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Plasma biochemical responses of the omnivorous crucian carp (*Carassius auratus*) to crude cyanobacterial extracts

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Abstract Healthy crucian carp (*Carassius auratus*) were treated by intraperitoneal (i.p.) injection of crude cyanobacterial extracts at two doses, 50 and 200 μg MC-LR equiv kg^{-1} BW. High mortality (100%) was observed within 60 h post injection in the high-dose group. In the treated fish, activities of four plasma enzymes, alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH), all showed substantial increases, with both dose and time-dependent effects. These increases of enzyme activity indicate severe impairment occurred in the liver of crucian carp over time. Plasma concentrations of energy-related biomolecules including glucose (GLU), cholesterol (CHO), triglyceride (TG), and total protein (TP) showed marked changes in the high-dose group, possibly a nutritional imbalance correlated with the liver injury caused by intraperitoneal exposure to crude cyanobacterial extracts.

Keywords *Carassius auratus* · Cyanobacterial extracts · Plasma · Biochemistry

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Introduction

Cyanobacterial blooms and their associated cyanotoxins have been documented in many eutrophic inland waters during past decades. Among cyanotoxins, the hepatotoxic microcystins (MCs), for example MC-LR and MC-RR, are considered to be one of the most dangerous groups. It is known that MCs accumulate mainly in the liver, but also in other organs for example the kidneys, gills, and intestine (Råbergh et al. 1991; Xie et al. 2004; Li et al. 2005; Malbrouck et al. 2006). In the subtropical eutrophic inland waters of China, massive cyanobacterial blooms occur in the summer. Crucian carp (*Carassius auratus* L), an omnivorous fish, is a dominant freshwater species in China. This carp can ingest a significant portion of toxic cyanobacteria in eutrophic lakes, leading to accumulation of MCs in various organs (Xie et al. 2004; Xie 2006).

Marked blood biochemical responses often occur after aquatic organisms have been exposed to microcystins, with injury of organs (Råbergh et al. 1991; Fischer and

Dietrich 2000; Malbrouck et al. 2003, 2006; Zhang et al. 2007). MC-induced liver damage has been correlated with the elevation of the activity of some plasma enzymes, for example alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) (Fischer and Dietrich 2000; Malbrouck et al. 2003, 2006; Molina et al. 2005). Pathological alterations in the blood activities of these enzymes are generally a result of hepatocyte leakage after cellular alteration or damage (Bernet et al. 2001; Malbrouck et al. 2006). The liver in fish plays an important role in several vital functions of basic metabolism, and levels of blood glucose (GLU), cholesterol (CHO), triglyceride (TG), and total protein (TP) are generally affected by the metabolism of carbohydrate, lipid, and protein, because of hepatic damage (Andenen et al. 1992). There have been a few studies documenting alterations of these indices after intraperitoneal injection with pure microcystins (Råbergh et al. 1991; Vajcová et al. 1998; Beasley et al. 2000).

Intraperitoneal (i.p.) injection is an important technique in toxicological studies. Many studies have used i.p. injection as the route of microcystin administration and purified MC-LR (concentration >95%) has been used as toxicant (Råbergh et al. 1991; Kotak et al. 1996; Vajcová et al. 1998; Beasley et al. 2000; Malbrouck et al. 2003, 2006). Alterations in blood biochemicals were documented in these studies. Compared to pure MC-LR, the toxic effects of crude cyanobacterial extracts are much more evident, as substances from crude extracts may increase the rate of uptake of toxins and the synergistic actions of toxins (Oberemm et al. 1997, 1999; Burýšková et al. 2006; Palíková et al. 2007; Falconer 2007). However, there have been no reports documenting blood biochemical responses of experimental animals to i.p. injection of crude cyanobacterial extracts. In view of these facts, the purpose of this study was, therefore, to evaluate the acute effects of i.p. injection of crude cyanobacterial extracts on plasma biochemical alterations in the crucian carp.

Materials and methods

Fish

Healthy crucian carp (mean body weight 265.0 ± 22.6 g) were purchased from a fish hatchery, and were transported to the laboratory. The experiment was conducted with a water temperature of 25 ± 1°C and a dissolved oxygen concentration between 6.0 and 7.1 mg l^{-1} by continuously aerating. Fish were allowed to acclimate for 14 days prior to experimentation in a 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. Fish were kept in a 12-h light/dark photoperiod.

Crude cyanobacterial extracts

Cyanobacteria (mainly composed of *Microcystis* spp.) were collected from surface blooms of Lake Dianchi, Yunnan of China; the predominant species was *Microcystis aeruginosa*. Crude microcystins in the cyanobacteria were extracted three times with 75% (*v*/*v*) methanol and were suspended in distilled water for the toxicity experiment. For determination of the MC content of the cyanobacteria, the extract was filtered through a glass microfibril filter (Whatman GF/C; Maidstone, UK) and the filtrate was diluted 1:5 with distilled water. The diluted filtrate was then applied to a 5-g reversed-phase ODS (octadecylsilane) column previously activated with 50 ml methanol (100%) followed by 50 ml distilled water. The cartridges were then washed with 50 ml distilled water. Microcystins were eluted from the cartridges with 100 ml 100% methanol and then evaporated to dryness. The residue was dissolved in 10 ml distilled water and used for final detection. Quantitative analysis of MCs was performed by reversed-phase high-performance liquid chromatography (HPLC; LC-10A; Shimadzu, Nakagyo-ku, Kyoto, Japan) on an ODS column (Cosmosil 5C18-AR, 4.6 × 150 mm; Nacalai, Japan); detection was with an SPA-10A UV–vis spectrophotometer set at 238 nm. MC concentrations were determined by comparing peak areas of the test samples with those of the standards (MC-LR and MC-RR; Wako Pure Chemical Industries, Japan).The MCs content was 1.34 mg g^{-1} dry weight (DW), among which MC-RR and -LR were 0.84 and 0.50 mg g^{-1} DW, respectively. The crude microcystins extracts was finally diluted with aqueous salt

solution (0.9% NaCl) to 136.5 μg ml^{-1} MC-RR and 22.7 μg ml^{-1} MC-LR.

Experimental protocol

Ten acclimated fish without administration were denoted 0 h and sampled 2 h prior to injection. A total of 180 acclimated crucian carp were injected intraperitoneally (i.p) under the ventral fin, into the peritoneum, by syringe. Fish in the low-dose ($n = 60$) and high-dose ($n = 60$) groups were injected i.p. with crude cyanobacterial extracts containing 600 and 150 μg microcystins (MC-LR + MC-RR) per kg body weight (BW). Since LD_{50} (i.p.) in mice for MC-RR is about five times higher than for MC-LR (Gupta et al. 2003), the doses of 600 and 150 μg kg^{-1} extracted MC-RR and MC-LR toxins injected in this study were equivalent to 200 and 50 μg kg^{-1}purified MC-LR, respectively. Control fish ($n = 60$) were injected i.p. with an equal volume of distilled water. The 60 fish in each dose group and in the control group were divided equally into 12 aquariums. Six sampling points were set during a period of 7 days in the experiment (1, 3, 12, 24, 48 h, and 7 days post treatment). At each sampling point, ten fish (from two tanks) for each dose group and the control were anaesthetized with 0.02% pH-neutralized tricaine methane sulfonate MS222 (ethyl ester 3-aminobenzoic acid; Sigma) and blood samples were taken by caudal puncture with heparinized syringes. Blood was centrifuged at 3,000*g* for 15 min at 4°C; and plasma was stored at −70°C until analysis.

Plasma biochemical analyses

For determinations, plasma enzyme activities (ALT, AST, LDH, and ALP) and concentrations of GLU, CHO, TG, and TP were measured with commercially available reagent kits (Zhongsheng, China) based on colorimetric reaction in an automatic analyzer (ACTA, Italy). According to the manufacturers' methods, activities of enzymes ALT (EC 2.6.1.2) and AST (EC 2.6.1.1) were determined by the method of Bergmeyer et al. (1976). LDH (EC 1.1.1.27) activity was measured by the method of Bergmeyer et al. (1974), and ALP (EC3.1.3.1) activity was determined according to method of McComb and Bowers (1972). Other plasma biochemical indices (GLU, CHO, TG, and TP) were measured by use of standard methods (Lowry et al. 1951; Hawk et al. 1954).

Statistical analysis

Values were expressed as mean ± SD. One-way Analysis of Variance (ANOVA) followed by Dunnett's test was used to determine the levels of difference between all doses groups over time, using the Statistica software package (Version 6.0, Statsoft). Differences were measured and considered to be statistically significant at $P < 0.05$.

Results

In the high-dose group (200 μg MC-LR equiv kg^{-1} BW), 3, 5, and 11 fishes died 12, 24, and 48 h post injection, respectively, and 100% fish mortality was observed 60 h post injection. In contrast, no mortality was found in the low-dose group (50 μg MC-LR equiv kg^{-1} BW) or the control group. In the high-dose group, ALT, ALP, and AST activities all increased significantly ($P < 0.05$) from 1 h post injection, with rises of 69.44 ± 5.72, 55.17 ± 4.33, and 34.24 ± 2.58%, respectively, while LDH activity increased significantly ($P < 0.01$) from 3 h post injection with an increase of 37.25 ± 7.36%. However, in the low-dose group, ALT and ALP activities increased significantly ($P < 0.05$) from 1 h post injection whereas AST and LDH activities increased significantly ($P < 0.01$) from 3 h post injection. ALT, AST, ALP, AST, and LDH activities exhibited significant differences ($P < 0.05$) at 48 h between the low-dose and high-dose groups. Activities of the four enzymes were apparently lower after 7 days than at 48 h post injection, and the fish exposed to the low dose of MC-LR exhibited a recovery trend. Enzymes AST, ALT, and ALP activities remained significantly ($P < 0.05$) higher than in the control group until the end of experiment and there were no significant changes ($P > 0.05$) in the control (Fig. 1).

Similar changes of GLU ($P < 0.01$) were observed in both dose groups, with maximum values at 12 h. TP, TG, and CHO levels in the high-dose group exhibited significant changes ($P < 0.01$), whereas no prominent changes ($P > 0.05$) were

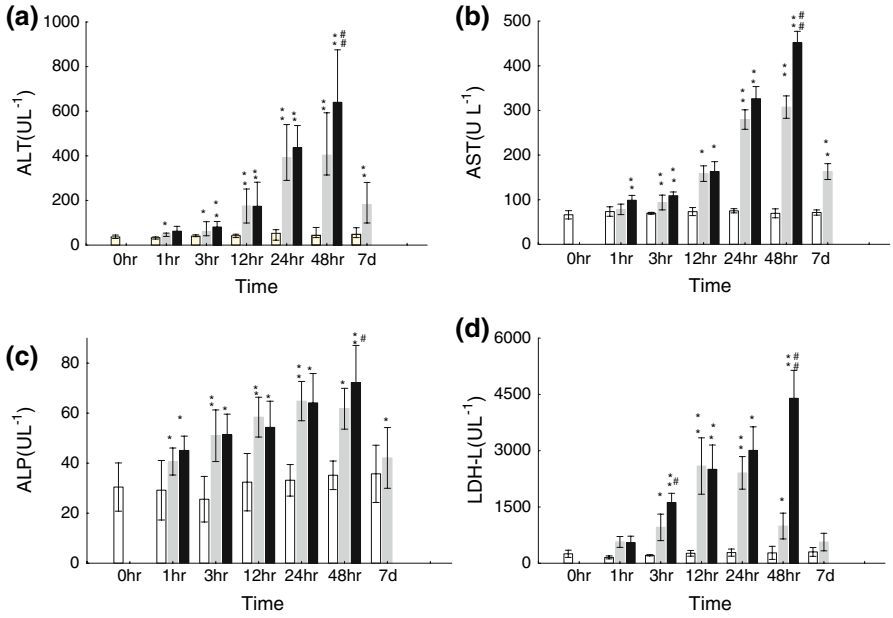

Fig. 1 Effect of i.p. injection of crude cyanobacterial extracts (50 and 200 μg MC-LR equiv kg^{-1} BW) on the activity of major plasma enzymes in the crucian carp. Values are expressed as mean ± SD. *Asterisks* indicate significant differences at $P < 0.05$ between MC-treated groups and the control group. *Hash symbols* indicate significant differences at $P < 0.05$ between the low dose and high-dose groups. *White bars* indicate control fish, *gray bars* indicate the 50 μg kg^{-1} dose, and *black bars* indicate the 200 μg kg^{-1} dose

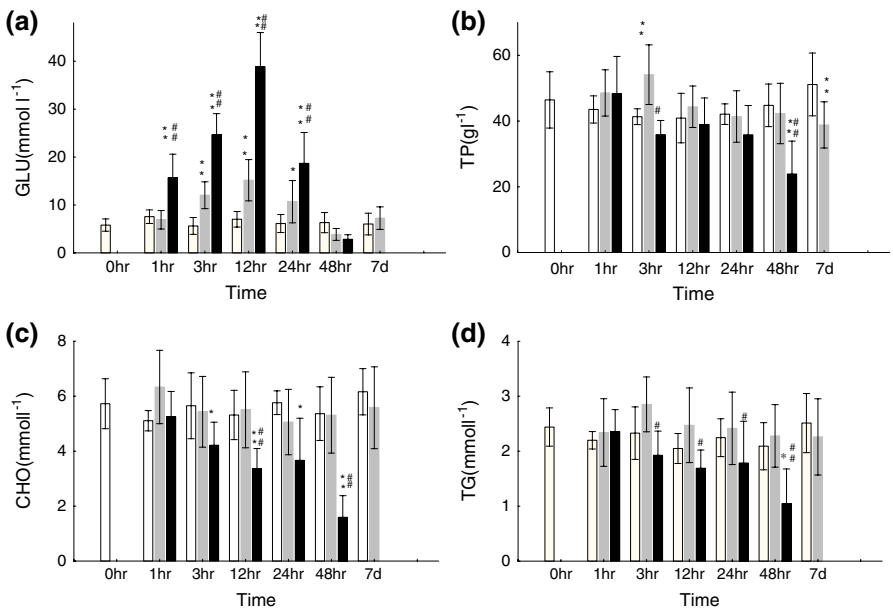

Fig. 2 Effect of i.p. injection of crude cyanobacterial extracts (50 and 200 μg MC-LR equiv kg^{-1} BW) on concentrations of basic metabolism-related plasma indices of crucian carp. Values are expressed as mean ± SD. *Asterisks* indicate significant differences at $P < 0.05$ between MC-treated groups and the control group. *Hash symbols* indicate significant differences at $P < 0.05$ between the low dose and high-dose groups. *White bars* indicate control fish, *gray bars* indicate the 50 μg kg^{-1} dose, and *black bars* indicate the 200 μg kg^{-1} dose

observed in the low-dose group and control (Fig. 2). TP, TG, and CHO levels exhibited significant differences at 48 h between the low-dose and high-dose groups.

Discussion

In this study, high mortality was observed in the high-dose group injected with crude cyanobacterial extracts (200 μg MC-LR equiv kg^{-1} BW). This dose is evidently lower than the lethal dose of 550 μg kg^{-1} in the common carp (*Cyprinus carpio*) and the LD_{50} for rainbow trout (*Oncorhynchus mykiss*), which is between 400 and 1,000 μg kg^{-1}, on injection of pure MC-LR (Råbergh et al. 1991; Kotak et al. 1996). These substantially different lethal doses might be attributed to the strong toxic effects of crude cyanobacterial extracts, as previous studies indicate that toxic effects of crude cyanobacterial extracts are

much more evident than those of pure MC-LR (Oberemm et al. 1997, 1999; Burýšková et al. 2006; Palíková et al. 2007). Burýšková et al. (2006) support the suggestion that microcystins are not the only or major toxic compounds in the complex cyanobacterial samples (at least for some species) and that more attention should be paid to other components of complex cyanobacterial biomass.

Blood parameters are useful and sensitive for diagnosis of diseases and monitoring of the physiological status of fish exposed to toxicants (Adhikari et al. 2004). MC-induced alteration of the activities of plasma enzymes (e.g. ALT, AST, ALP, and LDH) are frequently used to determine the toxic effects of microcystins (Råbergh et al. 1991; Vajcová et al. 1998; Malbrouck et al. 2003; Molina et al. 2005). These enzymes are present at low concentrations in plasma, and increases of their activities in plasma indicate the development of tissue lesions, particularly hepatic lesion (Malbrouck et al. 2003). In the current study, activities of the four liver-correlated enzymes progressively increased over time, an indicator of severe injury of the liver of crucian carp. Malbrouck et al. (2003) have demonstrated that significant increases in enzyme activity occurred in the order ALT > AST > LDH in goldfish (*Carassius auratus*) from 3, 24, and 24 h post injection with 125 μg pure MC-LR kg^{-1} BW. Råbergh et al. (1991) reported rather irregular changes in the activities of plasma enzymes (ALT, LDH, and AST) in common carp (*Cyprinus carpio*) after an intraperitoneal injection of 150 μg pure MC-LR kg^{-1} BW. Few studies have demonstrated ALP activity alterations caused by microcystins in fish (Molina et al. 2005). In the current experiment, significant increases in the activities of ALT, ALP, and AST were observed 1 h post injection, and LDH activity increased dramatically from 3 h; the magnitude of increase was in the order ALT > ALP > AST > LDH. On the other hand, the extents of the increase in enzyme activity in the current study exceeded those of the earlier studies (Råbergh et al. 1991; Vajcová et al. 1998; Malbrouck et al. 2003). It seems that crude cyanobacterial extracts have more prominent effects than purified MC-LR on alterations of the activity of plasma enzymes in crucian carp. Similar phenomena were also observed in the embryonic development of aquatic organisms treated with crude cyanobacterial extracts (Oberemm et al. 1999; Burýšková et al. 2006; Palíková et al. 2007). It has also been reported that the toxic effects of crude extracted microcystins are much more evident than those of pure MC-LR, probably because substances (not yet identified) in the crude extracts increase the rate of uptake of toxins; synergistic actions of toxins and unknown substances could also increase toxicity. Falconer (2007) reports that cyanobacterial toxins present in *Microcystis aeruginosa* extracts are more toxic than microcystins, and that the potentially numerous nonmicrocystin compounds with bioactivity will not be quantitated. In fact, it has been reported that lipopolysaccharides and microcystins have a synergistic relationship; lipopolysaccharides in the cell extracts might therefore enhance the toxicity of microcystins (Best et al. 2002; Ding et al. 2006). It is worth noting that in the current study, activities of all plasma enzymes in the low-dose (50 μg MC-LR equiv kg^{-1} BW) group were reversible, suggesting a possible trend of the fish to return to their normal status. This recovery was similar to results for juvenile *Carassius auratus* i.p. injected with 125 μg pure MC-LR kg^{-1} BW (Malbrouck et al. 2003).

In fish, mobilization of energy stores is required to maintain homeostasis during a chemical challenge. Blood concentrations of energy-related biomolecules may be useful biomarkers of toxicant stress, and GLU, TP, CHO, and TG may also be used as energy sources during severe stress (Vajcová et al. 1998; Rěhulka 2000). Plasma GLU is an indicator of metabolic stress, and the biological significance of elevated glucose levels in fish is probably related to an increased demand for metabolic substrates after exposure to a stress stimulus. Råbergh et al. (1991) and Malbrouck et al. (2003) explain alterations in plasma GLU levels as a consequence of disruption of glycogen homeostasis resulting from protein phosphatase inhibition. The gradually decreasing GLU level was probably a result of acute failure of hepatic gluconeogenesis and of altered glucose mobilization and metabolism (Beasley et al. 2000). In the current study, TP, CHO, and TG in the high-dose group gradually decreased, levels becoming very low compared with the control, indicating a possible nutritional imbalance after exposure to MC-LR; this result is in accordance with earlier studies (Vajcová et al. 1998; Beasley et al. 2000) in which it is concluded that

decreased TP implies a failure of protein synthesis because of liver injury caused by microcystins. In the low-dose group, TP, CHO, and TG levels exhibited no remarkable changes, indicating a dose-dependent effect.

In conclusion, this study demonstrates that i.p. injection of crude cyanobacterial extracts induced significant elevations in the activity of liver-related enzymes, and such increases were faster and greater in extent than in previous studies with i.p. injection of pure MC-LR. Plasma concentrations of energy-related biomolecules changed markedly in the high-dose group, possibly because of nutritional imbalance correlated with hepatic injury after exposure to the cyanobacterial extracts.

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