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SHORT RESEARCH AND DISCUSSION ARTICLE

Quantitatively evaluating detoxification of the hepatotoxic microcystin-LR through the glutathione (GSH) pathway in SD rats

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Abstract Glutathione (GSH) plays crucial roles in antioxidant defense and detoxification metabolism of microcystin-LR (MC-LR). However, the detoxification process of MC-LR in mammals remains largely unknown. This paper, for the first time, quantitatively analyzes MC-LR and its GSH pathway metabolites (MC-LR-GSH and MC-LR-Cys) in the liver of Sprague–Dawley (SD) rat after MC-LR exposure. Rats received intraperitoneal (i.p.) injection of 0.25 and 0.5 lethal dose 50 (LD_{50}) of MC-LR with or without pretreatment of buthionine-(S,R)-sulfoximine (BSO), an inhibitor of GSH synthesis. The contents of MC-LR-GSH were relatively low during the experiment; however, the ratio of MC-LR-Cys to MC-LR reached as high as 6.65 in 0.5 LD_{50} group. These results demonstrated that MC-LR-GSH could be converted to MC-LR-Cys efficiently, and this metabolic rule was in agreement with the data of aquatic animals previously reported. MC-LR contents were much higher in BSO+MC-LR-treated groups than in the single MC-LR-treated groups. Moreover, the ratio of MC-LR-Cys to MC-LR decreased significantly after BSO pretreatment, suggesting that the depletion of GSH induced by BSO reduced the detoxification of MCs. Moreover, MC-LR remarkably induced liver damage, and the effects were more pronounced in BSO pretreatment groups. In conclusion, this study verifies the role of GSH in the detoxification of MC-LR and furthers our understanding of the biochemical mechanism for SD rats to counteract toxic cyanobacteria.

Keywords Microcystin-LR-GSH/Cys · Detoxification · GSH pathway · Liver · Rat

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Introduction

Microcystins (MCs), a family of naturally occurring toxins produced by the toxigenic cyanobacteria, have drawn increasing attention as a public health concern over the last decades. To date, more than 90 analogues of MCs have been isolated and identified (Ufelmann et al. 2012), among which MC-LR is one of the most common and toxic variants (Gupta et al. 2003). MCs are known to be potent hepatotoxins (Dawson 1998) and tumor promoter (Nishiwakimatsushima et al. 1992), leading to serious liver damage through inhibition of the serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A) (MacKintosh et al. 1990; Fischer et al. 2010) and induction of intracellular reactive oxygen species (ROS) production (Ding et al. 1998). Consequently, MCs pose potential threats to both aquatic animals (Chen and Xie 2005; Malbrouck and Kestemont 2006) and mammals (Wang et al. 2008), as well as to humans (Azevedo et al. 2002). The most severe human intoxication happened in February 1996 in Brazil, where 100 of 131 patients developed acute liver failure due to MCs contamination of the water used for hemodialysis and 52 patients died (Azevedo et al. 2002; Yuan et al. 2006). More recently, MCs were first detected

in the serum (average 0.228 ng/mL MC-$LR_{equivalent}$) of fishermen in Lake Chaohu who were chronically exposed to hepatotoxic microcystins by drinking contaminated lake water and consuming aquatic products (Chen et al. 2009).

Glutathione (GSH), the major non-protein thiol abundant in the cells of most organisms, was demonstrated to play an important role in the detoxification of MCs in both mammals and aquatic organisms (Kondo et al. 1996; Pflugmacher et al. 1998, 2001; He et al. 2012; Li et al. 2014b). The glutathione and cysteine conjugates of MCs (MC-GSH and MC-Cys) were firstly synthesized under chemical conditions by Kondo et al. (1992) with Frit-FAB liquid chromatography–mass spectrometry (LC/MS), and then the resulting conjugates were identified as two metabolites of MCs in vivo in the livers of mouse and rat. Subsequently, MC-LR-GSH was proved to form in various aquatic animals via glutathione S-transferase (GST) in vitro, and it was suggested to be the first step in the detoxification of MCs in aquatic organisms, followed by degradation to the cysteine conjugate (Pflugmacher et al. 1998, 2001). In addition, Ito et al. (2002) further confirmed the distribution of MC-LR, MC-LR-GSH, and MC-LR-Cys in intestine and kidney of mice by immune-staining methods. However, the aforementioned studies merely focused on qualitative analyses. In recent years, some analytical methods were established for the quantitative determination of MCs and their metabolites in fish tissues (Dai et al. 2008; Wu et al. 2010), and on these bases, several investigations on detoxification of MCs in aquatic animals were performed under both field and laboratory conditions (Zhang et al. 2009, 2012; He et al. 2012; Li et al. 2014b).

Plenty of studies have shown that the toxicokinetic profiles of the toxin vary remarkably among different species, and mammals seem to be more susceptible to MCs than fishes. For instance, the median lethal dose (LD_{50}) of MC-LR via intraperitoneal (i.p.) injection is approximately 50–60 μg/kg body weight (BW) in mice (Botes et al. 1984; Carmichael et al. 1988) and 72 and 122 μg/kg BW for fasted and fed rats (Miura et al. 1991), respectively, but increases to 300–550 μg/kg BW in common carp (*Cyprinus carpio*) (Råbergh et al. 1991), to 400–500 μg/kg BW in rainbow trout (*Oncorhynchus mykiss*) (Tencalla et al. 1994; Kotak et al. 1996) and even to 1500 μg/kg BW in perch (*Pera fluviatilis*) (Ibelings et al. 2005). Nevertheless, so far, there is no literature on simultaneous quantification of MC-LR and its two main metabolites and the GSH detoxification dynamics of MCs in mammals, and the role of GSH in the detoxification of MCs in mammals remains largely unknown. Hence, a quantitative determination of MC-LR, MC-LR-GSH, and MC-LR-Cys in rats is essential to our better understanding of the important roles of GSH pathway in the detoxification of MCs in different animals. Until recently, we established an LC-electrospray ionization (ESI)-MS method to identify and quantify MC-LR and its GSH pathway metabolites (MC-LR-GSH and MC-LR-Cys) in Sprague–Dawley (SD) rats (Guo et al. 2014).

In this study, MC-LR and its two metabolites (MC-LR-GSH and MC-LR-Cys) were quantified for the first time in the liver of rat. Moreover, to better understand the roles of GSH in MC-LR-induced hepatotoxicity, the GSH contents in rat liver were modulated by the specific GSH synthesis inhibitor, buthionine-(S,R)-sulfoximine (BSO). The main purposes of the present study were to quantitatively describe the toxicokinetics of MC-LR and its two main GSH pathway metabolites in the liver of SD rat under exposure conditions of different MC-LR doses and GSH levels and to further identify the role of GSH pathway in the detoxification of MC-LR-induced hepatotoxicity in mammals.

Materials and methods

Materials

MC-LR was extracted and purified from freeze-dried surface blooms collected from Lake Dianchi in China according to the method of Ramanan et al. (2000). L-Glutathione and L-cysteine for chemosynthesis were purchased from Acros Organics (purity >99 %, Geel, Belgium, GR). MC-LR-GSH and MC-LR-Cys were prepared using the method of Dai et al. (2008) and Zhang et al. (2009).

MC-LR, MC-LR-GSH, and MC-LR-Cys were identified by LC-MS (Thermo Electron, Waltham, MA, USA) and quantified by HPLC (LC-20A, Shimadzu, Kyoto, Japan) with the purities all above 95 %, and the impure percentages (<5 %) were some unknown compounds that do not affect the qualitative and quantitative analysis of the three target compounds. All of them were stored at −80 °C before use.

D,L-Buthionine-(S,R)-sulfoximine (BSO) was purchased from Sigma (USA). Acetic acid (AR) and formic acid (FA) were purchased from Sinopharm Chemical Reagents (Shanghai, China) and Aldrich (Sigma, USA), respectively. Experimental ultra-pure water was collected from a Milli-Q water purification system from Millipore (Bedford, MA, USA). Methanol and acetonitrile were of HPLC grade (Tedia Company, Inc., Fairfield, OH, USA). Other reagents included in this study were all of analytical reagent grade.

Animals

Male Sprague–Dawley (SD) rats aged 8 weeks (weighting 180±20 g) were purchased from Animal Experiment Center of Wuhan University (Wuhan, China). The animals were acclimated for 7 days before treatment in laboratory animal center under constant conditions: 12/12-h light/dark cycle, 24±

2 °C, and 50±15 % humidity. They were housed in stainless steel cages with free access to rodent pellets and tap water. All procedures carried out on animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Huazhong Agricultural University, Wuhan, Hubei Province of China, and were in accordance with all the ethics guidelines. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Toxin exposure

The intraperitoneal (i.p.) lethal dose 50 (LD_{50}) of MC-LR was 82.7 μg/kg BW according to the improved Karber method determined in our previous study (Li et al. 2014a). Rats were randomly assigned to six groups with 18 animals in each group: (1) control group, (2) 0.25 LD_{50} of MC-LR (20.675 μg/kg) group, (3) 0.5 LD_{50} of MC-LR (41.35 μg/kg) group; (4) 1 mM/kg BSO control group, (5) 1 mM/kg BSO plus 0.25 LD_{50} of MC-LR group, and (6) 1 mM/kg BSO plus 0.5 LD_{50} of MC-LR group. All compounds were administered through i.p. injection. The rats in the fifth and sixth groups were injected with BSO 3 h before the injection 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 and 8 h and 1, 3, 5, and 7 days). Three rats from each group were weighed and killed at each time point, and the livers were quickly removed, weighed, minced, and stored frozen at −80 °C immediately. The hepatosomatic index (HIS) was calculated by the formula [*liver weight* (g)/*whole body weight* (g)]×100 %.

Sample preparation and quantitative analysis of MC-LR and its metabolites

All samples for quantitative analysis were lyophilized by a freeze dryer (Martin Christ, Alpha 2-4, Osterode, Germany). Lyophilized samples (200±10 mg) were homogenized and extracted three times with 5 mL of water containing EDTA-Na_2 (0.01 M)–NaCl (3 M)–5 % acetic acid by ultrasonication for 3 min (30 % amplitude, 60 W, 20 kHz, Branson Digital Sonifier, USA) at 0 °C, and then centrifuged at 12,000×*g* (Sigma, 3–18 K, USA) at 4 °C for 10 min. The supernatant was conducted according to the method described by our previous work (Guo et al. 2014) for the extraction and purification of MC-LR and its metabolites.

Qualitative and quantitative analysis of MC-LR, MC-LR-GSH, and MC-LR-Cys were conducted by using an LC-MS/MS system (Finnigan, USA), which consists of a Surveyor HPLC system and an LCQ Advantage MAX ion trap mass spectrometry equipped with an ESI (+) probe. The instrument control, data processing, and analysis were conducted by using Xcalibur software (Thermo Electron). The parameters of this Finnigan LC-MS system were set up according to the previously mentioned method (Guo et al. 2014). The limit of detections (LODs) of MC-LR, MC-LR-GSH, and MC-LR-Cys were 0.005, 0.007, and 0.006 μg/g dry weight (DW), respectively.

The mobile phase was composed of solvent A (water+0.05 % (*v*/*v*) formic acid) and solvent B (acetonitrile+0.05 % formic acid). The linear gradient programme was as follows: 0 to 7 min (solvent A/solvent B = 75:25), 8 min (solvent A/solvent B = 45:55), 13 min (solvent A/solvent B = 40:60), 14 min (solvent A/solvent B = 30/70), and 15 to 20 min (solvent A/solvent B = 75:25). The total flow rate was set at 0.2 mL/min during the analysis stage (0–14 min) and then increased to 0.3 mL/min for the last 6 min. The temperatures of vial tray and column oven were set at 10 and 25 °C, respectively. Ten microliters of sample solution was injected for each run.

The MS analytical conditions were used as follows: Nitrogen (purity ≥99.995 %) was used as the sheath and auxiliary gas with the flow rate set at 20 and 5 units, respectively. ESI spray voltage was 4.54 kV, capillary temperature was 250 °C, and multiplier voltage was −1336.28 V. The tube lens voltage was 55 V for MC-LR and 50 V for MC-LR-GSH and MC-LR-Cys.

Recovery experiment

Recovery experiments were carried out in triplicate by spiking 200 mg of freeze-dried rat liver samples with MC-LR, MC-LR-GSH, and MC-LR-Cys solution at 0.5 μg/g DW. The extraction and analysis were performed as described in section Sample preparation and quantitative analysis of MC-LR and its metabolites. The recovery and the relative standard deviation of the analytical method were calculated.

Optical microscopic observation

Tissue samples were routinely fixed in Bouin's solution at 4 °C for 24 h and washed in 70 % alcohol to remove excess picric acid, and then they were dehydrated, paraffin-embedded, and sectioned. Following deparaffinization in xylene, the sections of 4 μm were stained with hematoxylin and eosin (H&E) for later pathological assessment.

Statistics

The values were expressed as mean±standard deviation (S.D.). One-way ANOVA was carried out to determine whether the biochemical index and the content of MC-LR and its metabolites in rat liver were significantly different. All statistical tests were performed by using SPSS for Windows (Ver. 13.0, Chicago, IL, USA). Statistical significance was determined at $p<0.05$.

Results

General characteristics in experimental groups

No rats died during the experiment. Hepatosomatic indices showed no significant changes in SD rats after i.p. administration of MC-LR (Fig. 1), being independent of BSO pretreatment.

Chemical characterization of MC-LR and its metabolites

MC-LR, MC-LR-GSH, and MC-LR-Cys were identified by ESI LC-MS/MS in scan mode (Fig. 2). The peak at 10.45 min was confirmed to be MC-LR-GSH with the abundant precursor ion $[M+2H]^{2+}$ at *m*/*z* 652.03 and the corresponding product ions at *m*/*z* 587.94 and *m*/*z* 1168.48. Similarly, the peaks obtained at 10.65 and 12.04 min were MC-LR-Cys and MC-LR, respectively, with precursor ion $[M+H]^{+}$ at *m*/*z* 1116.57 and product ions at *m*/*z* 599.28, *m*/*z* 995.48, and *m*/*z* 1029.46 for MC-LR-Cys, and precursor ion $[M+H]^{+}$ present at *m*/*z* 995.62 and product ions at *m*/*z* 599.21 and *m*/*z* 977.39 for MC-LR, respectively.

Recoveries

The average recoveries from rat (n=4) liver were 71.5 % (ranging from 67.4 to 77.2 %), 81.0 % (ranging from 74.8 to 84.6 %), and 97.0 % (ranging from 91.3 to 99.8 %) for MC-LR-GSH, MC-LR-Cys, and MC-LR, respectively. The relative standard deviations (RSDs) of MC-LR-GSH, MC-LR-Cys, and MC-LR were 6, 5, and 4 %, respectively.

Dynamics of MC-LR and its metabolites

No MC-LR, MC-LR-GSH, and MC-LR-Cys were detected in the control groups during the experiment.

MC-LR treatment groups

The concentration–time profiles of MC-LR, MC-LR-GSH, and MC-LR-Cys in liver treated with doses of 0.25 and 0.5 LD_{50} of MC-LR are shown in Fig. 3a, b, respectively. The contents of three analytes, especially for MC-LR and MC-LR-Cys, displayed a significant dose–response relationship. MC-LR was detected in all the experimental points for the two doses groups, with concentrations between 4.931±0.314 and 12.341±1.692 ng/g DW for the low-dose (0.25 LD_{50} of MC-LR) group and between 7.791±0.688 and 23.453±3.707 ng/g DW for the high-dose (0.5 LD_{50} of MC-LR) group, respectively. The maximum concentrations for low- and high-dose groups were both detected at 1 day post-injection which were 12.341±1.692 and 23.453±3.707 ng/g DW, respectively.

MC-LR-Cys was abundant throughout the experiment; it increased gradually from 8.338±2.630 (1 day post-injection) to 24.935±2.319 ng/g DW (5 days post-injection) for the low-dose group. A similar trend was observed in the high-dose group with the concentration from 2.986±2.635 (8 h post-injection) to 54.880±7.140 ng/g DW (5 days post-injection), and then decreased as the experiment progressed.

The glutathione conjugate of MC-LR (MC-LR-GSH) was occasionally detected during the experiment.

BSO+ MC-LR treatment groups

The MC-LR, MC-LR-GSH, and MC-LR-Cys contents in the liver of SD rats from the BSO+MC-LR treatment groups are shown in Fig. 4. The dose–response relationship for three analytes was as remarkable as shown in the MC-LR treatment groups. However, in the BSO+MC-LR treatment groups, MC-LR content was significantly higher than in the single MC-LR treatment groups throughout the experiment with the concentration between 12.508±2.851 and 24.985±5.948 ng/g DW for the low-dose group and between 15.886±3.764 and 109.991±8.391 ng/g DW for the high-dose group, respectively. MC-LR accumulated abundantly in the liver after BSO pretreatment, as the MC-LR content reached as high as 109.991±8.391 ng/g DW (high-dose group) at 8 h post-injection.

MC-LR-Cys in the BSO+MC-LR treatment groups exhibited almost the same trend as the MC-LR treatment groups. However, its concentration was much lower than that of the MC-LR treatment groups during the whole study period. The maximum concentrations of MC-LR-Cys for the two dose groups were both observed at 3 days post-injection with the values of 15.898±2.550 (low-dose group) and 31.201± 12.587 ng/g DW (high-dose group), respectively. MC-LR-GSH was also occasionally detected during the experiment.

Biotransformation from MC-LR to its cysteine conjugate

In the MC-LR treatment groups, MC-LR-Cys was abundant throughout the experiment and the concentrations were always higher than MC-LR. Moreover, the biotransformation from MC-LR to MC-LR-Cys in the high-dose group was more efficient than that in the low-dose group (Fig. 5a). The ratios of MC-LR-Cys to MC-LR for the low- and high-dose groups were 2.40 and 6.65 at 5 days post-injection, respectively, indicating that MC-LR was successfully biotransformed to MC-LR-Cys.

In the BSO treatment groups, MC-LR-Cys contents were much lower than MC-LR with the progress of experiment, and the transformation efficiencies in the high-dose group were lower than those in the low-dose group (Fig. 5b). The ratios of MC-LR-Cys to MC-LR for the low- and high-dose groups pretreated with BSO were significantly lower than those in the single MC-LR treatment groups, with the ratios of only 0.04

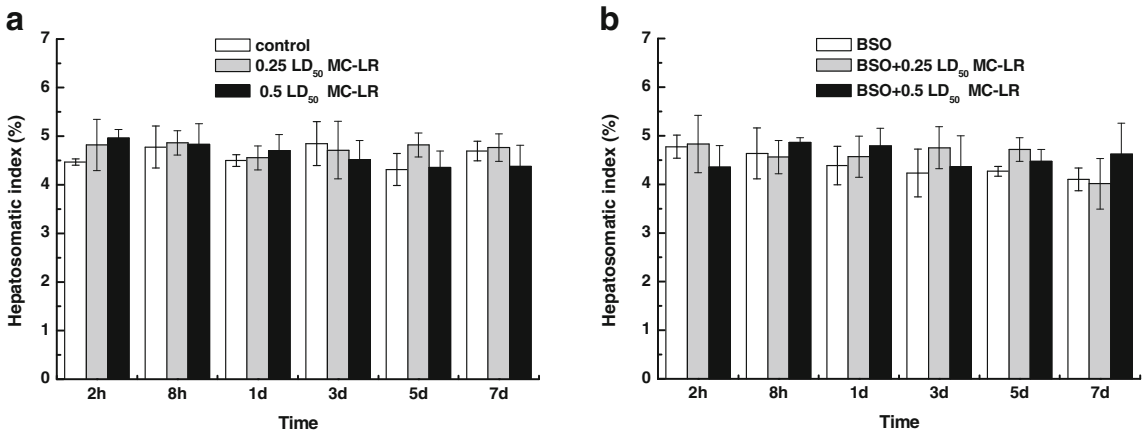

Fig. 1 Hepatosomatic index (HIS) of rats treated with (**a**) MC-LR and (**b**) BSO+MC-LR. Values are presented as mean±standard deviation (S.D.)

and 0.01 at 8 h post-injection, namely, MC-LR was present mainly in the form of unconjugated. Apparently, the biotransformation from MC-LR to MC-LR-Cys was strongly inhibited.

Based on the above results, 8 h post-injection was selected for the morphological observation to verify the hepatic injury induced by MC-LR.

Histopathology

Alterations in the structural organization of hepatocytes at 8 h post-injection are shown in Fig. 6. Control rat presented with normal histology (Fig. 6a, d). In contrast, rat treated with MC-LR presented with different degrees of loss of cytoplasm and membrane-bound vacuole in hepatocytes. These alterations revealed a significant dose–response relationship. With increasing doses, hepatocyte damage became more conspicuous and involved larger areas of the hepatocytes. Moreover, after BSO pretreatment, the changes were more conspicuous and the membrane-bound vacuoles were larger.

Discussion

Glutathione (GSH), the most abundant non-protein thiol present mainly in liver, was confirmed to play crucial roles in antioxidant defense and detoxification metabolism of many xenobiotics (Ketterer et al. 1983; Lu 2013). Microcystins (MCs) are known as hepatotoxins since liver of animals (both aquatic animals and mammals) is the most important target organ (Clark et al. 2007; Malecot et al. 2011). Our and others' previous studies demonstrated that GSH and GSH-related enzymes (GPX, GR, and GST) played an important role in the protection against MC-induced liver injury (Gehringer et al.

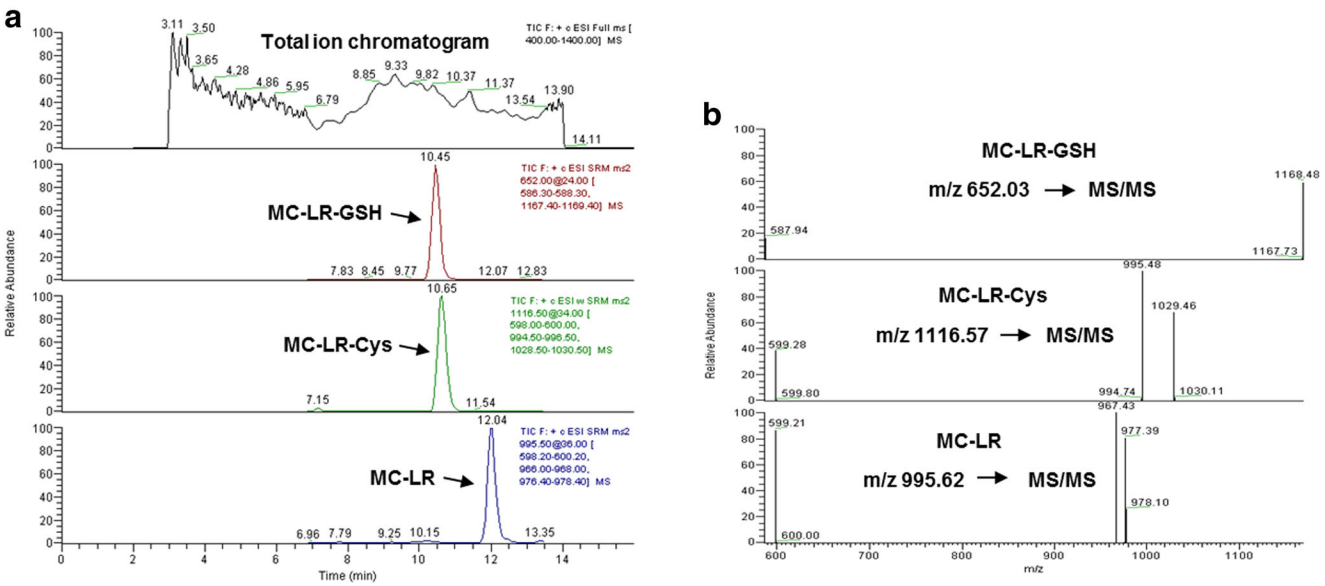

Fig. 2 ESI LC/MS/MS analysis of MC-LR and its two metabolites (MC-LR-GSH and MC-LR-Cys) in liver of SD rats. Shown are (**a**) total ion and SRM chromatograms for MC-LR-GSH, MC-LR-Cys, and MC-LR, and (**b**) product ion mass spectra for MC-LR-GSH, MC-LR-Cys, and MC-LR

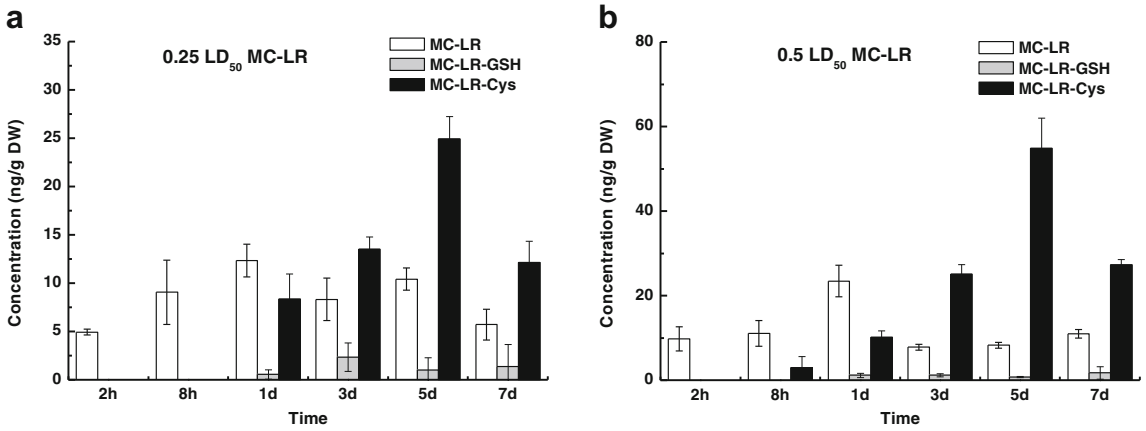

Fig. 3 The contents of MC-LR, MC-LR-GSH, and MC-LR-Cys in liver of SD rats treated with (**a**) 0.25 LD_{50} of MC-LR and (**b**) 0.5 LD_{50} of MC-LR. Values are presented as mean±standard deviation (S.D.)

2004; Chen et al. 2014; Li et al. 2014a). For a better understanding of the role of GSH in the detoxification process of MC-LR, in the present study, we first performed the quantitative determination of MC-LR and its glutathione and cysteine conjugates (MC-LR-GSH and MC-LR-Cys) in rats after MC-LR exposure with or without pretreatment of buthionine-(S, R)-sulfoximine (BSO), a specific GSH synthesis inhibitor.

In the present study, MC-LR was detected in all the experimental time points for all the treatment groups, and more MC-LR was accumulated in liver at 0.5 LD_{50} groups compared with that in 0.25 LD_{50} groups. Particularly, MC-LR was dominant in the liver after BSO pretreatment with the average content reaching as high as 47.48 ng/g DW (in 0.5 LD_{50} of MC-LR group). Interestingly, the maximum MC-LR contents were always detected at 1 day post-injection rather than in the initial stage of MC exposure. Additionally, the content fluctuated upward in 5 days post-injection, displaying a regular fluctuation. Meanwhile, Soares et al. (2006) reported that the serum MC concentrations of dialysis patients exposed to sublethal MCs ranged from <0.16 to 0.96 ng/mL during the 57-day monitoring period, with the highest values detected at 1 month after initial exposure. During an experiment of 96 h, the concentration of MC-LR in liver of juvenile gold fish (*Carassius auratus* L.) increased from 6 to 48 h after intraperitoneal (i.p.) injection with MC-LR at a dose of 125 μg/kg, but a significant decrease was observed between 48 and 96 h post-injection (Malbrouck et al. 2004). There are two alternative explanations for this observation. First, after exposure, MCs could be directly transported to the liver or indirectly transferred from other organs through blood circulation. So the levels of MCs in the liver indicated two phases in the intoxication process. Second, part of the MC-LR may covalently bind with protein phosphatases in liver and then released gradually over time. Covalent binding of MCs with protein phosphatases reduces extractability of microcystins (Craig et al. 1996), which could result in an underestimation of the amount of toxin really present into the liver (Malbrouck et al. 2004). Although the mechanism of sustained-release behavior of MC-LR is not well studied so far, the binding form and subsequent release may account for the fluctuation of MC-LR detected in the rat liver during the experimental process.

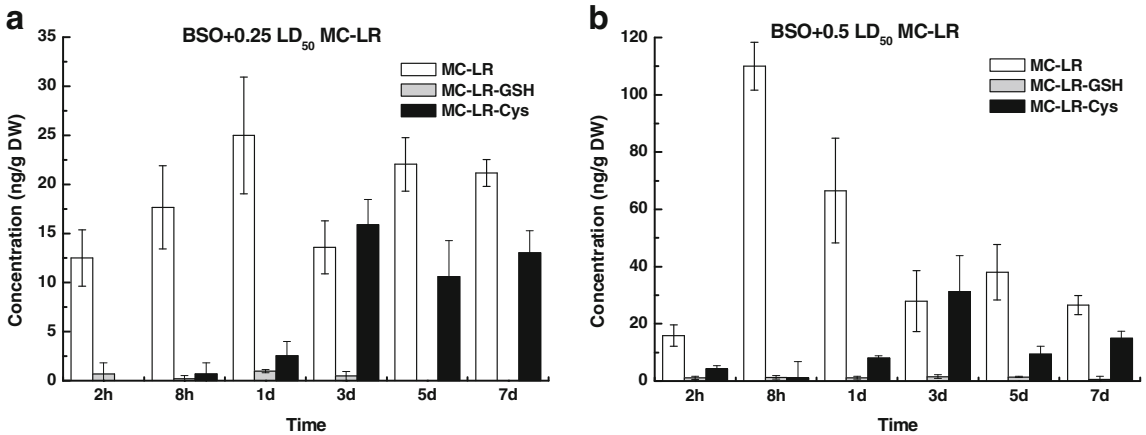

Fig. 4 The contents of MC-LR, MC-LR-GSH, and MC-LR-Cys in liver of SD rats treated with (**a**) BSO+0.25 LD_{50} of MC-LR and (**b**) BSO+0.5 LD_{50} of MC-LR. Values are presented as mean±standard deviation (S.D.)

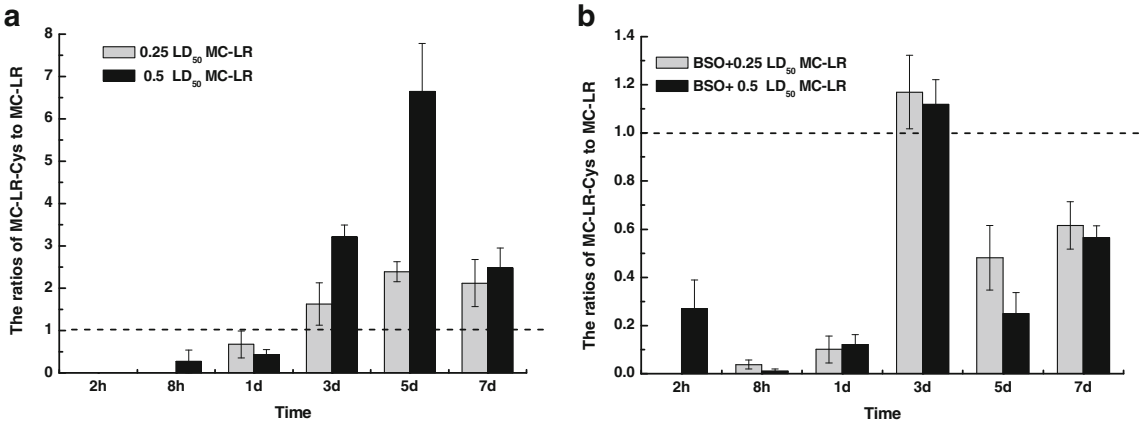

Fig. 5 The ratios of MC-LR-Cys to MC-LR in liver of SD rats treated with (**a**) MC-LR and (**b**) BSO+MC-LR. Values are presented as mean±standard deviation (S.D.)

The glutathione and cysteine conjugations have long been recognized as important metabolites of the GSH detoxification pathway (Wolfgang 2001). As for microcystin-LR (MC-LR), it is demonstrated that MC-LR conjugated with GSH via glutathione S-transferase (GST) and further degraded to MC-LR-Cys (Pflugmacher et al. 1998, 2001). In this work, both MC-LR-GSH and MC-LR-Cys were detected in rat liver, and the contents, especially for MC-LR and MC-LR-Cys, display a significant time–response and dose–response relationship in all treated groups. However, the contents of MC-LR-GSH were relatively low during the experiment, which was in agreement with previous reports on aquatic animals: GSH conjugates of MCs were almost undetectable in snail, shrimp, and bighead carp under both field and laboratory conditions (Zhang et al. 2009, 2012; He et al. 2012). Anders (1980) reported that S-substituted glutathione derivatives degraded very rapidly ($t_{1/2}$≈3.5 s) in microperfusion studies in rat kidneys. Meanwhile, in an MC-GSH exposure study, Li et al. (2014b) also showed that any MC-RR-Cys detected in bighead carp in vivo was derived from MC-RR-GSH with a 96.7-fold increase of contents in kidney from 0.25 to 0.5 h post-injection. All above results demonstrated that MC-GSH could act as reactive intermediates and could be effectively converted to MC-Cys in both aquatic animals and mammals.

In the current study, substantial MC-LR-Cys were detected throughout the experiment, suggesting the importance of cysteine conjugate, one product of the GSH pathway, in the detoxification of MC-LR in mammals. This is comparable to the results of previous studies in both wild and domestic aquatic animals (Zhang et al. 2009, 2012; He et al. 2012) and could be attributed to a number of factors. First, the high reactivity of MC-LR-GSH facilitates the formation of MC-LR-Cys. In addition, MC-LR was supposed to conjugate with the cysteine residues of polypeptides or proteins (mainly PP1 and PP2A), and subsequently, MC-LR-Cys is degraded from these polypeptide or proteins (He et al. 2012). These data suggest that

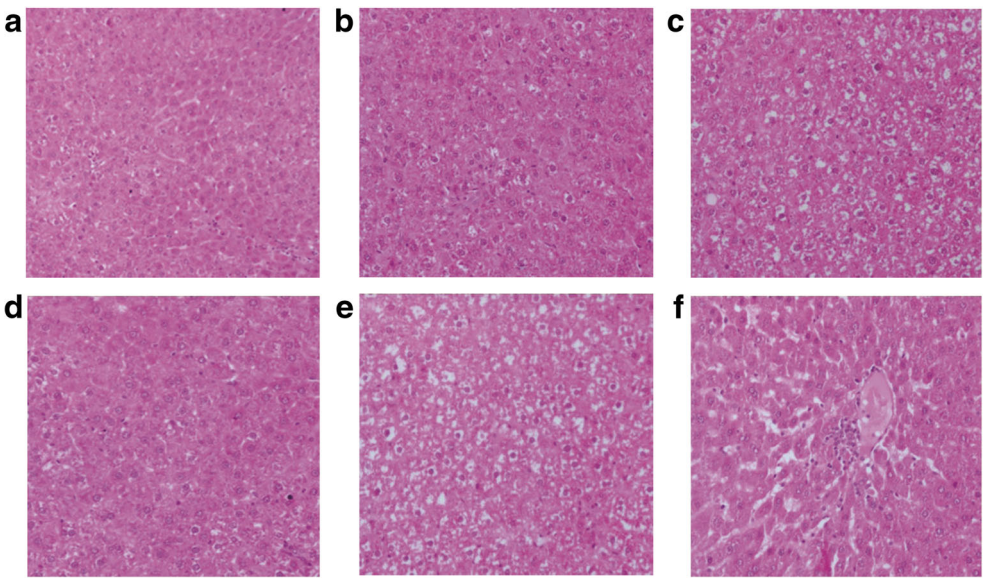

Fig. 6 Histopathological changes in liver of rats after MC-LR exposure at 8 h (×400). (**a**) and (**d**) showing the normal structure of hepatocytes in the control group and BSO group, respectively. (**b**) and (**c**) showing the loss of cytoplasm and membrane-bound vacuole in hepatocytes exposed to 0.25 and 0.5 LD_{50} of MC-LR, respectively. (**e**) and (**f**) showing the widening of loss of cytoplasm and membrane-bound vacuole exposed to 0.25 and 0.5 LD_{50} of MC-LR after pretreatment with BSO (1.0 mM/kg) for 3 h, respectively

Table 1 Effects of microcystins (MCs) on glutathione (GSH) level in liver of fish, mice, and rats

Test animals	Exposure route and dose	GSH basic level	Effects on GSH level	Reference
Female Balb/c mice	i.p. injection of 0.75 $LD_{50\text{-}24h}$ MC-LR	About 10 μmol/g tissue[a]	Decrease of GSH at 16 h post-exposure	Gehringer et al. (2004)
Goldfish (*Carassius auratus* L.)	i.p. injection of 125 μg/kg BW MC-LR	About 1–1.5 μmol/g tissue	No significant change at 6, 24, 48, and 96 h post-exposure	Malbrouck et al. (2004)
Female Swiss albino mice	i.p. injection of 0.5 $LD_{50\text{-}24h}$ MC-LR (38.31 μg/kg BW)	3.69±0.20 μmol/g tissue[b]	No significant change at 1, 3, and 7 days post-exposure	Jayaraj et al. (2006)
Female Swiss albino mice	i.p. injection of 1 $LD_{50\text{-}24h}$ MC-LR (76.62 μg/kg BW)	4.29±0.43 μmol/g tissue[b]	Significant decrease of GSH at 1 and 2 h post-exposure	Jayaraj et al. (2006)
Silver carp (*Hypophthalmichthys molitrix*)	Exposed to *Microcystis* blooms naturally	50.0±3.5 mg/g protein[a]	No significant change during and after *Microcystis* blooms	Li et al. (2007a)
Silver carp (*Hypophthalmichthys molitrix*)	i.p. injection of 1000 μg/kg BW MC-LR equivalents	7.95±0.55 μmol/g tissue[b]	No significant change at 1, 3, 12, 24, and 48 h post-exposure	Li et al. (2007b)
Silver carp (*Hypophthalmichthys molitrix*)	Exposed to *Microcystis* blooms naturally	59.1±3.9 μmol/g protein (18.1±1.2 mg/g protein)[a,c]	No significant change during and after *Microcystis* blooms	Qiu et al. (2007)
Bighead carp (*Aristichthys nobilis*)	Exposed to *Microcystis* blooms naturally	62.0±6.2 μmol/g protein (19.0±1.9 mg/g protein)[a,c]	No significant change during and after *Microcystis* blooms	Qiu et al. (2007)
Carassius auratus	Exposed to *Microcystis* blooms naturally	86.7±41.5 μmol/g protein (26.6±12.7 mg/g protein)[a,c]	No significant change during and after *Microcystis* blooms	Qiu et al. (2007)
Culter ilishaeformis	Exposed to *Microcystis* blooms naturally	94.8±48.5 μmol/g protein (29.1±14.9 mg/g protein)[a,c]	No significant change during and after *Microcystis* blooms	Qiu et al. (2007)
Bighead carp (*Aristichthys nobilis*)	i.p. injection of 200 and 500 μg/kg BW MC-LR equivalents	57.8±5.2 mg/g protein[a]	No significant change at 1, 3, 12, 24, and 48 h post-exposure	Li et al. (2008)
Bighead carp (*Aristichthys nobilis*)	Exposed to *Microcystis* blooms naturally	49.3±5.4 mg/g protein[a]	No significant change during and after *Microcystis* blooms	Li et al. (2008)
Cyprinus carpio	Immersion in 10 μg/L MC-LR	71.5±10.0 mg/g protein[a]	Significant decrease of GSH at 4 days post-exposure	Jiang et al. (2011)
Male Balb/c mice	i.p. injection of 50 μg/kg BW MC-LR	About 10 μmol/g protein (about 3.07 mg/g protein)[c]	Significant decrease of GSH at 24 h post-exposure	Sun et al. (2011)
Male Sprague–Dawley (SD) rat	i.p. injection of 0.25 and 0.5 $LD_{50\text{-}24h}$ MC-LR (20.675 and 41.35 μg/kg BW)	About 4 mg/g protein	Significant decrease of GSH at 8 h but increase at 5 days in 0.25 LD_{50} group; significant decrease at 2 h but increase at 3 days in 0.5 LD_{50} group	Chen et al. (2014)

i.p. intraperitoneal, *LD* lethal dose, *GSH* glutathione, *BW* body weight

[a] Values were expressed as mean±standard deviation (S.D.)

[b] Values were expressed as mean±standard error (S.E.)

[c] Data were calculated by the formula: mg/g protein values of GSH (mean/S.D./S.E.)=(μmol/g protein values×307 g/mol)/1000

the detoxification mechanism and metabolic pathway of MC-LR in liver of mammals were similar to those in aquatic animals: MC-LR conjugates with GSH to form MC-LR-GSH and rapidly degraded to MC-LR-Cys and finally excreted from the body in the form of MC-LR-Cys.

Due to the low contents of MC-LR-GSH and its rapid conversion to MC-LR-Cys, the ratio of MC-LR-Cys to MC-LR was selected to evaluate the biotransformation efficiency of MC-LR in vivo. It should be noted that the biotransformation from MC-LR to MC-LR-Cys was much lower in the BSO pretreatment groups, indicating that, as an active mid-metabolite, MC-LR-GSH was an important source of MC-LR-Cys. Moreover, in the MC-LR treated groups, the biotransformation from MC-LR to MC-LR-Cys in the high-dose group was more efficient than that in the low-dose group, which was in agreement with our previously published research on bighead carp to a certain extent (He et al. 2012). Pretreatment with BSO, an inhibitor of GSH synthesis, enhanced the accumulation of MC-LR compared to non-pretreatment group. In fact, BSO could decrease intracellular GSH and reduce the detoxification of MCs, which may increase the MCs in the liver, similar to the high-dose injections are performed. It is likely that the efficient biotransformation from MC-LR to MC-LR-Cys was triggered to perform as an important protection mechanism to resist MC-LR at relatively low-dose exposure. On the other hand, the balance between accumulation and detoxification was broken by high-dose exposure (BSO pretreatment), and the biotransformation was negatively influenced. Actually, with the increase of MC concentrations, the transformation efficiency from MC-LR to MC-LR-GSH and further to MC-LR-Cys was promoted initially because the GSH is sufficient; however, when the MC concentration is too high, the transformation will be slowed down for the limitation of GSH.

If the GSH conjugation might play an important role in the detoxification process in rat, it could result in a depletion of intracellular GSH level. In fact, we observed an initial decrease of GSH in rat liver in 0.5 LD_{50} group at 2 h and 0.25 LD_{50} group at 8 h (Chen et al. 2014) (Table 1), but the GSH level was increased in the 0.5 LD_{50} group at 3 days and in the 0.25 LD_{50} group at 5 days. Sun et al. (2011) also observed a decrease in GSH concentration in the liver of Balb/c mice following i.p. injection of 50 μg/kg body weight (BW) MC-LR for 10 days. A decrease of GSH content was also observed in liver of *Cyprinus carpio* after immersion of MC-LR in water at 10 μg/L (Jiang et al. 2011). Thus, it seems reasonable to conclude that glutathione conjugation of MC-LR is a critical bio-chemical mechanism for rat to resist toxic cyanobacteria. However, a significant increase in GSH level was recorded in liver of silver carp exposed to the living natural population of cyanobacterial water bloom for 25 days (Bláha et al. 2004). The juvenile goldfish i.p. injected with MC-LR at a dose of 125 μg/kg BW displayed no significant changes in GSH content in liver during an experiment of 96 h (Malbrouck et al. 2004). Li et al. (2007a, b, 2008) and Qiu et al. (2007) also reported that GSH contents showed no significant changes in the liver of fish after i.p. administration of MCs in laboratory and exposure to natural cyanobacterial blooms in field. Response of intracellular GSH content to MC exposure has been quite different so far, probably due to different animal species, different exposure doses, times (time), and routes, or different compositions of MCs

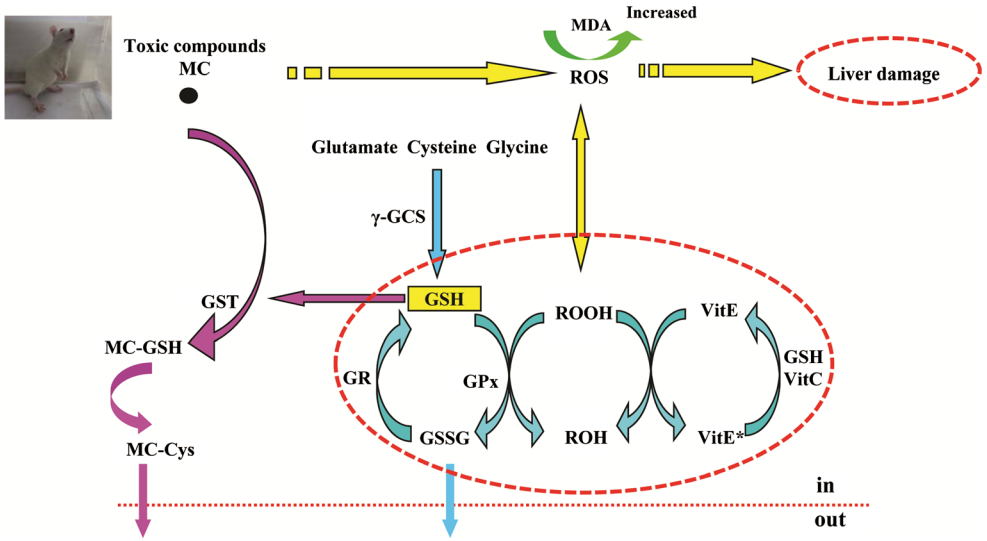

Fig. 7 Diagrammatic representation of the role of glutathione (GSH) pathway in the detoxification of microcystin-LR (MC-LR). Acute exposure to MC-LR promoted the production of reactive oxygen species (ROS), inducing oxidative stress and liver damage. GSH could bind to MC-LR forming conjugates (MC-LR-GSH) via glutathione S-transferase (GST) and further degraded to cysteine conjugates (MC-LR-Cys), thereby reducing the toxicity of MC-LR and enhancing its excretion. Additionally, the abundant GSH and its related antioxidant enzymes could also defend against oxidative stress to protect the liver

(Table 1). Malbrouck et al. (2004) attributed the lack of GSH alteration in liver of goldfish i.p. administered with purified MC-LR to its important basic GSH concentration. In our study, the basic level of GSH in rat liver was about 4 mg/g protein (Chen et al. 2014), similar to that in liver of mice (about 3 mg/g protein, Sun et al. 2011), but, however, far below that in liver of fish, especially silver carp and bighead carp (about 20–60 mg/g protein, Li et al. 2007a, 2008; Qiu et al. 2007) (Table 1). Also, Fu and He (2012) reported that five genes involved in the GSH biosynthesis were identified as experienced positive selection between silver carp and zebrafish, and the positive selection might be the result of the adaptation of silver carp to the eutrophied bodies of water. Also, rodent GST has a two-fold higher catalytic efficiency for MC and GSH conjugation than human ones (Buratti and Testai 2015). Therefore, we believe that the differences of GSH basic levels, GSH synthetic capacities, and GST catalytic efficiencies may be responsible for the sensitivity of some vertebral species to MCs.

Coincided with the contents of MC-LR and its biotransformation, in the present study, MCs significantly induced histopathological damage, and the effects were more pronounced by BSO injection prior to MC-LR treatment. Actually, our previous study (Chen et al. 2014) also detected the contents of GSH and malondialdehyde (MDA) and the activities of antioxidant enzymes including GSH peroxidase (GPX) and GSH reductase (GR). MC-LR induced time-dependent alterations of GSH levels in rat liver. Increased MDA and significant changes of GPX and GR activities were also observed. The results indicated that acute exposure to MC-LR induced oxidative stress, and GSH depletion (BSO pretreatment) enhanced the level of oxidative stress. The induction of enzymatic antioxidant defenses after MC exposure can be considered as an adaptive response, that is, a compensatory mechanism that enables the liver cells to overcome the oxidative imbalance (Chen et al. 2014). Previous studies also showed that BSO increased the cell susceptibility to MC-induced cytotoxicity by disrupting intracellular GSH balance (Ding et al. 2000; 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. 2000). Žegura et al. (2006) also demonstrated that BSO pretreatment dramatically increased the susceptibility of HepG2 cells to MC-induced DNA damage, while NAC pretreatment almost completely prevented MC-induced DNA damage. Thus, taking these studies into consideration, we suppose that GSH is responsible for cellular defence against MC-induced hepatotoxicity.

In conclusion, our study provided toxicokinetic and histopathological evidence for the role of the glutathione pathway in the detoxification of MC-LR in liver as presented in Fig. 7. MC-LR and its two main GSH pathway metabolites (MC-LR-GSH and MC-LR-Cys) displayed significant time–response and dose–response relationships in the liver of SD rat, and the metabolic rules were in agreement with those in aquatic animals to a certain extent: GSH conjugates with MCs to form the MC-GSH conjugates, followed by rapid degradation to cysteine conjugates (MC-Cys). The efficient biotransformation from MC-LR to MC-LR-Cys was inhibited at low GSH level induced by BSO, i.e., BSO pretreatment reduced the detoxification of MCs. Moreover, our results also demonstrated that MC-LR significantly induced liver damage, and the effects were more pronounced by BSO injection prior to MC-LR treatment. Apparently, all the results in the present study suggested that liver with low GSH concentration is highly vulnerable to MC-LR toxicity and GSH pathway is critical for the detoxification in MC-LR-induced hepatotoxicity in mammals.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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