Hepatic Histopathological Characteristics and Antioxidant Response of Phytoplanktivorous Silver Carp Intraperitoneally Injected with Extracted Microcystins[1]

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Objective To investigate the hispathological characteristics and antioxidant responses in liver of silver carp after intraperitoneal administration of microcystins (MCs) for further understanding hepatic intoxication and antioxidation mechanism in fish. **Methods** Phytoplanktivorous silver carp was injected intraperitoneally (i.p.) with extracted hepatotoxic microcystins (mainly MC-RR and -LR) at a dose of 1000 μg MC-LReq./kg body weight, and liver histopathological changes and antioxidant responses were studied at 1, 3, 12, 24, and 48 h, respectively, after injection. **Results** The damage to liver structure and the activities of hepatic antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxide (GPX) were increased in a time-dependent manner. **Conclusion** In terms of clinical and histological signs of intoxication and LD_{50} (i.p.) dose of MC-LR, silver carp appears rather resistant to MCs exposure than other fishes. Also, the significantly increased SOD activity in the liver of silver carp suggests a higher degree of response to MCs exposure than CAT and GPX.

Key words: Antioxidant response; Histopathological characteristics; Liver; Microcystins; Fish

INTRODUCTION

The occurrence of *Microcystis* blooms has been frequently reported in freshwater bodies all over the world. The blooms not only decrease water quality, but also increase the risk to animals, humans and plants as a large number of species of *Microcystis* (mainly *Microcystis aeruginosa*) are capable of producing a group of highly potent hepatotoxins called microcystins (MCs)[1-2]. There are more than 70 microcystin isoforms, but the most commonly studied variant is microcystin-LR (MC-LR), followed by MC-RR and MC-YR[2].

One of the well-studied toxic mechanisms of MCs is their ability to inhibit protein phosphatases 1 and 2A, inducing hyperphosphorylation of cytosolic and cytoskeletal proteins, which leads to disruption of the hepatocyte cytoskeleton in animals and fish[3-4]. At present, there is also evidence that oxidative damage is involved in the development of MCs toxicity in mammals[5-6]. To date, however, the effects of MCs on antioxidant systems of fish have only been observed in *Danio rerio* embryos[7], isolated hepatocytes of common carp (*Cyprinus carpio*)[4], liver of goldfish (*Carassius auratus*) and loach (*Misgurnus mizolepis*) either injected intraperitoneally (i.p.) with MC-LR or exposed to cyanobacterial blooms[8-9]. Little information is available on the toxic effects of MCs in phytoplanktivorous fishes that are more frequently exposed to cyanobacterial toxins under natural conditions because of habitat and feeding mode. It has been reported that silver carp (*Hypophthalmichthys molitrix*) accumulates MCs in intestine, blood, liver, kidney and muscle like other freshwater fish[10], at levels above the tolerable daily intake (TDI) of 0.04 μg/kg body weight (BW) per day proposed by WHO as a provisional guideline value[11]. This is of great concern for public health because chronic ingestion of trace amounts of MCs (MC-LR in particular) in food and drinking water has a considerable potential to promote cancer[2].

Silver carp, one of the most intensively cultured

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freshwater phytoplanktivorous fish in China, is especially important to human beings because of its roles in aquatic ecosystems as a direct consumer of phytoplankton and zooplankton, its importance as food fish, and its potential for biological management of cyanobacterial blooms[12-13]. In this context, the present study was to investigate the hispathological characteristics and antioxidant responses by assay of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxide (GPX) as well as reactive oxygen species (ROS) in liver of silver carp after intraperitoneal administration of MCs to further understand the hepatic intoxication and antioxidation mechanism in fish.

MATERIALS AND METHODS

Toxin

Freeze-dried cyanobacterial materials used in experiment were collected from Lake Dianchi, Yunnan of China. MCs were prepared and measured as previously described[14] with some improvements. Briefly, the materials were extracted three times with 10 mL of 75% methanol (v/v) for 3 h at 4 ℃. The extract was centrifuged at 10 000×g for 30 min, and supernatants were pooled and applied to a C_{18} cartridge (Dalian Institute of Chemical and Physical Research, China). The cartridge containing MCs was rinsed with 10 mL of water and MCs were finally eluted from the C_{18} cartridge with 10 mL of methanol. The elution was evaporated under a reduced pressure and the residue was dissolved in water. Quantitative and qualitative analyses 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 measured by comparing the peak areas of test samples with those of the standards available (MC-LR and MC-RR, Wako Pure Chemical Industries, Japan). The obtained microcystin was MC-RR+MC-LR+MC-YR with a purity >80%. The microcystin content was 1.41 mg g^{-1} dry weight (DW). MC-RR, -LR, and -YR accounted for 0.84, 0.50, and 0.07 mg g^{-1} DW, respectively.

Fish, Treatment and Sample Preparation

Silver carp with a mean weight of 25.5±1.4 g (n=21) was purchased from a local fish hatchery (Wuhan, China). Fish were acclimated for 3 days prior to experimentation in a 100-liter aquaria containing dechlorinated tap water 20±1 ℃ with its pH maintained at 7.4 and dissolved oxygen value set at 7.5 mg/L. No food was given to the fish throughout the experiment.

The treated fish were injected intraperitoneally (i.p.) with MC-LR+MC-RR+MC-YR at a dose of 2000 μg /kg BW. The control fish were injected i.p. with distilled water. The reported LD_{50} (i.p.) dose of MC-LR, -RR, and -YR for 24 h in mice is 43, 235.4, and 110.6 μg/kg BW, respectively[15]. Thus the toxicity of MC-RR and MC-YR in mice was nearly 20% and 40% of MC-LR, respectively. Comparably, the dose of 2000 μg/kg BW injected with extracted compound toxins of MC-LR, -RR, and -YR in this study, according to the toxicity of MC-RR and MC-YR, was equivalent to 1000 μg/kg of purified MC-LR.

Three MC-treated fish were killed at 1 h, 3 h, 12 h, 24 h, and 48 h post-injection, respectively. Three control fish were killed at 0 and 48 h. For each sampling, liver samples were excised and divided into two parts. One was immediately fixed in Bouin's fixative for microscopic analysis, and the other was frozen at -80 ℃ for biochemical analysis.

Histopathological Observation

Liver tissue (3 mm^3) was fixed in Bouin's fixative, processed routinely, embedded in paraffin, cut into 4-μm thick sections. The sections were stained with hematoxylin and eosin (HE) and observed under a light microscope at 400× magnification.

Measurement of Biochemical Parameters in Liver

Liver samples were homogenized (1:10, w/v) in a cold (4 ℃) buffer solution containing 20 mmol/L Tris base, 1 mmol/L EDTA, 1 mmol/L dithiothreitol (DTT, sigma), 0.5 mmol/L sucrose and 150 mmol/L KCl, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), with its pH adjusted to 7.6. Homogenates were centrifuged at 9 500×g for 20 min at 4 ℃ and the supernatant was used as an enzyme source. The activity of CAT was measured according as preciously described[16] and expressed as μmol H_2O_2 consumed/min/mg protein. SOD was assayed as previously described[17]. One SOD unit was defined as 50% inhibition of the nitroblue tetrazolium (NBT) photoreduction to blue formazan. Specific activity was expressed as unit SOD per mg protein. GPX activity was measured as previously described[18], using GSH as a substrate, and expressed as nmoles/min/mg protein. ROS formation (mainly hydroxyl radical) was detected spectrophotometrically by measuring the rate of color intensity change in a gress agent at 550 nm based on the Fenton reaction[19].

Protein contents were measured by Coomassie blue method[20] using bovine serum albumin as a standard. All experiments were carried out in triplicate.

Statistical Analysis

Analysis was undertaken using SPSS 11.5 for Windows. Since no significant difference was found in the major biochemical parameters of the control fish between 0 h and 48 h using an unpaired two-tailed Student's *t*-test ($P>0.05$), we took the mean value of 0 h and 48 h as a control. The effect of MCs exposure time was assessed by one-way analysis of variance (ANOVA 1) followed by Bonferroni's test. Results are presented as $\bar{x} \pm s$. $P<0.05$ was considered statistically significant.

RESULTS

Mortality and Gross Morphology

No death or clinical signs of intoxication were found in the MCs-treated fish 48 h after intraperitoneal injection of MC-LR+MC-RR+MC-YR. The liver of MCs-treated fish was wet, soft with hemorrhage. No overt gross pathological changes were observed in any of the other organs.

Histopathology

The liver of control silver carp showed a normal appearance (Fig. 1. I). In contrast, the liver of MC-treated fish had alterations in its structure, the severity of which increased with the time of exposure

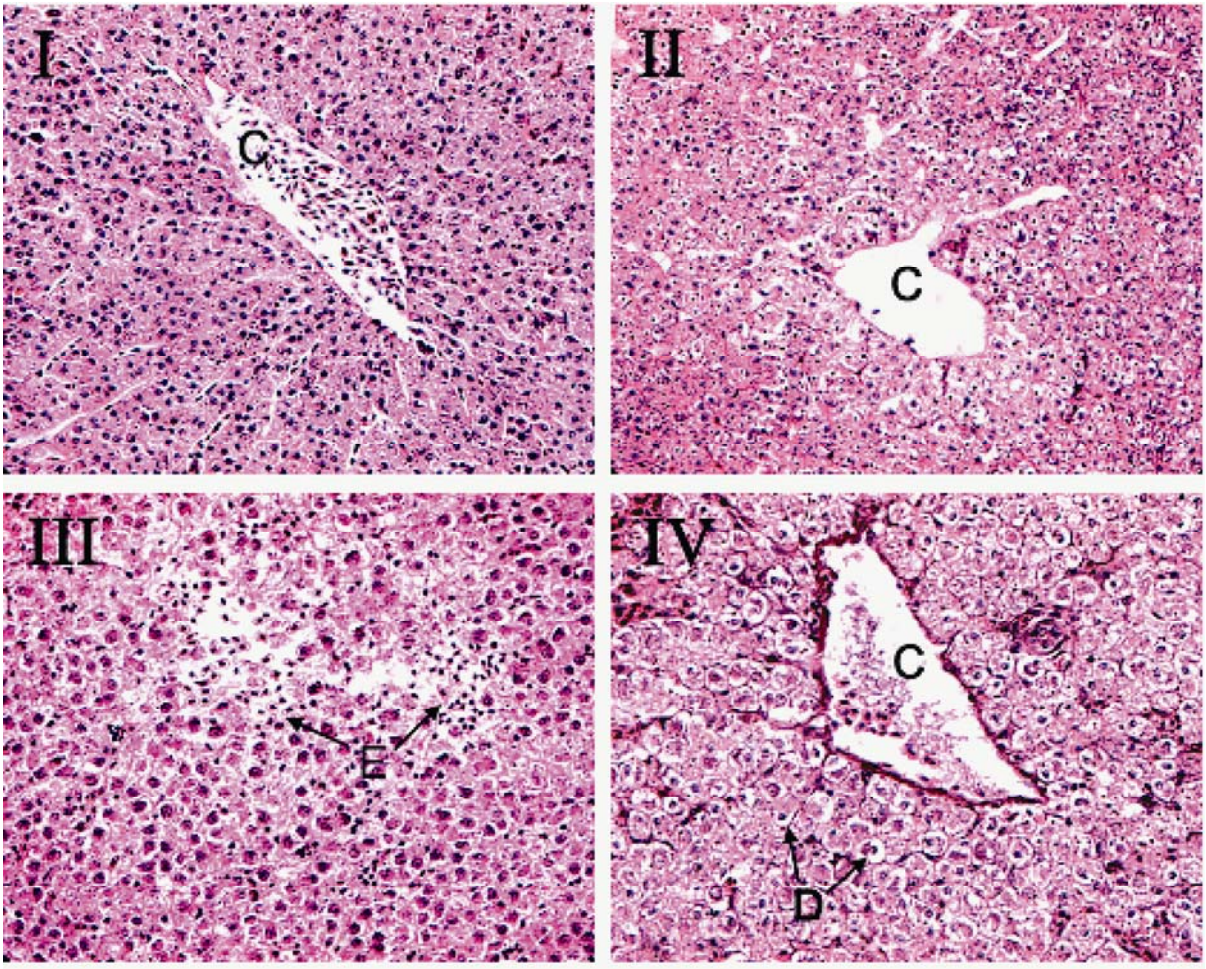

FIG. 1. H&E stained liver tissue sections of silver carp injected i.p. with 1 000 μg MC-LReq./kg BW. Ⅰ: control; Ⅱ: 1 h post-injection with rounding and vacuolation of pericentral hepatocytes; Ⅲ: 12 h post-injection with loss of typical chord liver structure and slight hemorrhage; Ⅳ: 48 h post-injection with hemorrhage and highly condensed chromatin (Magnification, 400×). C: central vein; D: condensed chromatin; E: erythrocytes.

to MCs (Fig. 1). One and three hours after injection of MCs, changes occurred mainly in the pericentral region of liver and were characterized by the appearance of hepatocytes with condensed cytoplasm (Fig. 1.II). Twelve hours after injection of MCs, the typical chord structure disappeared, condensation and vacuolation in the cytoplasm of hepatocytes became more conspicuous and extended to a larger area of the liver. In addition, slight hemorrhaging from ruptured vessels was observed (Fig. 1. III). Twenty-four and forty-weight hours after injection of MCs, hemorrhage and tissue damage involved the whole liver. Most of the cells were swollen and lost their granular appearance with chromatin clumping and condensation, which were most likely necrotic (Fig. 1. IV).

ROS Level and Antioxidant Enzymes

Temporal variations of ROS content and activities of SOD, CAT, and GPX were observed in the liver of silver carp after injection of 1 000 μg MC-LReq./kg BW (Fig. 2). ROS content was increased by about 150% 24 and 48 h after injection of MC-LReq compared with the control. A similar significant increase in SOD activity was also observed 24 and 48 h after injection of MC-LReq. The percentage was increased by about 150% as compared with the control. CAT activity was not significantly increased from 1 to 12 h and then returned gradually to the control value 48 h after injection of MC-LReq. GPX activity was increased in a time-dependent manner,

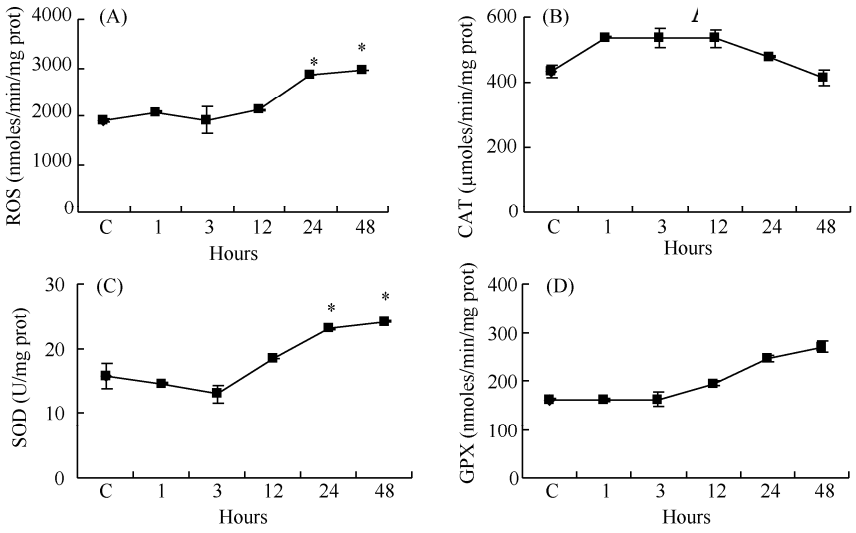

FIG. 2. Temporal variations of ROS content (A) and activities of CAT (B), SOD (C), and GPX (D) in liver of silver carp after injection of 1000 μg MC-LReq./kg BW. Data are represented as $\bar{x} \pm s$ (n=3). The mean value at 0 and 48 h was used as a control. $^{*}P<0.05$ *vs* control group.

but no significant difference was found between MC-treated and control fish.

DISCUSSION

In this study, injection of 1 000 μg MC-LReq./kg BW led to severe pathological changes in the liver of silver carp, whereas no death occurred within 48 h. In common carp, injection of 550 μg MC-LR/kg BW leads to 100% mortality, whereas fish injected with 130-300 μg MC-LR/kg BW survive a 7-day experiment[21]. In rainbow trout, all fish in 1 000 μg MC-LR/kg BW dose group (i.p.) died within 25 h but no death was reported at the dose of 400 μg/kg BW or less, suggesting that the 24 h LD_{50} ranges 400-1 000 μg/kg BW[22], and the mortality of rainbow trout is 100% within 24 h after injection of 550 μg MC-LR/kg BW[23]. It was reported that the LD_{50} of MC-LR in *Carassius auratus* is 380 μg MC-LR/kg BW[24]. Andersen *et al.*[25] have also reported a few deaths in Atlantic salmon (*Salmo salar*) injected three times with 550 pg/kg MC-LR (spaced 3 days apart) over a 36-day experiment. Therefore, as a fish species, silver carp is rather resistant to MCs in terms of LD_{50} (i.p.) dose of MC-LR.

In the present study, when silver carp was injected i.p. with 1 000 μg MC-LReq./kg BW, no clinical sign of intoxication was observed within 48 h and hepatocytes degenerated with loss of cytoplasm

and condensation of chromatin in a time-dependent manner. Råbergh *et al.*[21] have described the pathological alterations in common carp after intraperitoneal application of MC-LR. After a sublethal dose of the toxin (130-300 μg/kg BW) was used, large impairment of liver was found with dilation of intercellular space in the parenchyma due to the hydropic degeneration of hepatocytes. After a lethal dose of this toxin (550 μg/kg BW) was applied, the liver parenchyma was completely destroyed. It was reported that pathological alterations can be observed in rainbow trout injected i.p. with 400 or 1 000 μg MC-LR/kg BW[22], indicating that expressive dystrophic alterations occur in hepatocytes with karyopycnosis, karyorrhexis, and karyolysis, small focal necrosis, hemorrhage and edema. However, clinical and pathological signs of intoxication of silver carp in the present study were not significant, suggesting that silver carp is more resistant to the effect of MC-LR.

The worldwide occurrence of cyanobacterial blooms makes it necessary to perform environmental risk assessment to monitor the effects of MCs on fish and oxidative stress biomarkers are valuable tools in this regard[26]. In the present study, ROS content was significantly increased in the liver of silver carp 24 and 48 h after injection of MCs, suggesting that MCs exposure induces oxidative stress and damage. A continuous production of ROS in hepatocytes can attack lipid polyunsaturated fatty acids (PUFA) and lead to PUFA oxidation. PUFA oxidation is believed to be highly deleterious to cells by damaging cell membranes, inactivating cellular enzymes, and damaging DNA. It has been shown that ROS formation precedes the onset of mitochondrial permeability transition, loss of mitochondrial membrane potential and initiation of apoptosis[5]. In the present study, the significantly increased SOD activity indirectly verified that oxidative stress occurred in the liver of silver carp, indicating that toxin-induced oxidative stress activates antioxidant enzymes to eliminate ROS and protects cells from lesion. It was reported that CAT, SOD, and GPX activities as well as ROS levels are increased in hepatocytes of common carp exposed to MC-LR[4]. It has also been found that commercial eel food containing cyanobacterial cells can significantly increase the activity of CAT, SOD, and GPX in the liver of loach 28 days after oral exposure to 10.0 μg MC-RReq./kg/day[9]. In general, CAT and GPX act cooperatively in the liver as a scavenger of hydrogen peroxide, and SOD plays an important role in the scavenging of superoxide free radicals, which helps to maintain a balance between oxidants and antioxidants. In the present study, MCs exposure significantly increased the SOD activity in the liver of silver carp while CAT activity showed a biphasic change with an increase in the first 12 h followed by a decrease after injection of MCs. Bainy *et al.*[27] have also found similar results when comparing the CAT activity in gills of tilapida (*Oreochromis niloticus*) sampled from polluted and reference sites. Fish from the polluted site had a lower gill CAT activity, while the gill SOD activity was similar in fish from both sites. It was reported that singlet oxygen, superoxide and peroxyl radicals are CAT inhibitors[28]. These two facts should imply an exacerbation of tissue oxidative stress, since H_2O_2 production as a consequence of SOD activity remains stable but the capacity of H_2O_2 degradation is lower in fish from the polluted site due to its lower CAT activity. Consequently, a lack of CAT response to MCs exposure, as observed in our study, should favor CAT inhibition if ROS such as superoxide are generated. In this study, the SOD activity was significantly increased in the liver of silver carp, suggesting that fish has a higher degree of response to MCs exposure than to CAT and GPX exposure. However, our study was an acute study and could not reflect adequately the uptake route under natural environments. Therefore, the toxic effects of chronic exposure at a lower level of MCs need to be evaluated in future study in order to have a better understanding of its antioxidation mechanism underlying hepatic intoxication in fish.

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