Erythrocyte damage of crucian carp (*Carassius auratus*) caused by microcystin-LR: in vitro study

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Abstract Fish suffer from anemia and hypovolemic hypotensive shock after in vivo exposure with microcystins. However, except for in vivo causes for anemia and hypotension, an in vitro study of fish erythrocytes exposed to MC is necessary. For a better understanding of hematology toxicity of MC, the main aim of the present study was to investigate the toxic effects of microcystin on fish erythrocytes in vitro. Crucian carp erythrocytes were incubated in vitro with microcystin-LR (MC-LR) at doses of 0, 1, 10, 100 and 1,000 nM. The level of lipid peroxidate significantly increased in MC-LR treatment groups. Glutathione decreased after exposure to MC-LR. The activities of antioxidative enzymes, including superoxide dismutase, catalase, glutathione peroxidase and glutathione-S-transferase, were significantly increased after exposure with MC-LR. The hemolysis was significantly increased, while the activities of acetylcholinesterase, Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase were significantly decreased. In addition, pathological alterations in agglomerated and jagged erythrocytes were observed in blood smears. The findings indicate that damages to erythrocytes should also be responsible for anemia and hypotensive shock or even death.

Keywords Erythrocytes · Fish · Cyanotoxin · Antioxidative response · In vitro

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Introduction

The occurrence of toxic cyanobacterial blooms in eutrophic lakes has become a worldwide problem (Paerl et al. 2001). Microcystins (MCs), a group of cyclic heptapeptide compounds with specific hepatotoxins produced by cyanobacterial species, have received worldwide concern in these years (Cohen 1989). Up to now, more than 80 different structural analogues of MCs have been identified, and it was reported that MC-LR was the most toxic (Fastner et al. 2002). MCs have been already demonstrated as potent inhibitors of protein phosphatases 1 (PP1) and phosphatases 2A (PP2A) (Nishiwaki-Matsushima et al. 1992). MCs cause damage to different organs, and liver is the main target (Robinson et al. 1989). One of the most important toxic mechanisms of microcystin is attributed as oxidative damage (Prieto et al. 2006).

Fish can be easily exposed to the toxins either by feeding or passively when the toxins pass through gills during breathing (Malbrouck and Kestemont 2006). MCs exert profound impacts on fish including

behavior, physiological and biochemical processes as well as embryonic development (Zhang et al. 2007, 2008; Šetlíková and Wiegand 2009). Fish can use the antioxidant defense system to reduce the impairment of the oxidative stress, including radical-scavenging enzymes, i.e., superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR). And glutathione (GSH) is also an important antioxidant to clean out radicals (Prieto et al. 2006, 2007).

Blood plays an important role in the transportation and excretion of MCs in animals. MCs can be transported via the bile acid transport system or via the blood to the kidneys for excretion when MCs are absorbed and distributed into different organs (Cazenave et al. 2006). Several studies have demonstrated that MCs exposure could result in fish hematological changes, anemia as well as hypotension (Zhang et al. 2007; Li et al. 2009). Erythrocyte has been one of the important cellular models for toxicological study under laboratory condition (Anna et al. 1999). Paulina et al. (2006) have observed antioxidative responses of human erythrocytes in vitro, and they confirmed that damages to erythrocytes may be the result of direct covalent binding of MC-LR with –SH residues of proteins and indirectly be related with reactive oxygen species formation.

Some previous studies in vivo in our laboratory revealed that fish was suffered from anemia and hypovolemic hypotensive shock when crucian carp was injected intraperitoneally (i.p.) with extracted MCs (Zhang et al. 2007; Li et al. 2009). Red blood cell (RBC) counts, hematocrit (Ht) and hemoglobin (Hb) were significantly decreased, indicating the appearance of normocytic anemia of crucian carp (Zhang et al. 2007). Li et al. (2009) observed decreased circulating blood volume (BV). However, the underlying mechanisms of anemia and hypovolemic hypotensive shock still remain puzzling. Anemia generally results from blood loss, decreased RBC production, kidney failure, poor RBC maturation or increased RBC destruction (Greenburg 1996). Therefore, except for in vivo causes for anemia and hypotension, an in vitro study of fish erythrocytes after exposure to MCs is greatly necessary. The main goal of the present study was to assess the toxic effects of MC-LR on crucian carp erythrocytes by examining antioxidative response and biochemical parameters, as well as hemolysis. Our present work would provide a better understanding of anemia, hypotensive shock and even death in fish treated with MCs.

Materials and methods

Toxin and chemicals

MC-LR was purchased from Express Technology Co., Ltd, China, with a purity of 97%. All reagents were bought from various commercial sources and were of analytical grade.

Fish and erythrocytes

Healthy crucian carp (mean body weight 255.0 ± 21.5 g) were purchased from the local fish farm in Wuhan City, China. The fish were allowed to acclimate for 1 week prior to experimentation and were fed with commercial fish food at a rate of 4.0% of body weight. Feeding was terminated 2 days before the experiment. Experiments were performed according to the guidelines of Ethical Committee for Animal Experiments at Huazhong Agricultural University, Wuhan of China. Then, whole blood was taken by caudal puncture with heparinized syringes. The erythrocytes were isolated by centrifuging at 3,000 rpm for 10 min at 4°C and washed three times with ten volumes of an isotonic buffered (pH = 7.4) NaCl solution. After careful removal of the leukocytes, erythrocytes were incubated (RBC = 2.6×10^7 cells/ml) in the sterile incubation medium (90.5 mM NaCl, 3 mM KCl, 1.3 mM $CaCl_2$, 0.5 mM $MgSO_4$, 6 mM glucose, 1 mM pyruvate and 1 mM Tris–HCl, pH = 7.4). Each batch of erythrocytes was exposed for 1, 3, 12, 24 and 48 h with 1, 10, 100 and 1,000 nM MC-LR in a shaker at the temperature of 15°C. Control erythrocytes were simultaneously incubated with sterile incubation medium without any MC-LR.

Determination of lipid peroxidation and antioxidative response

Malondialdehyde (MDA), an end product of lipid peroxidation, was determined by measuring the formation of thiobarbituric acid–reactive substances (TBARS) according to the method described by Stock and Dormandy (1971). One milliliter of a freshly prepared solution (thiobarbituric reactant solution) containing 15% trichloroacetic acid (w/v), 0.375% TBA (w/v) and 2.5% hydrochloric acid (v/v) was added to supernatants and placed in a boiling water bath for

15 min. After cooling, absorbance was measured on a spectrophotometer at 532 nm.

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 and 50 μl hemolysate. 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 measured according to the method of Aebi ([1984]). To 3 ml H_2O_2 (54 mM H_2O_2 in 50 mM phosphate buffer pH 7.0), 5 μl loaf catalase solution was added and the decrease in H_2O_2 was measured spectrophotometrically at 240 nm, at 25°C for 60 s. One unit of catalase activity was defined as the activity required to degrade 1 μmol hydrogen peroxide in 60 s. 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 hemolysate. After 5 min of preincubation (20–25°C), the reaction was initiated by the addition of 100 μl 0.25 mM H_2O_2. Glutathione-S-transferase (GST; EC2.5.1.18) activity was measured according to the method of Habig et al. ([1974]), using 1 mM glutathione (GSH), 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 0.1 M potassium phosphate buffer, (pH 6.5). The rate of increase in product concentration was monitored by measuring the absorbance at 340 nm at 25°C for 3–5 min in a spectrophotometer. Glutathione (GSH) in incubation solutions was determined according to the method described by Beutler ([1975]). Erythrocyte suspension was deproteinized by the addition of trichloroacetic acid (TCA) to a final concentration of 2%. To the supernatant (with glutathione) cleared by centrifugation, 5, 5′-dithio-bis (2-nitrobenzoic acid) (DTNB) was added to a final concentration of 0.47 mM. The formation of 5-thio-2-nitrobenzoic acid, which is proportional to the concentration of acid-soluble thiols mainly glutathione, was monitored at 25°C. The absorbance was measured at 412 nm in a spectrophotometer.

Measurement of biochemical analyses

Acetylcholinesterase (AChE) activity of erythrocytes was assayed by the method of Ellman et al. ([1961]). The assay mixture contained 0.45 mM ATCh as substrate and 0.3 mM DTNB as chromogen in 0.1 M phosphate buffer (pH 7.4) at 37°C. Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase activities of cell membrane were determined by measuring the initial rate of release of Pi from ATP according to the method of Chauhan et al. ([2002]). The mixture contained 30 mM imidazole buffer (pH 7.0), membrane sample, 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.1 mM EGTA and 0.1 mM ouabain. The enzymatic reaction was initiated by the addition of 3 mM ATP and stopped by the addition of 1 ml of 15% TCA 60 min later. The amount of inorganic phosphorus release was measured using KH_2PO_4 as a standard and determined at 660 nm. The protein content was determined by the method of Lowry et al. ([1951]). Hemoglobin in blood was estimated using Drabkin's reagent by the method of Dacie and Lewis ([1984]).

Evaluation of hemolysis

The ratio of hemolysis was calculated from the equation:

$$H(\%) = A_{pb} \times 100\%/A_{water}$$

The $H\%$ is the hemolysis of erythrocytes incubated with MC-LR; A_{pb} is the absorbance of supernatant in all samples at 540 nm; A_{water} is the absorbance of erythrocytes completely hemolysed in distilled water at 540 nm.

Observation of blood smears

At 12 h, blood smears were observed in 100- and 1,000-nM dose groups and control group. Air-dried smears were fixed for 1 min in methanol, stained with Wright–Giemsa for 1 min and washed twice in distilled water for 1 min. Blood smears were examined using an Olympus compound microscope at 400 total magnifications.

Statistical analysis

Every sample was measured in triplicates to get an average value for statistical use. All results expressed as mean ± SD 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

Lipid peroxidation and antioxidative response

Lipid peroxidation levels showed significant increases at 12 h in 100- and 1,000-nM dose groups and at 24 and 48 h in all treatment groups (Fig. 1).

In all MC-LR treatment groups, GSH levels significantly decreased at 12 and 24 h after MC-LR incubation. The GPx activities showed significant increases at 12 and 24 h after incubation of MC-LR in 1-, 10- and 100-nM dose groups. Enzymatic activity of GST exhibited marked increase at 12 h in 1, 10 and 100 nM, and prominent increases were observed at 24 and 48 h in all MC-LR treatment groups. CAT activity significantly increased at 12 and 24 h in 1-, 10- and 100-nM dose groups. The significant increases in SOD activity were observed at 3 h in 10- and 1,000-nM dose groups. After incubation at 12, 24 and 48 h, there were distinct increases in 1-, 10- and 100-nM dose groups (Fig. 2).

Biochemical determination

The activity of AChE significantly decreased at 12 h in 100-nM dose group and at 24 h in 10-, 100-, 1,000-
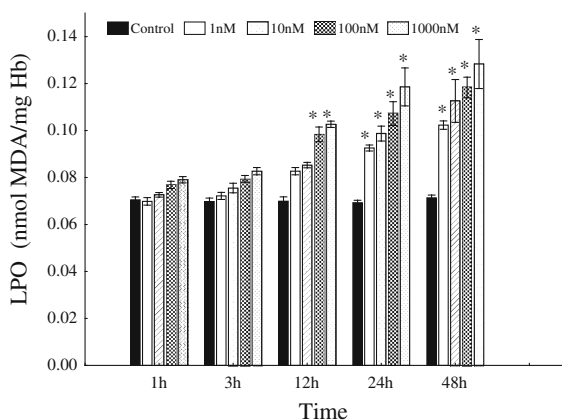
Fig. 1 Lipid peroxidation levels in erythrocytes incubated with microcystin-LR. The values are expressed as mean ± SD. *Asterisk* indicates significant differences at $P < 0.05$ in comparison with control group values

nM dose groups, as well as 48 h in 100-nM dose group. The decrease in the activity of Ca^{2+}–Mg^{2+}-ATPase showed significant decreases at 12 and 24 h in 10-, 100- and 1,000-nM dose groups and at 48 h in 10- and 100-nM dose groups. The activity of Na^+–K^+-ATPase significantly decreased at 12 and 24 h in 10-, 100- and 1,000-nM dose groups and at 48 h in 100-nM dose group (Fig. 3).

Hemolysis examination

Hemolysis of fish erythrocytes is shown in Table 1. Compared with the control, significant increases were observed from 12 to 48 h in 10-, 100- and 1,000-nM dose groups.

Blood smear observation

Morphological changes were observed in erythrocytes at 12 h in 100- and 1,000-nM dose groups. Erythrocytes in control group exhibited round shape with pink-stained cytoplasm and central, oval nucleus (Fig. 4a). Significantly agglomerated erythrocytes were observed at 12 h in 100-nM group, with swelled, blebbing and notched cell membrane (Fig. 4b). Disrupted or dissolving cellular membrane was recorded at 12 h in 1,000-nM group, and some erythrocytes exhibited jagged margin (Fig. 4c).

Discussion

One previous study of our laboratory has demonstrated that MCs were detected mainly in blood, liver, gonad and kidney when crucian carp were injected i.p. with extracted MCs. And the highest concentration of MCs was found in blood (Lei et al. 2008). Therefore, in some extent, blood is one of the most important target organs in animals after MC exposure. Blood indices are useful and sensitive for monitoring the physiological status of fish exposed to toxicants (Adhikari et al. 2004). Some in vivo studies focused on hematological changes in fish treated with MC (Zhang et al. 2007). However, as so far as we know, no study has been conducted to investigate the toxic effects of MC on fish erythrocytes in vitro. In fact, erythrocyte in vitro has been one of the most important materials for mechanism investigating blood toxicology.
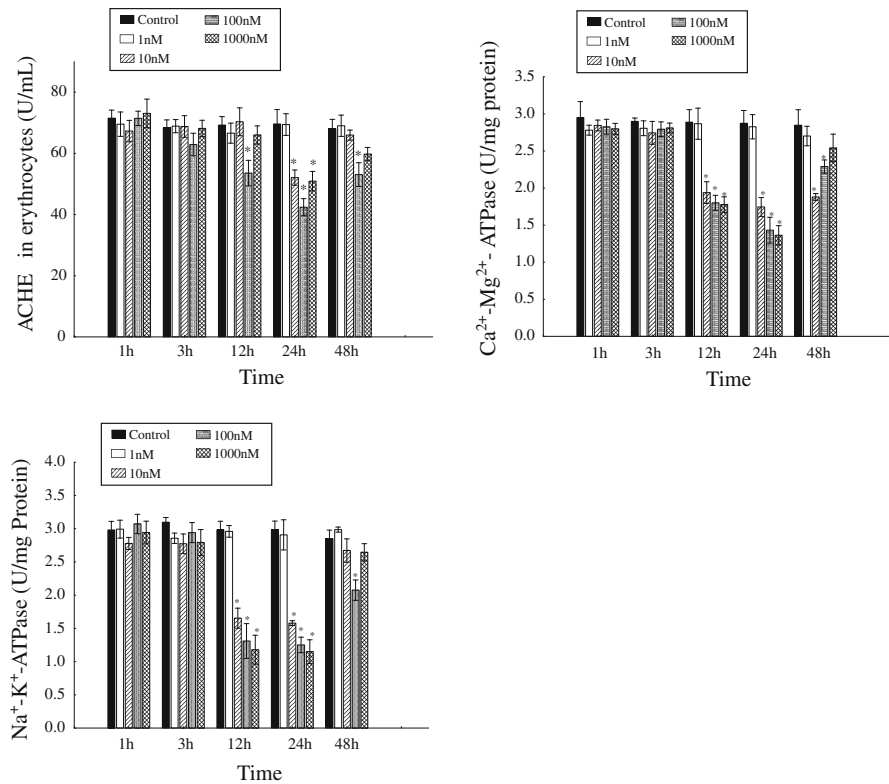
Fig. 2 Antioxidative response of erythrocytes incubated with microcystin-LR. The values are expressed as mean ± SD. *Asterisk* indicates significant differences at $P < 0.05$ in comparison with control group values
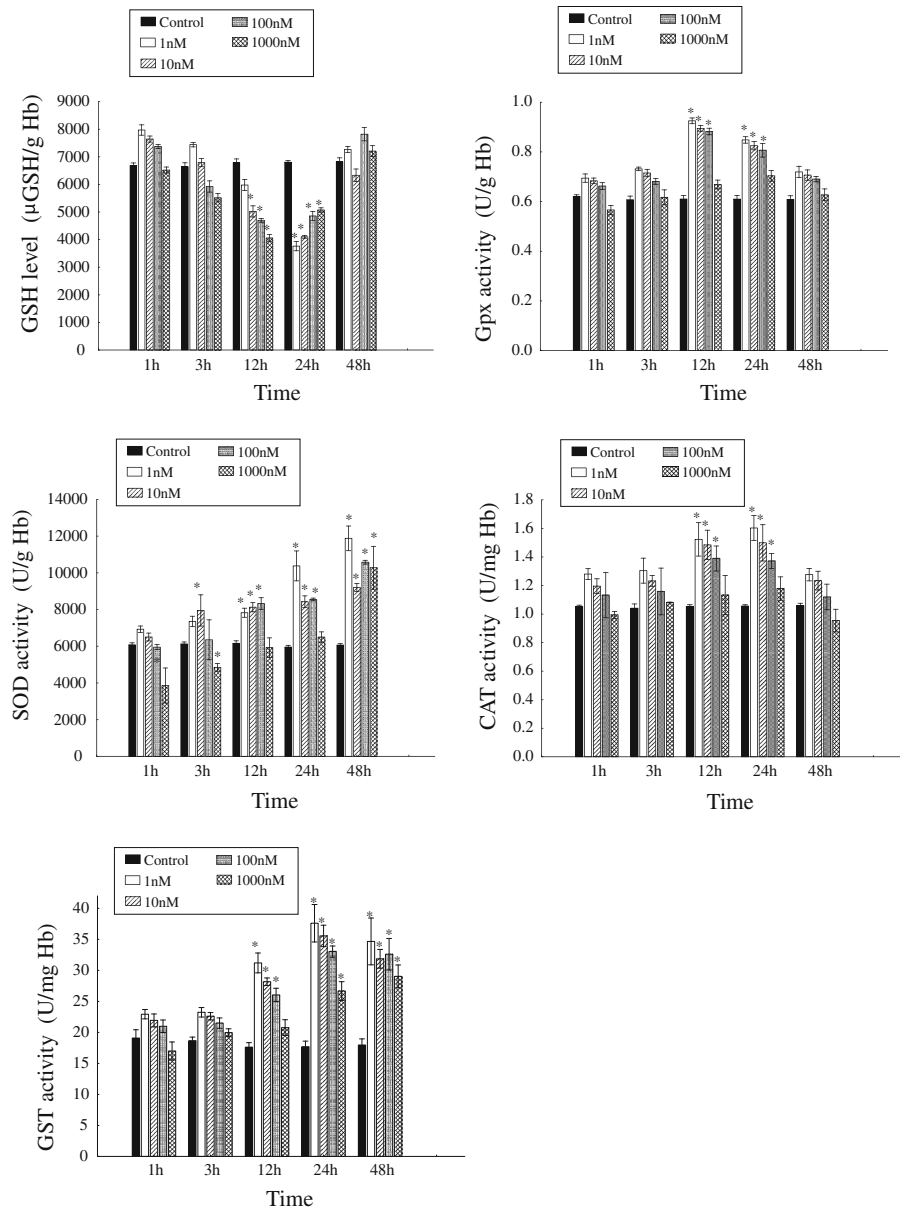
Many investigations demonstrated that various compounds exert toxic effect on erythrocytes via lipid peroxidation (Orhan and Sahin 2001; Bukowska and Kowalska 2004). Erythrocytes are prone to oxidative damage as a consequence of free radical attack, as they are exposed to high oxygen tension and are rich in iron and polyunsaturated fatty acids (Chiu and Lubin 1989). The spontaneous generation of superoxide, peroxide and hydroxyl radicals has been reported in RBC (Hebbel et al. 1982). Erythrocytes contain high concentrations of polyunsaturated fatty acids, molecular

oxygen and ferrous ions in the ligand state; however, they contain cellular antioxidants and enzymes, GSH, CAT, SOD and GPx (Ertabak et al. 2004).

Lipid peroxidant is a significant membrane-damaging event (Valenzeno 1987), and the MDA is a measure of the extent of lipid peroxidative damage. In the present study, the significant increases in MDA levels indicated that marked lipid peroxide occurred in erythrocytes after exposure to MC-LR. Our results are consistent with earlier reports which hypothesized that the interaction of hemoglobin with redox drugs/

Fig. 3 Biochemical alterations in erythrocytes incubated with microcystin-LR. The values are expressed as mean ± SD. *Asterisk* indicates significant differences at $P < 0.05$ in comparison with control group values

Table 1 Percent of hemolysis in erythrocytes incubated with microcystin-LR

	Control	1 nM	10 nM	100 nM	1,000 nM
1 h	0.87 ± 0.02	0.86 ± 0.04	0.88 ± 0.02	0.88 ± 0.03	0.87 ± 0.01
3 h	0.85 ± 0.02	0.88 ± 0.01	0.87 ± 0.03	0.85 ± 0.01	0.86 ± 0.05
12 h	0.89 ± 0.02	0.87 ± 0.03	1.21 ± 0.57*	1.29 ± 0.01*	1.99 ± 0.25*
24 h	0.85 ± 0.03	0.90 ± 0.03	1.28 ± 0.06*	6.58 ± 0.47*	1.70 ± 0.01*
48 h	0.89 ± 0.02	0.89 ± 0.03	3.33 ± 0.18*	2.62 ± 0.09*	1.78 ± 0.06*

The values are expressed as mean ± SD

* Significant differences at $P < 0.05$ in comparison with control group values

xenobiotics to be the source of radical generation leading to membrane LPO and lysis (French et al. 1978; Clemens et al. 1984).

In the present study, significantly higher values of GPx, CAT and GST were observed in samples with lower concentration of MC-LR than higher ones; it seems that animals may have more time to metabolize the toxins or to form an adaptive response to reduce the adverse effects when exposed to the low dose of MCs. And the enzymatic activities increased in erythrocytes incubated with MC-LR; however, the level of GSH decreased. SOD catalyzes the dismutation of superoxide anions to form hydrogen peroxide and oxygen. The SOD activity behavior could be related to an increased oxidative stress (Maiti and Kar 1997; Kale et al. 1999). The consequence of the dismutation reaction of superoxide anion, catalyzed by SOD, is an increase in H_2O_2 level. The CAT, a soluble protein in erythrocytes, plays a role in the decomposition of hydrogen peroxide to give H_2O. The presence of
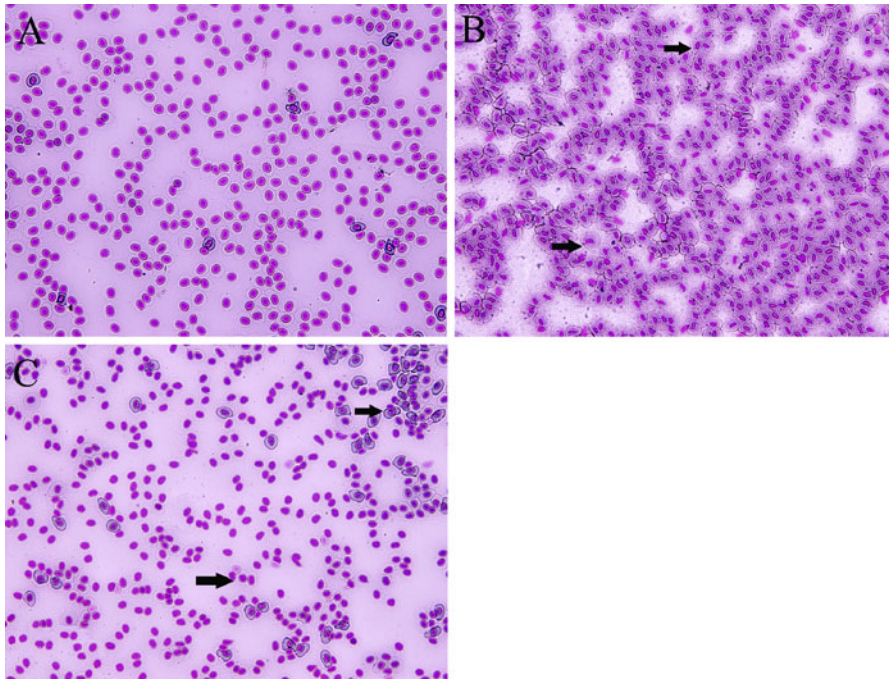
Fig. 4 Morphological change in erythrocytes incubated with microcystin-LR. **a** erythrocytes of control group (400×); **b** erythrocytes in 100-nM dose group at 12 h (400×); **c** erythrocytes in 1,000-nM dose group at 12 h (400×)

hydrogen peroxide could stimulate an increase in the CAT activity. GSH, one of the most potent biological molecules, affects scavenging of all functions including free radical reactions in the erythrocytes. One reason for the GSH decrease may be GSH oxidation to GSSG, and another reason is correlated with the increased activity of glutathione transferase (GST), which catalyzes the process of conjugation. In many aquatic organisms such as *Artemia salina*, fish and mussels, it was demonstrated that the metabolism of MCs begins with a conjugation reaction to GSH catalyzed by GST (Pflugmacher et al. 1998; Beattie et al. 2003). And this conjugation is mediated by the system of microsomal and cytosolic glutathione S-transferases. GST is considered to be an oxidative stress marker. The conjugation of electrophilic molecules with GSH is catalyzed by GST, and it is a part of the mechanism of detoxification of xenobiotics and peroxidation products (Neefjes et al. 1999). GPx is an enzyme capable of destroying the end products of ROS generation pathway involving H_2O_2 and organic peroxides (Ji 1999). Previous studies reported the changes in oxidative status in human erythrocytes under the influence of 2, 4-D and 2,4-DCP, and during oxidative stress, the increase in the activity of

glutathione peroxidase decreases the concentration of GSH (Bartosz 1995). The action of GPx is strictly linked with the concentration of GSH because it catalyzes the reaction between glutathione and hydrogen peroxide, resulting in the formation of glutathione disulfide (Mates 2000).

Some biochemical indicators are widely used in toxicology and in clinical chemistry to diagnose erythrocyte damage (Wang et al. 2007). In this study, a significant decrease in the activity of AChE was observed. The inhibition in erythrocytes' AChE activity was also found in erythrocytes treated with pyrethroid intoxication (Kale et al. 1999). They suggested that erythrocytes AChE can be a marker enzyme in pyrethroid toxicity due to the significant correlation of increased LPO level with the decreased AChE activity in erythrocytes. In the present study, the significant increased LPO levels should be responsible for the declined AChE activities. Some other studies also have demonstrated that there was a significant correlation between inhibition in AChE activity and increase in LPO level in erythrocytes (Su et al. 1971; Yang and Dettbarn 1996). In fact, some authors suggest that AChE inhibition induces cholinergic hyperactivity, initiating the accumulation of free radicals and leading

to lipid peroxidation, which in turn may lead to cell injury (Yang and Dettbarn 1996; Üner et al. 2006). The membrane-bound Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase activities in erythrocytes could be assessed as simple, safe and useful marker of intracellular damage (Ohta et al. 1989). It has been reported that the decrease in Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase activity was relative to the increase in the level of LPO when erythrocytes exposed to heavy metals and chemical medication (Wang et al. 2007; Vaddi et al. 2009). In this study, the significant decrease in Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase activity was observed in 10-, 100- and 1,000-nM dose groups from 12 to 24 h. Wang et al. (2007) found that Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase activities in rat erythrocytes were significantly decreased when it exposed to butenolide. And they demonstrated that the inhibition of ATPase activity may be ascribed to lipid peroxidation and/or the decrease in the number of –SH groups induced by butenolide. Paulina et al. (2006) have demonstrated that damages to erythrocyte membrane and antioxidative enzymes may be the result of direct covalent binding of MC-LR with –SH residues of protein.

The lysis of cells and death of erythrocytes were ascribed to changes in the shape of erythrocytes when the bilayer structure was disturbed by peroxidation process and incorporation of xenobiotic into membrane in the study by (Paulina et al. 2006). In this study, we observed that MC-LR induces significant increase in hemolysis in 10-, 100-, 1,000-nM dose groups from 12 to 48 h. Anna et al. (1999) observed that the level of hemolysis of carp erythrocytes significantly increased when it exposed to zinc in vitro. And they suggested that the increase in hemolysis may be due to the lipid peroxidative damage. Metal ions affect the phospholipid arrangement in the membrane by phospholipase A_2 action and finally increase the erythrocyte susceptibility to hemolysis (Anna et al. 1999). Paulina et al. (2006) also found that the level of hemolysis of human erythrocytes significantly increased when it exposed to MC-LR in vitro. They considered that the increase in hemolysis was ascribed to the lipid peroxidation that induced the membrane damage and hemolysis of erythrocytes.

Morphological changes in erythrocytes always occur in fish as a result of intoxication with inorganic or organic poisons (Malgorzata et al. 2005; Zeni et al. 2002). Malgorzata et al. (2005) observed that the share of cellular anomalies significantly increased when

juvenile tench was exposed to cadmium, including abnormal cell shape, vacuolization, swelling, chromatin disintegration in the nucleus and nucleus indentation. Vosyliene (1999) considered that morphology of erythrocytes is one of the most specific and sensitive indicators when fish exposed to various environmental factors. In this study, we observed that the cell membrane was swelled, notched and disrupted at 12 h after MC-LR exposure. Paulina (2006) observed changes in the shape of erythrocytes and echinocytes when human erythrocytes were incubated with MC-LR. Echinocytes were formed when the chemical compounds were binding to the outer part of membrane monolayer or may be formed when membrane skeleton was treated with oxidative stress (Bukowska and Zatorska 2003).

In conclusion, the present study focused on in vitro damage to erythrocytes of crucian carp treated with microcystin-LR. The results of antioxidative response, alterations in other biochemical parameters, hemolysis examination and morphological observation all indicate damages to erythrocytes. And the findings also validate the previous research that anemia and hypotensive shock result in fish damage or even death.

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