The Role of GSH in Microcystin-induced Apoptosis in Rat Liver: Involvement of Oxidative Stress and NF-κB

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ABSTRACT: Microcystins (MCs) are potent and specific hepatotoxins produced by cyanobacteria in eutrophic waters, representing a health hazard to animals and humans. The objectives of this study are to determine the relationship between oxidative stress and NF-κB activity in MC-induced apoptosis in rat liver and the role of glutathione (GSH). Sprague-Dawley rats were intraperitoneally (i.p.) injected with microcystin-LR (MC-LR) at 0.25 and 0.5 LD_{50} with or without pretreatment of buthionine-(*S*,*R*)-sulfoximine (BSO), a specific GSH synthesis inhibitor. MC-LR induced time-dependent alterations of GSH levels in rat liver. Increased malondialdehyde (MDA) and significant changes of antioxidant enzymes including GSH peroxidase (GPX) and GSH reductase (GR) were also observed, particularly at 24 h post-exposure. The results indicated that acute exposure to MC-LR induced oxidative stress, and GSH depletion (BSO pretreatment) enhanced the level of oxidative stress. Furthermore, the modulation of pro-apoptotic gene p53 and Bax and anti-apoptotic gene Bcl-2 was observed in 0.5 LD_{50} group at 24 h, and the alteration was more pronounced by BSO injection before MC-LR treatment, suggesting that GSH played a protective role against MC-induced toxicity. Additionally, electrophoretic mobility shift assay (EMSA) showed that NF-κB was induced at 0.25 LD_{50} but inhibited at 0.5 LD_{50}. The above results indicated that the possible crosstalk of oxidative stress and NF-κB activity was associated with MC-LR-induced hepatocytes apoptosis *in vivo*. Our data will provide a new perspective for understanding the mechanisms of MC-induced liver injury. Environ Toxicol 31: 552–560, 2016.

Keywords: microcystin-LR; oxidative stress; apoptosis; glutathione; NF-κB; liver

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INTRODUCTION

Cyanobacterial blooms, which are characterized by excessive proliferation of cyanobacteria, represent a worldwide environmental problem in freshwater, brackish, and marine ecosystems (Paskerová et al., 2012; Merel et al., 2013). Cyanobacteria are known to produce various toxins, i.e. cyanotoxins, with microcystins (MCs, mainly microcystin-LR and -RR) being one of the most toxic and abundant species

(Dittmann and Wiegand, 2006; van Apeldoorn et al., 2007). MCs primarily act as hepatotoxins, for they are preferentially and predominantly absorbed, transported, and accumulated into the liver (Svirčev et al., 2010). Acute exposure to MCs can lead to severe liver damage, including massive intrahepatic hemorrhage, liver swelling, and even animal death. The most severe human intoxication happened in 1996 in Brazil, where 100 of 131 patients developed acute liver failure due to MCs contamination of the water used for dialysis and 52 patients died (Jochimsen et al., 1998; Pouria et al., 1998; Carmichael et al., 2001; Azevedo et al., 2002).

Recent studies showed that oxidative stress was involved in MCs toxicity in rat hepatocytes *in vitro* (Ding et al., 1998) and rat liver *in vivo* (Guzman and Solter, 1999). MCs induced intracellular reactive oxygen species (ROS) formation, lipid peroxidation, and cell injury (Guzman and Solter, 1999; Ding and Ong, 2003; Jayaraj et al., 2006). Meanwhile, MCs can induce hepatocytes apoptosis both *in vivo* (Hooser, 2000) and *in vitro* (McDermott et al., 1998), characterized by cell membrane blebbing, cytoplasmic shrinkage, nuclear chromatin condensation, DNA fragmentation, and formation of apoptotic bodies. It has been shown that formation of ROS precedes the onset of mitochondrial permeability transition (MPT), loss of mitochondrial membrane potential (MMP), and initiation of apoptosis in rat hepatocytes exposed to microcystin-LR (MC-LR) *in vitro* (Ding et al., 2000a). Similarly, an *in vivo* study found that MC-LR treatment promoted large amount of ROS generation in mice liver, up-regulated the expression of Bax and Bid, and caused MMP loss and hepatocyte apoptosis as well as liver injury (Weng et al., 2007). While pretreatment with antioxidants, oral administration of vitamin C and E, significantly reduced the generation of ROS and effectively inhibited the MC-LR-induced hepatocyte apoptosis and liver injury (Weng et al., 2007). MC-LR could up-regulate the expression of pro-apoptotic gene p53 and Bax and down-regulate anti-apoptotic gene Bcl-2 in cultured hepatocytes and rat liver tissues (Fu et al., 2005). These data suggest that induction of oxidative stress and mitochondrial alterations may play a critical role in MC-induced apoptosis.

Free radicals or ROS generated in tissues can be effectively scavenged by the antioxidant defense system. Glutathione (GSH) is present in all mammalian tissues as the most abundant nonprotein thiol that defends against oxidative stress (Zhang and Forman, 2012). It acts directly as a free radical scavenger and as a substrate for GSH peroxidase (GPX, EC 1.11.1.9) to reduce hydrogen peroxide and lipid peroxide (i.e. $H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$), while oxidized glutathione (GSSG) can be converted to its reduced form catalyzed by glutathione reductase (GR, EC 1.6.4.2). GSH can also conjugate with xenobiotics and/or their metabolites and it plays an important role in the detoxification processes (Lu, 2013). MCs have been shown to conjugate with GSH in cell-free systems and in liver tissue *in vivo*, catalyzed by glutathione-*S*-transferase (GST, EC 2.5.1.18) (Kondo et al., 1996; Pflugmacher et al., 1998; Guo et al., 2014). Meanwhile, GSH played an important role in protecting against MC-induced toxicity *in vivo* (Hermansky et al., 1991) and *in vitro* (Ding et al., 2000b; Žegura et al., 2006). MCs led to lactate dehydrogenase (LDH) leakage, microfilament and microtubule disruption in primary cultured rat hepatocytes (Ding et al., 2000b), and DNA strand break in HepG2 cells (Žegura et al., 2006). Furthermore, the cytotoxicity and genotoxicity induced by MCs were enhanced by buthionine-(*S*,*R*)-sulfoximine (BSO), a specific GSH synthesis inhibitor (Ding et al., 2000b; Žegura et al., 2006). However, these studies were conducted by using *in vitro* system; in fact, an *in vivo* exposure should be more proper to evaluate the MC toxicity. Moreover, the role of GSH in MC-induced oxidative stress and apoptosis is not fully known.

Transcription factor nuclear factor-kappa B (NF-κB), which is sensitive to ROS production and oxidative stress, is thought to play a key role in the development of several liver diseases including hepatitis, liver fibrosis and cirrhosis, and hepatocellular carcinoma (Sun and Karin, 2008; Morgan and Liu, 2011). Recent studies have reported that MC-LR induced activation of NF-κB in HepG2 cells (Feng et al., 2011), rat insulinoma cells (INS-1) (Ji et al., 2011), melanoma cells (MDA-MB-435) (Zhang et al., 2012), human hepatoma cells (Huh7) (Christen et al., 2013), mice hepatocytes (Zhang et al., 2013a), and HeLa cells (Chen et al., 2014). MC-LR promoted nuclear translocation and activation of p65 subunit of NF-κB, resulting in cellular apoptosis. Meanwhile, this effect was inhibited by transfection of siRNA against p65 in HepG2 cells (Feng et al., 2011) and pretreatment with ammonium pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-κB activation, in INS-1 cells (Ji et al., 2011). Christen et al. (2013) demonstrated that MC-LR activates NF-κB, induces IFN-α and TNF-α expression, and may contribute to tumor promotion and induction of apoptosis in Huh7 cells. However, these studies were all conducted by using cultured cell lines, whether MCs can modulate NF-κB activity *in vivo* is unknown yet. Moreover, the relationship between oxidative stress and NF-κB activity in MC-induced apoptosis has not been studied.

Therefore, the objectives of this study are to determine the relationship between oxidative stress and NF-κB activity in MC-induced apoptosis in rat liver and the role of GSH. SD rats were intraperitoneally (i.p.) injected with MC-LR, and the effects of MC-LR on GSH levels, antioxidant system response, apoptosis induction, and NF-κB activation were evaluated. Additionally, in order to better explore the role of GSH in MCLR-induced oxidative stress and apoptosis, the intracellular GSH level in rat liver was modulated with the specific GSH synthesis inhibitor BSO. Our data will improve the understanding the mechanisms of MC-LR-induced liver injury from a fresh point and hold promise for planning new clinical strategies of MCs intoxication.

MATERIALS AND METHODS

Chemicals

MC-LR was isolated and purified from the freeze-dried surface blooms (mainly *Microcystis aeruginosa*) collected from Lake Dianchi (Yunnan, China), following the method described by Wang et al. (2008). MC-LR was separated by semiperformance preparative liquid chromatography system (Waters 600E) and pure MC-LR was obtained. MC-LR was analyzed for MCs content via a reverse-phase high performance liquid chromatography (HPLC) (LC-10A, Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan). MC-LR (purity >95%) concentration was determined by the UV spectra and retention time, and by using a commercial standard MC-LR, -RR (Wako Pure Chemical Industries, Japan) to compare the peak areas of the test samples. All reagents obtained from various commercial sources were analytical or higher grades.

Animals

Male Sprague-Dawley (SD) rats weighing 180 to 200 g were purchased from Wuhan University Center for Animal Experiment/A3-Lab. Rats were housed in stainless steel cages containing saw dust bedding at 24 ± 2°C and 55 ± 15% relative humidity with a regime of 12 h light/dark cycle. Rats were given free access to standard rodent pellet diet and water. All procedures carried out on animals were approved by the Institutional Animal Care and Use Committee (IACUC), and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory. All efforts were made to minimize animal suffering to reduce the number of animals used.

Toxins Exposure

The median lethal dose (LD_{50}) of MC-LR was determined in our previous study (Li et al., 2014). Rats were randomly assigned to the following six groups: (1) control group; (2) 0.25 LD_{50} MC-LR group (20.675 μg/kg); (3) 0.5 LD_{50} MC-LR group (41.35 μg/kg); (4) 1 mM/kg BSO group; (5) 1 mM/kg BSO plus 0.25 LD_{50} MC-LR group; (6) 1 mM/kg BSO plus 0.5 LD_{50} MC-LR group. Compounds were administered through intraperitoneal (i.p.) injection. The rats in the fifth and sixth groups were injected with BSO for 3 h before the exposure of MC-LR. An equivalent volume of 0.9% saline solution was applied to control ones. Six sampling points were set during a period of 7 days in the experiment (2, 8, 24, 72, 120, and 168 h). No rats died during the experiment. Three rats from each group were randomly sampled at each time point, and the livers were quickly removed, minced, and stored frozen at liquid nitrogen for later analysis.

Biochemical Studies

The livers were homogenized (1:10, w/v) in a cold (4°C) buffer solution (pH 7.5) containing sucrose (250 mM), PMSF (1 mM), DTT (1 mM), and EDTA (1 mM). Homogenates were centrifuged at 12,000 × *g* (4°C) for 15 min and the supernatants used as the enzyme source.

The contents of GSH and MDA, and the activities of GPX and GR were assayed by the kits purchased from Nanjing Jiancheng Bioengineering, Inc. (Nanjing, Jiangsu, China). In brief, GSH content was measured according to Griffith (1980). MDA was used as an index of lipid peroxidation (LPO) and was determined by the thiobarbituric acid (TBA) method (Ohkawa et al., 1979). GPX activity was determined according to Drotar et al. (1985), using H_2O_2 as substrate. GR activity was measured spectrophotometrically by measuring the oxidation of NADPH at 340 nm (Carlberg and Mannervik, 1975). Protein concentration was determined by the coomassie blue staining using bovine serum bovine serum albumin (BSA) as the standard (Bradford, 1976). The content or the activity of antioxidants was calculated in terms of the protein content of a sample. Each assay was carried out in triplicate. The authors declared that 24 h data has been published in Li et al. (2014).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear proteins were extracted using Nuclear Protein Extraction Kit (Beyotime, China) and kept at −80°C. Protein concentration was determined in the supernatant using coomassie blue staining (Bradford, 1976) and 5 μg nuclear protein was used for EMSA. The DNA-binding activity of NF-κB was detected using LightShift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer's protocol.

Western Blot

For Western blotting sample preparation, small tissue sections were homogenized in ice-cold protein extraction buffer (Beyotime, China). The tissue homogenates were centrifuged at 12,000 × *g* (4°C) for 10 min and supernatant was carefully collected. Protein concentrations were determined using the coomassie blue method (Bradford, 1976). All samples were stored at −80°C before electrophoresis.

Aliquots from supernatant containing 20 μg of proteins were mixed with equal volume of 2× sample buffer. The sample was boiled for 5 min and subjected to 12% SDS-PAGE. After electrophoresis, the resolved proteins were transferred to a polyvinylidenedifluoride (PVDF) membrane (Millipore) using an electro blotting apparatus (Bio-Rad). Membranes were blocked in 5% nonfat dry milk in PBS buffer for 1.5 h at room temperature to prevent nonspecific binding of reagents, and then incubated in specific antibody against p53 (Biowold), Bax (EPI, Germany), Bcl-2 (CST), and GAPDH (EPI, Germany). Then they were incubated secondary antibodies following the manufacturer's instructions. The protein signal was evaluated using the NBT/BCIP system. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was analyzed in samples, and the protein level was stable and unaffected by MC-LR exposure, similar to the results reported in previous studies (Weng

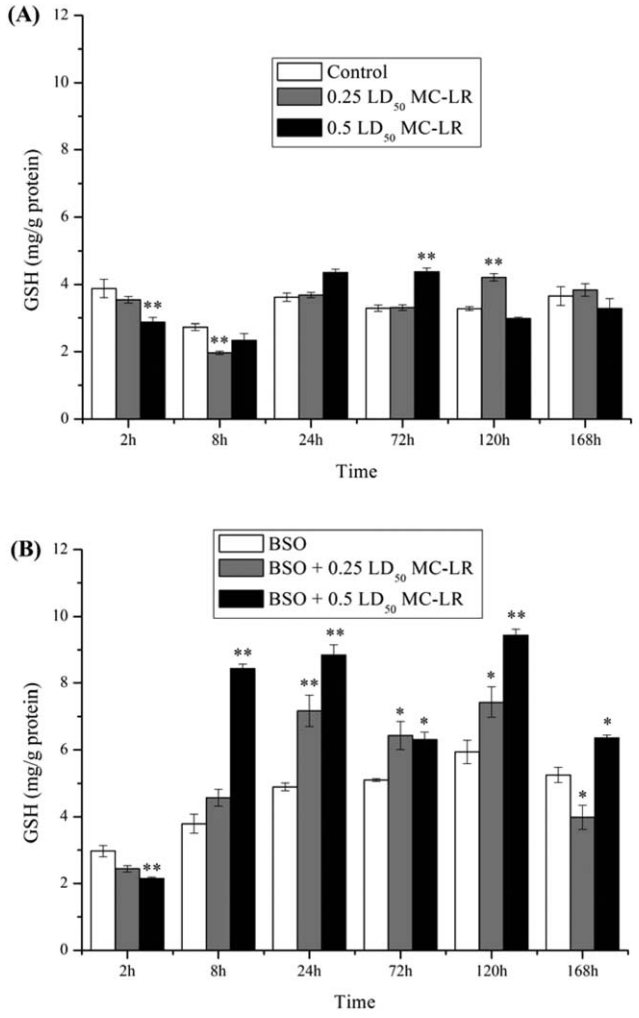

Fig. 1. Glutathione (GSH) levels in liver of rats exposed to microcystin-LR without (A) or with (B) buthionine-(*S,R*)-sulfoximine (BSO) pretreatment. Values are presented as the mean ± standard error (SE). *$p < 0.05$ *versus* control, and **$p < 0.01$ *versus* control.

et al., 2007; Sun et al., 2011), therefore GAPDH was used as the endogenous assay control.

Statistical Analysis

All values were expressed as the mean ± standard error (SE) and analyzed using one-way ANOVA and Turkey's multiple comparison tests with SPSS package13.0 (SPSS, Chicago, IL). Statistical differences between data of the control and MC-LR treatment groups were determined at the $p < 0.05$ or $p < 0.01$ levels for all analyses and indicated with * and **, respectively.

RESULTS

GSH and MDA Levels

Figure 1(A) showed the GSH levels in liver of rats after MC-LR exposure. There was an initial depletion of GSH in the liver tissue of rats in 0.5 LD_{50} group at 2 h and 0.25 LD_{50} group at 8 h; then the GSH level was increased in rat livers in 0.5 LD_{50} group at 72 h and 0.25 LD_{50} group at 120 h [Fig. 1(A)]. In the BSO pretreatment groups [Fig. 1(B)], the changes of hepatic GSH were more noticeable, with significant decrease in 0.5 LD_{50} group at 2 h; while significant increase of GSH was observed in 0.25 LD_{50} group at 24, 72, and 120 h and 0.5 LD_{50} group at 8, 24, 72, 120, and 168 h. No obvious change of MDA contents in rat livers after MCs exposure was observed at both two doses [Fig. 2(A)], except an increase occurred in liver treated with 0.25 LD_{50} MC-LR at 120 h post-exposure. However, BSO pretreatment significantly promoted MC-LR-induce MDA formation in 0.25 LD_{50} group at 8 and 24 h, and 0.5 LD_{50} group at 8, 24, and 120 h [Fig. 2(B)].

GR and GPX Activities

GR activity showed no changes before 24 h post-exposure [Fig. 3(A)]; however, a significant increase was observed in

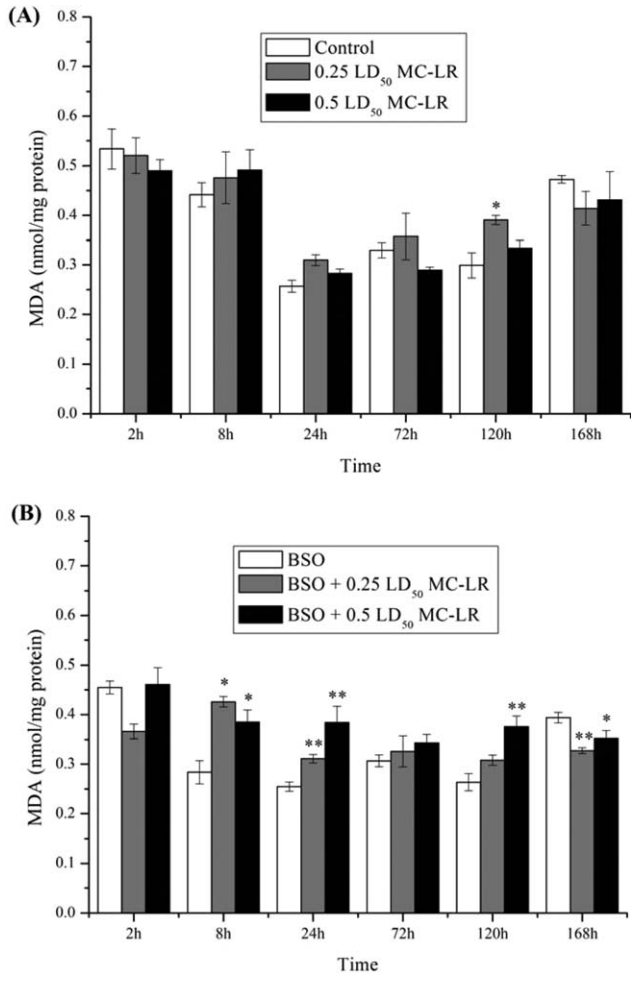

Fig. 2. Malondialdehyde (MDA) levels in liver of rats exposed to microcystin-LR without (A) or with (B) buthionine-(*S,R*)-sulfoximine (BSO) pretreatment. Values are presented as the mean ± standard error (SE). *$p < 0.05$ *versus* control, and **$p < 0.01$ *versus* control.

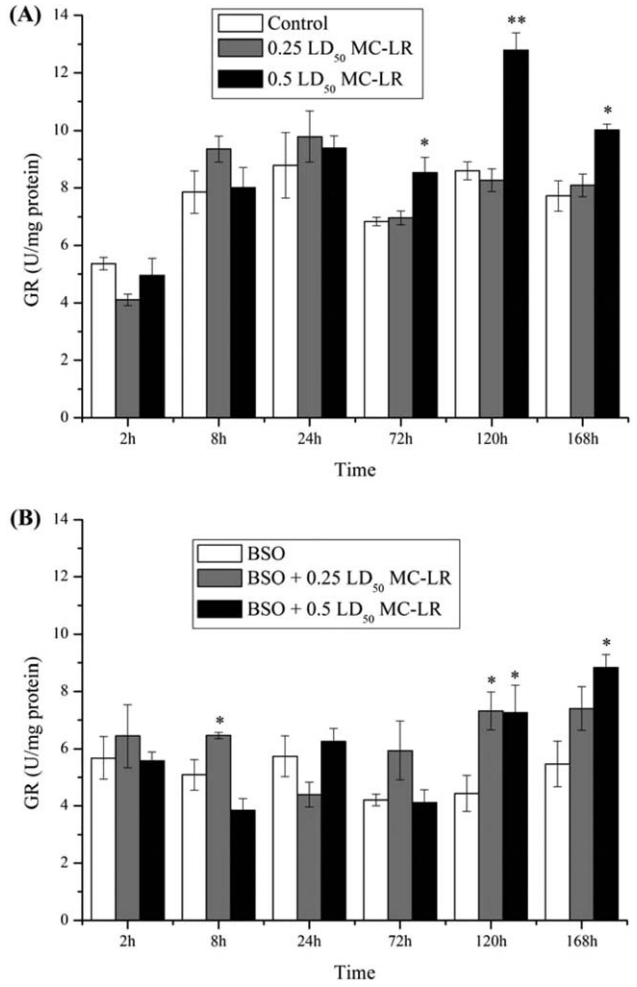

Fig. 3. Glutathione reductase (GR) activities in liver of rats exposed to microcystin-LR without (A) or with (B) buthionine-(*S,R*)-sulfoximine (BSO) pretreatment. Values are presented as the mean ± standard error (SE). $^{*}p < 0.05$ *versus* control, and $^{**}p < 0.01$ *versus* control.

0.5 LD_{50} group at 72, 120, and 168 h after MC-LR injection. GR activities in the BSO-pretreatment groups were also elevated in 0.25 LD_{50} group at 120 h and 0.5 LD_{50} group at 120 and 168 h [Fig. 3(B)]. GPX activity was initially induced in rat liver in 0.5 LD_{50} group at 2 h [Fig. 4(A)], but was inhibited then in 0.25 and 0.5 LD_{50} groups at 24 h, and it recovered to the control level at 72, 120, and 168 h post-exposure. In the BSO pretreatment groups [Fig. 4(B)], significant decreased GPX activity was also detected in 0.25 and 0.5 LD_{50} groups at 8 and 24 h, and it recovered to the normal level finally at 168 h. Based on the above results, and the maximum MC-LR contents were always detected at 24 h (Data not shown); 24 h post-exposure was selected for the subsequent research.

The DNA Binding Activity of NF-κB

Figure 5 showed the electrophoretic mobility shift assay of NF-κB at 24 h after MC-LR injection. NF-κB binding activity of the liver cells was enhanced in the 0.25 LD_{50} group, but was inhibited in the 0.5 LD_{50} group. Interestingly, in the BSO pretreatment groups, NF-κB binding activity revealed a similar variation trend, but there was an overall increase in the level of NF-κB activity in the livers compared with MC-LR exposure alone.

Expression of p53, Bax, and Bcl-2

The effects of MC-LR on protein expression of p53, Bax, and Bcl-2 were shown in Figure 6. No change of p53 protein expression was observed after MCs exposure alone. However, the p53 expression was induced in BSO pretreatment group. Bax protein level was up-regulated at 0.5 LD_{50} with or without BSO injection, but no apparent change was observed at 0.25 LD_{50}. The expression of Bcl-2 protein was inhibited at both 0.25 and 0.5 LD_{50}, and the inhibition was more pronounced by BSO injection before MC-LR treatment.

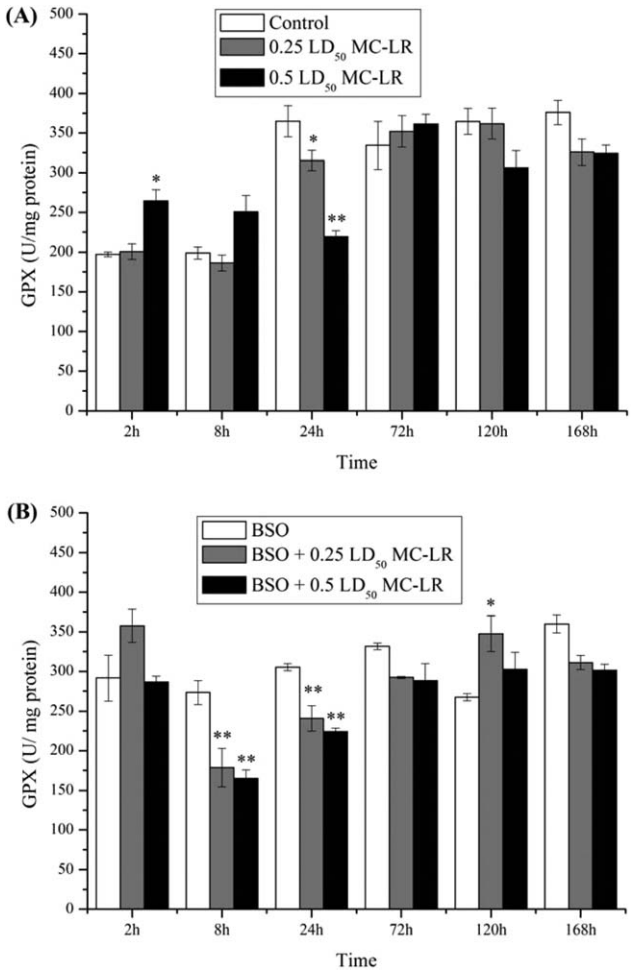

Fig. 4. Glutathione peroxidase (GPX) activities in liver of rats exposed to microcystin-LR without (A) or with (B) buthionine-(*S,R*)-sulfoximine (BSO) pretreatment. Values are presented as the mean ± standard error (SE). $^{*}p < 0.05$ *versus* control, and $^{**}p < 0.01$ *versus* control.

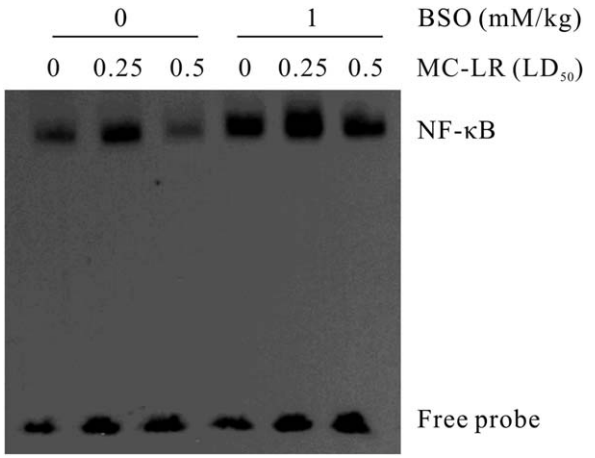

Fig. 5. NF-κB DNA-binding activity in liver of rats exposed to microcystin-LR without or with buthionine-(*S,R*)-sulfoximine (BSO) pretreatment by electrophoretic mobility shift assay (EMSA). Nuclear proteins were extracted before the detection. The upper band is NF-κB-probe specific band, and the lower band is free probe.

DISCUSSION

In recent years, cyanobacteria blooms and their toxins have drawn increasing attention as a worldwide public health concern. Many studies have reported that liver is the major target organ of microcystins (MCs) (Guzman and Solter, 1999; Svirčev et al., 2010; Li et al., 2014), but the mechanisms of MCs hepatotoxicity are not fully clarified. The current study demonstrated that acute exposure of MC-LR significantly induced oxidative stress, antioxidant system response, cellular apoptosis, and NF-κB activation, and GSH played an important role in the protection against MC-induced liver injury.

Oxidative stress can be defined as an adverse reaction resulting from the exposure of molecules, cells, or tissues to the excess level of oxidants, particularly to the free radicals, such as superoxide or hydroxyl radicals, which commonly refer to reactive oxygen species (ROS) (Ding and Ong, 2003). ROS could be efficiently scavenged by the antioxidant defense system, including enzymatic and nonenzymatic mechanisms. Glutathione peroxidase (GPX), superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR) are the important antioxidant enzymes. The nonenzymatic defenses include GSH and vitamin C and E. When the activity of cellular antioxidant defense system decreases or the production of ROS increases, oxidative stress may occur (Jayaraj et al., 2006). Recent studies suggest that MC-LR can produce a large amount of ROS and lipid peroxidation in primary hepatocytes and liver tissue (Ding et al., 2000a; Moreno et al., 2005). In this work, increased malondialdehyde (MDA) and significant changes of antioxidant enzymes (GPX and GR) were observed in liver of rat intraperitoneally (i.p.) injected with MC-LR at 0.25 and 0.5 LD_{50} (20.675 and 41.35 μg/kg, respectively). Similar results were also found in liver of female Balb/c mice i.p. injected with 0.75 LD_{50} MC-LR (μg/kg value not provided) (Gehringer et al., 2004), male wistar rats i.p. injected with 0.94 and 1.42 LD_{50} MC-LR (100 and 150 μg/kg, respectively) (Moreno et al., 2005), and female Swiss albino mice i.p. injected with 0.5 and 1 LD_{50} MC-LR (38.31 and 78.62 μg/kg, respectively) (Jayaraj et al., 2006). However, compared with these acute tests at a relatively higher dose of MCs, the level of oxidative stress in our study was low, indicated by the mild changes of GSH and MDA contents and GPX and GR activities. Actually, Jayaraj et al. (2006) also reported that 0.5 LD_{50} MC-LR induced a low level of oxidative stress in the liver of mice. It is likely that the animals have enough ability to reduce the toxic effects of MCs in the present and previous study (Jayaraj et al., 2006) when they are exposed to MCs at relatively lower doses.

GSH plays an important role in the protection of cellular constituents against ROS (Zhang and Forman, 2012); and it has been shown that MCs bind to GSH, forming conjugates via glutathione *S*-transferase (GST), as the first step in the detoxification process (Pflugmacher et al., 1998; Guo et al., 2014). In this study, a decrease of GSH was observed at the very beginning of the exposure (2 h), maintaining at this reduced level during the first 8 h, and then starting to rise at 24 h afterward. Also, the activity of GPX, catalyzing the reduction of hydrogen peroxide and organic peroxide at the expense of GSH, was significantly induced at 2 h, while it was inhibited at 24 h and recovered to the normal level finally. The correspondence of GPX activity and its substrate GSH level in the current study indicated that the depletion of GSH was due to its reaction with ROS and conjugation with MC-LR. In fact, the content of intracellular GSH is a function of the balance between its use and its synthesis, and cellular responses to MCs involve the alteration of GSH levels (Jayaraj et al., 2006; Žegura et al., 2006). Initially, GSH is consumed by conjugation with MC-LR via GST and by GSSG formation when GSH reacts with ROS. GSSG is then reduced back to GSH catalyzed by GR or by *de novo*

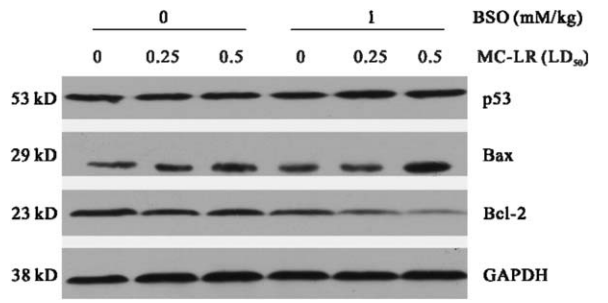

Fig. 6. Protein expression of p53, Bax, and Bcl-2 in liver of rats exposed to microcystin-LR without or with buthionine-(*S,R*)-sulfoximine (BSO) pretreatment. Western blot analysis was performed with antibody against p53, Bax, Bcl-2, and GAPDH.

synthesis (Žegura et al., 2006). In this study, GR activity showed no changes before 24 h post-exposure, but was induced at the following time-points (72, 120, and 168 h). The modest increase of GR activity detected only at the time-points after 24 h could be explained by the depletion of GSH was only to a minor extent, in the case of lower doses of MC-LR used in this experiment mentioned above.

In this study, MDA contents showed no changes in rat liver after MC-LR exposure at both two doses, except an increase occurred in 0.25 LD_{50} group at 120 h post-exposure. However, after pretreatment with buthionine-(*S*,*R*)-sulfoximine (BSO), MC-LR induced MDA increased in 0.25 LD_{50} group at 8 and 24 h, and 0.5 LD_{50} group at 8, 24, and 120 h, suggesting that MDA production was significantly enhanced by BSO pretreatment. Similarly, BSO pretreatment resulted in significant changes of antioxidant enzymes (GPX and GR) in liver of rat exposed to MC-LR. These results indicated that the level of oxidative stress was enhanced by BSO pretreatment before MC-LR exposure. Previous *in vitro* studies also showed that BSO increased the cell susceptibility to MC-induced cytotoxicity by disrupting intracellular GSH balance (Ding et al., 2000b; Žegura et al., 2006). Pretreatment with *N*-acetylcystein (NAC), a GSH precursor, significantly enhanced the intracellular GSH level and decreased the MC-induced cytotoxicity as well as cytoskeleton changes in rat hepatocytes; in contrast, BSO increased the cell susceptibility to MC-induced cytotoxicity (Ding et al., 2000b). Žegura et al. (2006) demonstrated that BSO pretreatment dramatically increased the susceptibility of HepG2 cells to MC-induced DNA damage, while the DNA damage was almost completely prevented by NAC pretreatment. Our previous study also showed that BSO reduced the detoxification of MCs and resulted in more severe histopathological damage (Li et al., 2014). Thus, taken together with these studies, we can conclude that GSH is responsible for cellular defense against MC-induced hepatotoxicity.

Additionally, we evaluated the consequences in the induction of apoptosis-related proteins, p53, Bax, and Bcl-2. Bcl-2 family consists of both pro-survival (Bcl-2, Bcl-xL, Bcl-w) and pro-apoptotic members (Bax, Bak, Bik, and Bim) (Donovan and Cotter, 2004). p53 is known to be a regulator of Bax as a p53-binding site has been found in the Bax gene promoter (Miyashita and Reed, 1995). Thus, p53 is responsible for regulating cell apoptosis through Bax/Bcl-2 imbalances. Fu et al. (2005) demonstrated that MC-LR could increase the expression of p53 and Bax significantly and decrease the expression of Bcl-2, both in rat liver tissues *in vivo* and cultured hepatocytes *in vitro*. It can be concluded that the expression of p53, Bax, and Bcl-2 are involved in the regulation of MC-LR-induced apoptosis (Fu et al., 2005). Our data also show that MCs induced the hepatocytes apoptosis at 0.5 LD_{50} but not at 0.25 LD_{50}; however, pretreatment with BSO promoted MC-induced apoptosis both at 0.25 and 0.5 LD_{50}. Although the detailed mechanisms by which BSO increases MC-LR-induced apoptotic protein changes are unclear, the results from this study suggest that oxidative damage may contribute to MC-LR-induced hepatocytes apoptosis, and GSH is responsible for cellular defense against MC-induced hepatotoxicity.

Redox-sensitive transcription factor NF-κB is well known for its paradoxical but important role in the regulation of cell proliferation, tumor development, and apoptosis (Morgan and Liu, 2011). Recent studies suggested that MCs could activate NF-κB and promote tumor development (Zhang et al., 2012, 2013a; Christen et al., 2013; Chen et al., 2014). In the current study, electrophoretic mobility shift assay (EMSA) showed that NF-κB binding activity of the liver cells was enhanced in the 0.25 LD_{50} group, but was inhibited in the 0.5 LD_{50} group. Actually, this is the first study investigating NF-κB activation responded to MCs exposure *in vivo*. Interestingly, the results coincided with our previous *in vitro* study (Chen et al., 2014). In that experiment, NF-κB and its downstream genes were also activated in HeLa cells at lower MC doses, while were inhibited at higher doses, and NF-κB may contributed to MC-induced cell proliferation, tumor development, and apoptosis (Chen et al., 2014). All these data demonstrated that NF-κB mediate the MC toxicity. In addition, both survival (anti-apoptotic NF-κB) and death signals (pro-apoptotic p53 and Bax) could be activated by MCs (Zhang et al., 2013b; Chen et al. 2014) not only *in vitro* but also *in vivo*, and the death signal was stronger than survival signal by higher MC doses and BSO pretreatment. In fact, BSO pretreatment could decrease intracellular GSH level and reduce the detoxification of MCs, which enhanced the accumulation of MC-LR in the liver compared to non-pretreatment group (Li et al., 2014), similar to the high-dose injections are performed.

Recent studies suggested that a relative low oxidative stress could modulate an NF-κB response and NF-κB target genes would attenuate ROS to prevent further oxidative damage and promote cell survival (Gloire et al., 2006; Nakano et al., 2006). However, when too much cellular oxidative damage has occurred, it is to the advantage of a multicellular organism to remove the cell for the benefit of the surrounding cells; ROS can therefore inhibit NF-κB activity and cells would undergo apoptosis (Morgan and Liu, 2011; Chen et al., 2014). The role of NF-κB in the regulation of cell proliferation, apoptosis, and hormesis in HeLa cells responded to MCs exposure *in vitro* has also been shown in our previous study (Chen et al., 2014). NF-κB could activate the GCLC, GCLM, and GC promoter, as a regulator of cellular GSH biosynthesis and redox state. All three genes are responsible for GSH biosynthesis, while the first step of GSH biosynthesis is rate limiting and catalyzed by GCL (glutamate-cysteine ligase, EC 6.3.2.2; formerly γ-glutamylcysteine synthetase, γ-GCS), which is composed of a heavy or catalytic (GCLC) and a light or modifier (GCLM) subunit (Zhang and Forman, 2012; Lu, 2013). While NF-κB may induce the GCLM and GS promoters indirectly via c-Jun, it exerts a direct effect on the GCLC

promoter (Lu, 2013). In our previous study, GCLC transcription in rat liver was significantly induced at 0.25 and 0.5 LD_{50} of MC-LR (Li et al., 2014), and the induced synthesis of GSH would display its function in the protection of liver cells against oxidative damage and apoptosis. Therefore, we believe that oxidative stress could interact with NF-κB activation in MC-induced apoptosis. The induction of enzymatic antioxidant defenses and NF-κB activation after MCs exposure can be considered as an adaptive response; that is, a compensatory mechanism that enables the liver cells to overcome the oxidative imbalance and avoid apoptosis. Further investigations are needed to better understand the interaction of oxidative stress and NF-κB activity in MC-induced apoptosis.

In summary, our *in vivo* study indicated that acute exposure to MC-LR lead to oxidative stress, and GSH depletion (BSO pretreatment) enhances the toxic effects of MCs. MCs can also modulate the activation of NF-κB and the expression of p53, Bax, and Bcl-2. Our data suggested that the crosstalk of oxidative stress and NF-κB activity may play a critical role in MC-induced apoptosis *in vivo*, and GSH is responsible for the protection of MC toxicity. More future works are required to elucidate the toxic mechanisms of MC-induced liver injury.

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REFERENCES

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.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Carlberg I, Mannervik B. 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J Biol Chem 250:5475–5480.

Carmichael WW, Azevedo SMFO, An JS, 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 L, Zhang X, Chen J, Zhang XZ, Fan HH, Li SC, Xie P. 2014. NF-κB plays a key role in microcystin-RR-induced HeLa cell proliferation and apoptosis. Toxicon 87:120–130.

Christen V, Meili N, Fent K, 2013. Microcystin-LR induces endoplasmatic reticulum stress and leads to induction of NFκB, interferon-alpha, and tumor necrosis factor-alpha. Environ Sci Technol 47:3378–3385.

Ding WX, Ong CN. 2003. Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity. FEMS Microbiol Lett 220:1–7.

Ding WX, Shen HM, Ong CN. 2000a. Critical role of reactive oxygen species and mitochondrial permeability transition in microcystin-induced rapid apoptosis in rat hepatocytes. Hepatology 32:547–555.

Ding WX, Shen HM, Ong CN. 2000b. Microcystic cyanobacteria extract induces cytoskeletal disruption and intracellular glutathione alteration in hepatocytes. Environ Health Perspect 108: 605–609.

Ding WX, Shen HM, Zhu HG, Ong, CN. 1998. Studies on oxidative damage induced by cyanobacteria extract in primary cultured rat hepatocytes. Environ Res 78:12–18.

Dittmann E, Wiegand C. 2006. Cyanobacterial toxins-occurrence, biosynthesis and impact on human affairs. Mol Nutr Food Res 50:7–17.

Donovan M, Cotter TG. 2004. Control of mitochondrial integrity by Bcl-2 family members and caspase-independent cell death. Biochim Biophys Acta 1644:133–147.

Drotar A, Phelps P, Fall R. 1985. Evidence for glutathione peroxidase activities in cultured plant cells. Plant Sci 42:35–40.

Feng G, Abdalla M, Li Y, Bai YS. 2011. NF-kappa B mediates the induction of Fas receptor and Fas ligand by microcystin-LR in HepG2 cells. Mol Cell Biochem 352:209–219.

Fu WY, Chen JP, Wang XM, Xu LH. 2005. Altered expression of p53, Bcl-2 and Bax induced by microcystin-LR *in vivo* and *in vitro*. Toxicon 46:171–177.

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.

Gloire G, Legrand-Poels S, Piette J. 2006. NF-kappa B activation by reactive oxygen species: Fifteen years later. Biochem Pharmacol 72:1493–1505.

Griffith O. 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem 106:207–212.

Guo XC, Xie P, Chen J, Tuo X, Deng XW, Li SC, Yu DZ, Zeng C. 2014. Simultaneous quantitative determination of microcystin-LR and its glutathione metabolites in rat liver by liquid chromatography-tandem mass spectrometry. J Chromatogr B 963:54–61.

Guzman RE, Solter PF. 1999. Hepatic oxidative stress following prolonged sublethal microcystin LR exposure. Toxicol Pathol 27:582–588.

Hermansky SJ, Stohs SJ, Eldeen ZM, Roche VF., Mereish KA. 1991. Evaluation of potential chemoprotectants against microcystin-LR hepatotoxicity in mice. J Appl Toxicol 11:65–73.

Hooser SB. 2000. Fulminant hepatocyte apoptosis *in vivo* following microcystin-LR administration to rats. Toxicol Pathol 28: 726–733.

Jayaraj R, Anand T, Rao PVL. 2006. Activity and gene expression profile of certain antioxidant enzymes to microcystin-LR induced oxidative stress in mice. Toxicology 220:136–146.

Ji Y, Lu G, Chen GQ, Huang B, Zhang X, Shen K, Wu S. 2011. Microcystin-LR induces apoptosis via NF-kappa B/iNOS pathway in INS-1 cells. Int J Mol Sci 12:4722–4734.

Jochimsen EM, Carmichael WW, An JS, Cardo DM, Cookson ST, Holmes CEM, Antunes MB, de Melo Filho DA, Lyra TM, Barreto VST, Azevedo, SMFO, Jarvis WR. 1998. Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. N Engl J Med 338:873–878.

Kondo F, Matsumoto H, Yamada S, Ishikawa N, Ito E, Nagata S, Ueno Y, Suzuki M, Harada K. 1996. Detection and identification of metabolites of microcystins formed *in vivo* in mouse and rat livers. Chem Res Toxicol 9: 1355–1359.

Li SC, Chen J, Xie P, Guo XC, Fan HH, Yu DZ, Zeng C, Chen L. 2014. The role of glutathione detoxification pathway in MCLR-induced hepatotoxicity in SD rats. Environ Toxicol. DOI: 10.1002/tox.22017.

Lu SC. 2013. Glutathione synthesis. Biochim Biophys Acta 1830: 3143–3153.

McDermott CM, Nho CW, Howard W, Holton B. 1998. The cyanobacterial toxin, microcystin-LR, can induce apoptosis in a variety of cell types. Toxicon 36:1981–1996.

Merel S, Walker D, Chicana R, Snyder S, Baurès E, Thomas O. 2013. State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. Environ Int 59:303–327.

Miyashita T, Reed JC. 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80:293–299.

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

Morgan MJ, Liu ZG. 2011. Crosstalk of reactive oxygen species and NF-κB signaling. Cell Res 21:103–115.

Nakano H, Nakajima A, Sakon-Komazawa S, Piao JH, Xue X, Okumura K. 2006. Reactive oxygen species mediate crosstalk between NF-κB and JNK. Cell Death Differ 13:730–737.

Ohkawa H, Ohishi N, Yagi K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358.

Paskerová H, Hilscherová K, Bláha L. 2012. Oxidative stress and detoxification biomarker responses in aquatic freshwater vertebrates exposed to microcystins and cyanobacterial biomass. Environ Sci Pollut Res 19:2024–2037.

Pflugmacher S, Wiegand C, Oberemm A, Beattie KA, Krause E, Codd GA, Steinberg CEW. 1998. Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: The first step of detoxication. Biochim Biophys Acta 1425:527–533.

Pouria S, de Andrade A, 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.

Sun B, Karin M. 2008. NF-κB signaling, liver disease and hepatoprotective agents. Oncogene 27:6228–6244.

Sun XY, Mi LX, Liu J, Song LR, Chung FL, Gan NQ. 2011. Sulforaphane prevents microcystin-LR-induced oxidative damage and apoptosis in BALB/c mice. Toxicol Appl Pharmacol 255: 9–17.

Svirčev Z, Baltić V, Gantar M, Juković M, Stojanović D, Baltić M. 2010. Molecular aspects of microcystin-induced hepatotoxicity and hepatocarcinogenesis. J Environ Sci Health Part C: Environ Carcinog Ecotoxicol Rev 28:39–59.

van Apeldoorn ME, van Egmond HP, Speijers GJA, Bakker GJI. 2007. Toxins of cyanobacteria. Mol Nutr Food Res 51:7–60.

Wang Q, Xie P, Chen J, Liang GD. 2008. Distribution of microcystins in various organs (heart, liver, intestine, gonad, brain, kidney and lung) of Wistar rat via intravenous injection. Toxicon 52:721–727.

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

Zhang HQ, Forman HJ. 2012. Glutathione synthesis and its role in redox signaling. Semin Cell Dev Biol 23:722–728.

Zhang XX, Fu ZY, Zhang ZY, Miao C, Xu PF, Wang T, Yang LY, Cheng SP. 2012. Microcystin-LR promotes melanoma cell invasion and enhances matrix metalloproteinase-2/-9 expression mediated by NF-κB activation. Environ Sci Technol 46: 11319–11326.

Zhang JY, Chen J, Xia ZP. 2013a. Microcystin-LR exhibits immunomodulatory role in mouse primary hepatocytes through activation of the NF-κB and MAPK signaling pathways. Toxicol Sci 136:86–96.

Zhang X, Xie P, Zhang XZ, Zhou WS, Zhao SJ, Zhao YY, Cai Y. 2013b. Toxic effects of microcystin-LR on the HepG2 cell line under hypoxic and normoxic conditions. J Appl Toxicol 33: 1180–1186.

Žegura B, Lah TT, Filipič M. 2006. Alteration of intracellular GSH levels and its role in microcystin-LR-induced DNA damage in human hepatoma HepG2 cells. Mutat Res 611: 25–33.