Involment of p53, Bax, and Bcl-2 Pathway in Microcystins-Induced Apoptosis in Rat Testis

Guang-Yu Li, Ping Xie, Hui-Ying Li, Le Hao, Qian Xiong, Tong Qiu

Donghu Experimental Station of Lake Ecosystems, State Key Laboratory for Freshwater Ecology and Biotechnology of China, Institute of Hydrobiology, The Chinese Academy of Sciences, Donghu South Road 7, Wuhan 430072, People's Republic of China

Received 19 May 2009; revised 18 July 2009; accepted 21 July 2009

ABSTRACT: It has been reported that microcystins (MCs) could accumulate in the gonads of mammals and MCs exposure exerts obvious toxic effects on male reproductive system of mammals. We have comfirmed that MCs could accumulate and induce apoptosis in rat testis. The p53, Bax, and Bcl-2 protein play important roles in mitochondria-dependent apoptotic pathway, and this study aimed to investigate whether the p53, Bax, and Bcl-2 pathway is involved in microcystins-induced apoptosis in rat testis and discussed the possible mechanisms. Our results show that MCs led to persistent increase of transcriptional and protein level of P53 and Bax expression but led to decrease of Bcl-2 expression, resulting in an increased ratio of Bax to Bcl-2, which might contribute to apoptotic cell death of rat testis following MCs treatment. The increased ratio of expression of Bax to that of Bcl-2 induced by MCs suggests their important role in MCs-induced apoptosis in rat testis tissue. © 2009 Wiley Periodicals, Inc. Environ Toxicol 26: 111–117, 2011.

Keywords: microcystin; apoptosis; real time PCR; Wistar rat; Bcl-2; Bax; P53

INTRODUCTION

In the last decade, public health concern about cyanobacteria and cyanotoxins has increased in many countries, owing to their frequent occurrence in aquatic ecosystems used for different purposes, including those used as drinking water supplies. Among cyanotoxins, microcystins (MCs) are considered to be one of the most dangerous groups and are known as a group of cyclic polypeptide hepatotoxins of varying potency (Chorus and Bartram, 1999). Their toxic effects have been described in organisms as diverse as macrophytes, zooplankton, fish, and mammals including humans (Sahin et al., 1996; Ferrão-Filho et al., 2002; Chen et al., 2009). Recently, Chen and Xie (2005) found that MCs could effectively accumulate in gonads which thus were regarded as the second target organs of MCs. Ding et al. (2006) also report that the testes of mice administered i.p. injection of *Microcystis* cell extract were damaged according to histological examination, and the quality of mature sperm in the seminiferous tubules decreased. Therefore, it could be concluded that MCs had toxic effects on the male reproductive system.

The mechanism of MCs toxicity is the inhibition of protein phosphatases 1 and 2A, which then leads to the increase in protein phosphorylation (Nishiwaki-Matsushima et al., 1991). These toxins penetrate cell membranes through a bile acid carrier and result in changes such as over phosphorylation of enzymes, necrosis, and even deadly intrahepatic bleeding (Falconer and Yeung, 1992; Macia-Silva and Garcia-Sainz, 1994). There is evidence that these adverse effects are closely related to oxidative stress processes and free radicals (Hermansky et al., 1990; Ding et al., 1998; Gehringer et al., 2003). DNA

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

Contract grant sponsor: National Basic Research Program of China (973 Program)

Contract grant number: 2008CB418101.

Published online 16 September 2009 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/tox.20532

damage has also been documented (Sekijima et al., 1999; Gehringer et al., 2003) as well as apoptosis, which is of great importance (Earnshaw, 1995).

Apoptosis is a tightly controlled process in which cell death is executed for maintaining steady state under physiological conditions and for responding to various stimuli. BCL-2 family members are of particular importance as they are potent regulators of apoptosis that can influence the permeability of the outer mitochondrial membrane (Borner, 2004; Sharpe et al., 2004; Donovan and Cotter, 2004). This family consists of both pro- and antiapoptotic members that elicit opposing effects on mitochondria, including the antiapoptotic protein Bcl-2 and the proapoptotic proteins Bax. Proapoptotic and antiapoptotic proteins of Bcl-2 family can heterodimerize and can modify one another's function. Since p53 gene is an important gene that regulates apoptosis (Symonds et al., 1994), apoptotic cell death is suppressed if p53 gene is mutated and its function is damaged. Wild-type 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 of cell death through Bax/Bcl-2 imbalances. Furthermore, p53 is a phosphoprotein, it has been shown that *in vivo* phosphorylated p53 can be dephosphorylated *in vitro* by PP2A (Scheidtmann et al., 1991). Besides, it has been found that PP2A inhibitor can induce hyperphosphorylation of p53 and increased apoptosis (Yan et al., 1997; Milczarek et al., 1999). Hence, there is the possibility that changes in the phosphorylation of p53 induced by PP2A inhibitor MC-LR have effects on its activity and turnover.

On the basis that MCs can induce rat testis cell apoptosis both *in vivo* and *in vitro* (Li et al., 2008; Zegura et al., 2008; Xiong et al., 2009), and that p53, Bax and Bcl-2 protein are vital regulator of apoptosis, it is of consequence to find out whether the expression of p53, Bax, and Bcl-2 protein are involved in the mechanisms of MCs induced apoptosis. The present study was undertaken to determine the expression level of p53 and Bax, Bcl-2 in rat testis, so the results will deepen our understanding about the toxicology mechanism of MCs on male rat reproductive system. This is the first study to investigate the molecular mechanism in MCs-induced apoptosis in reproductive system.

MATERIALS AND METHODS

Toxin

Cyanobacteria (mainly composed of *Microcystis aeruginosa*) were collected from surface blooms in Lake Dianchi, Yunnan Province, China, by a boat equipped with mechanical apparatus for cyanobacterial removal during the period of cyanobacterial blooms. In this study, freeze-dried algae cells were extracted three times with 75% methanol (V/V). The extract was centrifuged and the supernatant was applied to a C18 reversed phase cartridge, which had been preconditioned by washing with 100% methanol and distilled water. The cartridge was then washed with water and eluted with methanol. The elution was evaporated to dryness and the residue was dissolved in distilled water for the toxic experiment. MCs content in the extracts was determined by high performance liquid chromatography (HPLC, LC-10A, Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan). Determination of cellular MC was carried out according to Li et al. (2005). The microcystin content was 87 μg MC-LR equivalent/mL, among which MC-RR and -LR were 167.7 μg/mL and 47.0 μg/mL, respectively. Crude microcystins extracts were finally suspended in salt solution water (0.9% NaCl).

LD50 of Cyanobacterial Crude Extracts Containing MCs

Male Wistar rats ($n = 40$) were divided into five groups, and rats in each group were administered by an i.v. injection of MCs at different dose of MC-LR equivalent/kg body weight. Calculating the death number and mortality of rats in each group during 24 h, we obtained the LD50 level for 24 h by using the formula $LD_{50} = \log_{-1}[X_m - I(\Sigma p - 0.5)]$ (X_m: the log of max dose; p: mortality; Σp: the sum of mortality in each group; I: the difference between the log of adjacent two group dose). 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.

Experimental Protocol

Healthy male Wistar rats weighing 200 $\pm$ 20 g were divided into two groups randomly. One group received intravenous injection of 1 mL microcysitn extracts at LD_{50} of 87 μg MC-LR equivalent/kg body weight. As control, the other group was injected with the same volume of 0.9% saline solution. Rats in the two groups were sacrificed at 0, 1, 2, 4, 6, 12, 24 h postinjection, respectively. Five rats were sacrificed at each time point. The testis samples were quickly removed, minced, and stored frozen in liquid nitrogen until RNA isolation. All procedures carried out on animals were approved by the Institutional Animal Care and Use Committee, and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory.

Total RNA Isolation

Total RNA was isolated from 50 to 100 mg sections of testis using Trizol reagent (Invitrogen) and quantified by

TABLE I. Real-time PCR primers used in this experiment

Target gene	Primer Sequence (5′–3′)		Size (bp)
	Forward	Reverse	
p53	CTACTAAGGTCGTGAGACGCTGCC	TCAGCATACAGGTTTCCTTCCACC	106
Bax	CCAGGACGCATCCACCAAGAAGC	TGCCACACGGAAGAAGACCTCTCG	136
Bcl-2	GGATGACTTCTCTCGTCGCTACCGT	ATCCCTGAAGAGTTCCTCCACCAC	118
GAPDH	ATGGAGAAGGCTGGGGCTCACCT	AGCCCTTCCACGATGCCAAAGTTGT	209

determination at OD260. RNA was extracted according to the manufacturer's protocol, resuspended in 50 μL RNase-free water, and stored at -80°C. Quantification was done using Eppendorf Biophotometer (Germany). The purified total RNA (2 μg) was then reverse transcribed. Reverse transcription was performed with oligo (dT) 18 primer using first strand cDNA synthesis kit (TOYOBO, Japan). The resultant cDNA was then diluted 20-fold and kept at -20°C.

Quantitative Real-Time PCR (Q-PCR) to Determine the Levels of Gene Expression

All the primers used in Q-PCR were listed in Table I. The Gene-specific primers were designed based on the gene sequences of *Rattus norvegicus* present on the NCBI homepage (http://www.ncbi.nlm.nih.gov). The specification of each pair of primers was confirmed by randomly sequencing six clones, and further confirmed by the melting curve analysis using Q-PCR. The amplification efficiency of each pair of primers was tested by constructing corresponding plasmid. Only primers with similar amplification efficiency were used in this experiment. GAPDH was used as the internal control gene for Q-PCR assay. Q-PCR was conducted with the SYBR Green qPCR kit (Finnzymes, Finland) on a Chromo4 Real-Time Detection System (MJ Research, Cambridge, MA). The reactions were performed in a 20-μL volume mix containing 10 μL SYBR Green I mixture, 1 μL primers, 1 μL cDNA, and 1 μL sterile, distilled-deionized water. Cycling conditions were as follows: 3 min at 95°C, 44 cycles of 15 s at 95°C, 20 s at 60°C, and 15 s at 72°C. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected. Each sample was run in three tubes, and PCR reactions without the addition of the template used as blanks. After completion of the PCR amplification, data were analyzed with the Option Monitor software 2.03 version (MJ Research, Cambridge, MA).

Western Blotting

Tissues were homogenized for extract proteins in ice-cold protein extraction buffer (Wuhan Boster Biological Technology Company, China). The tissue homogenates were centrifuged at 12,000 $\times$ *g* for 10 min and supernatants were collected. Bradford method was used to determine the concentration of proteins. About 20 μg of protein from each sample was separated on SDS-PAGE and electrophoretically transferred on to a nitrocellulose membrane 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. Blocked membranes were incubated in rabbit polyclonal antibodies specific for rat p53, Bax, and bcl-2 (Santa Cruz, Santacruz, CA) or GAPDH (Wuhan Boster Biological Technology Company, China), in PBS (containing 1% nonfat dry milk) overnight at 4°C. Anti-rabbit secondary antibody (Wuhan Boster Biological Technology Company, China), at a concentration of 1:1000 in PBS (containing 1% nonfat dry milk) were added to membranes and incubated for 1.5 h at room temperature. The protein signal was developed using NBT/BCIP system. The results of Western blots were quantified with Gene Snap software (Syngene).

Statistics

All values are expressed as mean $\pm$ standard deviation. One-way ANOVA was used to elucidate if there are significant differences between the treatment groups and the control group ($P < 0.05$).

RESULTS

mRNA Level of p53, Bax, and Bcl-2

Figure 1 showed the transcriptional changes of p53, Bax, and Bcl-2 in the rat testes within 24 h. The transcription of p53 was significantly induced since 1 h postinjection, and reached a peak value about sixfold at 12 h and did not return to the original level even at 24 h. Bax mRNA level was induced at a time-dependent way from 1 h to 6 h postinjection and reached to the maximum about four-fold to the control at 6 h, after then it did not reverted to the control level and was induced about three-fold even at 24 h. The transcription of Bcl-2 was relatively stable from 1 h to 4 h postinjection, but decreased from 6 h. Generally, the

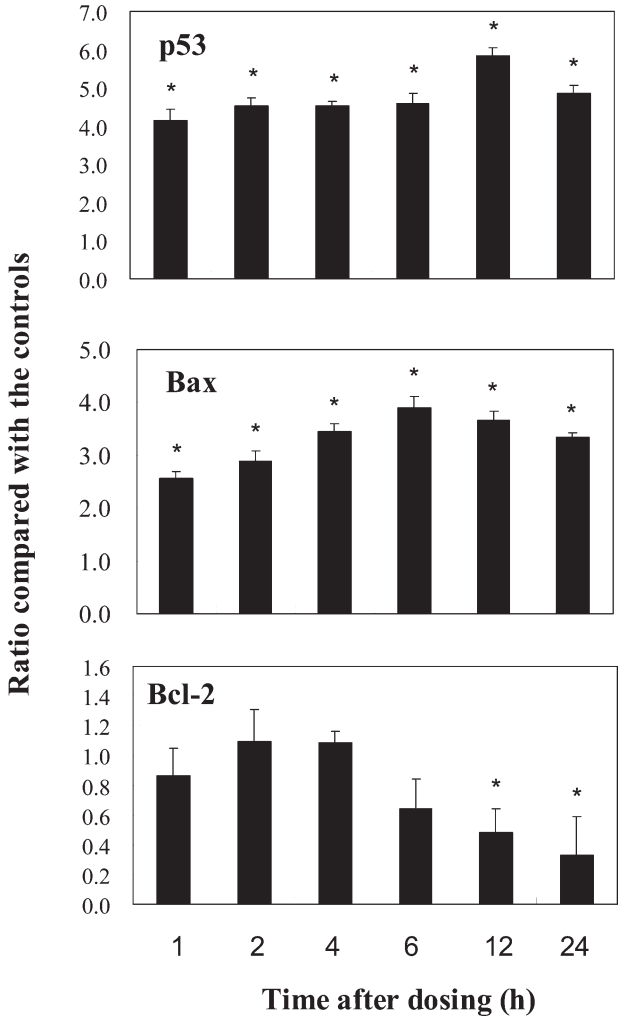

Fig. 1. Time course of p53, Bax, and Bcl-2 mRNA expression by Q-PCR analysis in rat testis after treatment with 87 μg MC-LRequivalent/kg body weight. (*indicates significant change at $P < 0.05$).

mRNA expressions of p53 and Bax were all significantly induced, while Bcl-2 expression was suppressed.

Western Blot Analysis

The effect of MCs on protein expressions of p53, Bax, and Bcl-2 in Wistar rat testes was shown in Figure 2(A). Both P53 and Bax proteins were induced obviously since 1 h after MCs treatment, and the high expression level remained throughout the experiment. Quantification of the two proteins revealed that the expressions of both p53 and Bax reached the maximum at 6 h and 4 h, respectively. In addition, Bcl-2 expression was inhibited from 1 h to 24 h postinjection with MCs and decreased sharply from 6 h to 24 h Figure 2(B).

Figure 2(C) illustrated the effect of MCs on expression of in rat testes tissue. The Bax/Bcl-2 ratio showed a

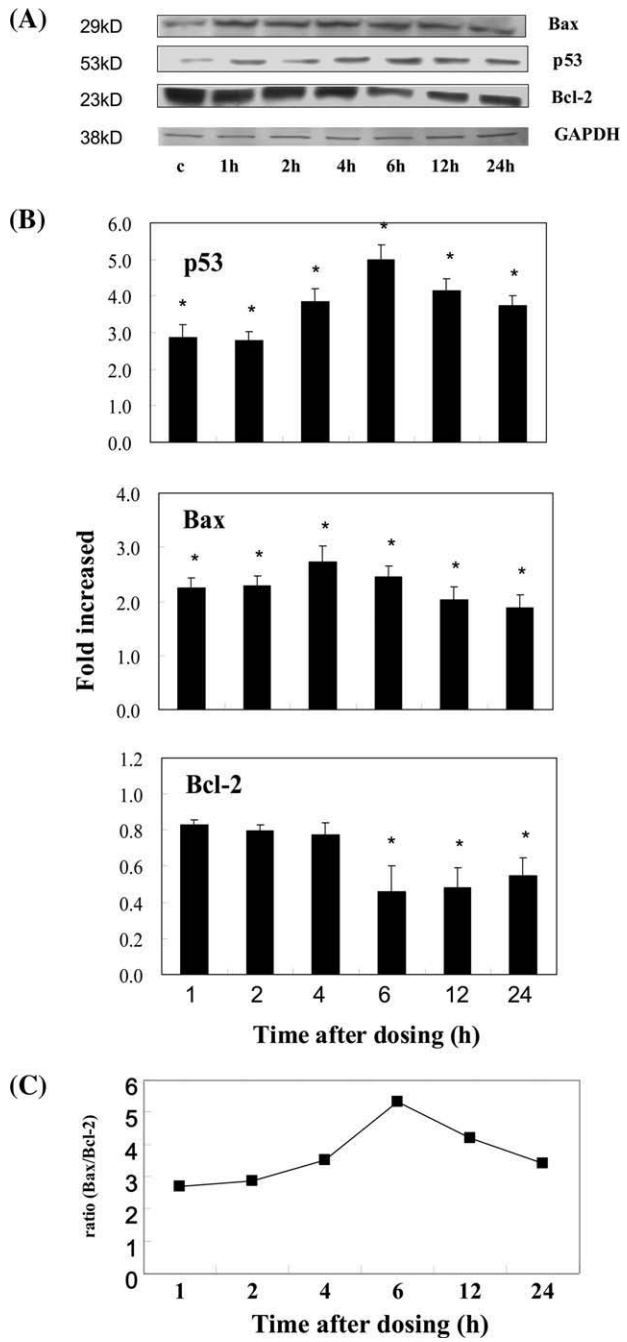

Fig. 2. Western blot analysis of proteins from rat testes with antibodies to p53, Bax, Bcl-2, and GAPDH. (A) Western blot analysis was performed with antibody against p53, Bax, Bcl-2, and GAPDH. Each figure corresponds to a representative experiment out of three experiments. Each column and bar represents the mean ± SD of three individual samples. (B) Mean protein expression in each treated groups is shown as increased compared with mean expression in control groups which has been ascribed an arbitrary value of 1 (*indicates significant change at $P < 0.05$). (C) Results are expressed as ratio of optical density presents in Bax *versus* Bcl-2 band.

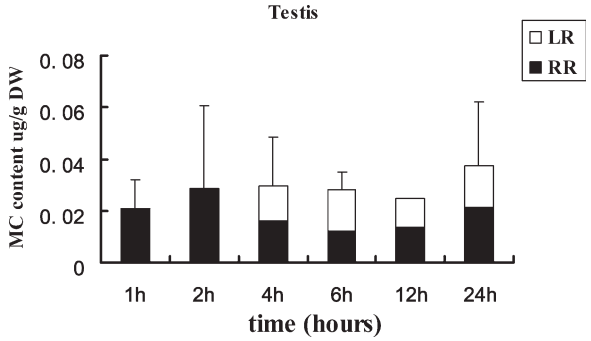

Fig. 3. Microcystin (MC) contents in Wistar rat testis after i.v. injection with 80 μg MC-LRequivalent/kg body weight. (Reproduced with permission from Wang et al., Toxicon, 2008, 52, 721-727, © Pergamon Press).

significant increase over control value. There was a time-dependent increase of the Bax/Bcl-2 ratio assayed from 1 h to 6 h exposure and reached to the maximum at 6 h and a little decrease from 12 h to 24 h. The elevation of the Bax/Bcl-2 ratio was evident from 1 h onward.

DISCUSSION

In recent years, numerous cosmopolitan lethal animal poisonings and a number of cases of human illness caused by cyanobacteria bloom and their toxins have drawn the attention of the World Health Organization, an increasing scientific community, and the public to cyanobacteria bloom and their toxins (Hallegeff, 1993). Several recent studies indicated that MCs could effectively accumulate in gonads and damage male reproductive system and the gonads have been thought to be the second target organ of MCs (Chen and Xie, 2005; Ding et al., 2006; Wang et al., 2008). It was also found by our previous results that MCs could accumulate in testis (Fig. 3) and induce rat testis cell apoptosis (Wang et al., 2008; Xiong et al., 2009), but the mechanisms of MCs-induced apoptotic cell death in rat testis are little known. The present study was designed to evaluate the time-dependent responses of the apoptosis related genes such as Bcl-2, Bax, and p53 after exposed to MCs, and further to elucidate the underlying toxicological mechanisms. Our results demonstrate that (1) accumulation of MCs in rat testis (Fig. 3) lead to persistent increase of p53 expression, which may regulate the expression of Bax and Bcl-2; (2) Bax is up-regulated and Bcl-2 is down-regulated following exposure of MCs, resulting in an increased ratio of Bax to Bcl-2, which may contribute to MCs-induced apoptotic cell death in rat testis.

It has been well studied that many stimuli such as ischemia, irradiation, excitotoxicity can induce an increased expression of p53, consequently leading to tissues or cell line apoptosis (Hughes et al., 1996; Zegura et al., 2008; Xiong et al., 2009). p53 is a multifaceted nuclear phosphoprotein. Under normal conditions, p53 levels are maintained at a low state by virtue of the extremely short half-life of the polypeptide but were induced in response to cellular stress, functioning as a transcriptional transactivator in DNA repair, apoptosis, and tumor suppression pathways (Lakin and Jackson, 1999). Induction of p53 is associated with a rapid increase in its levels and with an increased ability to bind DNA and mediate transcriptional activation (Lakin and Jackson, 1999). The products encoded by p53 can regulate the expression of Bax, which may mediate p53-dependent cell apoptosis (Xiang et al., 1998; Chen et al., 2005a,b). It was reported that p53 might lead to apoptosis indirectly by down-regulating expression of bcl-2 (Miyashita et al., 1994). Bax can promote apoptosis by homodimerizing or heterodimerizing with Bcl-2. Therefore, the alteration of Bax to Bcl-2 ratio appears to determine whether some cells live or die (Oltavi et al., 1993). In this study, we found that MCs induced a prolonged expression of P53, and also caused the continuous increase of Bax, but led to decrease of Bcl-2 after its transient stable after MCs treatment. The expression of P53, Bax, and Bcl-2 was significantly altered in rat testis following MCs treatment, suggesting their important roles in testis damage elicited by MCs. The up-regulation of Bax expression after MCs exposure in rat testis may be associated with the increased expression of p53, which functions in a pathway to promote the transcription and translation of Bax (Miyashita et al., 1994); on the other hand, increase of p53 products may down regulate the expression of Bcl-2 (Miyashita et al., 1994), consequently leading to an increased ratio of Bax to Bcl-2.In the present study, the Bax/Bcl-2 ratio showed a time-dependent increase and peaked at 6 h, which was in consistent with our previous study that the level of apoptosis rate peaked at 6 h after treatment and the elevation of the incidence of apoptosis was evident from 1 h onward in the rat testis exposed to MCs (Xiong et al., 2009). Therefore, it suggests that the increased ratio of Bax to Bcl-2 may play important roles in MCs-induced apoptosis in rat testis. Our findings are similar to several reports from other groups with respect to the mechanisms of apoptosis in testicular germ cells induced by ischemia, irradiation, and excitotoxicity. For example, Maheshwari et al. (2009) demonstrated that H_2O_2-elicited apoptosis of testicular germ cells is associated with activation of the p53, Bax, Fas/Fasl, and a simultaneous decrease in the Bcl-2 expression. It was also observed by Chaki et al. (2006) that an elevated expression of cell death effector Bax may contribute to the rat germ cell apoptosis with estradiol-3-benzoate administered.

Although the signal pathways underlying MCs-induced testis apoptosis and alterations in expression of apoptosis-related proteins p53, Bax, and Bcl-2 in rat testis are poorly understood, there are several mechanisms that can be suggested. In liver of mice, it was found that low doses (50 μg/kg) of MC-LR lead to apoptosis primarily through

the Bid-Bax-Bcl-2 pathway, whereas high doses of MC-LR (70 μg/kg) caused apoptosis via a reactive oxygen species pathway (Chen et al., 2005a,b). Clark et al. (2007) demonstrated increased hepatic upregulation of p21 and Bax in mice after 28 days exposure to MC-LR at sublethal levels. There was also a study that suggests that the testicular apoptosis might be closely related to nitric oxide (NO) in rats (El-Gohary et al., 1999). Moreover, we previously confirmed that MCs induce the expression of Fas/FasL system related genes at both mRNA and protein level in rat testis, indicating the likely participation of the Fas/FasL system in MCs-induced germ cell apoptosis (Xiong et al., 2009).

In summary, the results of the present study indicate that MCs lead to persistent increase of transcriptional and protein level of p53 and Bax expression but led to decrease of Bcl-2 expression, resulting in an increased ratio of Bax to Bcl-2, which may contribute to apoptotic cell death of rat testis following MCs treatment. However, further investigations remain to be performed to establish the major mechanisms underlying the altered expression of apoptosis-related genes and the roles played by these genes in testis damage induced by MCs.

The authors express their sincere thanks to Dr. Paul Tchounwou and two anonymous reviewers for their useful comments and suggestions on our manuscript.

REFERENCES

Borner C. 2004. Bcl-2 family members: Integrators of survival and death. Biochim Biophys Acta 1644:71–72.

Chaki SP, Misro MM, Gautam DK, Kaushik M, Ghosh D, Chainy GB. 2006. Estradiol treatment induces testicular oxidative stress and germ cell apoptosis in rats. Apoptosis 11:1427–1437.

Chen J, Xie P. 2005. Tissue distributions and seasonal dynamics of the hepatotoxic microcystins-LR and -RR in two freshwater shrimps, *Palaemon modestus* and *Macrobrachium nipponensis*, from a large shallow, eutrophic lake of the subtrophical China. Toxicon 45:615–625.

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

Chen J, Xie P, Guo LG, Zheng L, Ni LY. 2005b. Tissue distributions and seasonal dynamics of the hepatotoxic microcystins-LR and -RR in a freshwater snail (*Bellamya aeruginosa*) from a large shallow, eutrophic lake of the subtropical China. Environ Pollut 134: 423–430.

Chen J, Xie P, Li L, Xu J. 2009. First identification of the hepatotoxic microcystins in the serum of a chronically exposed human population together with indication of hepatocellular damage. Toxicol Sci 108:81–89.

Chorus I, Bartram J. 1999. Toxic Cyanobacteria in Water: A Guide to Public Health Consequences, Monitoring, and Management. London: E&FN Spon on behalf of WHO. pp 416.

Clark SP, Davis MA, Ryan TR, Searfoss GR, Hooser SB. 2007. Hepatic gene expression changes in mice associated with prolonged sublethal microcystin exposure. Toxicol Pathol 35:594–605.

Ding XS, Li XY, Duan HY, Chung IK, Lee JA. 2006. Toxic effects of microcystis cell extracts on the reproductive system of male mice. Toxicon 48:973–979.

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

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.

Earnshaw WC. 1995. Nuclear changes in apoptosis. Curr Opin Cell Biol 7:337–343.

El-Gohary M, Awara WM, Nassar S, Hawas S. 1999. Deltamethrin-induced testicular apoptosis in rats: The protective effect of nitric oxide synthase inhibitor. Toxicology 132:1–8.

Falconer IR, Yeung DS. 1992. Cytoskeletal changes in hepatocytes induced by Microcystis toxins and their relation to hyperphosphorylation of cell proteins. Chem Biol Interact 81:181–96.

Ferrão-Filho AS, Kozlowsky-Suzuki B, Azevedo SMFO. 2002. Accumulation of microcystins by a tropical zooplankton community. Aquat Toxicol 59:201–208.

Gehringer MM, Govender S, Shah M, Downing TG. 2003. An investigation of the role of vitamin E in the protection of mice against microcystin toxicity. Environ Toxicol 18:142–148.

Hallegeff GM. 1993. A review of harmful algal bloom and their apparent global increase. Phycologia 32:79–99.

Hermansky SJ, Stohs SJ, Markin RS, Murray WJ. 1990. Hepatic lipid peroxidation, sulfhydryl status, and toxicity of the blue-green algal toxin microcystin-LR in mice. J Toxicol Environ Health 31:71–91.

Hughes PE, Alexi T, Yoshida T, Schreiber SS, Knusel B. 1996. Excitotoxic lesion of rat brain with quinolinic acid induces expression of p53 messenger RNA and protein and p53-inducible genes Bax and Gadd-45 in brain areas showing DNA fragmentation. Neuroscience 74:1143–1160.

Lakin ND, Jackson SP. 1999. Regulation of p53 in response to DNA damage. Oncogene 18:7644–7655.

Li L, Xie P, Chen J. 2005. In vivo studies on toxin accumulation in liver and ultrastructural changes of hepatocytes of the phytoplanktivorous bighead carp i.p.-injected with extracted microcystins. Toxicon 46:533–545.

Li Y, Sheng J, Sha Jh, Han XD. 2008. The toxic effects of microcystin-LR on the reproductive system of male rats in vivo and in vitro. Reprod Toxicol 26:239–245.

Macia-Silva M, Garcia-Sainz JA. 1994. Inhibition of hormone-stimulated inositol phosphate production and disruption of cytoskeletal structure. Effects of okadaic acid, microcystin. Chlorpromazine, W7 and nystatin. Toxicon 32:105–112.

Maheshwari A, Misro MM, Aggarwal A, Sharma RK, Nandan D. 2009. Pathways involved in testicular germ cell apoptosis induced by H_2O_2 in vitro. FEBS J 276:870–881.

Milczarek GJ, Chen W, Gupta A, Martinez JD, Bowden GT. 1999. Okadaic acid mediates p53 hyperphosphorylation and

growth arrest in cells with wild-type p53 but increases aberrant mitoses in cells with non-functional p53. Carcinogenesis 20:1043–1048.

Miyashita T, Krajewski S, Krajewska M, Wang HG., Lin HK, Liebermann DA, Hoffman B, Reed JC. 1994. Rumor suppressior p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene 9:1799–1805.

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

Nishiwaki-Matsushima R, Nishiwakl S, Ohta T, Yoshizawam S, Suganuma M, Harada K, Watanabe MF, Fujiki H. 1991. Structure-function relationships of microcystins, liver tumor promoters, in interaction with protein phosphatase. Jpn J Cancer Res 82:993–996.

Oltavi ZN, Milliman CL, Korsmeyer SJ. 1993. Bcl-2 heterodimerizes in vivo with a conserved homolog. Bax that accelerates programmed cell death. Cell 74:609–619.

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

Sahin A, Tencalla FG, Dietrich DR, Naegeli H. 1996. Biliary excretion of biochemically active cyanobacteria (blue-green algae) hepatotoxins in fish. Toxicology 106:123–130.

Scheidtmann KH, Mumby MC, Rundell K, Walter G. 1991. Dephosphorylation of simian virus 40 large-Tantigen and p53 protein by protein phosphatase 2A: Inhibition by small-t antigen. Mol Cell Biol 11:1996–2003.

Sekijima M, Tsutsumi T, Yoshida T, Harada T, Tashiro F, Chen G, Yu SZ, Ueno Y. 1999. Enhancement of glutathione *S*-transferase placental-form positive liver cell foci development by microcystin-LR in aflatoxin B1-initiated rats. Carcinogenesis 20:161–165.

Sharpe JC, Arnoult D, Youle RJ. 2004. Control of mitochondrial permeability by Bcl-2 family members. Biochim Biophys Acta 1644:107–113.

Symonds H, Krall L, Remington L, Saenz-Robles M, Lowe S, Jacks T, Van Dyke T. 1994. p53-dependent apoptosis suppresses tumor growth and progression in vivo. Cell 78:703–711.

Wang Q, Xie P, Chen J, Liang G. 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.

Xiang H, Kinoshita Y, Knudson CM, Korsmeyer SJ, Schwartzkroin PA, Morrison RS. 1998. Bax involvement in p53-mediated neuronal cell death. J Neurosci 18:1363–1373.

Xiong Q, Xie P, Li HY, Hao L, Li GY, Qiu T, Liu Y. 2009. Involvement of Fas/FasL system in apoptotic signaling in testicular germ cells of male Wistar rats injected i.v. with microcystins. Toxicon 54:1–7.

Yan Y, Shay JW, Wright WE, Mumby MC. 1997. Inhibition of protein phosphatase activity induces p53-dependent apoptosis in the absence of p53 transactivation. J Biol Chem 272:15220–15226.

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