as the experimental fish to study copper effects on different enzyme activities in toxicity testing.

In this study, the changes of SOD and CAT activities in kidney, gill, spleen and brain of *C. auratus* after 96-h copper exposure were investigated. Furthermore, recovery process of these enzyme activities was evaluated after the removing of copper exposure. At present, some studies have focused on the reversibility of enzyme activity caused by different contaminant exposure in fish (Begum, 2004; De Menezes et al. 2011; Oruc, 2012). However, very few studies focused on the recovery of the copper induced changes in enzyme activity. In summary, the objective of this study is to investigate the responses of SOD and CAT in different organs of *C. auratus* to copper exposure, to assess the reversibility of these enzyme activities after removing copper exposure and to evaluate the effectiveness of these enzymes as early biomarkers to monitor copper pollution in aquatic ecosystems.

2. Material and Methods

Experimental fish and conditions

Healthy crucian carp (*C.auratus*) were obtained from the pond of farming of Henan Normal University (Xinxiang, China). The average wet weight of the fish was 50.58±2.15 g, and the average body length was 10.36±1.25 cm. Fish had been acclimatized for 14 days in 100 L tanks containing dechlorinated tap water. The tap water used for the experiments is pH value of 7.9±0.20, conductivity of 578.8±17.8μs/cm, total hardness of 305.2±19.3 mg $CaCO_3$/L and alkalinity of 140.1±12.5 mg $CaCO_3$/L. The fish were fed with commercial pellet at 2.0 % of body weight a day during acclimation, and without food during experimentation. The experiment was conducted at 25±1°C, with a 12 h light/ dark photoperiod under continuously aerating with an air pump.

Experimental protocol

Based on 96 h LC_{50} value (5.04 mg/L) of copper for *C. auratus* obtained by the acute toxicity test, in this study, sublethal copper concentrations were designed as 0, 0.05, 0.1, 0.2, 0.5 and 1.0 mg/L (corresponding to 0%, 1%, 2%, 4%, 10% and 20% of 96 h LC_{50}). The nominal copper concentrations were prepared in the different exposure tanks using the stored solution of $CuSO_4$, prepared by analytical grade $CuSO_4.5H_2O$ (from Chemical Reagent Company of China), 50% of the experimental solution was replaced daily to ensure the relative stabilization of copper concentrations. The tap water without adding copper ions was considered as the control. In pre experiment, fish exposed to 1.0 mg/L of copper concentrations for 96h did not show acute and subacute copper intoxication. Fish were randomly grouped into six, which were respectively exposed to the different copper concentrations for 96 h, with the duplication. Then, 5 fish of each group were sampled for biochemical analyses. The surplus fish exposed to 1.0 mg Cu^{2+} /L for 96h were transferred to the control water to conduct the recovery experiment, and then 5 fish were sampled on Day 1, 5 ,10, 15 and 30 respectively, and the fish without copper exposure were used as the control.

Sample preparation

The experimental fish were dissected carefully on ice, and the gill, spleen, kidney and brain were taken out and rinsed in ice-cold physiological saline immediately. The samples were homogenized in ice-cold physiological saline using a glass homogenizer (1g tissue: 9 ml buffer solution). Homogenates were centrifuged at 10000 *g* for 10 min at 4°C in a Universal 30RF centrifuge (Hettich, Tuttlingen, Germany). The supernatant was collected and stored at -80°C until biochemical analyses. All the above operations were carried out at 4°C.

Enzyme assays

SOD activity was measured according to the method of Masood et al. (1991), based on the inhibition of enzyme activity on the rate of NADH oxidation. Catalase (CAT) activity was determined by measuring the decomposition of H_2O_2 at 230 nm according to the method of Aebi (1984). One unit of SOD activity was defined as the amount of enzyme producing 50% inhibition of SOD per milligram of protein. One unit of CAT activity was defined as the quantity of enzymes to decompose 1μM H_2O_2 per minute per milligram of protein. Protein concentration of supernatant solution was determined by the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as the standard protein.

Statistical analysis

Experimental data are presented as Mean ± Standard Deviation (Mean±SD). Statistical analysis was implemented in SPSS statistical package programs. One-way ANOVA was used to compare variations among the different groups. An unpaired two-tailed Student's *t*-test was used to analyze significant differences. Significant level was assigned

at P=0.05 (significant difference) and P=0.01(extremely significant difference).

3. Results

Response of SOD activity in four organs to copper exposure

As seen in Fig.2, compared with the control, SOD activities in gill and spleen all increased firstly and then decreased with the increase of copper concentration. SOD activity in gill significantly increased at 0.10 and 0.20 mg/L (P<0.05), but it significantly decreased at 1.00 mg/L (P<0.01). SOD activity in spleen significantly increased at 0.05 mg/L (P<0.05), while it significantly decreased at 0.20, 0.50 and 1.00 mg/L (P<0.01). SOD activity in kidney significantly increased at all copper exposures (P<0.01). SOD activity in brain significantly decreased at all copper exposures (P<0.05 or P<0.01). Analysis also demonstrates a significant negative correlation between SOD activity in brain (Y) and the exposure concentrations (X), and the regress equation is $Y = -1.9327X+30.117$ (R^2=0.9447). SOD activity in gill, spleen and brain all reached the least value at 1.00 mg/L, with inhibition rate of 21%, 18% and 36% respectively. SOD activity in kidney reached the maximum value at 0.10 mg/L, with an activation rate of 63%.

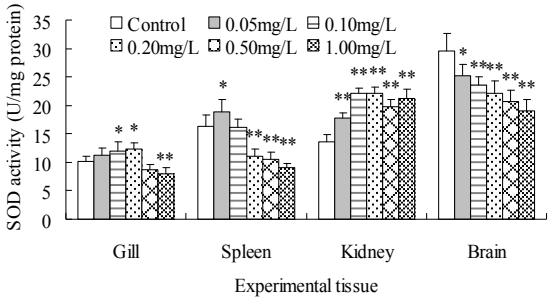


Fig.1. Changes of SOD activities in four organs of *C. auratus* after a 96-h copper exposure.
The values are expressed as mean±SD (n=5). Compared with the control, "*" represents the significant difference (p <0.05), "**" respresents the extremely significant difference (p <0.01).

Response of CAT activity in four organs to copper exposure

Changes of CAT activity are shown in Fig.2. CAT activity in gill and spleen all increased firstly and then decreased with the increase of copper concentration. Compared with the control, CAT activities in gill significantly decreased at 0.20, 0.50 and 1.00 mg/L (P<0.01). CAT activity in spleen significantly increased at 0.05 mg/L (P<0.01), while it significantly decreased at 0.10, 0.20, 0.50 and 1.00 mg/L (P<0.01). CAT activity in kidney and brain all significantly decreased at all concentrations (P<0.05 or P<0.01). Besides, a significant negative correlation was demonstrated between the CAT activity in brain (Y) and the exposure concentrations (X). The regress equation is $Y = -1.0223X+8.0371$ (R^2=0.9915). CAT activities in gill, kidney and brain all reached the minimum value at 1.00 mg/L, with inhibition rate of 31%, 55% and 71%, respectively. CAT activity in spleen reached the least value at 0.20 mg/L, with an inhibition rate of 53%.

Changes of SOD activity in four organs after removed 1.0 mg/L exposure

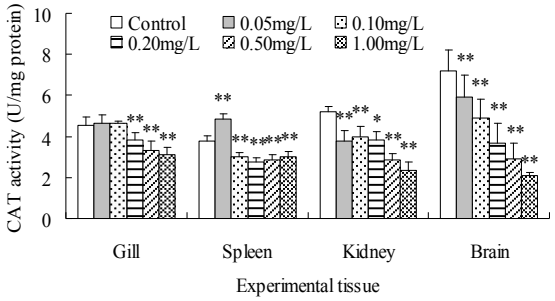


Fig.2. Changes of CAT activities in four organs of *C. auratus* after a 96-h copper exposure.
The values are expressed as mean±SD (n=5). Compared with the control, "*" represents the significant difference (p <0.05), "**" respresents the extremely significant difference (p <0.01).

As shown in Fig.3, compared with 96h exposure, along with recovery time extension, SOD activity in gill and spleen rised gradually, SOD activity in kidney rised firstly and then decreased, whereas SOD activity in brain decreased firstly and then increased. Compared with the control, there was no significant change in SOD activity in gill during the whole recovery period (P>0.05). SOD activity in kidney in recovery was significantly higher than in control on Day1, 5, 10 and 15 (P<0.01), while it was close to the control (P>0.05) and significantly lower than in 96h exposure (P<0.01) on Day 30. SOD activities in spleen and brain in recovery were significantly lower than in control on Day1, 5 and 10 (P<0.05 or P<0.01), while they were close to the control (P>0.05) and significantly higher than in 96h exposure (P<0.01) on Day15 and 30. The deta indicated that SOD activity in gill normalized in the fastest speed and the enzyme activity in kindey normalized in the slowest speed.

Changes of SOD activity in four organs after removed 1.0 mg/L exposure

As shown in Fig.3, compared with 96h exposure, along with recovery time extension, SOD activity in gill and spleen rised gradually, SOD activity in kidney rised firstly and then decreased, whereas

SOD activity in brain decreased firstly and then increased. Compared with the control, there was no significant change in SOD activity in gill during the whole recovery period (P>0.05). SOD activity in kidney in recovery was significantly higher than in control on Day1, 5, 10 and 15 (P<0.01), while it was close to the control (P>0.05) and significantly lower than in 96h exposure (P<0.01) on Day 30. SOD activities in spleen and brain in recovery were significantly lower than in control on Day1, 5 and 10 (P<0.05 or P<0.01), while they were close to the control (P>0.05) and significantly higher than in 96h exposure (P<0.01) on Day15 and 30. The deta indicated that SOD activity in gill normalized in the fastest speed and the enzyme activity in kindey normalized in the slowest speed.

Changes of CAT activity in four organs after removed 1.0 mg/L exposure

During the recovery period, changes of CAT activity in four organs are depicted in Fig.4. Compared with 96h exposure, along with recovery time extension, CAT activities in gill and kidney rised gradually, whereas CAT activities in spleen and brain decreased firstly and then increased. CAT activity in gill in recovery was significantly lower than that in control on Day1 (P<0.01), while it was close to the control (P>0.05) and significantly higher than in 96 h exposure (P<0.01) on Day 5, 10, 15 and 30. CAT activities in spleen and brain in recovery were significantly lower than in control on Day1, 5 and 10 (P<0.05 or P<0.01), while they were close to the control (P>0.05) and significantly higher than in 96h exposure (P<0.01) on Day 15 and 30. CAT activity in kidney in recovery was significantly lower than in control on Day1, 5, 10 and15 (P<0.01), but it was close to the control (P>0.05) and significantly higher than in 96h exposure (P<0.01) on Day 30. The deta indicated that CAT activity in gill normalized in the fastest speed and the enzyme activity in kindey normalized in the slowest speed.

4. Discussions

In the field of ecotoxicology, antioxidant enzymes are considered as sensitive biomarkers in environmental stress before hazardous effects occur in fish (Heath, 1987; Geoffroy et al., 2004). SOD and

CAT are the two primary antioxidant enzymes, which are involved in protective mechanisms within tissue injury following oxidative process and phagocytosis. Some laboratory studies have reported the increase or decrease in their activities in fish exposed to xenobiotics, and suggested their activities were related to the status of the organisms affected by different factors including dietary nutrition, environmental factors etc (Livingstone, 2001; Winstonand Giulio,1991).

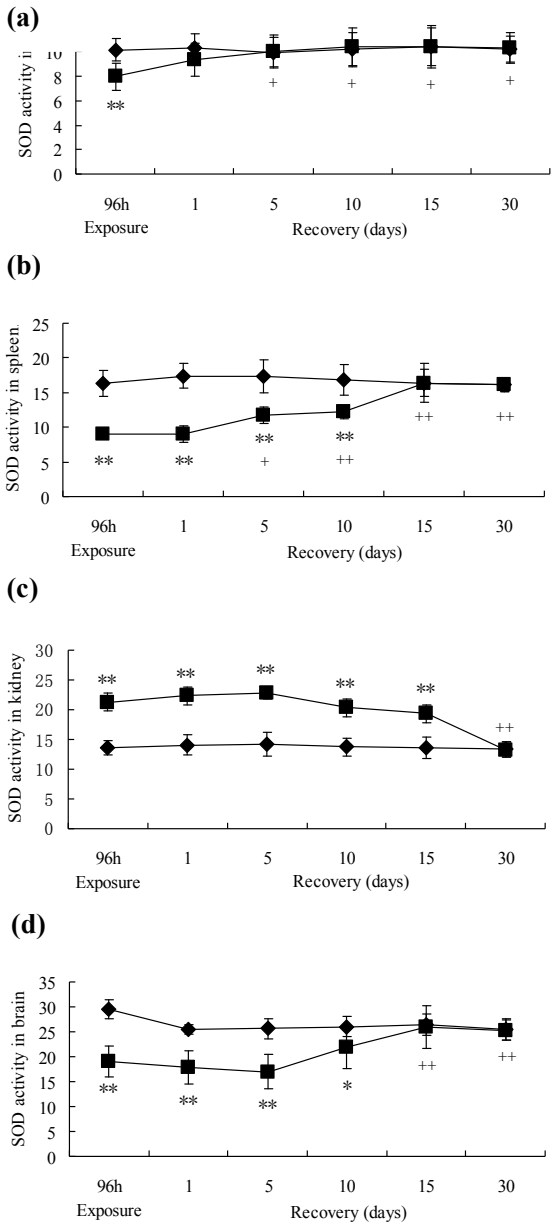
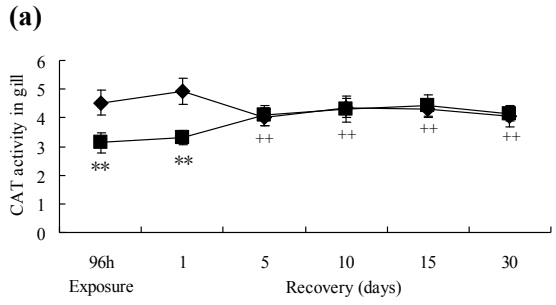


Fig.3. Changes of SOD activities in four organs of *C. auratus* from control group (◆) and group exposed to 1.0mg/L of copper for 96h and subsequent recovery in clean water (■). Data are expressed as mean±SD (n=5). Enzyme activity unit is U/mg protein. Compared with the control, "*" represents significant difference (p <0.05) and "**" represents the extremely significant difference (p <0.01). Compared with the 96h exposure, "$_+$" refers to significant difference (p <0.05) and "$_{++}$" respresents the extremely significant difference (p <0.01).

The two major antioxidant defense enzymes are inducible enzymes. They can be induced by a slight

oxidative stress due to compensatory response. However, a severe oxidative stress suppresses the activities of the both enzymes due to oxidative damage and a loss in compensatory mechanisms. Usually, higher SOD and CAT activities indicate there are more radicals need to be reacted (Andersen et al., 1998; Chien et al., 2003; Ross et al., 2001).Therefore, the increases in SOD and CAT activities in gill and spleen at lower copper concentrations might indicate that the copper stress resulted in an accumulation of radicals in fish. If these radicals induced by copper were not scavenged, the organisms would suffer from serious oxidative damage (Winston and Giulio, 1991).So, the enhanced activities of both SOD and CAT at low copper concentrations may enable fish to maintain health by scavenging the radicals produced. This increase may be an adaptive mechanism ensuring the organism survival, while it only occurred in a certain copper concentration extent. At high copper concentrations, SOD and CAT activities in gil and spleen decreased. The reason for this decrease may be that copper took the place of essential metals located in the active center of amylase; or it combined with functional groups located on the enzyme molecules, such as the hydroxyl group, peptidyl, and hydrosulfide groups (Von Borell 2000; Muhlia and García 2002), and hence decreased enzymatic activities.

The present observation of an increase in SOD activity in kidney at all copper concentrations suggests a physiological adaptation for the elimination of ROS generation. On the other hand, this increase in SOD activity also can probably be explained by the modification of the SOD isoform pattern. Géret et al. （2002）have revealed the existence of the most acidic isoforms SOD (SOD-3). This SOD isoform is oxidative and still possesses catalytic activity, and it is prone to induced by chemical environment (Sharonov and Churilova 1992). In the present study, crucian carp were exposed to copper contaminants, which probably induced a modification of the SOD pattern in kidney, which further explains the increase of SOD enzymatic activities in kidney. The inhibition of SOD activity in kidney under high pollutant concentration



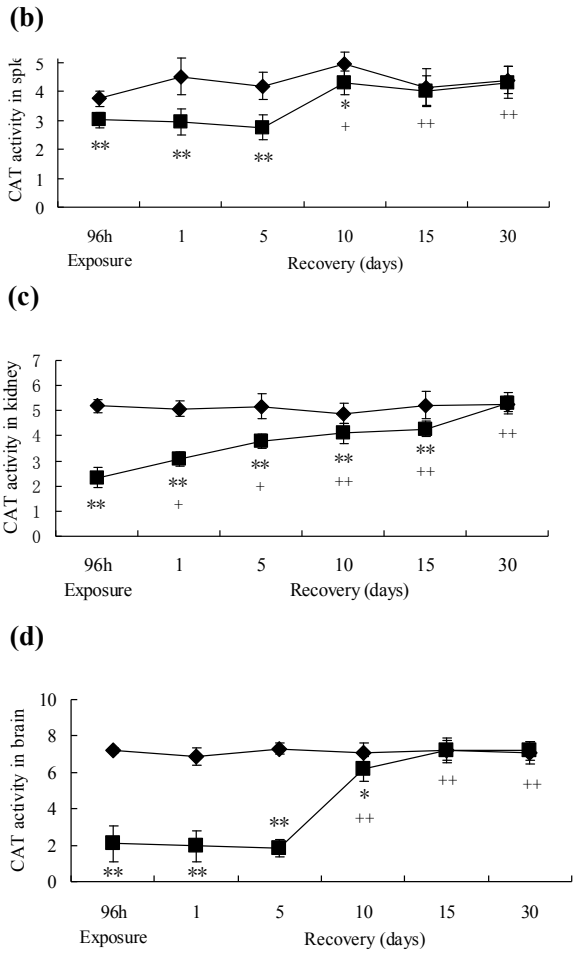


Fig.4. Changes of CAT activities in in four organs of *C. auratus* from control group (◆) and group exposed to 1.0mg/L of copper for 96h and subsequent recovery in clean water (■). Data are expressed as mean±SD (n=5). Enzyme activity unit is U/mg protein. Compared with the control, "*" represents significant difference (p <0.05) and "**" represents the extremely significant difference (p <0.01). Compared with the 96h exposure, "$_+$" refers to significant difference (p <0.05) and "$_{++}$" represents the extremely significant difference (p <0.01).

did not occur in our study. A possible explanation for this is that the concentration series may be within the tolerance range of SOD in kidney. On the contrary, CAT activity in kidney decreased at all copper concentrations. This may be due to the consequence of a complementary effect between antioxidative enzymes. Oruç and Usta (2007) reported that a decline in CAT activity can be attributed to high SOD activity.

Appreciable declines in SOD and CAT activities were observed in brain at all copper concentrations, suggesting impaired antioxidant defense mechanisms as a result of the excess generation of superoxide

radicals by copper in this organ. The decrease in SOD activity could be due to its inhibition by the excess production of ROS, the decrease in CAT activity could be due to its inactivation by the superoxide radical or due to decreases in the rate of the reaction as a result of the excess production of H_2O_2. SOD and CAT activities in brain all showed the highest inhibition rate among the investigated organs at 1.00 mg/L and a significant negative correlation was demonstrated between the two enzymatic activities in brain and the exposure concentrations. This indicates that the both enzymatic activities in brain are very sensitive to copper exposure and they also could be considered as the valuable early indicators of environmental copper exposure. Fish brain is the major component of the central nervous system, while water contaminants can effect the activities of various enzymes in brain (Bagnyukova et al., 2005; Modesto and Martinez, 2010) and even lead to the neurodegenerative damage (Berntssen et al., 2003). Several transporters have been suggested to transport copper into brain across the blood-brain barrier (Choi and Zheng, 2009). The sensitivity of antioxidant enzyme activities in brain in this study indicated that the brain of *C. auratus* is the main target of copper.

Generally speaking, the response of SOD and CAT activities in different organs exposed to different copper concentrations was found to be variable depending on copper concentrations, enzymic molecule structure, the interaction of enzyme molecule and copper, physiological functions and metal bioaccumulation of organs. Nevertheless, all enzyme activities in different organs of the fish in 1.0 mg/L group normalized within 30-day recovery span. It was demonstrated that these enzyme activities were reversible. In recovery process, enzyme activities restored at different speeds. SOD and CAT activities in gill normalized in the fastest speed and the both enzyme activities in kindey normalized in the slowest speed. Fish gill is the main site for gas exchange and has the other important functions such as ionic and osmotic regulation and acid-basee quilibrium. At the same time, fish gill is in direct contact with water environment and also is one of the excretory organs, this made the copper in it discharged rapidly during the recovery period, and thus the enzyme activities in this organ also restored fastly. On the other hand, after 96 h copper exposure, the decrease in SOD and CAT activities in gill at 1.0 mg/L would have led to the damage of respiratory function caused by the ROS which was not scavenged by SOD and CAT in time. Reduction in critical O_2 and O_2 diffusion capacity due to the respiratory disturbances have been reported in fish exposed to copper (Boeck et al.1995; Mazon et al.1999). So, the faster restoration of antioxidant enzymes activities in gill during the recovery period will facilitates the rapid recovery of respiratory function, which will allow fish to get more O_2 and energy and contribute to the restoration of the other organs. This is an adaptive mechanism, thus avoiding the death of the fish. The antioxidant enzymes activities in kidney restored slowly. This may be associated with the slow discharge of the copper from this organ caused by the high copper bioaccumulation of it, because fish kidney is the important compartments of heavy metal accumulation (Fallah et al.2011; Palaniappan and Karthikeyan 2009).

In recovery process, the enzymes in differernt organs exhibited different recovery pattern. SOD activity in kidney increased significantly at 1.0 mg/L compared with the control after 96-h copper exposure. While, after copper exposure was free, it did not decrease immediately compared with the 96-h exposure, but increased continuously from Day 1 to Day 5. The reason might be that the higher level of SOD mRNA made the enzyme synthesis continue in the first few days, although environmental factor to induce SOD activity had been eliminated. After 96-h copper exposure, SOD and CAT activities in gill, spleen and brain and CAT activity in kidney all significantly decreased at 1.0 mg/L compared with the control. However, during the recovery period, SOD activity in gill and spleen, CAT activity in gill and kidney increased continuously compared with 96-h exposure, suggesting the consistency between the change of enzyme activities and the elimination of copper. SOD activity in brain, CAT activity in spleen and brain decreased firstly and then increased compared with 96-h exposure. The reason might be that, in the first few days, the copper accumulated in fish organs was not excreted immediately and still inhibited the enzyme activities although copper in water had been eliminated. However, with recovery time extending, copper was gradually eliminated from organs, the enzyme activities were also gradually restored. The recovery pattern in this study is very similar with the result of recovery study on *Carassius auratus gibelio var* conducted by Jiang et al (2012). This different recovery pattern may be relate to the different physiological functions of organs. However, it is often difficult to explain the recovery responsiveness of enzymes to chemical stress because various factors will affect the activities of enzymes (Oost et al., 2003; Regoli et al., 2003). So, its detailed mechanism is still in need for further research.

Conclusions

In summary, the changes of the two antioxidant enzymes activities in different organs of *C. auratus* exposed to copper are different, SOD and CAT activities in gill and spleen all displayed the phenomenon of low-dose stimulation and high-dose

inhibition. SOD and CAT activities in brain were all inhibited by copper. While SOD activity in kiney was induced by copper. After removing 1.0 mg/L copper exposure, all the antioxidant enzyme activities recovered to the normal levels within 30 days suggesting that the 30 days of recovery period was long enough for the complete repair. In recovery process, SOD and CAT activities in gill normalized in the fastest speed and the both enzyme activities in kindey normalized in the slowest speed. Moreover, this study unambiguously demonstrates firstly that SOD and CAT activities in brain of *C.auratus* are more sensitive to copper exposure, which can be used as sensitive biomarkers to assess copper contamination in aquatic ecology. However, fish usually lives in a complex water environment. so, the answer to whether these two parameters are good as early biochemical markers of copper pollution relies on the nature of water environment in which fish live. Further studies are still needed.

Acknowledgments

This work was sponsored by Program for Science & Technology Innovation Talents in Universities of Henan Province (Grant no. 2011HASTIT012), Research Program of Application Foundation and Advanced Technology in Henan (Grant no. 102300410258), and partly supported by the Key Subjects of Fisheries in Henan Province, China.

Corresponding Author:
Dr. Xianghui Kong
College of Fisheries,
Henan Normal University,
Xinxiang, Henan 453007, China
E-mail: xhkong@htu.cn

References

1. Handy RD. Chronic effects of copper exposure versus endocrine toxicity: two sides of the same toxicological process? Comp Biochem Physiol 2003;135A:25–38.
2. Stohs SJ, Bagchi D. Oxidative mechanisms in the toxicity of metal ions. Free Radical Biology and Medicine 1995;18:321–336.
3. WHO. Copper: Environmental Health Criteria 200. World Health Organization, Geneva, Switzerland. 1998.
4. Shariff M, Jayawardena PA, Yusoff FM, Subasinghe R. Immunological parameters of Javanese carp *Puntius gonionotus* (Bleeker) exposed to copper and challenged with Aeromonas hydrophila. Fish Shellfish Immunol 2001;11: 281–291.
5. Cerqueira CCC, Fernandes MN. Gill tissue recovery after copper exposure and blood parameter responses in the tropical fish *Prochilodus scrofa*. Ecotoxicol Environ Safe 2002 ;52: 83–91.
6. Kolok AS, Hartman MM, Sershan J. The physiology of copper tolerance in fathead minnows: insight from an intraspecific, correlative analysis. Environ Toxicol Chem 2002;21: 1730–1735.
7. Oliveira M, Santos MA, Pacheco M. Glutathione protects heavy metal-induced inhibition of hepatic microsomal ethoxyresorufin O-deethylase activity in *Dicentrarchus labrax* L. Ecotoxicol Environ Saf 2004;58: 379–385.
8. Antognelli C, Romani R, Baldracchini F, De Santis A, Andreani G,Talesa V. Different activity of glyoxalase system enzymes in specimens of *Sparus auratus* exposed to sublethal copper concentrations.Chem Biol Interact 2003;142: 297–305.
9. Carvalho CS, Fernandes MN. Effect of copper on liver key enzymes of anaerobic glucose metabolism from freshwater tropical fish *Prochilodus lineatus*.Comp Biochem Physiol 2008:151A: 437–442.
10. Atli G, Alptekin O, Tukel S, Canli M. Response of catalase activity to Ag^{+},Cd^{2+},Cr^{6+},Cu^{2+} and Zn^{2+} in five tissues of freshwater fish *Oreochromis niloticus*.Comp Biochem Physiol 2006;143C:218–224.
11. Mates JM. Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology.Toxicology 2000;153:83–104.
12. Vutukuru SS, Chintada S, Madhavi K R, Rao JV, Anjaneyulu Y. Acute effects of copper on superoxide dismutase, catalase and lipid peroxidation in the freshwater teleost fish, *Esomus danricus*. Fish Physiol Biochem. 2006;32:221–229.
13. Di Giulio, RT, Benson WH, Sanders BM, Van Veld PA. Biochemical mechanisms: metabolism, adaptation, and toxicity. In: Rand, G. (Ed.), Fundamentals of Aquatic Toxicology, Effects, Environmental Fate, and Risk Assessment. Taylor and Francis, London, 1995;pp. 523–561.
14. Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine. Oxford University Press, Oxford, UK. 2001;936 pp.
15. Hidalgo MC, Expósito A, Palma JM, de la Higuera M. Oxidative stress generated by dietary Zn-deficiency: studies in rainbow trout (*Oncorhynchus mykiss*). Int J Biochem Cell Biol 2002;34, 183–193.
16. Yi XH, Ding H, Lu YT, Liu HH, Zhang M, Jiang W. Effects of long-term alachlor exposure on hepatic antioxidant defense and detoxifying enzyme activities in crucian carp (*Carassius auratus*). Chemosphere 2007;68 ,1576–1581.
17. Eyckmans M, Celis N, Horemans N, Blust R, De Boeck G. Exposure to waterborne copper reveals differences in oxidative stress response in three freshwater fish species. Aquat Toxicol 2011;103, 112–120.
18. Begum G. Carbofuran insecticide induced biochemical alterations in liver and muscle tissues of the fish *Clarias batrachus* (linn) and recovery response. Aquat Toxicol 2004;66: 83–92.
19. De Menezes C C, Loro V L, Da Fonseca M B, Cattaneo R, Pretto A, Dos Santos Miron D, Santi A. Oxidative parameters of *Rhamdia quelen* in response to commercial herbicide containing clomazone and recovery pattern. Pestic Biochem Phys 2011;100: 145–150.
20. Oruc E. Oxidative stress responses and recovery patterns in the liver of oreochromis niloticus exposed to

chlorpyrifos-Ethyl. Bull Environ Contam Toxicol 2012; 88:678–684.
21. Masood KJ, Rahman MF, Narsaiah J, Mustafa M. Haematological and spectrophotometric method for the determination of superoxide dismutase activity of giant freshwater prawn, *Macrobrachium rosenbergii*. Aquat Toxicol 1999;64:25–37.
22. Aebi H. Catalase in vitro. In: Packer, L. (Ed.), Methods in Enzymology, vol. 105. Academic Press Inc., San Diego, 1984; pp. 121–126.
23. Bradford M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye-binding. Anal Biochem 1976;72, 248.
24. Heath AG. Water Pollution and Fish Physiology. CRC Press, Florida, USA 1987; pp. 245.
25. Geoffroy L, Frankart C, Eullaffroy P. Comparison of different physiological parameter responses in *Lemna minor* and *Scenedesmus obliquus* exposed to herbicide flumioxazin. Environ Pollut 2004;131, 233–241.
26. Livingstone DR. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar Pollut Bull. 2001;42, 656–666.
27. Winston GW, Giulio RT. Prooxidant and antioxidant mechanisms in aquatic organisms. Aquat Toxicol. 1991;19, 137–161.
28. Andersen F, Lygren B, Maage A, Waago R. Interaction between two dietary levels of iron and two forms of ascorbic acid and the effect on growth, anti oxidant status and some non-specific immune parameters in Atlantic salmon (*Salmo salar*) smolts. Aquaculture 1998;161, 437–451.
29. Chien YH, Pan CH, Hunter H. The resistance to physical stresses by *Penaeus monodon* juveniles fed diets supplemented with astaxanthin. Aquaculture 2003;216, 177–191.
30. Ross SW, Dalton DA, Kranmer S, Christensen BL. Physiological (antioxidant) responses of estuarine fishes to variability in dissolved oxygen. Comp Biochem Physiol 2001;130C, 289–303.
31. Winston GW, Giulio RT. Prooxidant and antioxidant mechanisms in aquatic organisms. Aquat Toxicol 1991;19, 137–161.
32. Von Borell E. Stress and coping in farm animals. Arch Tierz (Sonderheft) 2000;43:144–152
33. Muhlia-Almazán A, García-Carreño FL. Influence of molting and starvation on the synthesis of proteolytic enzymes in the midgut gland of the white shrimp Penaeus vannamei. Comp Biochem Physiol 2002;133:383–394.
34. Géret F, Jouan A, Turpin V, Bebianno MJ, Cosson RP. Influence of metal exposure on metallothionein synthesis and lipid peroxidation in two bivalve mollusks: the oyster (*Crassostrea gigas*) and the mussel (*Mytilus edulis*). Aquat Living Resour 2002;15:61–66.
35. Sharonov BP, Churilova IV. Inactivation and oxidative modification of Cu/Zn superoxide dismutase by stimulated neutrophils: the appearance of new catalytically active structures. Biochem Biophys Res Commun 1992;189:1129–1135.
36. Oruç EÖ , Usta D. Evaluation of oxidative stress responses and neurotoxicity potential of diazinon in different tissues of Cyprinus carpio. Environ Toxicol Pharmacol 2007;23:48–55.
37. Bagnyukova TV, Vasylkiva O Yu, Storey KB, Lushchak VI. Catalase inhibition by aminotriazole induces oxidative stress in goldfish brain. Brain Res 2005; 1052:180 –186.
38. Modesto KA, Martinez CBR. Roundup causes Oxidative stress in liver and inhibits acetylcholinesterase in muscle and brain of the fish *Prochilodus lineatus*. Chemosphere 2010;78: 294–299.
39. Berntssen MHG, Aatland A, Handy RD. Chronic dietary mercury exposure causes oxidative stress, brain lesions, and altered behaviour in Atlantic salmon (*Salmo salar*) parr. Aquat Toxicol 2003; 65:55–72.
40. Choi BS, Zheng W. Copper transport to the brain by the blood-brain barrier and blood-CSF barrier. Brain Res 2009;1248:14-21.
41. Boeck G, Smet H, Blust R. The effect of sublethal levels of copper on the oxygen consumption and ammonia excretion in the common carp, *Cyprinus carpio*. Aquat Toxicol. 1995;32,127-141.
42. Mazon A F, Fernandes M N. Toxicity and differential tissue accumulation of copper in the tropical freshwater fish, *Prochilodus scrofa* (Prochilodontidae). Bull Environ Cont Toxicol. 1999;63,797-804.
43. Fallah AA, Saei-Dehkordi SS, Nematollahi A, Jafari T. Comparative study of heavy metal and trace element accumulation in edible tissues of farmed and wild rainbow trout (*Oncorhynchus mykiss*) using ICP-OES technique. Microchem J 2011;98: 275–279.
44. Palaniappan PR, Karthikeyan S. Bioaccumulation and depuration of chromium in the selected organs and whole body tissues of freshwater fish *Cirrhinus mrigala* individually and in binary solutions with nickel. J Environ Sci 2009;21:229–236.
45. Jiang HX, Yang HM, Kong XH, Wang SP, Liu DQ, Shi SJ. Response of Acid and Alkaline Phosphatase Activities to Copper Exposure and Recovery in Freshwater Fish *Carassius auratus gibelio var.* Life Sci J 2012;9(3):233-245.
46. Oost, RVD, Beyer J, Vermeulen NPE. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol Pharmacol 2003;13, 57– 149.
47. Regoli F, Winston GW, Gorbi S, Frenzilli G, Nigro M, Corsi I, Focardi S. Integrating enzymatic responses to organic chemical exposure with total oxyradical absorbing capacity and DNA damage in the European eel Anguilla anguilla. Environ Toxicol Chem 2003;22, 2120– 2129.