Toxicon 53 (2009) 638–644

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Intraperitoneal injection of extracted microcystins results in hypovolemia and hypotension in crucian carp (*Carassius auratus*)

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ARTICLE INFO

Article history:
Received 15 October 2008
Received in revised form 23 January 2009
Accepted 26 January 2009
Available online 4 February 2009

Keywords:
Microcystins
Fish
Blood pressure
Hemorrhage
Shock

ABSTRACT

Circulatory responses of crucian carp injected intraperitoneally with extracted microcystins (MCs) were studied at sublethal and lethal doses (150 and 600 μg MC kg^{-1} body mass, respectively). Mean arterial blood pressure (MAP), heart rate, hematocrit (Hct), red blood cell (RBC) counts, and circulating blood volume (BV) were assayed at 0, 1, 3, 12, 24, and 48 h post-toxin administration. MAP decreased significantly in a dose-dependent manner over time. Within the 48-h test period, the lethal dose as well as the sublethal dose resulted in a steady decline of MAP without recovery. Heart rate significantly increased within 24 h post-injection as blood pressure significantly dropped, then showed a terminal decline to the control level. The dose-dependent decreases in BV and Hct were directly related to the drop in MAP. Intraperitoneal injection of a lethal dose of MCs led to hepatic and gill hemorrhage. Consequently, crucian carp given MCs suffered from hypovolemic hypotensive shock.

1. Introduction

Microcystins (MCs), known as monocyclic heptapeptides, constitute one of the major cyanobacterial toxin classes involved in a variety of mammalian, avian, and teleost poisonings (Carmichael, 1994; Dawson, 1998; De Figueiredo et al., 2004; Zhang et al., 2007). MCs are produced by species of freshwater cyanobacteria, primarily of the genus *Microcystis* (Chorus and Bartram, 1999). Over 75 structural variants of MCs are known (Svrcek and Smith, 2004), and MC-LR is the most commonly encountered and most studied member of the group (Dawson, 1998; MacKintosh et al., 1990). Problems related to toxigenic cyanobacteria seem to be of increasing importance due to their potential risk to animal and human health, and in a recent study by Chen et al. (2009), microcystins were identified for the first time in the serum of a chronically exposed human population (fishermen at Lake Chaohu, China) together with indication of hepatocellular damage. Extracts of toxic cyanobacteria given orally, intraperitoneally, or intravenously in acutely and subacutely toxic doses may cause hepatotoxicity or even death of fish and mammals (Ernst et al., 2006; Falconer et al., 1988; Fischer and Dietrich, 2000; LeClaire et al., 1995; Li et al., 2005; Lovell, 1989; Miura et al., 1989; Solter et al., 1998).

Acute MC poisoning in mammals is characterized by disruption of hepatic architecture, which leads to massive intrahepatic hemorrhage and death within a few hours to a day (Carmichael, 1994; Sasner et al., 1984; Theiss et al., 1988). Hypotension is usually secondary to massive hemorrhage, which reduces circulating blood volume (Randall et al., 1997). Cardiac and circulatory effects of MCs observed in rats and swine include severe reductions in arterial blood pressure (Beasley et al., 2000; Berg and

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0041-0101/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2009.01.027

Soli, 1985; LeClaire et al., 1995; Theiss et al., 1988), which Ostensvik et al. (1981) attributed to sequestration of blood in the liver of mammals. Increased heart rate of swine and mice (Lovell, 1989; Oishi and Watanabe, 1986) and depression of heart rate in rats caused by MC-LR have been observed (LeClaire et al., 1995). MC-LR also decreases hepatic and renal perfusion and causes circulatory shock in intravascular dosed swine (Beasley et al., 2000). Previous investigations indicate that MCs are potent and rapid-acting hepatotoxins, with the immediate cause of death being hemorrhagic shock secondary to massive hepatocellular necrosis and collapse of hepatic parenchyma. Therefore, lethality in mammals exposed to MCs has been attributed to hypovolemic shock. In addition, a cardiogenic component may exist in rats because of the decrease of heart rate and cardiac output (LeClaire et al., 1995).

There has been only limited information on the cardiovascular responses of fish to MCs exposure. Best et al. (2001) reported an increased heart rate and cardiac output in brown trout (*Salmo trutta*) exposed to environmentally relevant MC concentrations of aqueous *Microcystis* extracts, while heart rate changed inappreciably when the trout were exposed to purified MC-LR. To date, no study has documented changes in blood pressure of fish exposed to MC or MC-containing cyanobacteria. Thus nothing is known about the impact of MCs on the arterial blood pressure and circulating blood volume in fish, although Zhang et al. (2007) recently observed anemia in carp intraperitoneally exposed to extracted MCs. As a consequence, the MC-mediated mechanisms responsible for fish death are still under debate. In fact, the main cause of MC-mediated fish death is ascribed to hepatic malfunction, rather than to hypovolemic shock as in mammals (Bury et al., 1997; Kotak et al., 1996).

Therefore, the purposes of this experiment were to study the cardiovascular responses and changes in circulatory blood volume of crucian carp (*Carassius auratus*) injected intraperitoneally with extracted MCs at sublethal and lethal doses over a 48-h period and to establish possible relationships among changes in arterial blood pressure, heart rate, circulating blood volume, and fish mortality and physiological disturbances induced by MCs.

2. Materials and methods

2.1. Fish

Crucian carp weighing 265 ± 22.6 g, collected from a local fish hatchery in Wuhan City, China, were transported to the College of Fishery, Huazhong Agricultural University. Fish were acclimated for 7 days before the start of the experiment. During the acclimation and experimental period, water temperature (25 ± 1 °C), dissolved oxygen (6.8 ± 0.7 mg l^{-1}), and ammonia concentration (range: trace to 0.5 mg l^{-1}) were recorded daily. Fish were fed with pellet food at a rate of 2.0% of the body weight per day. Feeding was terminated 2 days before initiation of the experiment, and no food was supplied to fish during the experimental period.

2.2. Toxin

Cyanobacteria (mainly composed of *Microcystis* spp.) were collected from surface blooms on Lake Dianchi, Yunnan. Purification of toxin and quantity analysis were performed as described by Li et al. (2008). Crude MCs in the cyanobacteria were extracted three times with 75% (v/v) methanol. Quantitative analysis of MCs was performed using HPLC (LC-10A, Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan). MC concentration was determined by comparing the peak areas of the test samples with those of the standards (MC-LR and MC-RR, Wako Pure Chemical Industries, Japan). The total MC content was 1.41 mg g^{-1} dry weight (DW), with MC-RR and MC-LR values of 0.84 and 0.50 mg g^{-1} DW, respectively. The extracted MCs (mainly MC-RR and -LR) were resuspended in 0.75% NaCl solution to 75 μg ml^{-1} and 300 μg ml^{-1}, respectively.

2.3. Toxin administration

Fish were exposed to sublethal and lethal concentrations of extracted MCs (Zhang et al., 2007). Fish in the sublethal group ($n = 200$) and the lethal group ($n = 200$) were injected intraperitoneally with *Microcystis* extracts containing 150 and 600 μg MC per kg body mass (BM), which were equivalent to 50 and 200 μg purified MC-LR respectively (Gupta et al., 2003). The injection volume used in each treated fish was 2 ml kg^{-1}. Fish ($n = 200$) in the control tank were injected intraperitoneally with 0.5 ml of 0.75% NaCl. Fish in each group were distributed equally into twenty 200 l tanks. Thirty fish (from three tanks) from each group were sampled at 0, 1, 3, 12, 24, and 48 h post-injection.

2.4. Surgery for arterial blood pressure and heart rate measurements

Fish were anaesthetized with 150 mg MS-222 l^{-1} (buffered with $NaHCO_3$ at 300 mg l^{-1}) to achieve a rapid and deep anesthesia. Then, fish were placed on an operation sling where they were artificially ventilated with buffered anesthetic solution (50 mg l^{-1} MS-222 buffered with 100 mg $NaHCO_3$ l^{-1}). A heparinized cannula (PE 50 tube, 0.5 mm ID, 1.0 mm OD) was implanted in the dorsal aorta using a trocar (Soivio et al., 1975). Afterwards, the trocar was removed. The cannula was exteriorized through the snout via a larger diameter cannula (1.05 mm ID, 1.5 mm OD). The fish were then placed in the experimental tank. Following this surgical procedure, fish were allowed a 48 h recovery period before toxin administration.

Arterial blood pressure and heart rate of the fish in each treatment group and in the control group were measured via the cannula using a blood pressure transducer (Model PT-100, Chengdu Technology and Market Co. Ltd., Chengdu). These parameters were monitored and logged electronically using the physiological signals collection system (Model BL-420F, Chengdu Technology and Market Co. Ltd.) at 0, 1, 3, 12, 24, and 48 h ($n = 8$ in each group at each time point) post-injection. Pressure calibrations were performed with reference to a static water column. The zero point was set to the same level as the water surface of

the experimental tank. The arterial blood pressure and heart rate were calculated by a data acquisition and analysis system (TM-WAVE, BL-420F, Chengdu Technology and Market Co. Ltd).

2.5. Clinical investigations

Fish behavior and clinical signs in the control and treatment groups were observed throughout the experiment. Dead fish were registered to calculate mortality and were immediately autopsied. At the end of the experiment, 10 fish from each group were dissected to determine if hemorrhage of liver and other organs had occurred.

2.6. Circulating blood volume determination

Circulating blood volume (BV) was measured using Evans Blue (EB) dye (Gallaugher et al., 1995), which initially requires a preparation of plasma-bound EB (PBEB). To prepare the PBEB, 40 ml blood samples from 20 fish were taken by caudal puncture using heparinized syringes and then centrifuged at 3000 × *g* for 15 min at 4 °C before the start of the experiment. The separated plasma was collected and combined. EB was added to the plasma and mixed to make PBEB (1 mg ml^{-1}) and then stored at −25 °C until the BV assay could be performed. PBEB was thawed at 25 °C immediately before use. An absorbance calibration curve of PBEB at the concentrations of 0.125, 0.25, 0.5, 0.75, 1.5, 3.0, 6.0, 12.0, 24.0, and 48.0 mg ml^{-1} was produced to calculate the concentration of EB in each blood sample.

To measure BV, fish were anaesthetized in buffered MS222 solution and then placed on a V-shaped operation sling. The second afferent branchial artery on the left side of the fish was cannulated using a PE 50 tube, which penetrated the artery approximately 0.5 cm. The cannula was connected to a three-way stopcock with two 1 ml syringes, one for administering 1% heparinized saline solution and the other for drawing 0.8 ml blood samples to measure hematocrit and spectral absorption in dye-free plasma. Surplus blood was returned to the fish, followed by an additional 0.5 ml heparinized saline solution to replace loss blood. Before returning excess blood from the dye-free sample, the stopcock was removed and PBEB was injected directly into the cannula. The mean dye concentration used was 1.02 ± 0.08 mg kg^{-1} BM. The stopcock then was replaced and surplus blood was used to flush the cannula, which was subsequently filled with heparinized saline solution. Each blood sample was taken 5 min after injection of dye and then centrifuged at 3000 × *g* for 10 min. The spectral absorption of the dye plasma of each blood sample was read at 620 nm. BV was calculated according to the following formula:

$$BV(\%) = 100 \times \left\{ [PBEB] \times [Plasma_{EB}]^{-1} \times (1 - Hct)^{-1} \times BD \right\} \times BM^{-1}$$

where [PBEB] is the concentration (mg ml^{-1}) of PBEB injected into the fish, [$Plasma_{EB}$] is the concentration (mg ml^{-1}) of EB in the plasma of the blood sample, BD is blood density (g ml^{-1}), and BM is the body mass of the fish (g). BV measurements were performed at 0, 1, 3, 12, 24, and 48 h after MCs administration ($n = 8$ in each group at each time point).

2.7. Hematological parameters assay

At each time point, blood samples (1.0 ml) were taken via the cannula using heparinized syringes after blood pressure determination and used for the measurements of hematocrit (Hct) and red blood cell (RBC) counts. Hct was determined using the microhematocrit method and RBC count was determined by the hemocytometer method.

2.8. Statistical analyses

The data from control fish and MC-exposed fish were expressed as means ± SD. All data were tested for statistical differences using one-way ANOVA and Tukey's HSD post-hoc tests (Statistica software package, version 6.0). To clarify the relationships among MAP, BV, and Hct in MC-exposed fish, regression analyses on these parameters were performed using general regression models (Statistica, version 6.0). Statistical differences were determined at $p < 0.05$.

3. Results

3.1. Mortality and clinical signs

No deaths occurred in either the control or the sublethal dose group during the experimental period. However, 6.0%, 8.4%, and 18.4% fish mortalities were found at 12, 24, and 48 h post-injection in the lethal dose group, respectively.

In the lethal dose group, focal hemorrhage in the liver and the gills occurred after 12 h post-injection, with a large area of petechiae visible beneath the liver of post-mortem fish (Fig. 1). This was not found in the sublethal dose and control groups. However, the livers of fish in both dose groups became pale and swollen during the experimental period, whereas the livers in control fish had no hemorrhages.

3.2. Arterial blood pressure

At the lethal dose, mean arterial blood pressure (MAP) of the fish dropped significantly at 1 h and reached a minimum at 12 h post-injection, with a drop of 61 ± 41% from the initial value, and remained lower than that of the control fish. At the sublethal dose, a significant decrease in MAP occurred at 3 h post-injection, with a minimum drop of 51 ± 3% from the control (Fig. 2), and MAP remained significantly lower than that in control fish.

3.3. Heart rate

There was no significant chronotropic change in heart rate in the control group during the experimental period (Fig. 3). In the lethal dose group, heart rate increased, reaching a maximum at 1 h post-injection and then recovered with time by 48 h. In the sublethal dose group, heart rate increased significantly after 3 h, reaching

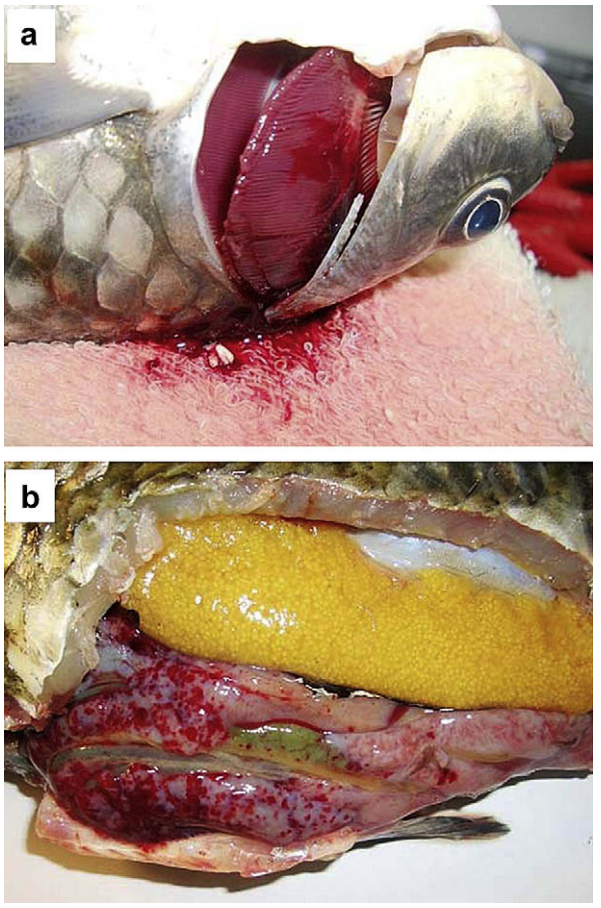

Fig. 1. Hepatic hemorrhage and gill hemorrhage in crucian carp exposed to microcystins (MCs). (a) Gill hemorrhage in MC-exposed fish at concentrations of 150 μg MC kg^{-1} BM; (b) focal intrahepatic hemorrhage in pale white liver of MC-exposed fish injected intraperitoneally with 600 μg MC kg^{-1} BM.

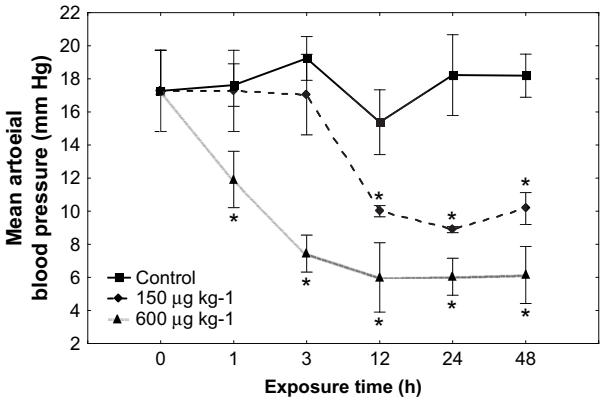

Fig. 2. Changes in mean arterial blood pressure (MAP) of crucian carp injected intraperitoneally with 0.75% NaCl (control) and *Microcystis* extract corresponding to 150 and 600 μg MC kg^{-1} BM. Values are expressed as means ± SD. There was no significant change in MAP in the control group during the experimental period. * indicates significant differences between MC-administration groups and the control group at each time point ($p < 0.05$).

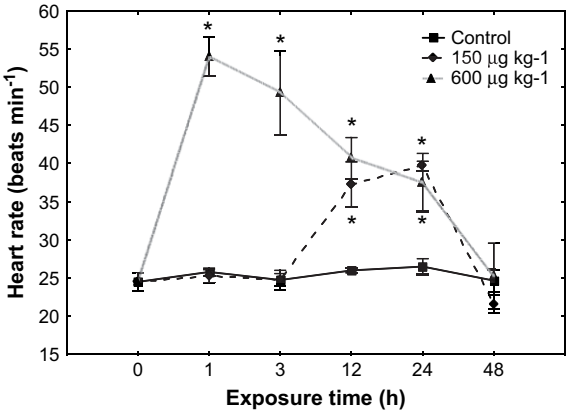

Fig. 3. Changes in heart rates of crucian carp injected intraperitoneally with 0.75% NaCl (control) and *Microcystis* extract corresponding to 150 and 600 μg MC kg^{-1} BM. Values are expressed as means ± SD. There was no significant change in heart rate in the control group during the experimental period. * indicates significant differences between MC-administration groups and the control group at each time point ($p < 0.05$).

a maximum at 24 h post-injection and recovering at 48 h post-injection.

3.4. Hct and RBC counts

No significant change in Hct and RBC counts occurred in the control group during the experimental period. In contrast, Hct and RBC counts of MC-exposed fish significantly decreased as of 3 h post-injection (Table 1). Hct and RBC counts in the lethal dose group were significantly lower than those in the sublethal dose group as of 12 h post-injection.

3.5. Circulating blood volume

Significant declines in BV were observed in both dose groups (Fig. 4). BV decreased significantly from 1 h post-injection in the lethal dose group, with a minimum drop of 56 ± 5% from the initial value. In the sublethal dose group, BV decreased significantly from 3 h post injection, with a drop of 36 ± 4% as the minimum value. The dose of toxin given and the magnitude of reductions in BV were significantly related. At each sampling point, the BV in the lethal dose group was significantly lower than that in the sublethal dose group.

3.6. Correlation between MAP, BV, and Hct

A drop in MAP was significantly correlated to the decrease of Hct in the sublethal dose group (regression equation: $MAP = -1.259 + 0.644Hct$, $r^2 = 0.6274$, $p < 0.05$) and the lethal dose group (regression equation: $MAP = -1.441 + 0.532Hct$, $r^2 = 0.7099$, $p < 0.01$). MAP also was linearly correlated to BV in the sublethal dose (regression equation: $MAP = -6.265 + 4.373BV$, $r^2 = 0.7840$, $p < 0.05$) and lethal dose group (regression equation: $MAP = -4.091 + 3.761BV$, $r^2 = 0.9866$, $p < 0.01$).

Table 1
Changes in red blood cell (RBC) counts and hematocrit (Hct) of *Carassius auratus* injected intraperitoneally with 0.75% NaCl (control) and *Microcystis* extract corresponding to 150 and 600 μg MC kg^{-1} BM.

	Groups	Exposure time (h)					
		0 ($n = 8$)	1 ($n = 8$)	3 ($n = 8$)	12 ($n = 8$)	24 ($n = 8$)	48 ($n = 8$)
RBC (10^9 cells ml^{-1})	Control	1.39 ± 0.13	1.32 ± 0.08	1.36 ± 0.12^a	1.40 ± 0.17^a	1.43 ± 0.16^a	1.34 ± 0.14^a
	150 μg kg^{-1}	1.33 ± 0.16	1.35 ± 0.13	1.20 ± 0.19^a	1.07 ± 0.09^b	0.83 ± 0.04^b	1.04 ± 0.13^b
	600 μg kg^{-1}	1.36 ± 0.23	1.22 ± 0.15	1.10 ± 0.05^b	0.93 ± 0.06^c	0.87 ± 0.08^b	0.61 ± 0.06^c
Hct (%)	Control	28.50 ± 1.50	27.33 ± 1.76	28.02 ± 2.29^a	27.50 ± 1.04^a	26.35 ± 3.05^a	27.83 ± 2.25^a
	150 μg kg^{-1}	29.10 ± 1.78	26.33 ± 1.44	24.00 ± 1.32^b	22.00 ± 1.32^b	18.33 ± 2.84^b	16.83 ± 3.28^b
	600 μg kg^{-1}	29.01 ± 1.34	26.50 ± 1.53	23.00 ± 4.54^b	18.33 ± 3.97^c	15.50 ± 2.78^c	13.00 ± 2.36^c

Values are means ± SD. There were no significant variations in Hct and RBC counts in the control group during the experimental period. Values indicated with different letters at the same time point are significantly different at $p < 0.05$.

4. Discussion

In the present study, prominent hypotension, increase in heart rate, decreases in Hct and RBC counts, and reduction in blood volume were observed in crucian carp exposed to sublethal and lethal doses of MCs. The acute drop in MAP of the MC-exposed fish in a dose-dependent manner suggests a primary inadequacy in the circulatory system. This is the first study to examine the change in the arterial blood pressure of fish exposed to MCs. Studies on mammals have demonstrated that MCs can cause circulatory shock and acute hypotension (Beasley et al., 2000; LeClaire et al., 1995; Theiss et al., 1988), which is similar to our results in terms of changes in MAP in toxin-exposed fish. Previous studies found that steady declines in MAP to less than 50% of the pre-dosing values often occurred by 45–60 min in mammals (rat and swine) after a lethal parenteral dose of MCs, usually at 50 and 100 μg kg^{-1} (Beasley et al., 2000; Berg and Soli, 1985; Ostensvik et al., 1981; Theiss et al., 1988). Results from this study demonstrate that an acute intraperitoneal dose of MCs eventually may cause irreversible hypotension in fish. However, declines in MAP to 50% were observed after 3 h in fish exposed to MC doses of 150 or 600 μg kg^{-1}, suggesting that the cardiovascular MC -response in fish is less sensitive than that in mammals. Additionally, declines in MAP were positively related to drops in BV and Hct of MC-exposed carp. Thus, these results demonstrate that hypovolemia might be the cause of the hypotension induced by extracted MCs.

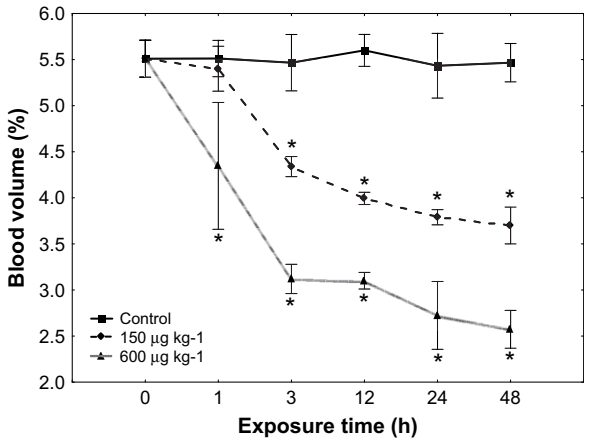

Fig. 4. Changes in circulating blood volume (BV) of crucian carp injected intraperitoneally with 0.75% NaCl (control) and *Microcystis* extract corresponding to 150 and 200 μg MC kg^{-1} BM, respectively. Values are expressed as means ± SD. There was no significant change in BV in the control group during the experimental period. * indicates significant differences between MC-administration groups and the control group at each time point ($p < 0.05$).

In the present study, fish exhibited the initial chronotropic effects in parallel with the drop in MAP after MC administration. Best et al. (2001) also found that increased heart rate of brown trout occurred upon acute exposure to MCs containing water. This indicates that fish heart may retain the ability to react to MC-induced hypotension via cardiac compensatory mechanisms during the early exposure period. However, due to hypovolemia, insufficient venous return may occur in MC-treated fish, which in turn aggravates the hypotension and leads to cardiac insufficiency (Ebert et al., 1955). The terminal lack of tachycardia during hypotension observed in this study seemed to represent a functional cardiac impairment.

Massive loss of circulating blood volume can result in hypotension, inducing hypovolemic shock, and eventually leads to physiological disturbances and animal death because of nutritive insufficiency from low tissue perfusion (Baue, 2006; Kelman, 1980; Randall et al., 1997). In this study, loss of BV in fish given MCs far exceeded 30%, especially in the lethal dose group. Acute loss of over 30% of the estimated blood volume can lead to irreversible prominent hypotension and animal death (Phyllis, 1976). This is why the chronotropic responses in crucian carp exposed to MCs seemed to be incapable of preventing a decrease in blood pressure. Therefore, it can be inferred that crucian carp exposed to MCs suffered from hypovolemic shock, which might cause fish death, as reported for mammals (Beasley et al., 2000; LeClaire, et al., 1995). This viewpoint differs from that proposed by previous studies (Bury et al., 1997; Kotak et al., 1996; Malbrouck et al., 2003). Even though losses of over 30% of BV occurred in the sublethal dose group, fish survived at the end of the experiment. This result differed from those of mammalian studies (Beasley et al., 2000; Theiss et al., 1988). It can be inferred that fish are more resistant to hypovolemic shock induced by MCs than mammals, even if the cause of death in crucian carp was considered to be similar to that in mammals.

The hepatic damage caused by MCs usually led to intrahepatic hemorrhage in mammals (Carmichael, 1994). Hepatotoxic effects of MC administration in fish seem to be

analogous to those in mammals (Fischer et al., 2000; Li et al., 2005; Solter et al., 1998; Tencalla and Dietrich, 1997; Råbergh et al., 1991). Li et al. (2005) reported that intrahepatic hemorrhage occurred in bighead carp (*Aristichthys nobilis*) injected intraperitoneally with crude MC extracts. Rainbow trout gavaged with MC-producing cyanobacteria (amounting to an equivalent of 5700 μg MC-LR kg^{-1} body weight) also displayed intrahepatic hemorrhage (Fischer et al., 2000). Furthermore, marked drops in Hct and RBC counts accompanied by severe hypovolemia suggested the existence of massive hemorrhage in fish (Duff and Olson, 1989). Hct, RBC, and BV also declined in this study, thus indicating the existence of hemorrhage. The latter was corroborated by the examination of the liver and gills of dissected, MC-exposed carp. Although the exposure routes in these studies are different, hemorrhage in MC-treated fish were all observed, indicating that the exposure routes do not affect the mechanism of action of MCs on the circulatory system. Considering the results from this study, circulatory failure is a key factor to physiological disturbances in fish exposed to MCs.

In summary, this study demonstrates that intraperitoneal injection of MCs extracts leads to severe hypovolemic hypotensive shock which is likely the main cause of fish death.

Acknowledgements

The authors would to thank Dr Alan Harvey and two anonymous reviewers for their comments and suggestions on the manuscript. The authors acknowledged Ms Guirong Zhang, Professor Xiuping Yang, and Xiaoming Wang for their technical assistances in the experiment. This work was supported by a fund for lecture professors from Huazhong Agricultural University and by a fund from the National Basic Research Program of China (973 Program) (Grant No. 2008CB418101).

Conflicts of interest

The authors declare that there are no conflicts of interest.

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