# The Role of Glutathione Detoxification Pathway in MCLR-Induced Hepatotoxicity in SD Rats

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*Received 27 January 2014; revised 13 June 2014; accepted 15 June 2014*

**ABSTRACT:** In the present study, we investigated the role of glutathione (GSH) and its related enzymes in Sprague Dawley (SD) rats subjected to microcystin-leucine-arginine (MCLR)-induced hepatotoxicity. SD rats were intraperitoneally (i.p.) injected with MCLR after pretreating with or without buthionine-(*S,R*)-sulfoximine (BSO), an inhibitor of GSH synthesis. The depletion of GSH with BSO enhanced MCLR-induced oxidative stress, resulting in more severe liver damage and higher MCLR accumulation. Similarly, the contents of malondialdehyde (MDA), total GSH (T-GSH), oxidized GSH (GSSG) and GSH were significantly enhanced in BSO pretreated rats following MCLR treatment. The study showed that the transcription of GSH-related enzymes such as glutathione-*S*-transferase (GST), γ-glutamylcysteine synthetase (γ-GCS), glutathione reductase (GR) varied in different ways (expect for glutathione peroxidase (GPx), whose gene expression was induced in all treated groups) with or without BSO pretreatment before MCLR exposure, suggesting an adaptative response of GSH-related enzymes at transcription level to combat enhancement of oxidative stress induced by MCLR when pretreated with BSO. These data suggested the tissues with low GSH concentration are highly vulnerable to MCLR toxicity and GSH was critical for the detoxification in MCLR-induced hepatotoxicity *in vivo*.  Environ Toxicol 30: 1470–1480, 2015.
**Keywords:** microcystin-LR; glutathione; oxidative stress; liver; BSO; SD rat

## INTRODUCTION

Blooms of cyanobacteria in aquatic environments have dramatically increased and become a worldwide concern in the past decades. Cyanobacteria cause serious environment and health problems because of their ability to produce hepatotoxic microcystins (MCs) (Falconer, 2001; Dietrich et al., 2008). Up to now, more than 90 variants of MCs have been reported (Ufelmann et al., 2012), with microcystin-LR (MCLR) being one of the most toxic and commonly encountered variants (Miura et al., 1989). MCs mainly accumulated in liver, kidney, heart, testis, brain, and spleen, resulting in hepatotoxicity, nephrotoxicity, cardiotoxicity, neurotoxicity, immunotoxicity, and reproductive toxicity (Fischer and Dietrich, 2000; Zhang et al., 2007; Li et al., 2008, 2012a,b; Qiu et al., 2009; Zhao et al., 2012). Recently, Chen et al. (2009) reported that MCs were identified in the serum of a chronically exposed human population, together with the indication of

Additional Supporting Information may be found in the online version of this article.

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Contract grant sponsor: National Natural Science Foundations of China.

Contract grant number: 31070457

Published online 25 June 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/tox.22017

hepatocellular damage. Therefore, the detoxification mechanism of MCLR in mammals is a cause for concern.

MCs are potent inhibitors of protein phosphatases 1 and 2A, leading to increased protein phosphorylation, which is directly related to their cytotoxicity and tumor promoting activity (Kondo et al., 1992; Nishiwaki-Matsushima et al., 1992; Humpage et al., 2000). Recent studies also found that oxidative stress was involved in MCs toxicity in rat hepatocytes and HepG2 cells (Ding et al., 1998; Ding and Nam Ong, 2003; Žegura et al., 2004). MCs can induce intracellular reactive oxygen species (ROS) formation, lipid peroxidation, and cell injury (Guzman and Solter, 1999; Jayaraj et al., 2006). Ding et al. (2001) suggested that ROS formation played a crucial role in MCLR-induced disruption of cytoskeleton organization and consequent hepatotoxicity. The results of Chen et al. (2005) indicated that acute exposure to MCLR may cause mice hepatocytes apoptosis and liver damage, partly through regulating the oxidative stress-associated proteins.

Free radicals or ROS generated in tissues can be effectively scavenged by the antioxidant defense system. The glutathione (GSH) and its related enzymes, which include enzymes responsible for GSH synthesis and redox status regulation, such as γ-glutamylcysteine synthetase (γ-GCS), glutathione-S-transferase (GST), glutathione peroxidase (GPx), and glutathione reductase (GR) were important constituents of the intracellular antioxidant defense system (Filomeni et al., 2002; Masella et al., 2005). Many studies have demonstrated that GSH played an important role in the detoxification of MCs in mice, rats, and fishes. After GSH conjugates was first identified as metabolite of MCs *in vivo* in the liver of mouse and rats (Kondo et al., 1992, 1996), the first step in detoxification of MCs was suggested to occur via conjugation to GSH and formation of MCLR-GSH conjugate by GST extracted from various aquatic organisms (Pflugmacher et al., 1998). Gehringer et al. (2004) provided molecular and biochemical evidence of the importance of the GSH detoxification pathway for the removal of MCLR from the liver in Balb/c mice. However, the role of GSH detoxification pathway in MCLR-induced hepatotoxicity *in vivo* was still incompletely understood.

To directly elucidate the potential role of GSH pathway in MCLR-induced hepatotoxicity *in vivo* and to further identify metabolic characteristics of the GSH pathway, Sprague Dawley (SD) rats were randomly divided into two groups. In the group A, the rats were exposed to 0.25 and 0.5 $LD_{50}$ MCLR through i.p. injection. In the group B, 1.0 mM/kg buthionine-(*S*,*R*)-sulfoximine (BSO), an inhibitor of GSH synthesis, was injected 3 h before MCLR exposure with the same MC doses in the group A. Control groups of both group A and group B were received the same volume of 0.9% saline solution. Pathological changes, the contents of MCLR and its conjugates, the enzymatic activity and gene expression of GSH-related enzymes (such as GST, γ-GCS, GPx, and GR) as well as the contents of malondialdehyde (MDA), total GSH (T-GSH), oxidized GSH (GSSG), and GSH in the liver of rats were examined in the present study. Furthermore, the results hold promise for further application to clinical treatment of human diseases induced by MCs intoxication.

## MATERIALS AND METHODS

### Chemicals

MCLR was extracted and purified from the freeze-dried surface blooms collected from Lake Dianchi, Yunnan, China. The MCs were extracted by the method of Wang et al. (2008). MCLR was separated by semi-performance preparative liquid chromatography system (Waters 600E). MCLR content was analyzed via HPLC (LC-10A, Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan). The purity of MCLR was over 95% and its identity was confirmed by liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS, Thermo Electron Corporation, Waltham, MA) (Supporting Information Fig. S1). MCLR was finally suspended in salt solution (0.9% NaCl). D,L-buthionine-(*S*,*R*)-sulfoximine (BSO, Sigma) was dissolved in 0.9% NaCl. L-Glutathione (L-GSH) and L-cysteine (L-Cys) were purchased from Acros Organics (Geel, Belgium) and the purity of GSH and Cys was greater than 99%.

### Animals

Healthy male SD rats aged 8 weeks (200 ± 20 g) were purchased from Wuhan University Center for Animal Experiment/A3-Lab. The rats were kept in stainless steel cages containing saw dust bedding at suitable temperature (24 ± 2°C) and relative humidity (55 ± 15%) with a regime of 12-h light-dark cycle. The animals were allowed free access to food and water. All animal procedures 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.

### *$LD_{50}$ of Purified MCLR*

To determine the median lethal dose ($LD_{50}$) and select relevant MCLR exposure dose for the following experiments, SD rats ($n = 40$) were divided into five groups and i.p. injected with MCLR at different doses of $MCLR_{equivalent}$ $kg^{-1}$ body weight (BW). Calculating the death number and mortality of rats in each group during 24 h, we obtained the $LD_{50}$ level for 24 h by using the formula $LD_{50} = \log_{-1}[X_m - I(\sum p - 0.5)]$ ($X_m$: the log of max dose; $p$: mortality; $\sum p$: the sum of mortality in each group; $I$: the difference between the log of adjacent two group dose).

### Experimental Protocol

To determine the time of BSO injection, twenty-one rats were i.p. injected with 1.0 mM/kg BSO for 0, 0.5, 1, 2, 3, 4,

and 5 h. Three rats from each group were killed at each time point. Liver tissues were excised for determination of intracellular GSH. The results of GSH contents were showed in Supporting Information Figure S2. Based on the results, 3 h postexposure was selected for the subsequent research.

Eighteen rats were randomly divided into group A ($n$ = 9) and group B ($n$ = 9). Both group A and group B were further divided into three subgroups with three animals in each subgroup. In the group A, the rats were exposed to 0.25 and 0.5 $LD_{50}$ MCLR through i.p. injection. Control group received the same volume of 0.9% saline solution. In the group B, 1.0 mM/kg BSO was injected 3 h before MCLR exposure with the same doses in the group A. Control group received the same volume of 0.9% saline solution as mentioned above. All the subjects were sacrificed through spinal transection after 24 h of MCLR exposure, livers were collected and conserved at −80°C.

## Histological and Ultrastructural Analysis

The histological and ultrastructural analysis was according to our previous study (Chen et al., 2013). Briefly, Livers from SD rats were fixed in Bouin's solution and embedded in paraffin wax, and blocks were sectioned on a microtome. The 10 μm thick liver sections were stained with hematoxylin and eosin (H&E). Histological analysis was performed using light microscopy.

For transmission electron microscopic (TEM) study, liver tissues were diced into 1 $mm^3$, prefixed in 2.5% glutaraldehyde solution and fixed in aqueous osmium tetroxide. The specimens were then embedded in Epon 812. Ultrathin sections were sliced with glass knives on a LKB-V ultramicrotome (Nova, Sweden), stained with uranyl acetate and lead citrate and examined under a HITACHI, H-600 electron microscope.

## Determination of MCLR and its GSH Conjugate

The method for the preparation of MCLR-GSH and MCLR-Cys conjugates was described by Dai et al. (2008) and Zhang et al. (2009). Liver tissues were lyophilized by a Christ@ Alpha 2–4 freeze dryer (Martin Christ, Osterode, Germany). The lyophilized samples (0.2 g dry weight for each sample) were processed and analysed by a Finnigan LC-MS/MS according to our previous study (Guo et al., 2014).

## Biochemical Analysis

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 T-GSH, GSH, and MDA and the activities of γ-GCS, GR, GST, and GPx were assayed by the kits supplied by the Nanjing Jiancheng Bioengineering Institute, China. GST activity was detected by evaluating the conjugation of GSH with the standard model substrate 1-chloro-2,4-dinitrobenzene (CDNB) according to the method of Habig et al. (1974). γ-GCS activity was determined by measuring the rate of formation of ADP from the NADH oxidation by using the coupled method described by Seelig and Meiste (1985). We used $H_2O_2$ as the substrate to determine the GPx activity according to Drotar et al. (1985). GR activity was measured spectrophotometrically by measuring the oxidation of NADPH at 340 nm (Carlberg and Mannervik, 1975). T-GSH and GSH contents were determined by the methods of Habig et al. (1974) and Giffith et al. (1980). GSSG was calculated by using the formula T-GSH equivalent = 2 GSSG + GSH (Sani et al., 2014). The content of MDA was used as an index of lipid peroxidation and was determined according to Ohkawa et al. (1979). Protein contents were determined by the coomassie blue method using bovine serum as the standard (Bradford, 1976). All the experiments were carried out in triplicate.

## RNA Extraction and Quantitative Real-Time PCR (Q-PCR)

Total RNA was isolated from liver tissues and reverse transcribed as described before (Xiong et al., 2010). Q-PCR was performed based on SYBR green (SYBR Green qPCR kit; Finnzymes). All the primers used were listed in Table I. The primers were designed based on the gene sequences of *Rattus norvegicus* present on the GenBank. GAPDH was used as the internal control gene for Q-PCR assay. The amplification conditions were 95°C for 3 min, 40 cycles of 95°C for 15 s, 60°C or 64°C for 20 s and 72°C for 15 s. All PCR reactions were performed with a Chromo4 Real-Time Detection System (MJ Research, Cambridge, MA) and results were analyzed with the Option Monitor software 2.03 version (MJ Research, Cambridge, MA).

## Statistical Analysis

Statistical analysis was undertaken using SPSS 13.0 for Windows. All values expressed as mean ± SE were subjected to one-way analysis of variance (ANOVA). Differences in mean values between groups were assessed and were considered statistically different at $*p < 0.05$, $**p < 0.01$.

# RESULTS

## *$LD_{50}$ of MCLR in SD Rats*

The effects of MCLR on SD rats' mortality were shown in Table II. Accordingly, the 24 h $LD_{50}$ of MCLR (i.p.) in SD rats was about 82.7 μg/kg BW.

**TABLE I. Q-PCR primers used in this experiment**

| Target Gene | Primer Sequence(5′-3′) | Accession No. |
|---|---|---|
| GPx | (+) CAGTTCGGACATCAGGAGAAT | NM_030826.3 |
| | (−) AGAGCGGGTGAGCCTTCT | |
| GR | (+) GGGCAAAGAAGATTCCAGGTT | NM_053906.2 |
| | (−) GGACGGCTTCATCTTCAGTGA | |
| γ-GCS | (+) ATCTGGATGATGCCAACGAGTC | NM_012815.2 |
| | (−) CCTCCATTGGTCGGAACTCTACT | |
| GST | (+) TTGAGGCACCTGGGTCGCTCTTTAG | NM_012577.2 |
| | (−) GGTTCTGGGACAGCAGGGTCTCAAA | |
| GAPDH | (+) ACAGCAACAGGGTGGTGGAC | NM_017008.4 |
| | (−) TTTGAGGGTGCACGAACTT | |

## Histological and Ultrastructural Observation

Histopathological observations showed that lobular architecture was normal in the livers of control, with hepatocytes arranged in neat rows and clear nuclei [Fig. 1(A,D)]. In the group A, the livers of MCLR-treated rats showed the loss of cytoplasm compared with control [Fig. 1(B,C)]. More serious damages were found in the treated rats of group B, which showed apparently the loss of cytoplasm [Fig. 1(E,F)].

The TEM images showed that the livers of control in the group A and group B were normal, which contain the normal organelle, clear cytoplasm and a nucleus enveloped by smooth, intact karyotheca [Fig. 2(A,C)]. Condensed nuclear and swollen mitochondria were observed in liver of rats after 0.5 $LD_{50}$ MCLR exposure with BSO pretreatment in the group B [Fig. 2(D)], while the liver of the rats treated with 0.5 $LD_{50}$ MCLR showed no change compared with the control in the group A [Fig. 2(B)].

## Determination of MCLR Contents and its GSH Conjugate in Liver

The contents of MCLR, MCLR-GSH, and MCLR-Cys were shown in Figure 3. No MCLR and its GSH conjugates were detected in the control rats in the group A and group B. The contents of MCLR were 16.7 (0.25 $LD_{50}$) and 20.7 ng/g dry weight (DW) (0.5 $LD_{50}$) in the group A, and the contents of MCLR were 25.0 (0.25 $LD_{50}$) and 66.6 ng/g DW (0.5 $LD_{50}$) in the group B. For the same dose of MCLR exposure, the contents of MCLR in the group B were significantly higher than those in the group A. The contents of MCLR-GSH were 0.4 (0.25 $LD_{50}$) and 1.3 ng/g DW (0.5 $LD_{50}$) in the group A, and 1.0 (0.25 $LD_{50}$) and 1.2 ng/g DW (0.5 $LD_{50}$) in the group B. The contents of MCLR-Cys were 8.3 and 8.1 ng/g DW for 0.25 and 0.5 $LD_{50}$ MCLR in the group A and the contents of MCLR-Cys were 8.5 and 8.0 ng/g DW in the group B, respectively.

**TABLE II. The effect of MCLR on SD rats**

| Groups | No. Rats | Dose (μg/kg) | No. Death | Mortality |
|---|---|---|---|---|
| 1 | 8 | 19 | 0 | 0 |
| 2 | 8 | 34 | 0 | 0 |
| 3 | 8 | 61 | 3 | 37.5% |
| 4 | 8 | 111 | 5 | 62.5% |
| 5 | 8 | 200 | 8 | 100% |

## Biochemical Analysis

The contents of T-GSH, GSSG, and GSH were shown in Figure 4. The contents of T-GSH, GSSG, and GSH showed no significant change in the MCLR-treated rats of group A, whereas GSH contents significantly increased 46% and 81% for 0.25 and 0.5 $LD_{50}$ MCLR in the group B ($p < 0.01$). T-GSH contents significantly increased 51% (0.25 $LD_{50}$) and 94% (0.5 $LD_{50}$), and GSSG contents increased 66% (0.25 $LD_{50}$) and 137% (0.5 $LD_{50}$) ($p < 0.01$).The contents of MDA in rats were shown in Figure 5. The contents of MDA, as an index of lipid peroxidation, showed no change in the group A. However, the MDA contents were significantly increased 22% (0.25 $LD_{50}$) and 51% (0.5 $LD_{50}$) in the group B ($p < 0.01$). The enzymatic activities of GST, γ-GCS, GPx, and GR were shown in Figure 5(B–E). In the group A, a significant decrease in GPx activities was observed and the change was dependent on the doses of MCLR administered (16 and 40% for 0.25 and 0.5 $LD_{50}$ MCLR, respectively). Similar alterations were also observed in the group B, with about 21 to 26% reduction of this enzyme activity at 0.25 and 0.5 $LD_{50}$ MCLR groups. In the group A, the activities of γ-GCS did not show any change in rats treated with 0.25 $LD_{50}$ MCLR, but a reduction of about 35% was observed in rats treated with 0.5 $LD_{50}$ MCLR compared with the control. In the group B, the percentages of γ-GCS reduction were dose-dependent, 30 and 50%, in rats treated with 0.25 and 0.5 $LD_{50}$ MCLR, respectively, when compared with control values. No change of GST and GR activities was observed in treated rats of group A and group B, regardless of doses used.

## Gene Expression

In the group A, the transcription level of GST gene was significantly up-regulated 1.8-fold at 0.25 $LD_{50}$ MCLR, but its

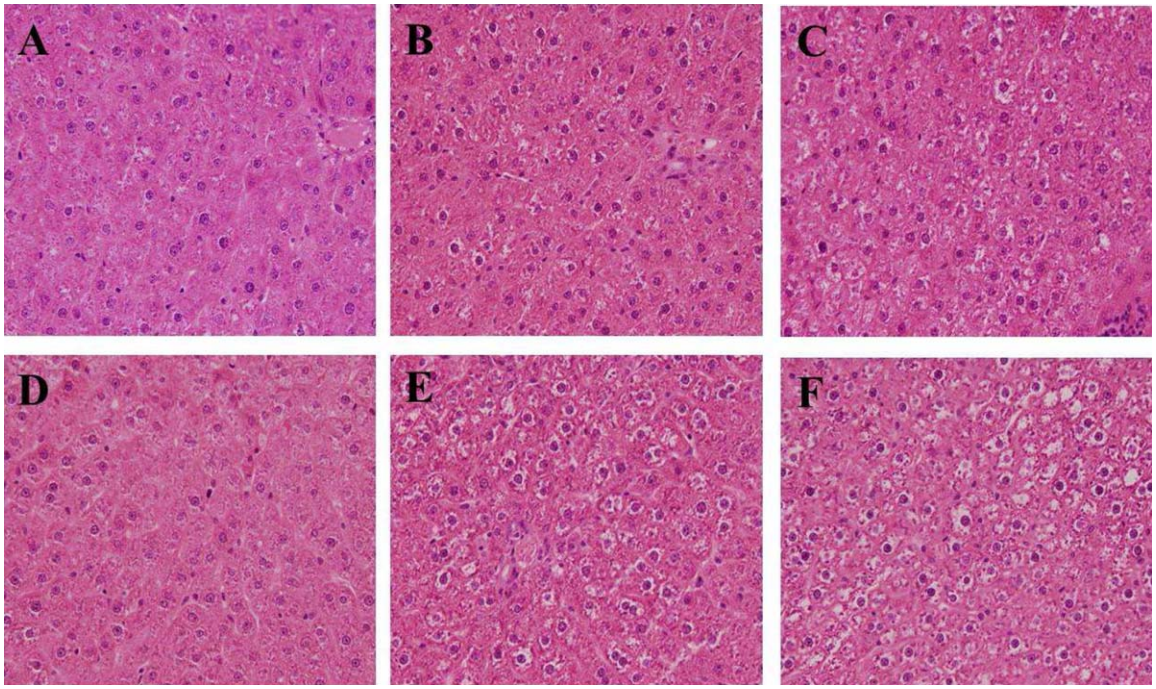


**Fig. 1.** Haematoxylin and eosin (H&E) stained sections of liver from rats after MCLR exposure. (A) and (D) Showing normal structure of hepatocyte in the liver of control in the group A and group B, respectively. (B) and (C) Showing the loss of cytoplasm exposed to 0.25 and 0.5 $LD_{50}$ MCLR, respectively. (E) and (F) Showing the widening of loss of cytoplasm in the liver exposed to 0.25 and 0.5 $LD_{50}$ MCLR after pretreatment with BSO (1.0 mM/kg) for 3 h, respectively. Magnification: ×600. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

expression showed no change at 0.5 $LD_{50}$ MCLR compared with the control. GPx was significantly induced 1.3- and 1.8-fold in a dose-dependent manner in 0.25 and 0.5 $LD_{50}$ MCLR treated groups compared with the control. γ-GCS was significantly upregulated 4.9-fold at 0.25 $LD_{50}$ MCLR exposure. The gene expression of γ-GCS was slightly decreased compared with 0.25 $LD_{50}$ MCLR, but its expression was significantly up-regulated 1.8-fold compared with the control after administration of 0.5 $LD_{50}$ MCLR. GR showed no change at 0.25 $LD_{50}$ MCLR and down-regulated 0.8-fold at 0. 5 $LD_{50}$ MCLR compared with the control [Fig. 6(A)].

In the group B, the mRNA expression of GST was significantly down-regulated 0.5- and 0.6-fold in 0.25 and 0.5 $LD_{50}$ MCLR-treated groups. GPx transcription was significantly up-regulated 1.7- and 3.0-fold in a dose-dependent manner compared with the control. The expression of GR was also up-regulated 1.3- and 5.4-fold in a dose-dependent manner. γ-GCS expression was significantly down-regulated 0.3- and 0.4-fold in 0.25 and 0.5 $LD_{50}$ MCLR-treated groups [Fig. 6(B)].

## DISCUSSION

Recent studies found that oxidative stress has an important function in the pathogenesis of MCs toxicity. GSH is involved in the detoxification of hepatotoxicity induced by MCLR (Gehringer et al., 2004). BSO is a specific inhibitor of γ-GCS, a rate-limiting enzyme of GSH synthesis (Griffith and Meister, 1979) and can therefore decrease the GSH level, even *in vivo* (Watanabe et al., 2003). In the present study, SD rats were i.p. injected with different doses of MCLR after pretreating with or without BSO, to investigate the role of GSH in hepatotoxicity induced by MCLR. The results demonstrated that BSO pretreatment enhanced oxidative stress in MCLR-treated rats, resulting in severe liver damage, accumulation of MCLR and its conjugates, and the increase of MDA, T-GSH, GSSG, and GSH contents. Meanwhile, we detected the activity and gene expression of GSH-related enzymes to further study the role of GSH detoxification pathway in MCLR-induced hepatotoxicity in SD rats.

MCs could accumulate in fish and mammals, which lead to the damage of tissues (Wang et al., 2008; Yan et al., 2012). GSH is the main intracellular antioxidant and the formation of MCLR-GSH conjugate has been proved in various aquatic animals *in vitro* and is suggested to be the first step in detoxification of MCs (Pflugmacher et al., 1998). In the present study, higher MCLR contents were found in rat liver from BSO pretreatment groups compared with the rats from BSO non-pretreatment groups (Fig. 3). In addition, although MCLR-GSH could be qualitatively detected in liver of both groups, the contents were very low, while substantial amount of MCLR-Cys were detected, confirming that the GSH

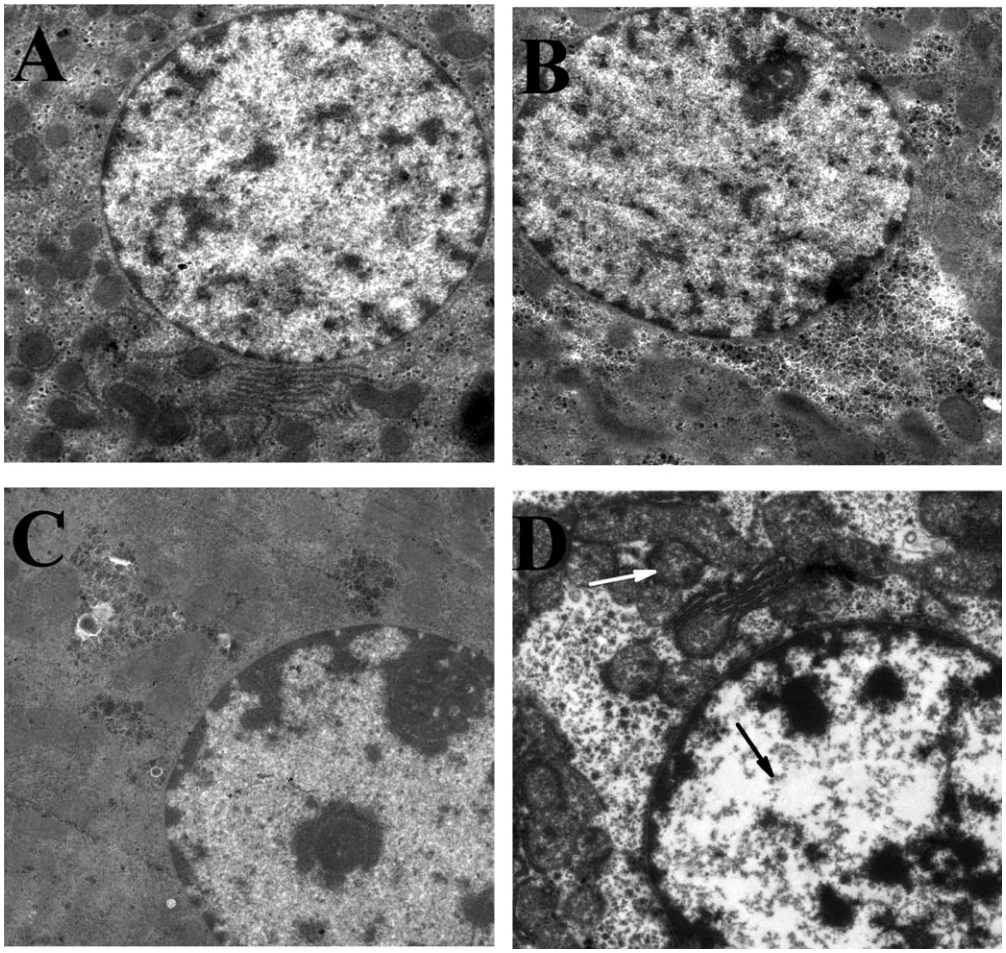


**Fig. 2.** Ultrastructure changes in liver of rats after MCLR exposure. (A) and (C) showing normal structure of hepatocyte in the liver of control in the group A and group B, respectively. (B) Showing no change in the liver exposed to 0.5 $LD_{50}$ MCLR. (D) Showing condensed nuclear (black arrow) and swollen mitochondria (white arrow) in the liver exposed to 0.5 $LD_{50}$ MCLR after pretreatment with BSO (1.0 mM/kg) for 3 h. Magnification: ×2500.

conjugate of MCLR might act as mid-metabolites and change rapidly to the more stable metabolite MCLR-Cys (Li et al., 2014). Furthermore, contrary to MCLR, lower contents of MCLR-GSH and MCLR-Cys (the important detoxification metabolites of MCLR) were found in rat liver from BSO pretreatment groups compared with the rats from BSO non-pretreatment groups, suggesting the biotransformation from MCLR to MCLR-GSH and MCLR-Cys in BSO pretreatment group was not as efficient as that in BSO non-pretreatment groups.

It's implicated that MCLR could induce more serious damage of tissues with higher MCLR concentration (Li et al., 2012b). Meanwhile, hemorrhaging with a high degree of coagulative necrosis was observed in Balb/c mice exposed to 75% $LD_{50}$ MCLR for 8 h (Gehringer et al., 2004). MCLR can also cause noticeable damage to liver ultrastructure. Early studies have shown that MCLR causes significant damage to intracytoplasmic organelles, such as mitochondria and endoplasmic reticulum, and loss of microvilli and desmosomes in hepatocytes of rats (Hooser et al., 1990; Wickstrom et al., 1996), mice (Hermansky et al., 1993), and rabbits (Zhao et al., 2008). Therefore, we further detected pathological changes of livers. Results showed apparently the loss of cytoplasm, karyopyknosis and mitochondria swelling in the group B, but only loss of cytoplasm was observed in the group A. Similarly, Žegura et al. (2006) also reported that pretreatment with BSO increased the susceptibility of HepG2 cells to MCLR-induced DNA damage. Taken together with these data, we supposed that BSO pretreatment enhanced toxin accumulation in the liver and resulted in more severe liver damage in MCLR-treated rats.

MCs could induce cell injury by intracellular ROS formation and lipid peroxidation. There are several reports of MCLR-induced lipid peroxidation in the liver of mice (Jayaraj et al., 2006; Weng et al., 2007). Ding et al. (1998) reported that increased lipid peroxidation measured as MDA production was observed in rat hepatocytes and hepatic microsomes following exposure to MCs. In the present

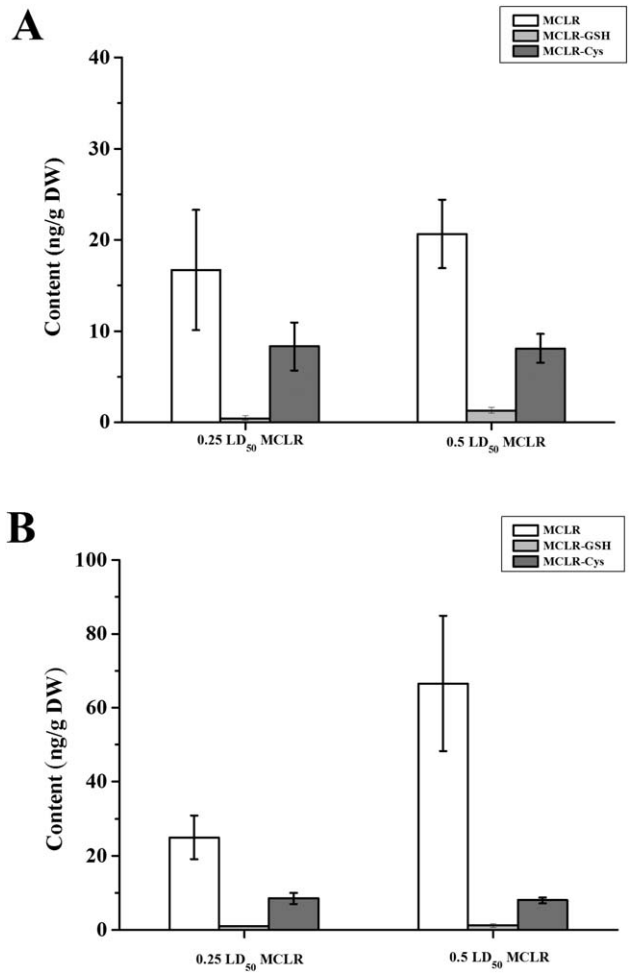


**Fig. 3.** The contents of MCLR and its GSH conjugates in the liver tissues of rats after MCLR exposure (0.25 $LD_{50}$ and 0.5 $LD_{50}$). (A) group A, (B) group B. MCLR and its GSH conjugates were not detected in the control group of both group A and group B. The values are expressed as mean ± SD ($n = 3$), $^{*}p < 0.05$, $^{**}p < 0.01$.

study, MDA contents showed no significant change in the 0.25 and 0.5 $LD_{50}$ MCLR-treated rats when compared with the control in the group A. However, the contents of MDA showed a dose-dependent increase in the group B. As we know, GSH, the major intracellular antioxidant, is critical for preserving normal cellular redox balance and protecting the liver against oxidative stress. The product of GSH oxidation, GSSG, can be reduced back to GSH by GR (Lu, 2009). T-GSH, GSSG, and GSH contents showed no change in the group A. By contrast, we found the levels of GSSG and GSH were increased synchronously; as a result, the T-GSH level was also increased in the 0.25 and 0.5 $LD_{50}$ MCLR exposure rats pretreating with BSO in the group B. There were also several lines of evidence suggesting that MCLR induced oxidative stress and up-regulation of GSH levels in mice (Gehringer et al., 2004; Sedan et al., 2010, 2013) and fish (Zhao et al., 2012), by acute, sub-chronic and prolonged exposure. The induction of GSH synthesis after MCLR exposure could be dependent on increased transcriptions and activities of the enzymes involved in GSH synthesis (Gehringer et al., 2004). In the present study, we found the accumulation of MCLR in both group A and group B. Pretreatment with BSO, an inhibitor of GSH synthesis, enhanced the accumulation of MCLR compared with 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 consecutive injections are performed. Both a single administration (Gehringer et al., 2004) and repeated administrations (Sedan et al., 2010, 2013) of MCs could lead to oxidative stress. Therefore, the significant increases in the contents of MDA and alternations of GSH redox state in the group B indicated that BSO pretreatment enhanced oxidative stress in MCLR-treated rats. BSO reduced the detoxification and resulted in more severe oxidative stress and liver damage. Our data also suggested that the tissues with low GSH concentrations are highly vulnerable to MCLR toxicity.

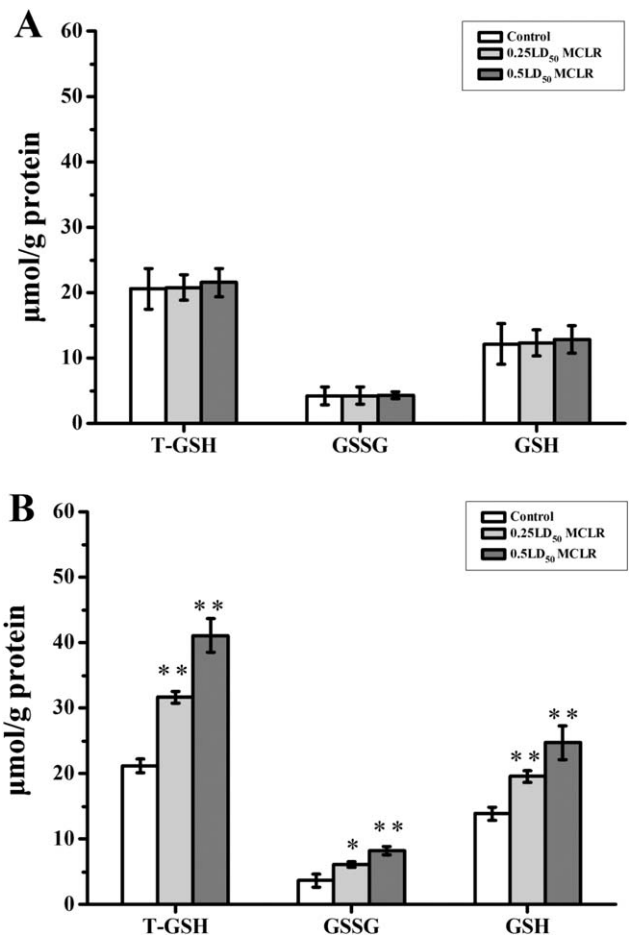


**Fig. 4.** Total (T-GSH), oxidized (GSSG) and reduced (GSH) glutathione contents in liver after MCLR exposure. (A) group A, (B) group B. The values are expressed as mean ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$.

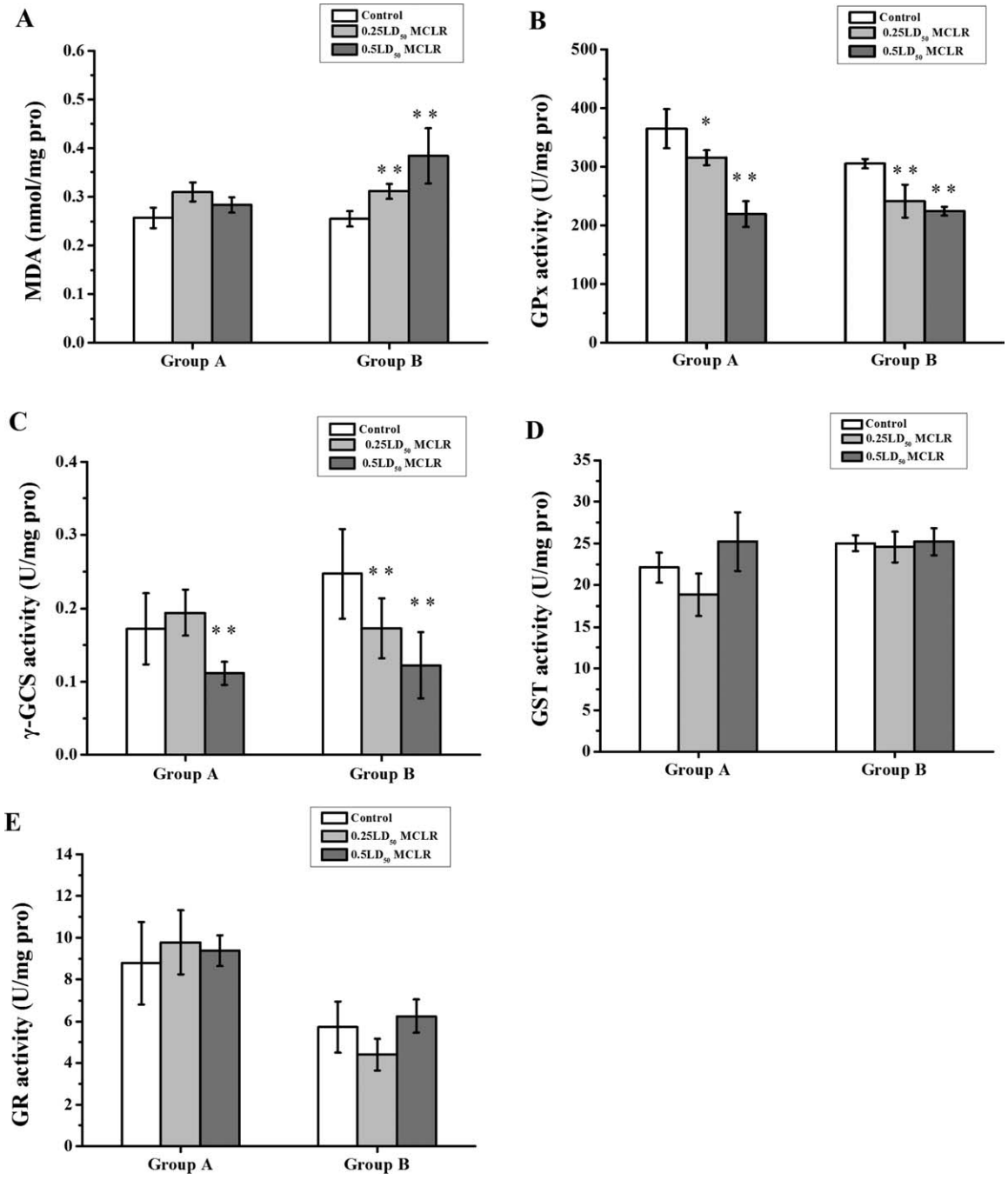


**Fig. 5.** The levels of MDA and the activities of GSH-related enzymes (GST, γ-GCS, GPx, and GR) in livers after MCLR exposure. The values are expressed as mean ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$.

Intracellular GSH is synthesized by two enzymes: γ-GCS and glutathione synthetase, of which γ-GCS is the rate-limiting enzymes for GSH biosynthesis (Lu, 2009). Acute exposure to MCLR modulated γ-GCS expression in the liver of Wistar rats at the transcriptional level (Xiong et al., 2009). In the present study, MCLR exposure resulted in decrease of γ-GCS activity in all treated groups except 0.25 $LD_{50}$ MCLR treatment in the group A. However, the gene expression of γ-GCS was significantly up-regulated in the group A while down-regulated in the group B. Meister and Anderson (1983) pointed out that GSH could regulate the activity of γ-GCS by negative feedback activity. Therefore, the down-regulated gene expression of γ-GCS in the group B might be due to the decreased GSH level. GPx catalyzes the reduction of free radicals at the expense of GSH (Masella et al., 2005). Our results showed that liver GPx activity was decreased in all treated groups, confirming MCLR induced free radicals production. Meanwhile, the up-regulation of

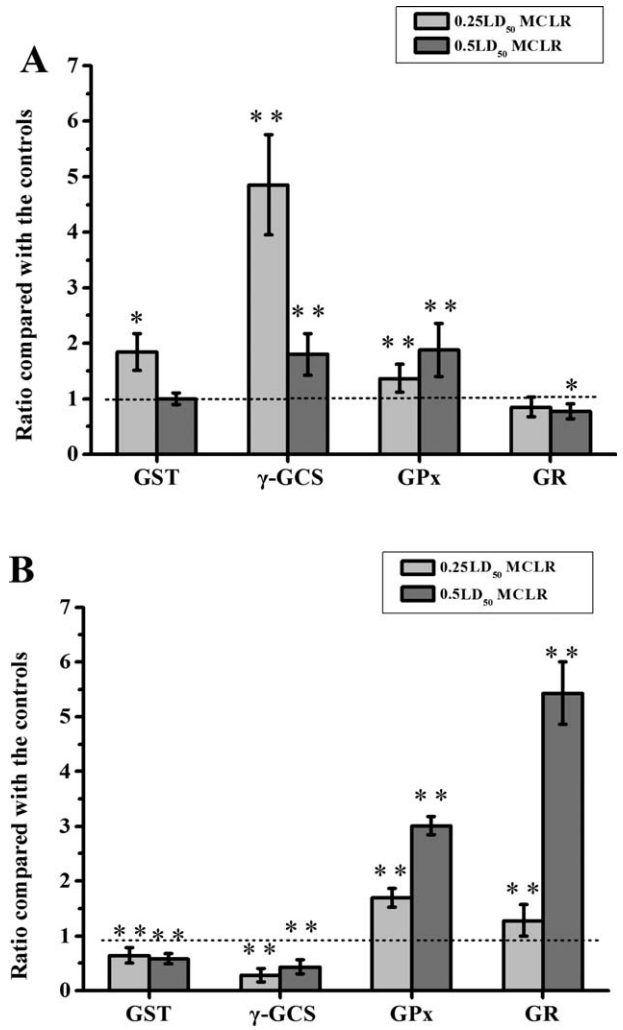


**Fig. 6.** Gene expression profile of GSH-related enzymes (GST, γ-GCS, GPx, and GR) after MCLR exposure. (A) group A, (B) group B. The values are expressed as mean ± SD ($n = 3$). *$p < 0.05$, **$p < 0.01$.

GPx gene expression in all treated groups could be resulted from the inhibition of GPx activity. It should be noted that gene expression of GPx was significantly induced 1.7- and 3.0-fold in BSO pretreating group; while in another group (group A, without BSO pretreatment), the corresponding data were only 1.3- and 1.8-fold, respectively. As it is well known, MCLR could be conjugated with GSH via GST and subsequently degraded to MCLR-Cys (Pflugmacher et al., 1998; Dittmann and Wiegand, 2006). The gene expression of GST was up-regulated in 0.25 $LD_{50}$ MCLR treatment of the group A while down-regulated in both 0.25 and 0.5 $LD_{50}$ MCLR treatments of the group B, suggesting that BSO pretreatment enhanced oxidative stress induced by MCLR, and MCLR conjugated with a greater amount of GSH via GST when compared with administration of MCLR alone. GR is a key enzyme in the regeneration of GSH from the oxidized form of glutathione (GSSG). Previous studies showed MCLR exposure induced changes in GR activity in the liver of mice and rats (Moreno et al., 2005; Jayaraj et al., 2006). In the present study, no change of GR activity was observed in all treated groups, while the gene expression of GR was down-regulated at 0.25 $LD_{50}$ MCLR treatment in the group A, but its expression was significantly up-regulated in both 0.25 and 0.5 $LD_{50}$ MCLR-treated rat livers of the group B. The significantly up-regulated gene expression of GR in the group B might be due to maintain intracellular GSH level under the inhibition effect of BSO.

In conclusion, our results confirmed MCLR-induced hepatotoxicity, and that BSO pretreatment induced more severe liver damage and higher MCLR accumulation. The significant increase of MDA, T-GSH, GSSG, and GSH was also observed compared with the control in BSO pretreated rats. Our results also showed that the transcription of GSH-related enzymes such as GST, γ-GCS, GR varied in different ways (expect for GPx whose gene expression was induced in all treated groups) with or without BSO pretreatment before MCLR exposure, suggesting an adaptative response of GSH-related enzymes at transcription level to combat enhancement of oxidative stress induced by MCLR when pretreated with BSO. These data suggested the tissues with low GSH concentration are highly vulnerable to MCLR toxicity and GSH detoxification pathway plays an important role in the resistance to MCLR-induced hepatotoxicity *in vivo*.

The authors greatly appreciate the constructive and useful comments and suggestions by Paul Tchounwou and anonymous reviewers.

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