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# Time-Dependent Oxidative Stress Responses of Crucian Carp (*Carassius auratus*) to Intraperitoneal Injection of Extracted Microcystins

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**Abstract** This study was conducted to investigate time-dependent changes in oxidative enzymes in liver of crucian carp after intraperitoneally injection with extracted microcystins 600 and 150 μg $kg^{-1}$ body weight. The results showed that activities of antioxidant enzymes, including superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase generally exhibited a rapid increase in early phase (1–3 h post injection), but gradually decreased afterwards (12–48 h) compared with the control, with an evident time-dependent effect. These zigzag changes over time contributed a better understanding on oxidative stress caused by microcystins in fish.

**Keywords** Crucian carp · Extracted microcystins · Oxidative stress · Time-dependent

With the progress of eutrophication in aquatic environments, massive freshwater cyanobacterial blooms and the associated cyanotoxins contamination have been documented worldwide (Xie 2006). Among cyanotoxins, the hepatotoxic microcystins (MCs) are the most common group and more than 70 variants are known (Fastner et al. 2002). It is well known that MCs are able to inhibit protein phosphatases 1 and 2A with liver as target organ (Falconer and Yeung 1992).

Recent studies show that microcystins can generate oxidative stress, through increasing the concentration of reactive oxygen species (ROS) and oxidative damage products such as lipid peroxides, and evidences suggest that oxidative stress may play a significant role in the pathogenesis of microcystin toxicity in animals (Moreno et al. 2005; Prieto et al. 2006, 2007). Fish, like many other vertebrates, try to reduce the damage of the oxidative stress using the antioxidant defense system, including radical scavenging enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR). Also, the defense system includes many other antioxidants such as glutathione (GSH), vitamins E, C, A and carotenes. Some studies have demonstrated that microcystins, including cyanobacterial bloom cells, cyanobacterial extracts and pure MCs (LR/RR), can induce oxidative stress with increases of ROS, lipid peroxidation and alterations of antioxidant system in aquatic animals (Prieto et al. 2006, 2007). However, these previous studies only sampled and measured oxidative stress responses at the end of experiments, without a continuous recordation.

Crucian carp (*Carassius auratus* L.), an omnivorous fish, is a dominant fresh-water species in China. This carp can ingest a significant portion of toxic cyanobacteria in eutrophic lakes, leading to MC accumulation in liver and hepatic damage. Our previous studies have demonstrated that extracted microcystins can induce time-dose dependent haematological and plasma biochemical changes in crucian carp (Zhang et al. 2007, 2008). Intraperitoneal (i.p.) injection is an important means for toxicological studies under laboratory conditions. Therefore, the main aim of this study was to evaluate dynamics of the liver oxidative

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stress responses over time (with six sampling points) in curcian carp intraperitoneally injected with extracted microcystins, contributing to a better understanding on the time course effect of oxidative damage of liver organ.

## Materials and Methods

The cyanobacterial material used in this experiment was collected from surface blooms (phytoplankton cells) of Lake Dianchi, Yunnan in China during May and June, 2006. According to microscopic examinations, the predominant species was *Microcystis aeruginosa*. Freeze-dried crude algae were extracted three times with 75% (V/V) methanol. Quantitative analysis of MCs were performed using a reverse-phase high-performance liquid chromatography (HPLC, LC-10A, Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan) equipped with an ODS column (Cosmosil 5C18-AR, 4.6 × 150 mm, Nacalai, Japan) and a SPA-10A UV–Vis spectrophotometer set at 238 nm. MC concentrations were determined by comparing the peak areas of the test samples with those of the standards available (MC-LR MC-RR, and MC-YR, Wako Pure Chemical Industries, Japan). The obtained microcystins were a mixture (MC-RR + MC-LR + MC-YR) with purity >80%. The microcystin content in the cyanobacterial material was 1.41 mg $g^{-1}$ dry weight (DW), among which MC-RR, -LR and -YR were 0.84, 0.50 and 0.07 mg $g^{-1}$ DW, respectively. Extracted microcystins (EMCs) were finally diluted with salt solution water (0.9% NaCl).

Healthy crucian carp weighing 250 ± 30 g were obtained from a fish hatchery affiliated to College of Fisheries, Huazhong Agricultural Univeristy in Wuhan City, China. Fish were acclimatized for 2 weeks prior to experimentation in 150 L aquarium containing dechlorinated tap water and were fed with commercial crucian carp food at a rate of 2.0% of body weight per day. Feeding was terminated 48 h before initiation of the experiment, and no food was supplied to fish throughout the experiment. The experiment was conducted with a water temperature of 25 ± 1°C, a dissolved oxygen concentration between 6.0 and 7.1 mg $L^{-1}$ by continuously aerating, and a 12 h light/dark photoperiod. Five acclimated fish without administration were denoted 0 h and sampled 2 h prior to injection. Acclimated crucian carp were injected intraperitoneally (i.p) under ventral fin into the peritoneum by syringe. In the present study, doses of 600 and 150 μg EMCs $kg^{-1}$BW were designed for toxin exposure, and were expressed as high and low dose groups (n = 60). The control fish (n = 60) were injected i.p. with equal volume of salt solution water (0.9% NaCl). Six sampling points were set in the experiment (1, 3, 12, 24, 48 h and 7 days post treatment). At each sampling point, five fish for each dose group and the control were anaesthetized with 0.02% pH-neutralized tricaine methane sulfonate MS222 (ethyl-ester.3.aminobenzoic acid, Sigma). Crucian carp were killed by transection of the spinal cord, and the liver was removed and rinsed with ice-cold saline and kept at −85°C until analysis. The tissues were homogenized in chilled TRIS buffer (100 mM, pH 7.8; 1:10 w/v) using tissue homogenizer. The homogenates were centrifuged at 10,000×*g* for 20 min at 4°C to obtain the post-mitochondrial supernatant (PMS) for various biochemical analyses. Experiments were performed according to the guidelines of Ethical Committee for Animal Experiments at Huazhong Agricultural University, Wuhan of China.

Total superoxide dismutase (SOD; EC 1.15.1.1) activity was measured using method as described by McCord and Fridovich (1969). The assay mixture contained 0.51 mL potassium phosphate–EDTA buffer (pH 7.8), 0.1 mL xanthine 50 μM, 0.1 mL cytochrome *c* 500 μM, 50 μL homogenized tissue and $H_2O$. The reaction was initiated by the addition of 10 μL xanthine oxidase. SOD Activity was determined spectrophotometrically at 505 nm. One unit of SOD activity is the amount of enzyme needed to inhibit 50% of cytochrome *c* reduction. Catalase (CAT; EC1.11.1.6) activity was assayed by the method of Beers and Sizer (1952), consisting of the spectrophotometric measurement of $H_2O_2$ breakdown at 240 nm. The assay mixture contained 0.3 mL phosphate buffered saline (PBS), 2.07 mL $H_2O$, 30 μL homogenized tissue and 0.6 mL $H_2O_2$ (10 mM). The final concentrations in the cuvettes were thus 50 mM potassium phosphate (pH 7), 20 mM $H_2O_2$ and 30 μL homogenized tissue. Glutathione peroxidase (GPx; EC 1.11.1.9) activity was assayed by method of Lawrence and Burk (1976), following the rate of NADPH oxidation at 340 nm by the coupled reaction with glutathione reductase. The assay mixture contained 600 μL buffer with 50 mM potassium phosphate + 1 mM EDTA + 1 mM $NaN_3$ (pH 7.5); 100 μL 0.2 mM reduced glutathione (GSH), 100 μL 0.1 mM NADPH, 8 μL glutathione reductase and 20 μL homogenized tissue. After 5 min of preincubation (20–25°C), the reaction was initiated by the addition of 100 μL 0.25 mM $H_2O_2$. The specific activity was determined using the extinction coefficient of 6.22 $mM^{-1}$ $cm^{-1}$. Glutathione reductase (GR; EC 1.6.4.2) activity was determined spectrophotometrically measuring NADPH oxidation at 340 nm (Carlberg and Mannervik 1975). The reaction mixture contained 600 μL of the buffer containing 0.1 M potassium phosphate + 0.5 mM EDTA + 0.1 mM KCl (pH 7.5), 100 μL 0.1mMNADPH, 100 μL $H_2O$, and 100 μL homogenized tissue. After 5 min of preincubation (37°C), the reaction was initiated by addition of 100 μL 1 m MGSSG. All enzymatic activities are expressed in U/mg protein. The malondialdehyde (MDA), an end product of

lipid peroxidation, was measured by the thiobarbituric acid (TBA) method (Ohkawa et al. 1979). Briefly, 1 mL of a freshly prepared solution (thiobarbituric reactant solution) containing 15% trichloracetic acid (w/v), 0.375% TBA (w/v) and 2.5% hydrochloric acid (v/v) was added to 0.5 mL of 10% (w/v) homogenized tissue. After stirring, tubes were incubated in boiling water for 45 min, cooled on ice, and then subjected to centrifugation at 4,000×*g* for 15 min at 4°C to remove particulate matter. The absorbance of the supernatant was read on a spectrophotometer at 532 nm. Values were presented as nmol/mg protein. Protein contents in the samples were estimated by the method of Bradford (1976) using bovine-γ-globulin as standard. Every sample was measured in triplicates to get an average value for statistical use. All results expressed as mean ± SE were subjected to one-way analysis of variance (ANOVA) and Dunnett's Post Test using STATISTICA software package (Version 6.0, Statsoft, Inc.). Differences were measured against control values and considered to be statistically significant at $p < 0.05$.

## Results and Discussion

In the high dose group (600 μg $kg^{-1}$), 3, 5 and 11 fishes died at 12, 24 and 48 h post injection, respectively, and 100% fish mortality was observed at 60 h post injection. However, no mortality was found in both the low dose group (150 μg $kg^{-1}$) and the control. Activities of antioxidant enzymes (SOD, CAT, GPx and GR) changed with time in both dose groups (Fig. 1). SOD activities of both dose groups significantly increased at 3 h post injection ($p < 0.01$), however, the enzymatic activities of both dose groups exhibited marked decreases at 24 h ($p < 0.05$). SOD activities of the high dose group reached minimum values at 48 h ($p < 0.05$). CAT activities of both dose groups showed prominent increases at 3 h ($p < 0.01$), and CAT activities of low dose group kept the increase at 12 h post injection ($p < 0.05$). However, CAT activities of both dose groups reached the lowest values at 48 h post injection ($p < 0.05$). For GPx activities, significant increases ($p < 0.05$) were observed at 1 h in high dose group, as well as at 3 h in low dose group. However, GPx activities of both dose groups showed significant decreases at 24 h ($p < 0.05$), as well as at 48 h in comparison to the control ($p < 0.01$). GR activities of both dose groups showed significant increases at 1 h ($p < 0.05$), but marked decreases were observed during 24–48 h ($p < 0.05$). It was noticeable that all the four enzymatic activities of 7 day showed no significant difference compared with the control ($p > 0.05$). For both doses group, lipid peroxidation (LPO) levels showed no significant increase during 1–3 h ($p > 0.05$), but exhibited significant increases during 24–48 h compared with the control ($p < 0.05$).There was no significant alteration ($p > 0.05$) at 7 day between the low dose group and the control (Fig. 2).

Toxic cyanobacterial blooms can be associated with fish mortality. In the present study, a high mortality rate was observed in the high dose group. Liver, as main target organ of microcystins, is often used to determine oxidative stress response of fish after exposure to microcystins (Prieto et al. 2006, 2007). However, in previous studies, fish are sacrificed and sampled at the end of the experiment, without consideration of time factor. Although Prieto et al. (2007) have verified that the antioxidant status of tilapia fish varies with time in the experimental conditions with acute exposure toxic cyanobacterial cells, indicating a time-dependent effect, they only determined the responses of tilapia fish at 24 and 72 h after acute exposure of microcystins, no information about antioxidant responses on other time points was described. To the extent of our knowledge this is the first study focused on fish liver oxidative stress responses with time (seven sampling time points were designed). In this study, intraperitoneally injection of cyanobacterial extracts induced oxidative stress responses in crucian carp liver showing a time-dependent effect response against microcystins exposure, with an evident characteristic of zigzag changes.

In the experiment, exposure to MCs caused elevation in antioxidant enzymatic activities during 1–3 h in crucian carp liver, and the induction of the enzymatic antioxidant defenses after MC exposure could be considered as an adaptive response; that is, a compensatory mechanism that enables the fish to overcome the threat (Prieto et al. 2006). In the present study, SOD and CAT activities of crucian carp significantly increased within 3 h post MC injection. This is consistent with alterations in livers of loach (*Misgurnus mizolepis*), tilapia fish (*Oreochromis* sp.) after exposure to the purified microcystin (Prieto et al. 2006). Li et al. (2003) also demonstrated similar enhancement of SOD and CAT activities caused by MC-LR within 6 h exposure in hepatocytes of common carp (*Cyprinud carpio* L.). Prieto et al. (2006) showed that i.p.injection of pure MC-LR (500 μg $kg^{-1}$) induced a significant increase in the activities of GR in the liver of tilapia. GPx activity also showed a marked increase in both doses group. And the enhancement of GPx activity after exposure with microcystins was also observed in tilapia (*Oreochromis* sp.) (Prieto et al. 2006). And the increases in GPx activity are explained as an adaptation to the oxidative conditions to which the fish had been exposed.

On the other hand, liver oxidative stress responses parameters also showed prominent decline, especially in the late phase of experiment (12–48 h). A decrease in activities of antioxidant enzyme suggests an increased production of oxygen free radicals by microcystins (Moreno et al. 2005).

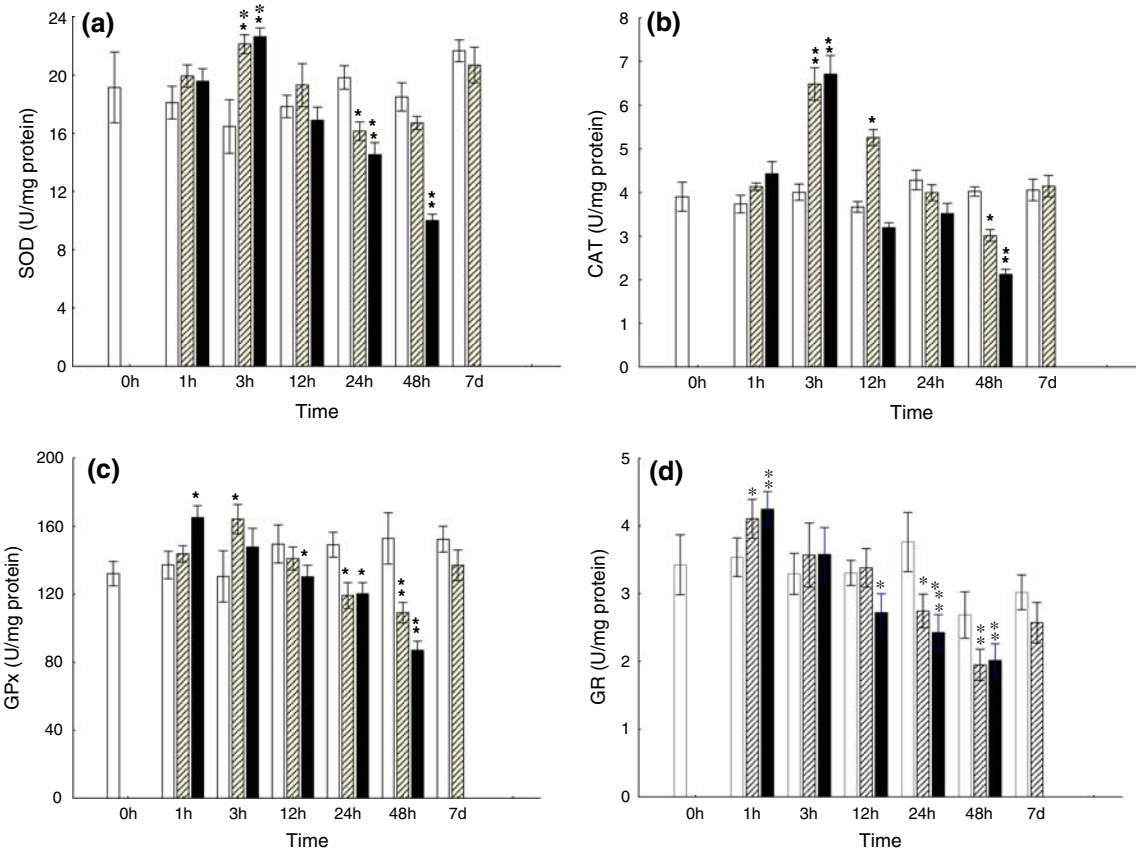


**Fig. 1** Antioxidant enzymes responses of liver in crucian carp after intraperitoneal injection of extracted microcystins with 600 and 150 μg $kg^{-1}$ body weight. Data are expressed as mean ± SE. □ Control ▨ 150 μg $kg^{-1}$ ■ 600 μg $kg^{-1}$. *White bars* indicate control fish, *gray bars* indicate the 150 μg $kg^{-1}$ BW dose, and *black bars* indicate the 600 μg $kg^{-1}$ BW dose

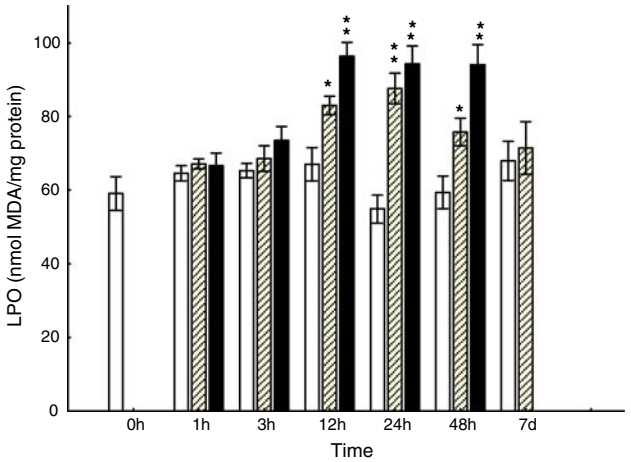


**Fig. 2** Lipid peroxidation (LPO) levels of liver in crucian carp after intraperitoneal injection of extracted microcystins with 600 and 150 μg $kg^{-1}$ body weight. Data are expressed as mean ± SE. □ Control ▨ 150 μg $kg^{-1}$ ■ 600 μg $kg^{-1}$. *White bars* indicate control fish, *gray bars* indicate the 150 μg $kg^{-1}$ BW dose, and *black bars* indicate the 600 μg $kg^{-1}$ BW dose

In fact, rats exposed intraperitoneally to an acute dose of pure MC-LR showed a significant decrease in the antioxidant enzymes, including CAT, SOD, GPx and GR (Moreno et al. 2005). Prieto et al. (2007) also observed a decrease in antioxidant enzyme (SOD and CAT) activities in tilapia fish, and attributed the decrease to different exposure periods and MC doses used in the experiments, i.e., higher MC dose may damage the enzyme proteins while lower dose, given for a longer period of time, may induce a defensive response. Reduction of antioxidant enzyme activities may be a consequence of decreased de novo synthesis of enzyme proteins or oxidative inactivation of enzyme protein (Kaushik and Kaur 2003).

One of the main manifestations of oxidative stress is lipid peroxidation. In this study, LPO levels significantly increased within 12–48 h in both dose groups, an indicator that marked oxidative damage have occurred. And the prominent increases of liver LPO value are also consistent with high mortalities of crucian carp after 12 h exposure. Prieto et al. (2006, 2007) have verified the enhancement of LPO values in tilapia fish (*Oreochromis* sp.) with acute exposure of microcystins.

At the end of this experiment (the 7th day), fish of the low dose group exhibited a recovery trend, which was indicated by the fact that there were no significant alterations of antioxidant enzyme values in comparison with

those of the control, as well as distinct drop of LPO levels. Similar recovery from oxidative damage was also observed in tilapia (*Oreochromis* sp.) at 72 h after acute exposure to toxic cyanobacterial cells containing microcysitins (Prieto et al. 2007). It should be pointed out that in the present study, most values of the antioxidant enzyme activities of the 7th day in the low dose group had not returned to the levels of the control fish, which is possibly because a longer time is required for the de novo synthesis of proteins (Prieto et al. 2007). Sun et al. (2006) reported that antioxidant enzymes such as CAT, SOD and glutathione-*S*-transferase (GST) in fish exposed to 0.05 mg $L^{-1}$ phenanthrene, needed 14 days to recover their activity after an initial reduction.

Although it is still relatively unknown about the causal relationship between microcystins and oxidative stress, some studies have tried to infer the possible toxicity mechanism of microcystin at this point (Li et al. 2003). MC can potently inhibit protein phosphatase type-1 and 2A after the toxin was transported to cytoplasm by the bile acid transporter in the cell membrane of hepatocytes and the inhibition may disturb the cellular phosphorylation balance, caused the marked increase of ROS contents and the depletion of GSH in hepatocytes. As a result, these changes would lead to oxidant stress in hepatocytes. Although antioxidant enzymes (SOD, CAT and GPx) had played their roles in eliminating ROS and regenerating GSH, they could not prevail over the oxidant stress induced by the toxin. In conclusion, the activities changes of antioxidant enzymes indicate that time courses are essential factors when evaluating oxidative stress responses caused by microcystins in fish. And further studies are needed to reveal the relationship between microcystins and oxidative stress.

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