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Hematological and plasma biochemical responses of crucian carp (*Carassius auratus*) to intraperitoneal injection of extracted microcystins with the possible mechanisms of anemia

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Abstract

Alterations in hematological indices such as decreases in blood cell counts (RBC), hematocrit (Ht) and hemoglobin (Hb) concentrations are key symptoms of anemia. However, few experiments were conducted to examine changes in hematological indices of fish exposed to microcystins that are believed to be fatal to circulatory systems of vertebrates. An acute toxicological experiment was designed to study hematological changes of crucian carp injected intraperitoneally (i.p.) with extracted microcystins at two doses, 50 and 200 μg MC-LReq kg^{-1} body weight. After being i.p. injected with microcystins, the fish exhibited behavioral abnormity. There were significant decreases in RBC in the high-dose group, and in Ht and Hb concentrations in both dose groups, while erythrocyte sedimentation rate (ESR) significantly increased, indicating the appearance of normocytic anemia. There were no prominent changes in the three red cell indices, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Increases in blood urea nitrogen (BUN) and creatinine (CR) in both dose groups suggest the occurrence of kidney impairment. Alteration in blood indices was reversible at the low dose group. Conclusively, anemia induced by kidney impairment was a key factor to cause abnormity of swimming behaviors and high mortality of crucian carp.

Keywords: Microcystins; Crucian carp; Hematological indices; Plasma biochemical parameters; Normocytic anemia; Kidney impairment

1. Introduction

Occurrence of cyanobacterial blooms and the associated cyanotoxins have been documented in many eutrophic inland waters worldwide (Paerl et al., 2001). Among cyanotoxins, the hepatotoxic microcystins (MCs) such as MC-LR and MC-RR are considered to be one of the most dangerous groups (Zimba et al., 2001). MCs are potent inhibitors of protein phosphatases 1 and 2A (Falconer and Yeung, 1992) due primarily to toxin uptake by the bile acid transport system (Runnegar et al., 1993). A variety of aquatic animals such as fish, mollusks and shrimps are able to bioaccumulate MCs

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in natural water bodies with toxic cyanobacteria (Magalhães et al., 2003; Chen and Xie, 2005; Xie et al., 2005). 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 its tissues (Xie et al., 2004).

There have been many toxicological experiments to examine MC bioaccumulation in organs of fish (Xie et al., 2004; Soares et al., 2004; Li et al., 2005) and to evaluate toxic effect of MCs on physiological status and histopathology of fish (Råbergh et al., 1991; Kotak et al., 1996; Fischer and Dietrich, 2000). It is known that MCs accumulate mainly in the liver, but also in other organs such as the kidney, gill and intestine. In mammals, acute deaths following MC administration have been attributed to hemorrhagic shock related to liver damage and intrahepatic hemorrhage (Beasley et al., 2000), as well as cardiac failure (Le Claire et al., 1995). MCs damnify aquatic organism with the liver as main target organ, and also cause impairment of kidney (Kotak et al., 1996; Fischer and Dietrich, 2000), which is one of the most important hematogenic organs for teleost fish (Ozaki, 1982). The disruptive action of a toxicant on the erythropoietic tissue may decrease erythrocyte number and hemoglobin content as an anemic sign, and even lead to death of European catfish (Köprücü et al., 2006). In fish, blood urea nitrogen (BUN) is the second important nitrogenous excretion product after ammonia and often used as an indicator of kidney dysfunction (Bernet et al., 2001). Increased concentrations of BUN occur due to renal lesions (Burtis and Ashwood, 1996). Creatinine (CR) is a waste product of the phosphorylation of ADP at the expense of the high-energy compound creatine phosphate and increased concentrations may reflect kidney dysfunction due to structural damage (Burtis and Ashwood, 1996).

Blood parameters are useful and sensitive for the diagnosis of diseases and monitoring of the physiological status of fish exposed to toxicants, which has been shown by Adhikari et al. (2004). A large amount of studies have focused on toxic effects of heavy metals and chemical medication on fish hamatology under laboratory condition (Harikrishnan et al., 2003; Ribeiroa et al., 2006). Many studies have defined anemia as decreased RBC, Hb and Ht (Haney et al., 1992; Greenburg, 1996; Hendy et al., 2001). Hematological parameters in fish including red blood cell counts (RBC), hematocrit (Ht), hemoglobin (Hb), mean corpuscular volume (MCV) may be influenced by intrinsic and external factors (Nespolo and Rosenmann, 2002; Rios et al., 2002). Only a few reports have documented alterations in hematological parameters caused by MCs in fish (Vajcová et al., 1998; Kopp and Hetesa, 2000). Vajcová et al. (1998) reported that microcystins can lead to alterations in hematological indices of silver carp (*Hypophthalmichthys molitrix*.); however, they only measured hematological parameters at 48 h post injection without any discussion on anemia caused by MCs. Until now, there have been no information available on anemia induced by microcystins.

The purposes of this study were to evaluate the acute toxicity of MCs on anemia by determining hematological and biochemical indices, and to establish a possible relationship among alterations in hematological indices, anemia, plasma biochemical and hypofunction of hemopoiesis due to kidney impairment. Our hypothesis was that anemia caused by kidney impairment is a key factor to influence behaviors and mortality of crucian carp.

2. Materials and methods

2.1. Fish

Healthy crucian carp (mean body weight 265.0 ± 22.6 g) were purchased from a fish hatchery affiliated to College of Fisheries, Huazhong Agricultural Univeristy in Wuhan City, China, 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 150 l aquarium (95 cm L × 55 cm W × 40 cm H) 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.

2.2. Toxin

Cyanobacteria (mainly composed of *Microcystis* spp.) were collected from surface blooms of Lake Dianchi, Yunnan of China. Crude microcystins in the cyanobacteria were extracted three times with

75% (v/v) methanol and were suspended in distilled water for the toxic experiment. For the determination of MC content in cyanobacteria, the extract was filtered through a glass microfibre filter (Whatman GF/C, Maidstone, UK) and the filtrate was diluted 1:5 with distilled water. Then the diluted filtrate was applied to a 5 g reversed phase octadecylsilance (ODS), which was previously activated with 50 ml of 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 the final detection. 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 and MC-RR, Wako Pure Chemical Industries, Japan). The MC content 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.

2.3. Experimental protocol

Ten acclimated fish without administration were expressed as 0 h and sampled 2 h prior to injection. A total of acclimated crucian carp ($N = 180$) were injected intraperitoneally (i.p) under ventral fin into the peritoneum by syringe. Fish in the low-dose group ($n = 60$) and high-dose group ($n = 60$) were injected i.p. with crude extracted microcystins (mainly MC-RR and -LR) at 50 and 200 MC-LR eq μg kg^{-1} BW, respectively; and the control fish ($n = 60$) were injected i.p. with equal volume of distilled water. The 60 fish in each dose group and the control were, respectively, 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, 10 fish (from two tanks) for each dose group and the control were anesthetized with 0.02% pH-neutralized tricaine methane sulfonate MS222 (ethyl-ester.3.aminobenzoic acid, Sigma). Blood samples were taken by caudal puncture with heparinized syringes. Blood was centrifuged at 3000*g* for 15 min at 4 °C and plasma was stored at −70 °C until analysis.

2.4. Hematological and biochemical indices

Hematological and biochemical parameters were measured in triplicates, and averaged for statistical use. RBC: 10^6 cells μl^{-1} and WBC: 10^4 cells μl^{-1} were determined by hemocytometer method (Stevens, 1997). Hematocrit (Ht v/v ratio or %) was determined by microhematocrit method (Goldenfarb et al., 1971) and hemoglobin concentrations (Hb g l^{-1}) were determined by cyanometahemoglobin method (Lee et al., 1998). ESR (mm h^{-1}) was determined by the Wintrobetule method (Chen and Yan, 1995). Red cell indices, MCV (μm^3 $cell^{-1}$), MCH (pg $cell^{-1}$), and MCHC (g l^{-1}) were calculated from RBC, Ht, and Hb according to Lee et al. (1998) as follows:

$$\mathrm{MCV}(\mu m^3\,\mathrm{cell}^{-1}) = \mathrm{Ht}(v/v\ \mathrm{ratio}) \times 1000/\mathrm{RBC}(10^6\,\mathrm{cells}\,\mu l^{-1}),$$

$$\mathrm{MCH}(\mathrm{pg\ cell}^{-1}) = \mathrm{Hb}(\mathrm{g}\,l^{-1})/\mathrm{RBC}(10^6\,\mathrm{cells}\,\mu l^{-1}) \text{ and}$$

$$\mathrm{MCHC}(\mathrm{g}\,l^{-1}) = \mathrm{Hb}(\mathrm{g}\,l^{-1}) \times 10/\mathrm{Ht}(v/v\ \mathrm{ration}).$$

The biochemical indices were determined with commercially available reagent kits (Zhongsheng, China) including creatinine (CR: kit no. 320251) and blood urea nitrogen (BUN: kit no. 280021) based on colorimetric reaction in automatica analyzer (ACTA, Italy) according to the instruction of the manufacturers.

2.5. Statistical analysis

Hematological and biochemical parameters were examined in duplicates, and every sample was measured in triplicates to get an average value for statistical use. Values were expressed as mean ± SD. Data were analyzed by repeated measures 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 $P \leqslant 0.05$.

3. Results

3.1. Mortality and behavioral observations

In the high-dose (200 μg kg^{-1}) group, 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 (50 μg kg^{-1}) and the control. Fish of the high-dose group died with swollen belly facing upward, and much ascites was observed with wet, soft and pale livers and kidney when we dissected the carcases. In both dose groups, fish exhibited uneasiness and frantic swimming behavior during 1–3 h post injection, and then showed sluggish swimming interrupted by quick bursts of frenetic swimming imbalance during 12–24 h post injection. Treated fish also lost swimming coordination and buoyancy control with elevation of opercular beat rate, which increased with time. The depression and lethargy became more pronounced in the high-dose group with increasing duration of the experiment. During 24–48 h post injection, fish of the high-dose group frequently floated near the surface of the water, either on their side or upright with the head facing down; and terminally, opercular ventilatory movements gradually decreased in frequency and magnitude. However, in fish of the low-dose group, such behavioral changes were seldom observed. On the contrary, control fish became slightly uneasy following injection but resumed normal swimming activity approximately 1 h after injection. These behavioral differences became indiscernible in control fish over time.

3.2. Hematological and plasma biochemical indices

Compared with the control, fish in the high-dose group (200 μg kg^{-1}) showed a persistent decrease in RBC, Hb and Ht ($P<0.05$) (Fig. 1), but a significant progressive increase in ESR ($P<0.01$) (Fig. 1d); statistically significant alterations in these four hematological indices were observed at 12 h post injection. WBC, CR and BUN levels all showed significant increases ($P<0.01$) (Figs. 1(e)–(g).

Compared with the control, fish in the low dose group (50 μg kg^{-1}) did not show significant difference in RBC count ($P>0.05$), but presented a significant decrease trend in Hb and Ht ($P< 0.05$) and a significant increase in ESR level ($P< 0.05$). Significant alterations in these four hematological indices were also observed at 12 h post injection. WBC was constantly higher in the low-dose group than in the control ($P<0.01$). There were no significant difference ($P>0.05$) in CR level between the low-dose group and the control, while significant increase in BUN levels was observed in the treated fish. In the low-dose group, hematological and biochemical indices exhibited a general tendency to approach the levels of the control on the seventh day post injection. It should be noted, however, that there is no information on the indices at 7 h post injection in the high-dose group due to fish mortality.

Red blood cell indices (MCV, MCH, and MCHC) are indicated in Table 1. There were no significant changes in red blood cell indices between the treatments and the control.

4. Discussion

In the present study, abnormity of swimming behaviors of crucian carp indicates severe damnification in response to MC injection in both time and dose-dependent manner, with a reduction of $14.8\pm3.9\%$ to $53.9\pm6.2\%$ in hematocrit. Therefore, the lethargic behavior of crucian carp might be caused by the sharply reduced hematocrits, which is similar to the results of Katharios et al. (2002). Jones (1971) also demonstrates that a reduction of the hematocrit to one-third of normal level caused a 34–40% reduction in the maximum sustained swimming speed of rainbow trout (Jones, 1971). In the present study, the clinical signs of the treated crucian carp included abdominal distension with ascites, pale gill filaments, hemorrhage in bases of fins, and wet, soft and pale white liver and kidney, while no intrahepatic hemorrhage were present based on the change in liver color. Similarly, Kotak et al. (1996) report a lack of hemorrhage in rainbow trout (*Oncorhynchus mykiss*) injected i.p. with MC-LR.

Decreases in RBC count, Ht level and Hb content are symptoms of anemia (Pathiratne and Rajapakshe, 1998; Köprücü et al., 2006). Our hematological results documented that i.p. injection of MCs could result in severe anemia of crucian carp, especially, in the high dosed group. Vajcová et al. (1998) report that silver carp (*Hypophthalmichthys molitrix*) administrated i.p. with 250 μg MC-LR eq kg^{-1} BW showed significant decreases in Hb, Ht and RBC. Beasley et al. (2000) also observed significant decreases of Hb and Ht in swine treated with 72 μg MC-LR kg^{-1} via the cranial vena cava. On the other hand, the increase of ESR is a sign of overlapping of red blood cells, and can also contribute to anemia, and even induces death of sea perch (*Lateolabrax japonicus*) (Qian et al., 2002;

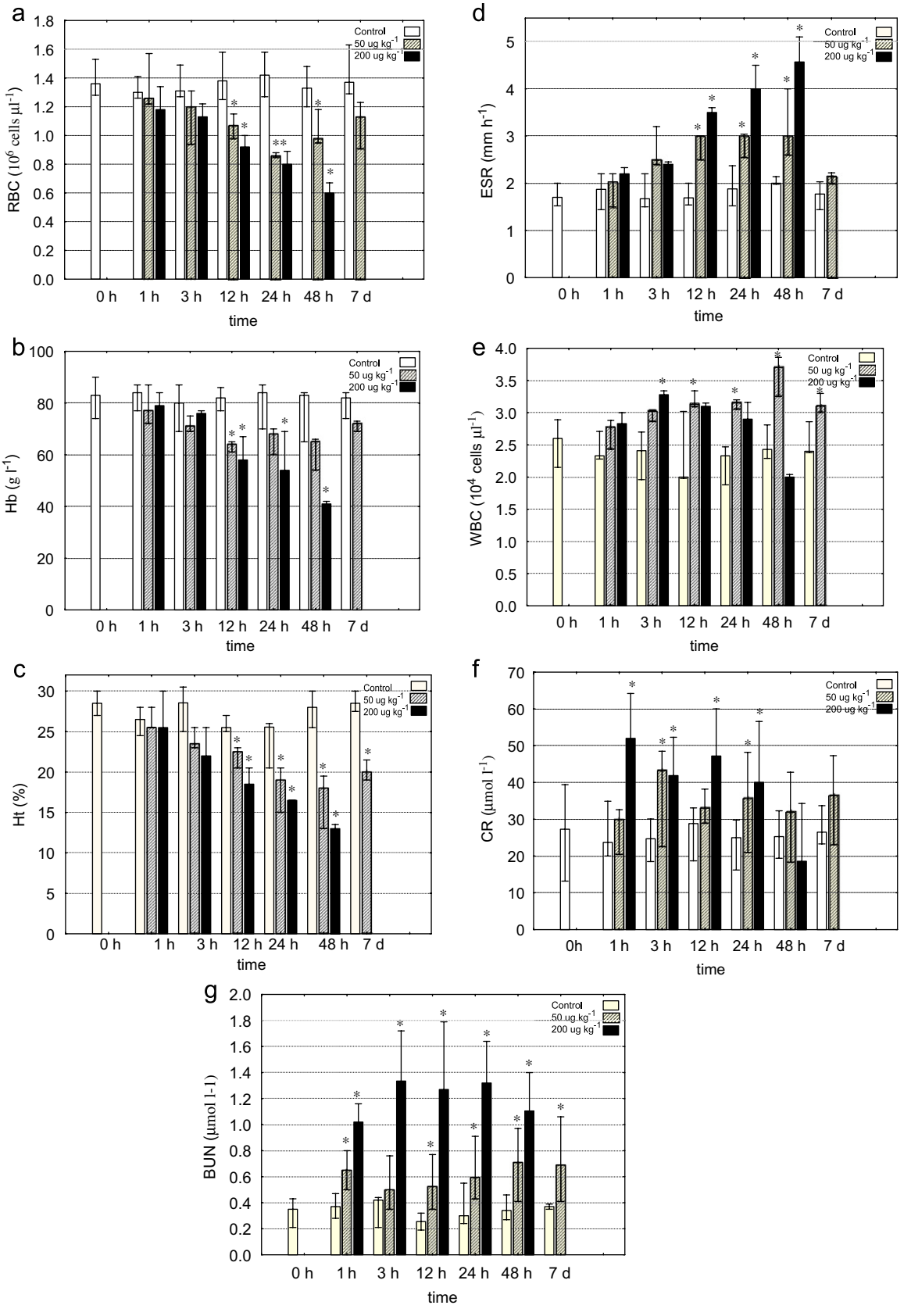
a
RBC (10^6 cells µl^{-1})
Control
50 ug kg^{-1}
200 ug kg^{-1}
0 h 1 h 3 h 12 h 24 h 48 h 7 d
time
b
Hb (g l^{-1})
c
Ht (%)
d
ESR (mm h^{-1})
e
WBC (10^4 cells µl^{-1})
f
CR (µmol l^{-1})
g
BUN (µmol l-1)

Table 1
Mean erythrocyte indices of crucian carp injected i.p. with 50 and 200 μg kg^{-1} MC-LReq. BW and distilled water (control)

		0 h	1 h	3 h	12 h	24 h	48 h	7 d
Mean corpuscular volume (MCV) (μm^3 cell^{-1})	Control	206.57±26.09	199.94±24.47	208.47±35.46	185.35±16.95	170.03±28.80	210.31±34.40	202.33±23.98
	Low dose		196.58±16.14	212.19±32.65	206.60±9.91	219.48±37.34	166.371±49.85	189.24±42.25
	High dose		224.15±20.90	209.98±25.70	197.92±33.38	197.56±24.74	215.40±18.48	
Mean corpuscular hemoglobin (MCH) (pg cell^{-1})	Control	59.22±1.65	62.22±5.57	58.57±10.76	58.61±6.09	57.24±11.46	58.32±10.74	56.25±4.27
	Low dose		59.17±11.27	63.83±13.92	59.73±6.64	79.80±9.60	60.51±12.78	66.51±10.95
	High dose		66.21±12.97	62.92±4.93	61.97±5.64	72.17±6.46	64.28±3.54	
Mean corpuscular hemoglobin concentration (MCHC) (g l^{-1})	Control	28.99±3.86	31.47±2.64	28.05±0.46	31.60±0.71	34.08±7.83	27.71±2.09	27.91±1.37
	Low dose		29.95±3.63	29.96±2.78	28.88±2.47	36.67±3.42	37.18±4.13*	35.47±2.61*
	High dose		29.66±6.11	30.26±4.25	32.22±8.41	36.80±4.62	30.02±3.45	

Values are expressed with mean±SD. * Indicates significant differences at $P<0.05$ between MC-treated groups and the control group.

Chen et al., 2005). Furthermore, an increase in the number of leucocytes also exhibited alterations in physiological status of fish when they are attacked by microcystins, (Nussey et al., 1995). There were no prominent changes in red blood cell indices (MCV, MCH and MCHC) of crucian carp in both dose groups. It is apparent that no cubage change in erythrocyte occurred, indicating that MCs-induced anemia in fish is characterized as normocytic anemia. Pouria et al. (1998) report normocytic anemia in patients who suffered from fatal microcystin intoxication in a hemodialysis unit in Caruaru, Brazil.

There are various factors to cause anemia in animals, such as hemorrhage, hemolysins, and renal diseases (Roberts, 1978; Laidley et al., 1988; Rand and Cone, 1990). In the present study, we did not observe obvious hepatic hemorrhage in crucian carp in both dose groups. Kotak et al. (1996) suggest that in contrast to mammals, liver hemorrhage is rare in fish as they found that hemorrhage was never a prominent feature in rainbow trout treated with microcystins. McCloskey and Oris (1993) suggest that hemolysis is observed with significant increase in blood hemoglobin concentration. In the present study, significant decrease of hemoglobin indicates that severe hemolysis did not occur in crucian carp of both dose groups. Pouria et al. (1998) also find no evidence of hemolysis for patients who received hemodialysis following the use of water from a lake with massive growth of cyanobacteria. Therefore, excluding the possible factors hepatic hemorrhage and hemolysis, we ascribe anemia of crucian carp to MC-induced kidney impairment that was convinced by the increases of CR and BUN in plasma. As kidney is the most important hematogenic organ for fish (Ozaki, 1982), it is quite possible that i.p. injection of MC-LR resulted in hypofunction of hemopoiesis, leading to severe anemia in crucian carp, and that the gradually aggravating anemia caused abnormity of swimming behaviors and the high mortality of crucian carp injected with MCs. It was worthy to note that in the present study, most blood indices of the low-dose group was reversible, suggesting a possible trend of crucian carp to return to their normal status during a period of 7 days,

Fig. 1. Effect of microcystins on major hematological and biochemical indices of crucian carp injected i.p. with 50 and 200 μg kg^{-1} MC-LReq. BW. Values are expressed with mean±SD. Ten samples were examined at every sampling point for each dose group and the control. Compared with the control, significant decreases ($P<0.05$) in red blood cell (RBC: 10^6 cells μl^{-1}) in the high-dose group, and in, hematocrit (Ht%) and hemoglobin (Hb g l^{-1}) occurred in both dose groups occurred; while erythrocyte sediment rate (ESR mm h^{-1}) and white blood cell (WBC: 10^4 cells μl^{-1}) significantly increased ($P<0.05$) in both dose groups. Significant increases ($P<0.05$) in creatinine (CR: μmol l^{-1}) in the high-dose group and blood urea nitrogen (BUN: μmol l^{-1}) in both dose groups occurred compared with the control. * Indicates significant differences at $P<0.05$ between MC-treated groups and the control group.

which is similar to the results of juvenile crucian carp i.p. injected with MC-LR (Malbrouck et al., 2003).

In conclusion, anemia caused by kidney impairment is a key factor to cause abnormity of swimming behaviors and the high mortality of crucian carp exposed to a dose of 200 μg MC-LR eq kg^{-1} BW by i.p. injection. Further evidences on the pathology of hematogenic organs (e.g., kidney and spleen) are needed in our future studies.

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