Anemia Induced by Repeated Exposure to Cyanobacterial Extracts with Explorations of Underlying Mechanisms

Xuezhen Zhang,[1] Ping Xie,[1,2] Dapeng Li,[1] Zechao Shi,[3] Jun Wang,[1] Gailing Yuan,[1] Yanyan Zhao,[1] Rong Tang[1]

[1]Key Lab of Agricultural Animal Genetics, Breeding and Reproduction of Ministry of Education, Fisheries College of Huazhong Agricultural University, Wuhan 430070, People's Republic of China

[2]Donghu Experimental Station of Lake Ecosystems, State Key Laboratory of Freshwater Ecology and Biotechnology of China, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, People's Republic of China

[3]Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Jinzhou 434000, People's Republic of China

Received 19 December 2009; revised 10 February 2010; accepted 11 February 2010

ABSTRACT: Hematological abnormalities or derangements have been demonstrated in patients suffering form microcystins (MCs) in hemodialysis unit in Caruaru, Brazil, 1996. While experimental study on hematological effect of microcystins has been rare and the underlying mechanisms are still puzzling. In the present study, microcystins were repeatedly intraperitoneally injected with a dose of 6 μg/kg/day in rabbits (*Oryctolagus cuniculus*) for 14 days, and the prolonged effects of extracted microcystins on hematotoxicology were investigated. Significant decreases were observed in the hematological indices red blood cell counts, hematocrit, hemoglobin, and platelet count, while an obvious anemia occurred in rabbits after 14-day exposure. Moreover, red blood cell volume distribution width, mean corpuscular volume, and mean corpuscular hemoglobin did not vary significantly, indicating that rabbits suffered from normocytic anemia. In bone marrow, on the 14th day after toxin exposure, the frequency of micronucleus increased significantly, and the viability of bone marrow cells decreased markedly compared with the control. Serum erythropoietin levels declined on the 7th and 14th day, which suggested that the ability to regulate differentiation and maturation of erythrocytes was impaired. These results indicate that repeated exposure of microcystins can result in normocyte anemia, and the bone marrow injures and the sharp decreases of erythropoietin levels were responsible for the anemia. Environ Toxicol 26: 472–479, 2011.

Keywords: microcystins; anemia; bone marrow; erythropoietin; hematotoxicology

Correspondence to: P. Xie; e-mail: xieping@ihb.ac.cn

Contract grant sponsor: National Natural Science Foundation of China.

Contract grant number: 30800160.

Contract grant sponsor: State Key Laboratory of Freshwater Ecology and Biotechnology.

Contract grant number: 2008FB015.

Contract grant sponsor: Huazhong Agricultural University.

Published online 31 August 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/tox.20583

Conflict of Interest: We declare that we have no conflict of interest.

INTRODUCTION

With the progress of eutrophication, cyanobacterial blooms in natural waters have been a worldwide problem, and cyanotoxins have posed a great threat to human health through contaminated drinking waters and fisheries products (Paerl et al., 2001; Xie, 2006). Among cyanotoxins, the hepatotoxic microcystins (MCs) are the most common and dangerous group, and microcystin-LR, RR, and YR are the

most toxic and abundant species (Fastner et al., 2002; Gupta et al., 2003). It is well known that MCs are cyclic heptapeptides, and are able to inhibit protein phosphatases 1 and 2A with liver as target organ (Falconer and Yeung, 1992). The high selectivity to liver is believed to be due primarily to toxin uptake by the bile acid transport system (Runnegar et al., 1993). The most tragic incident associated with MCs was reported in Caruaru, Brail in 1996, when renal dialysis patients were exposed to MC-contained water (Jochimsen et al., 1998; Pouria et al., 1998; Carmichael et al., 2001; Azevedo et al., 2002). Increasing evidences have demonstrated that microcystin is a genotoxic carcinogen, and is considered to induce DNA damage (Rao and Bhattacharya, 1996; Lankoff et al., 2003; Gaodin et al., 2008; Žegura et al., 2008). Epidemiological studies have suggested that microcystins are also suspected to be involved with promotion of primary liver cancer in humans exposed to long-term doses of microcystins through drinking water (Yu et al., 1995). In a recent study, microcystins were found to be transferred from contaminated lake water and aquatic animals to a chronically exposed human population (fishermen at Lake Chaohu, China) together with indication of hepatocellular damage (Chen et al., 2009).

Hematotoxicology is the study of adverse effects of drugs, chemicals, and other agents in environment on blood and blood forming tissues (Shashi and Mary, 2007). Hematological investigations have demonstrated that microcystins exposure caused a normocytic anemia in patients in a hemodialysis unit in Caruaru, Brazil in 1996 (Pouria et al., 1998). Additionally, hematological changes have been observed in swine after cranial vena cava exposure of microcystins, and the results indicate that major hematological parameters (hemoglobin, Hb and hematocrit, Ht) decreased significantly (Beasley et al., 2000). Similarly, Vajcová et al. (1998) demonstrated that blood values (red blood cell counts, RBC, Hb, and Ht) significantly decreased in *Hypophthalmichthys molitrix* after injection of microcystins. In a previous study, we observed normocytic anemia in crucian carp injected with microcystins at a dose of 200 μg kg^{-1}, and attributed the possible mechanism of anemia to the damage of kidney, the most important hematopoietic organ for crucian carp (Zhang et al., 2007).

Bone marrow, the crucial hematopoietic organ of mammals, has been approved as an important target organ of DNA damage induced by microcystin (Gaodin et al., 2008). The micronucleus test reflects chromosomal damage as well as damage to the mitotic spindle apparatus in polychromatic erythrocytes (PCE) in bone marrow, and is a sensitive method in the assessment of genotoxicity. Ding et al. (1999) conducted an *in vivo* study to investigate the toxicity of microcystins using micronucleus test, however, they sacrificed animal and determined micronucleated polychromatic erythrocytes of bone marrow only at the end of experiment, without consideration of time course effect. In fact, study on toxic effects of microcystin on bone marrow has been very rare.

Intraperitoneal (i.p.) injection is an important means for toxicological studies under laboratory conditions, and exposure quantity of toxicants can be accurately controlled. To observe the accumulative toxic effects, repeated injections of toxicants are often administrated in toxicological studies. Repeated intraperitoneal injection of microcystin-LR was conducted in mouse (Ito et al., 1997). It was reported that for oral exposure, animal has absorption difficulty of microcystin from the small intestine (Ito et al., 1997). According to the study of Gehringer et al. (2004), where the LD_{50} of intraperitoneal injection of extracted microcystins for rat is 82.5 μg kg^{-1} we set, in the present experiment, an exposure dose of 6 μg (extracted microcystins)/kg/day through a repeated injection of 14 days considering the accumulative effects of toxicity.

Therefore, the main aims of the present study are (1) to determine alterations of hematologcial parameters of rabbits after a prolonged exposure of repeated intraperitoneal injection of microcystins, (2) to investigate the genotoxic effects of microcystins on bone marrow by determining the micronucleus formation, (3) to evaluate changes of proliferate levels of bone marrow cells, and (4) to detect the variety of erythropoietin (EPO) levels of blood using method of enzyme-linked immunosorbent assay (ELISA). The present study was also to test the hypothesis that prolonged exposure of microcystins could lead to anemia in mammal, and the impairments of hematopoietic organs are responsible for the anemia.

MATERIALS AND METHODS

Cyanobacterial Extracts

The cyanobacterial material used in this experiment was collected from surface blooms of Lake Dianchi, Yunnan in China. According to microscopic examinations, cyanobacterial material refers to the phytoplankton cells from the surface of the water in which the predominant species belongs to Cyanophyta, and *Microcystis* was the predominant species. Freeze-dried crude algae were extracted three times with 5% acetic acid and were suspended in distilled water for the toxic experiment. Quantitative analysis of MC was performed using a reverse-phase high-performance liquid chromatography (HPLC, LC-20A, Shimadzu Corporation, Kyoto, Japan). 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 obtained extracted microcystins was a compound (MC-RR + MC-LR + MC-YR) with purity $\geq$80%. The microcystin content in the cyanobacterial material 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. The toxin-containing solution was finally diluted with phosphate-buffered saline (PBS) solution.

Experimental Animal

Healthy male rabbits (*Oryctolagus cuniculus*; 3 months old) weighing about 2000 ± 250 g were obtained from a local rabbit warren in Wuhan City, China. Rabbits were allowed to acclimate for 1 week prior to experimentation. Rabbits were housed with free access to water and food under the laboratory conditions: at (22 ± 2)°C and on a 12-h light/12-h dark cycle. Food and water offering was supplied throughout the experiment. Experiments were performed according to the guidelines of Ethical Committee for Animal Experiments at Huazhong Agricultural University, Wuhan of China.

Exposure and Sampling

Five acclimated rabbits without administration were expressed as 0 h. Acclimated rabbits were injected repeatedly (once daily with an interval of 24 h) by intraperitoneal route. Ten rabbits were injected i.p. with cyanobacterial extracts with the dose of 6 μg kg^{-1} BW per day, and equal volume of PBS solution for the control ($n = 10$). The whole injection process was finished within 3 min. Five rabbits were sacrificed both in MC-treated group and the control group at two different periods of time, 7 and 14 days postinjection, respectively.

Measurement of Hematological Parameters

Blood was sampled from femoral artery with anticoagulant (EDTA-K_2) syringes. The hematological indices were determined with blood cells analyzer (MEK-6318, Japan) according to the instruction of the manufacturers. Blood parameters were measured in triplicates, and averaged for statistical use, including red blood cell counts (RBC), hematocrit (Ht), hemoglobin (Hb), red blood cell volume distribution width (RDW), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

Bone Marrow Micronucleus Test

Immediately after the animals were sacrificed, femurs of the rabbit were freed from adherent tissues and were dissected out. The bone marrow was sampled by injection of filtered fetal calf serum using a syringe. The collected cells were smeared on glass slides. Slides were then dried in air, fixed with methanol for 10 min, and stained with Giemsa stain solution (phosphate buffered saline, 0.15 M, pH 7.4) for 15 min. The slides were scored under 1000× magnification using an optical microscope. Two slides were prepared from each animal, 1000 polychromatic erythrocytes (PCE) were examined from each slide for the

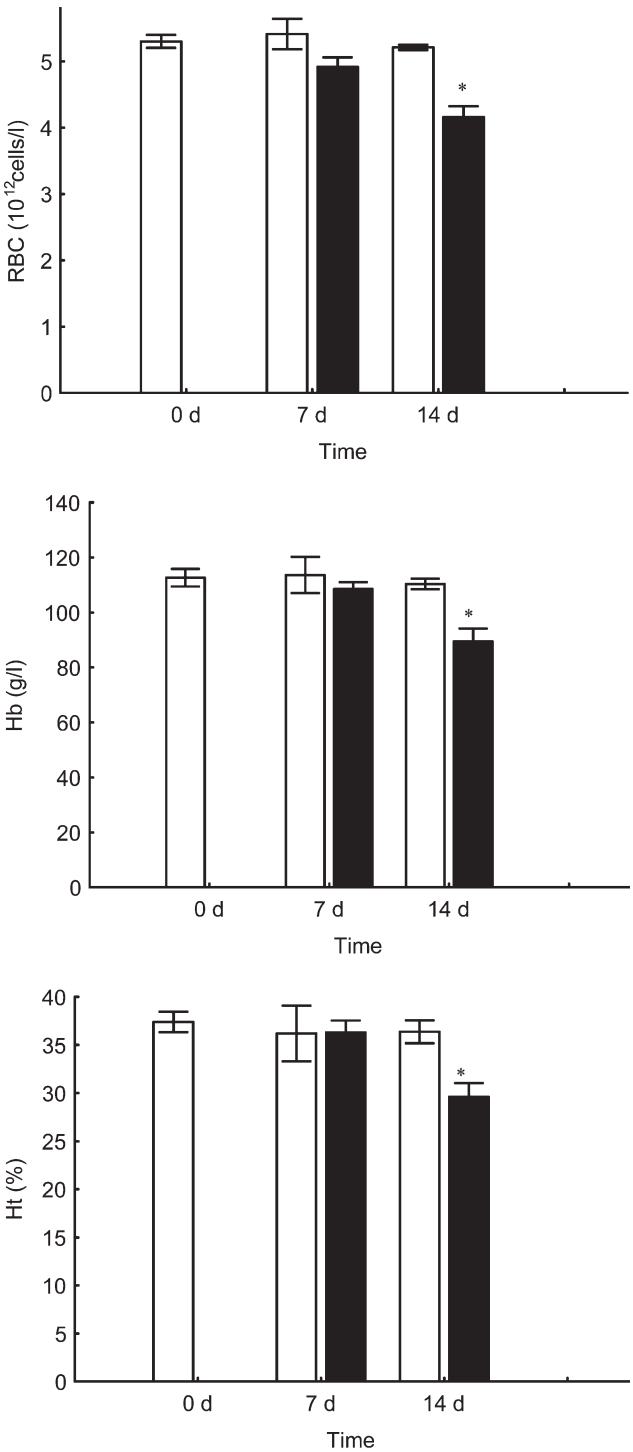

Fig. 1. Alterations of hematological indices (RBC, Hb and Ht) in rabbit after repeated intraperitoneal injection of microcystins (6 μg/kg/day) for 7 and 14 days. Values are expressed with mean ± SE ($n = 5$). □ White bars indicate control rabbits and ■ black bars indicate the MC-treated group. * indicates significant differences at $P < 0.05$ between MC-treated groups and the control group. ** indicates significant differences at $P < 0.01$.

TABLE I. No significant differences between cyanobacterial extracts treatment (6 μg/kg/day) and the control group

Indices	Treatments	0 h	7 days	14 days
RDW (%CV)	Control	16.03 ± 0.64	16.47 ± 0.93	16.69 ± 0.41
	6 μg kg^{-1}		16.57 ± 1.46	16.68 ± 1.38
MCV (fl)	Control	70.64 ± 2.73	70.54 ± 4.35	72.46 ± 2.57
	6 μg kg^{-1}		68. 26 ± 3.61	69.12 ± 3.48
MCH (pg)	Control	1.23 ± 0.24	1.31 ± 0.20	1.34 ± 0.36
	6 μg kg^{-1}		1.19 ± 0.22	1.26 ± 0.32
MCHC (g L^{-1})	Control	271.01 ± 24.21	298.43 ± 21.90	298.93 ± 10.70
	6 μg kg^{-1}		296.95 ± 34.09	299.91 ± 34.58

Values are expressed with mean ± SE ($n = 5$).

presence of micronuclei. The results were expressed as the average number of micronucleated polychromatic erythrocyte (MNPCE) cells per 1000 cells. The ratio of PCE to total (immature + mature) erythrocytes was also recorded. The size of each micronucleus was evaluated according to the size and shape criteria of Yamamoto and Kikuchi (1980). The unit of scoring was the micronucleated cell, not the micronucleus; thus, the occasional cell with more than one micronucleus was counted as one MNPCE.

Evaluation of Proliferation of Bone Marrow Cells

The proliferation ability of the bone marrow cells was determined by the MTT (3[4, 5-dimethylththiazoyl- 2-yl]-2, 5-diphenyltetrazolium bromide) colorimetric assay method (Mosmann, 1983). The collected cells suspension was adjusted to 1–3 $\times$ 10^6 cells mL^{-1} with RPMI 1640 buffer supplemented with 10% fetal calf serum. The 200 μL cells suspensions with culture medium were plated on a 96-well culture plate, and the proliferation of bone marrow cells was performed in triplicates. The assay control consisting 200 μL of RPMI 1640 medium was also plated in triplicate. The plates were incubated for 72 h at 37°C in a 5% CO_2. Four hours before the culture ultimate, 100 μL supernatant was discarded, and cultures were incubated for an additional 4 h after addition of 10 μL MTT (with concentration of 5mg mL^{-1}) per well. At the end of culture durations, the 100 μL SDS (10%) were plated per well, and the culture plate was placed 4 h at 37°C. Microtiter plates were then read on a microplate reader (BIO-RAD, Japan) using a test wavelength of 570 nm. Cell viability was estimated as optical density (OD) values.

Determination of Serum EPO Levels

The levels of EPO in the serum of rabbit were determined using a commercially available kit based on ELISA method (Sigma, USA).

Statistical Analysis

All results expressed as mean ± SE were subjected to one-way analysis of variance (ANOVA) and Dunnett's Test using STATISTICA software package (Version 6.0, Statsoft). Differences were measured against control values and considered to be statistically significant at $P < 0.05$.

RESULTS

Behavioral Observations

During the experimental period, clinical signs and behavior were monitored. No mortality was found in both the MC-treated group and the control group throughout the experiment. In the control group, there was no distinct abnormal behavior, except for some uneasiness during the injection treatments. However, in the MC-treated group, rabbits exhibited sluggishness, lethargy, as well as weak desire of food and water. At the end of the experiment, three rabbits from the MC-treated group presented obvious hair loss, and two rabbits exhibited severe paralysis of hindlimb.

Hematological Indices

After 7 days postmicrocystins exposure, no significant changes ($P > 0.05$) were observed in other hematological parameters (Fig. 1). On the 14th days after repeated injections of MCs, on the contrary, there were significant decreases ($P < 0.05$) in RBC, Hb, and Ht. Other red blood cell indices, including RDW, MCV, MCH, and MCHC all showed no significant changes (Table I).

Micronucleus Test and Cells Proliferation in Bone Marrow

Repeated injection of cyanobacterial extracts resulted in significant increases ($P < 0.01$) in frequency of micronucleus after 7- and 14-day toxin exposure compared with the

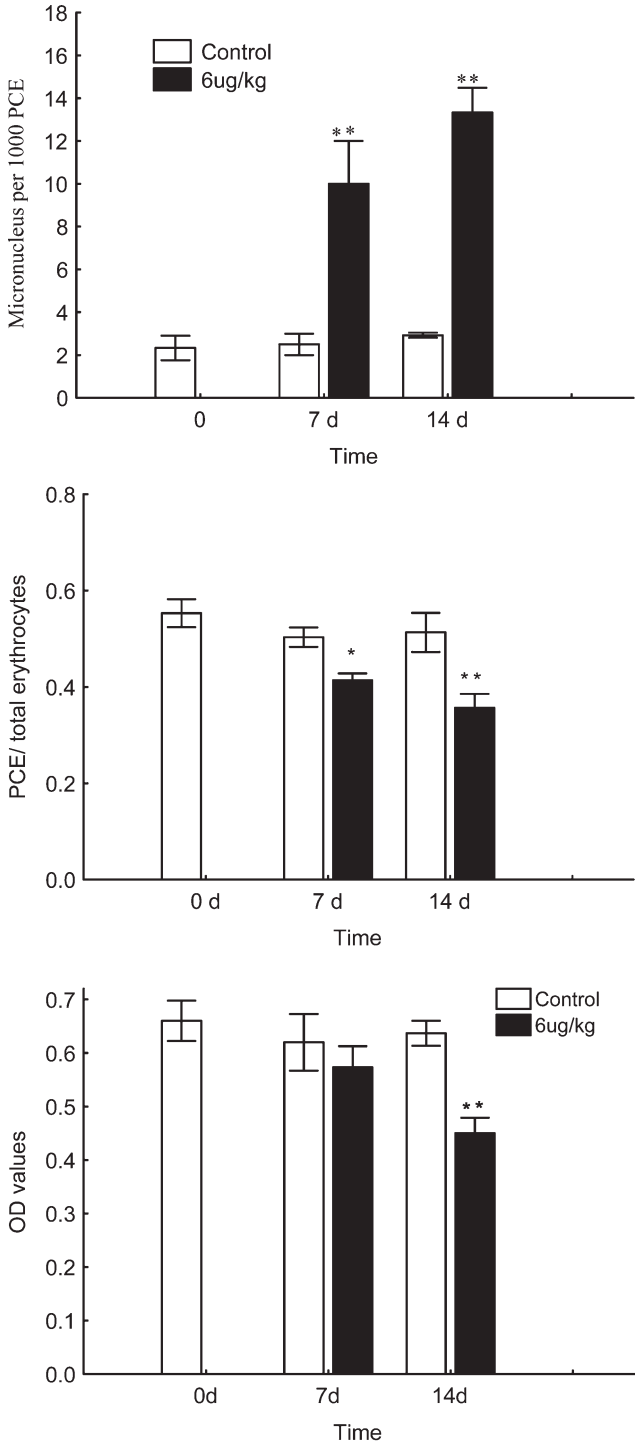

Fig. 2. Frequency of micronucleated polychromatic erythrocytes, ratio of polychromatic erythrocytes to total erythrocytes and changes of cell proliferation in bone marrow in rabbit after repeated intraperitoneal injection of microcystins (6 μg/kg/day) for 7 and 14 days. Values are expressed with mean $\pm$ SE (n = 5). □ White bars indicate control rabbits and ■ black bars indicate the MC-treated group. * indicates significant differences at $P < 0.05$ between MC-treated groups and the control group. ** indicates significant differences at $P < 0.01$.

control group (Fig. 2). The frequency of micronucleus on the 14th day was five-fold higher than that of the control.

The ratio of PCE/total erythrocytes of bone marrow in rabbit significantly decreased on the 7th day ($P < 0.05$), as well as on the 14th day ($P < 0.01$) (Fig. 2). Significant decreases were observed in the proliferation ability of bone marrow cells in rabbit on the 14th day ($P < 0.01$) post repeated injections of microcysitns (Fig. 2).

EPO Levels of Serum

The serum EPO levels significantly decreased both on the 7th day ($P < 0.05$) and the 14th day ($P < 0.01$) post microcystins exposure (Fig. 3).

DISCUSSION

Microcystins have been demonstrated to induce hematological alterations such as marked decreases of RBC, Hb, and Ht (Vajcová et al., 1998; Beasley et al., 2000; Kopp and Heteša, 2000; Zhang et al., 2007). In the present study, the hematological indices RBC, Hb, and Ht all showed pronounced decreases on the 14th day after a repeated exposure of microcystins. Anemia is characterized by prominent decreases of RBC and Hb (Greenburg, 1996). Microcystins have caused anemia in human in Brazil (Pouria et al., 1998). In the present study, rabbits after 14 days' repeated exposure of microcystins exhibited severe anemia symptoms. In addition, other indices (RDW, MCV, MCH, and MCHC) all showed no significant alterations between the

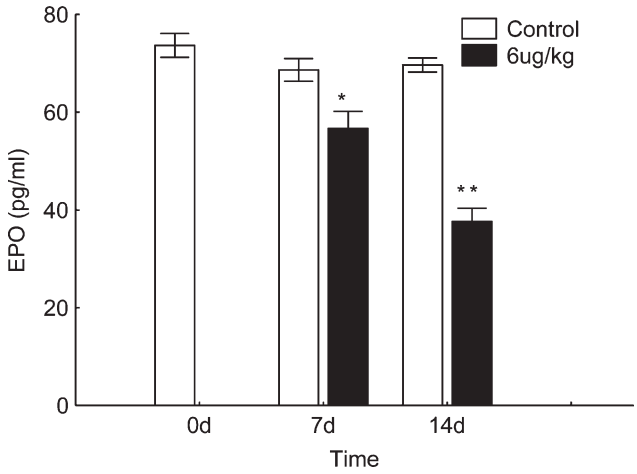

Fig. 3. Changes of serum EPO levels in rabbit after repeated intraperitoneal injection of microcystins (6 μg/kg/day) for 7 and 14 days. Values are expressed with mean $\pm$ SE ($n = 5$). □ White bars indicate control rabbits and ■ black bars indicate the MC-treated group. * indicates significant differences at $P < 0.05$ between MC-treated groups and the control group. ** indicates significant differences at $P < 0.01$.

control and the treatment. No changes occurred in cubage and hemoglobin of erythrocytes, therefore, rabbits suffered from normocytic anemia after repeated exposure of microcystins. Our findings are similar to those patients of hemodialysis unit of Brazil, where most patients had a normocytic anemia with a low normal platelet count (Pouria et al., 1998).

Anemia generally results from blood loss, decreased RBC production, kidney failure, poor RBC maturation, or increased RBC destruction (Greenburg, 1996). In our studies, we did not observe evident hemorrhage or hemolysis when we dissected rabbits. Hematopoiesis obstacles often cause anemia with decreased RBC or poor RBC maturation. It has been demonstrated that bone marrow can be damaged in mammals after exposure to microcystins, and obvious DNA damages in bone marrow have been observed in mice after intraperitoneal exposure of microcystin with a single dose (Gaodin et al., 2008). The micronucleus test, an *in vivo* short-term genotoxicity assay developed by Schmid (1975) and Heddle (1973), is widely used to detect chemically-induced damage to chromosomes or the mitotic apparatus in erythropoietic cells of mammal bone marrow (Asanami and Shimono, 2000). In the present study, repeated injection of microcystins induced significant increases in frequency of MNPCE in bone marrow on the 7th and 14th day, with a time-dependent effect. Ding et al. (1999) have demonstrated that induction of micronuclei by microcystic cyanobacteria extract in mouse was in a dose-dependent manner. Suzuki et al. (2008) explained that the increased opportunity for MNPCE induction by mutagens was due to the decreases of the efficiency of DNA repair. An induction of chromosomal aberrations was reported with purified extracts of hepatotoxin (Repavich et al., 1990). Moreover, MC-LR could affect the mitotic spindle apparatus (induction of hypercondensed chromosomes in G2/M, as wells as abnormal anaphases with defective chromosome separation and polyploidy cells) and provoke both necrosis and apoptosis in different cell types (Lankoff et al., 2003; Chen et al., 2005).

Microcysitns are reported to cause inhibition of proliferations of cells, including lymphocyte and other cultured cells (KB, HeLa, HepG2, and BY-2), and MTT method are usually used to evaluate the inhibition (Chong et al., 2000; Shen et al., 2003; Lankoff et al., 2004; Rymuszka et al., 2007; Žegura et al., 2008). In our studies, we observed marked decrease of proliferation of bone marrow cells post 14 days exposure. The viability of bone marrow cells maybe partly correlated with genotoxicity of microcystins. This is the first study focused on inhibition of proliferations of bone marrow cells.

Erythropoietin, a heavily glycosylated 34-kDa protein, regulates the final two steps for differentiation and maturation of erythrocytes (Jelkmann, 1992; Guzmán et al., 2008). In our study, there was marked decline in EPO levels in treatment group compared with the control. The decreased EPO levels are consistent with the reductions of RBC, Hb, and Ht levels. EPO is produced in the fetal liver and adult kidney cortex in mammals. When renal function is severely impaired and the ability to produce EPO will be also reduced. Moreover liver is a major extrarenal organ of EPO synthesis, when the hepatic damage occurs and the ability of EPO synthesis will also be declined (Hiratsuka et al., 1996). In fact, it is well known that liver and kidney are the main target organs of microcystins (Runnegar et al., 1993; Nobre et al., 2003; Moreno et al., 2005; Weng et al., 2007). In a previous study, we have demonstrated that after i.p. injection of microcystins, it exerts toxic effects on mitochondria of liver in rabbits (Zhao et al., 2008). Frangež et al. (2000) also observed obvious histological changes in liver of New Zealand rabbits after subchronic exposure to microcystins. Accordingly, we induce that drop of serum EPO levels resulted from injuries of kidney and liver, and there maybe some correlation between the viability of bone marrow cells and EPO levels.

In conclusion, after repeated injection of microcystins for 14 days, rabbits exhibited obvious normocytic anemia. In bone marrow, the most important hematopoiesis organ in mammal, DNA damages occurred with significant increase of frequency of micronucleus, and MTT assay indicated the ability of proliferation of bone marrow cells was markedly inhibited. We induce that the normocytic anemia is closely related with damage of bone marrow. In addition, the serum EPO levels on the 7th and 14th day decreased significantly. It is presumed that the damage of the main target organs of microcystins, i.e., kidney and liver, lead to the drop of the serum EPO. Therefore, repeated exposure of microcystins can result in anemia, and multiple organ injuries could be responsible for this anemia.

REFERENCES

Asanami S, Shimono K. 2000. Effects of chemically- and environmentally-induced hypothermia on micronucleus induction in rats. Mutat Res 471:81–86.

Azevedo SMFO, Carmichael WW, Jochimsen EM, Rinehart KL, Lau S, Shaw GR, Eaglesham GK. 2002. Human intoxication by microcystins during renal dialysis treatment in Caruaru, Brazil. Toxicology 181:441–446.

Beasley VR, Lovell RA, Holmes KR, Walcott HE, Schaeffer DJ, Walcott HE, Schaeffer DJ. 2000. Microcystin-LR decreases hepatic and renal perfudion, and causes circulatory shoch, severe hypoglycemia, and terminal hyperkalemia in intravascularly dosed swine. J Toxicol Env Health Part A 61:281–303.

Carmichael WW, Azevedo SMFO, An J, Molica RJR, Jochimsen EM, Lau S, Rinehart KL, Shaw GR, Eaglesham GK. 2001. Human fatalities from cyanobacteria: Chemical and biological evidence for cyanotoxins. Environ Health Perspect 109:663–668.

Chen J, Xie P, Li L, Xu J. 2009. First identification of the hepatotoxic microcystins in the serum of a chronically exposed human

population together with indication of hepatocellular damage. Toxicol Sci 108:81–89.

Chen T, Wang Q, Cui J, Yang W, Shi Q, Hua Z, Ji J, Shen P. 2005. Induction of apoptosis in mouse liver by microcystin-LR: A combined transcriptomic, proteomic, and simulation strategy. Mol Cell Proteomics 4:958–974.

Chong M, Gu K, Lam P, Yang M, Fong W. 2000. Study on the cytotoxicity of microcystin-LR on cultured cells. Chemosphere 41:143–147.

Ding WX, Shen HM, Zhu HG, Lee BL, Ong CN. 1999. Genotoxicity of microcystic cyanobacteria extract of a water source in China. Mutat Res 442:69–77.

Falconer IR, Yeung DSK. 1992. Cytoskeletal changes in hepatocytes induced by Microcystis toxins and their relation to hyperphosphorylation of cell proteins. Chem Biol Interact 81:181–196.

Fastner J, Codd GA, Metcalf JS, Woitke P, Wiedner C, Utkilen H. 2002. An international intercomparison exercise for the determination of purified microcystin-LR and microcystins in cyanobacterial field material. Anal Biochem Chem 374:437–444.

Frangež R, Kosec M, Sedmak B, Beravs K, Demsar F, Jutes P, Pogačnik M, Šuput D. 2000. Subchronic liver injuries caused by microcystins. Eur J Physiol 440:103–104.

Gaodin J, Huet S, Jarry G, Fessard V. 2008. *In vivo* DNA damage induced by the cyanotoxin microcystin-LR: Comparison of intra-peritoneal and oral administrations by use of the comet assay. Mutat Res 652:65–71.

Gehringer MM, Shephard EG, Downing TG, Wiegand C, Neilan BA. 2004. An investigation into the detoxification of microcystin-LR by the glutathione pathway in Balb/c mice. Int J Biochem Cell Biol 36:931–941.

Greenburg AG. 1996. Pathophysiology of anemia. Am J Med 101 (Suppl 2A):7S–11S.

Gupta N, Pant SC, Vijayaraghavan R, Rao PVL. 2003. Comparative toxicity evaluation of cyanobacterial cyclic peptide toxin microcystin variants (LR, RR, YR) in mice. Toxicology 188:285–296.

Guzmán A, García C, Marína C, Tortajadaa A, Ruiza MT, Henestrosaa AR, Marcosb R. 2008. Formation of micronucleated erythrocytes in mouse bone-marrow under conditions of hypothermia is not associated with stimulation of erythropoiesis. Mutat Res 656:8–13.

Heddle JA. 1973. A rapid *in vivo* test for chromosomal damage. Mutat Res 18:187–190.

Hiratsuka H, Katsuta O, Toyota N, Tsuchitani M, Umemura T, Marumo F. 1996. Chronic cadmium exposure-induced renal anemia in ovariectomized rats. Toxicol Appl Pharmacol 137:228–236.

Ito E, Kondo F, Harada K. 1997. Hepatic necrosis in aged mice by oral administration of microcystin-LR. Toxicon 35:231–239.

Jelkmann W. 1992. Physiol Rev 72:449.

Jochimsen EM, Carmichael WW, An JS, Cardo DM, Cookson ST, Holmes CEM, Antunes MBC, Melo-Filho DA, Lyra TM, Barreto VST, Azevedo SMFO, Jarvis WR. 1998. Liver failure and death after exposure to microcystins at a haemodialysis center in Brazil. N Engl J Med 33:873–878.

Kopp R, Heteša J. 2000. Changes of haematological indices of juvenile carp (*Cyprinus carpio* L.) under the influence of natural populations of cyanobacterial water blooms. Acta Vet Brno 69: 131–137.

Lankoff A, Banasik A, Obe G, Deperas M, Kuzminski K, Tarczynska M, Jurczak T, Wojcik A. 2003. Effect of microcystin-LR and cyanobacterial extract from Polish reservoir of drinking water on cell cycle progression, mitotic spindle, and apoptosis in CHO-K1 cells. Toxicol Appl Pharmacol 189: 204–213.

Lankoff A, Krzowski L, Glab J, Banasik A, Lisowska H, Kuszewski T, Gozdz S, Wojcik A. 2004. DNA damage and repair in human peripheral blood lymphocytes following treatment with microcystin-LR. Mutat Res 559:131–142.

Moreno I, Pichardo S, Jos á, Gomez-Amores L, Mate A, Vazquez CM, Caemean AM. 2005. Antioxidant enzyme activity and lipid peroxidation in liver and kidney of rats exposed to microcystin-LR administered intraperitoneally. Toxicon 45: 395–402.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63.

Nobre ACL, Martins AMC, Havt A, Benevides C, Lima AAM, Fonteles MC, Monteiro HSA. 2003. Renal effects of supernatant from rat peritoneal macrophages activated by microcystin-LR: Role protein mediators. Toxicon 41:377–381.

Paerl HW, Fulton RS, Moisander PH, Dyble J. 2001. Harmful freshwater algal blooms, with an emphasis on cyanobacteria. Sci World J 1:76–113.

Pouria S, Andrade A de, Barbosa J, Cavalcanti RL, Barreto VTS, Ward CJ, Preiser W, Poon GK, Neild GH, Codd GA. 1998. Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. Lancet 352:21–26.

Rao PVL, Bhattacharya R. 1996. The cyanobacterial toxin microcystin-LR induced DNA damage in mouse liver in vivo. Toxicology 114:29–36.

Repavich WM, Sonzogni WC, Standridge JH, Wedepohl RE, Mesner LF. 1990. Cyanobacteria (blue-green algae) in Wisconsin waters: Acute and chronic toxicity. Water Res 24: 225–231.

Runnegar MT, Kong S, Berndt N. 1993. Protein phosphatase inhibition and in vivo hepatotoxicity of microcystins. Am J Physiol 265:224–230.

Rymuszka A, Sierosławska , Bownik A, Skowroński T. 2007. *In vitro* effects of pure microcystin-LR on the lymphocyte proliferation in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immun 22:289–292.

Schmid W. 1975. The micronucleus test. Mutat Res 31:9–15.

Shashi KR, Mary BN. 2007. Blood and bone marrow toxicity. In: Gupta R, editor. Veterinary Toxicology Basic and Clinical Principles. New York: Elsevier, pp. 277–288.

Shen P, Zhao S, Zheng W, Hua Z, Shi Q, Liu Z. 2003. Effects of cyanobacteria bloom extract on some parameters of immune function in mice. Toxicol Lett 143:27–36.

Suzuki Y, Takagi R, Kawasaki I, Matsudaira T, Yanagisawa H, Shimizu H. 2008. The micronucleus test and erythropoiesis: Effects of cyclic adenosine monophosphate (cAMP) on micronucleus formation. Mutat Res 655:47–51.

Vajcová V, Navrati S, Palikova M. 1998. The effect of intraperitoneally applied pure microcystin-LR on haematological, biochemical and morphological indices of silver carp

(*Hypophthalmichthys molitrix Val.*). Acta Vet Brno 67: 281–287.

Weng D, Lu Y, Wei Y, Liu Y, Shen P. 2007. The role of ROS in microcystin-LR induced hepatocyte apoptosis and liver injury in mice. Toxicology 232:15–23.

Xie P. 2006. Microcystins in Aquatic Animals with Potential Risk to Human Health. Bejing: Science Press.

Yamamoto K, Kikuchi Y. 1980. A comparison of diameters of micronuclei induced by clastogens and by spindle poisons. Mutat Res 71:127–131.

Yu S. 1995. Primary prevention of hepatocellular carcinoma. J Gastroenterol Hepatol 10:674–682.

Žegura B, Zajc I, Lah TT, Filipič M. 2008. Patterns of microcystin-LR induced alteration of the expression of genes involved in response to DNA damage and apoptosis. Toxicon 51:615–623.

Zhang X, Xie P, Li D, Shi Z. 2007. Haematological and plasma biochemical responses of crucian carp (*Carassius auratus*) to intraperitoneal injection of extracted microcystins with the possible mechanisms of anemia. Toxicon 49:1150–1157.

Zhao Y, Xie P, Rong T, Zhang X, Li L, Li D. 2008. In vivo studies on the toxic effects of microcystins on mitochondrial electron transport chain and ion regulation in liver and heart of rabbit. Comp Biochem Physiol C 148:204–210.