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A Cyanobacterial Toxin, Microcystin-LR, Induces Apoptosis of Sertoli Cells by Changing the Expression Levels of Apoptosis-Related Proteins

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Toxic cyanobacterial blooms in freshwater have been considered as threats to human health. Microcystins are a family of cyclic polypeptides produced by cyanobacteria and are toxic to plants and animals. Microcystin-LR (MC-LR) is the most toxic variant among the microcystin family and could cause oxidative stress in various organs, including the reproduction system. The aim of this study was to investigate the effect of MC-LR on apoptosis of Sertoli cells that play an essential role in the development and maturation of sperm cells. Sertoli cells were isolated from healthy immature rats and cultured with MC-LR. The viability of Sertoli cells was decreased after treatment with MC-LR at 10 μg/ml for 24 h ($P < 0.05$). Moreover, the MC-LR-treated cells exhibited condensed chromatin and fragmented nuclei, features of apoptosis, as judged by Hoechst 33258 staining. We also analyzed the mRNA and protein levels of three apoptosis-related genes, p53, bax and bcl-2, using reverse transcription-polymerase chain reaction and Western blot analyses, respectively. Both p53 and bax function as promoters of apoptosis, while bcl-2 is an apoptotic suppressor. The mRNA and protein expression levels of p53 and bax were increased in Sertoli cells treated with MC-LR at 10 μg/ml compared with the control group ($P < 0.05$), while the bcl-2 protein levels were decreased in cells treated with MC-LR at 10 μg/ml ($P < 0.05$). Moreover, caspase-3 activity that is involved in the induction of apoptosis was significantly increased in Sertoli cells treated with MC-LR. These results indicate that MC-LR induces apoptosis of Sertoli cells.

Keywords: apoptosis; apoptosis-related gene; gene expression; microcystin-LR; Sertoli cell
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Toxic cyanobacterial blooms in freshwater have been threats to human health by causing diarrhea, vomiting, nausea and other damage (Botha et al. 2004). Microcystins are a family of cyclic polypeptides produced by various species of cyanobacteria, which can form water blooms under favorable conditions in lakes, ponds and reservoirs (Chorus et al. 2000). According to current reports, microcystins are a widely distributed group of cyanotoxins, and are also a frequent cause of acute human and animal poisonings (Carmichael 2001). Microcystin-LR (MC-LR) is the most toxic variant in the microcystin family, and has been studied most extensively (Hoeger et al. 2005). A previous study on fish revealed that microcystins could cause a massive weight loss and poor overall fitness of whitefish, suggesting that even a low-dose but long-term exposure to microcystin can cause health problems (Ernst et al. 2001).

A previous study has suggested that the toxicity of MCs is associated with liver disruption, and damage to the kidneys and intestines (Towner et al. 2002). MCs can result in cytoskeleton disruption in hepatocytes because of the disorganization of cytoplasmic microtubules, cytokeratin intermediate filaments and actin microfilaments (Falconer and Yeung 1992; Ding et al. 2000). In the MC-LR-treated primary human hepatocytes the actin mesh collapsed into the center of the cell, which was similar to what occurred in rat hepatocytes (Batista et al. 2003). It has been suggested that MCs are capable of inducing apoptosis in rat hepatocytes (Zegura et al. 2002). The capability of MCs to induce liver cell apoptosis *in vivo* has been confirmed in previous studies using mouse as a model (Guzman and Solter 1999; Hooser 2000; Chen et al. 2005; Weng et al. 2007). MC-LR exposure can cause apoptosis in mouse liver through two independent pathways (Chen et al. 2005). MCs are mainly excreted by the liver, although a small proportion (9%) can be eliminated through urine (Bischoff 2001). In addition, the toxic effects of MCs on kidney cells have been reported

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(Nobre et al. 1999; Milutinovic et al. 2003).

The distribution and accumulation of MC-LR in tissues have been investigated in various organs of freshwater snails (*Sinotaia histrica*) in a temperate eutrophic lake, Lake Suwa in Japan (Xie et al. 2007). The results revealed marked temporal variations in the microcystin content of various organs of the snails, and the gonad exhibited the second highest content of MCs.

The Sertoli cell is a highly specialized cell found in the testes. Sertoli cells play an important role in the development and maturation of sperm cells, and thus sometimes are referred to as a nurse cell. Chemical injury to Sertoli cells may lead to impaired male fertility (Zhang et al. 2008). In this study, we used a primary Sertoli cell culture model to study the toxicity of MC-LR in the reproduction system.

The molecular mechanisms underlying cell death have become one of the most significant topics in most fields, such as biology, immunology, and pathology. Apoptosis plays an important role in the physiological proceeding of cell development and growth, which represents the culmination of naturally occurring, or highly programmed mechanisms. Apoptosis is not only involved in the pathogenesis of some diseases, but can also induce pathological cell death (Thompson 1995). The synthesis of new proteins is required for apoptosis to proceed, indicating that genes including bax, bcl-2, p53, and caspase-3 are probably involved in apoptosis (Estus et al. 1994).

The Bcl-2 family of proteins function in regulating cell apoptosis. These proteins are located on the mitochondrial membrane, and regulate apoptosis partly by controlling the release of cytochrome c into the cytosol (Strasser et al. 2000). Bcl-2 is the first protein identified as a repressor of apoptosis (Reed 1997). Bcl-2 always binds to other proteins to form a complex network of homo- and heterodimers in order to not only prevent the increase of the mitochondrial permeability and release of cytochrome c, but also inhibit various caspases (Armstrong et al. 1996; Chinnaiyan et al. 1996).

Bax is a 21-kDa protein that can bind to Bcl-2 to form Bax/Bcl-2 heterodimers, which act as a promoter of cell death. The degree of bax binding to bcl-2 determines the susceptibility of a cell to apoptosis (Oltvai et al. 1993; Yin et al. 1994).

p53 is a tumor suppressor protein that is encoded by the TP53 gene in humans (Matlashewski et al. 1984; Isobe et al. 1986) and it plays an important role in the regulation of cell cycle in multicultural organisms. In addition, p53 plays an essential role in programmed cell death (PCD) because overexpression of p53 can lead to apoptosis in many types of cells.

Caspase-3 is a member of the caspase family of cysteine proteases. Caspases sequentially activate some signaling pathways to induce apoptosis. Caspases include initiator caspases and effector caspases, and caspase-3 is an effector caspase. In the early stage of apoptosis, caspase-3 cleaves substrates at their aspartate residues and activates the proteolytic activity (Harvey et al. 1998; Sakahira et al. 1998).

The aim of the present study was to determine the apoptosis-inducing effects of MC-LR in Sertoli cells and the underlying mechanisms of MC-LR toxicity to the reproductive system. The morphological changes of Sretoli cells were examined using the Hoechst 33258 staining method. The expression levels of three genes, bax, bcl-2, and p53 in Sertoli cells were determined using RT-PCR and western blot analyses. The caspase-3 activity in Sertoli cells was also measured.

Materials and Methods

Animals

Male Sprague-Dawley rats (18- to 20-day old) were purchased from the Experimental Animal Center of Henan Province (Zhengzhou, China). All experiments were approved by the Animal Ethics Committee of Zhengzhou University, China, and were conducted according to the ethical guidelines of the Laboratory Animal Care and Use Committee of the Association for Research in Vision and Ophthalmology. Efforts were made to minimize suffering and the number of animals used in the study.

Isolation and primary culture of Rat Sertoli cells

Primary culture of rat Sertoli cells was prepared using sequential enzymatic procedures that had been previously described (Mather et al. 1990) with modifications. Briefly, testes from 18- to 20-day old Sprague-Dawley rats (day of birth = day 0) were collected, excised rapidly, decapsulated, cut into small fragments, and washed twice in Hanks' balanced salt solution (HBSS). The fragments were then digested sequentially in 10 ml HBSS containing 0.25% trypsin (Amresco, Solon, OH, USA) and 0.1% collagenase (type I, Invitrogen, Grand Island, NY, USA) in a shaking water bath (35°C, 120 cycles/min) for 30 min. The digested cell suspension was washed extensively with no-phenol red-Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY, USA) to remove peritubular cells, followed by filtration using the B-D Falconcell strainers (nylon mesh size, 70 μg/ml). The final Sertoli cells suspension was supplemented with 5% fetal bovine serum (Invitrogen, Grand Island, NY, USA), and incubated in culture bottles in 5% CO_2 at 35°C. After 24 h, these cells were extensively washed twice with HBSS to remove the unattached cells, treated with 20 mM Tris-HCl (pH 7.4) for 5 min and with serum starvation for 24 h. The medium was changed every other day.

Measurement of cell viability

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide) assay was used to measure the viability of cells by measuring the activity of dehydrogenases, as described previously with modifications (Mosmann 1983). Briefly, Sertoli cells were seeded at $3\sim5\times10^3$ cells per well into a 96-well plate (Sarstedt, Shanghai, China) and allowed to attach. After 3~4 h, the initial viability was measured. The culture medium was aspirated. Cells were washed once with PBS-A (PBS without calcium and magnesium, with 5.4 mM EDTA, and 50 μl 0.5% MTT (resuspended in DMEM without FCS) was added to each well. After incubation for 2 h at 37°C, the MTT was aspirated. DMSO (200 μl) was added to each well, and gently swirled for 3 min. DMSO was transferred to a new

96-well plate and the optical density was measured at 570 nm.

Hoechst 33258 staining

After treated with MC-LR, cell cultures were washed twice with PBS and incubated with 2 μg/ml Hoechst 33258 (Beyotime, Nantong, China) for 1 h in dark at 37°C. After washed twice with PBS, the cells were viewed under a fluorescence microscope (Nikon, Tokyo, Japan) equipped with a UV filter. The images were recorded on a computer with a digital camera (DXM 1200, Nikon, Japan) attached to the microscope, and the images were processed with computer.

RT-PCR

Total RNA was extracted from the cells according to the manufacturer's suggested protocol. The first single-strand cDNA was synthesized according to the protocol of AMV RT kit. In the reverse transcription reaction system, 2 μg total RNA, 10 μl AMV buffer, 1.5 μg oligo(dT) (18) primer, and 1 μl of dNTPs (1.0 mM of each four deoxynucleotide triphosphates) were contained in a 50 μl reaction mixture. The mixture was incubated at 95°C for 5 min followed by addition with 40 U RNase, 10U AMV reverse transcriptase, and 7.5 μl 25 mM $MgCl_2$, and incubated at 42°C for 60 min. After that, the reverse transcriptase was inactivated at 95°C for 5 min. The PCR system contained the following: 6.0 μl cDNA product, 1.5 μl 10 × PCR buffer, 0.2 μl Taq polymerase, 0.5 μl primer 1 (1.0 mg/ml), 0.5 μl primer 2 (1.0 mg/ml), 1 μl dNTPs (10 mM), and 10.3 μl water. PCR was performed with 30 cycles of denaturation (95°C, 30 s), annealing (60°C, 45 s), and extension (72°C, 60 s). After PCR, the reaction mixture (2.5 μl) was electrophoresed in 2% agarose gel with ethidium bromide (0.5 mg/ml). The expression levels of p53, bax, and bcl-2 mRNAs were measured using densitometric analysis, and standardized to the β-actin control, using a digital imaging and analysis system (BiocaptMV software).

Western blot analysis

Collected cells were homogenized for extracting proteins in an ice-cold mild lysis buffer consisting of 1% Nonidet P40, 1 mM EDTA, 125 mM sodium fluoride, 0.5 mM sodium vanadate, 2.5 μg/ml of aprotinin, 5 μg/ml of pepstatin, 50 μg/ml of leupeptin, 25 mM PMSF, and 25 μg/ml of trypsin inhibitor. The tissue homogenates were centrifuged at 13,000 rpm for 15 min and the supernatants collected. SDS–polyacrylamide gel electrophoresis (PAGE) was performed with an equal amount of protein samples using precast Tris–HCl gels (12% resolving, 4% stacking) (Bio-Rad, Hercules, CA). Separated proteins were transferred to NC membranes (Millipore, Shanghai, China). Membranes were blocked in 5% nonfat dry milk in PBS buffer containing 0.1% Tween-20 for 1.5 h at room temperature. Blocked membranes were incubated in rabbit polyclonal primary antibodies specific for rabbit P53 (1:300), bax (1:200), bcl-2 (1:200), or β-actin (1:400) (Santa Cruz, Santacruz, CA) in 1 × PBS containing 0.1% Tween-20 and 5% BSA at 4°C overnight. Membranes were added with anti-rabbit secondary antibodies (1:6000; Jackson ImmunoResearch Laboratories, Shanghai, China) in 1 × PBS containing 0.1% Tween and 5% nonfat dry milk, and incubated at room temperature for 1.5 h. The protein signal was amplified and visualized via chemiluminescence using the ECL Western Blotting Detection System and Hyper film ECL autoradiography film (Amersham Pharmacia Biotech, Inc.). Images were quantified using the Lab works v3.0.2 image scanning and analysis software (Ultra-Violet Products Lds., Cambridge, UK).

Table 1. Primer sequences for p53, bcl-2, bax, and β-actin.

p53	Forward	5-GTCG GCTC CGAC TATA CCAC TATC-3
	Reverse	5-CTCT CTTT GCAC TCCC TGGG G-3
Bcl-2	Forward	5-CGGG AGAA CAGG GTAT GAT-3
	Reverse	5-CAGG CTGG AAGG AGAA GAT-3
Bax	Forward	5-CAGG GTTT CATC CAGG-3
	Reverse	5-TAGC AAAG TAGA AGAG GG-3
β-actin	Forward	5-GCCA TGTA CGTA GCCA TCCA-3
	Reverse	5-GAAC CGCT CATT GCCG ATAG-3

Measurements of caspase-3 enzymatic activity

The Caspase-3 activity was measured using the colorimetric ApoAlert caspase 3 assay kit (ClonTech, Shanghai, China) according to the manufacturer's instructions. Briefly, following animal dissection, collected cells were immediately washed in ice-cold PBS. Cell suspensions were centrifuged at 400 g for 5 min. Cell pellets were resuspended in 50 μl chilled Cell Lysis Buffer and were incubated on ice for 10 min. Cell lysates were centrifuged at 14,000 g at 4°C for 10 min, to precipitate cellular debris. Supernatants were transferred to a 96-well plate placed on ice, added with 50 μl reaction buffer and 5 μl caspase-3 substrate-pNA (Ac-DEVD-pNA) to each well, and incubated at 37°C for 1 h. Samples were measured at 405 μg/ml in a microplate reader (B-R Model 550; Bio-Rad, Beijing).

Statistical analysis

All data are shown as mean ± S.D. Significance was assessed by ANOVA. Mean values were compared by subsequent student-Newman-Keuls (SNKs) using SPSS 12.0 software (SPSS Inc., Chicago, IL, USA). A difference at $P < 0.05$ was considered statistically significant. All assays were performed three times.

Results

The effects of MC-LR on the viability of rat Sertoli cells

Sertoli cells were treated with 0, 0.5, 1, 10, or 20 μg/ml MC-LR for 24 h, respectively, and the viability of Sertoli cells was assessed using MTT assay. MC-LR could accumulate for a long time in vivo, and might reach these ranges although the concentration is very low (Yu et al. 2009). Different cells have their different responses to MC-LR (Yi et al. 2011). As shown in Fig. 1, the viability of Sertoli cells was decreased after treatment with 10 or 20 μg/ml MC-LR ($P < 0.05$). Based on this result, MC-LR at concentration of 1 or 10 μg/ml was used in subsequent experiments.

Morphological changes of cell nuclei

The results of Hoechst 33258 staining showed that there were significant morphological changes in the nuclear chromatin (Fig. 2). When cells were treated with MC-LR for 24 h, the blue emission light in apoptotic cells was much brighter than the control cells. Furthermore, in contrast to the homogeneous staining in the control cells, many MC-LR-treated cells exhibited condensed chromatin and

fragmented nuclei that are hallmark characteristics of apoptosis.

MC-LR changes the expression levels of p53, bax and bcl-2

The mRNA expression levels of p53, bax and bcl-2 were determined using RT-PCR in Sertoli cells exposed to different doses of MC-LR (0, 1, and 10 μg/ml). The results from densitometric analysis of the band intensity were illustrated. The p53 mRNA level in cells treated with 10 μg/ml MC-LR was significantly increased compared with the control group ($P < 0.05$) (Fig. 3A). The bax mRNA levels were significantly higher in Sertoli cells treated with 10 μg/mL MC-LR than those in the control group ($P < 0.05$) (Fig. 3B). Moreover, compared with the control group, the mRNA level of bcl-2 was increased in the Sertoli cells treated with 1 μg/ml MC-LR, but decreased in cells treated with 10 μg/ml MC-IR. These changes were statistically significant ($P < 0.05$) (Fig. 3C).

The expression levels of p53, bax and bcl-2 proteins were determined in Sertoli cells exposed to 0, 1, or 10 μg/ml MC-LR by western blot assay. The intensity of each band was shown in Fig. 4A. The expression levels of p53 were significantly higher in Sertoli cells treated with 1 μg/ml or 10 μg/ml MC-LR than those in the control group ($P < 0.05$) (Fig. 4B). Compared with the control group, the expression level of bax was increased in the Sertoli cells treated with MC-LR at 10 μg/mL (Fig. 4C; $P < 0.05$), but the expression of bcl-2 protein was decreased (Fig. 4D; $P < 0.05$).

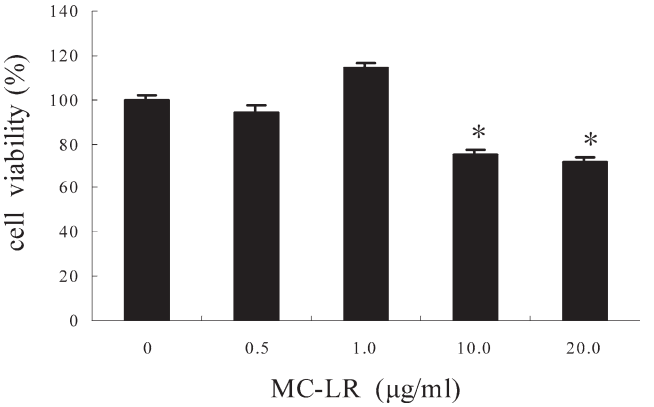

Fig. 1. Effect of MC-LR on the viability of Sertoli cells. Sertoli cells were exposed to MC-LR (0-20 μg/ml) for 24 h. *Indicates a significant difference compared to control ($p < 0.05$).

Caspase-3 enzymatic activities

The caspase-3 activity was measured in Sertoli cells treated with MC-LR (at 0, 1, or 10 μg/ml, Fig. 5). Treatment with MC-LR at 10 μg/ml caused the significant increase in the caspase-3 activity of Sertoli cells, compared with the control group ($P < 0.05$).

Discussion

Microcystins are a group of heptapeptide toxins produced by cyanobacteria in eutrophic freshwater. MC-LR is one of the most toxic and abundant variants of microcystin in blooms. Microcystins have been suggested to be threats

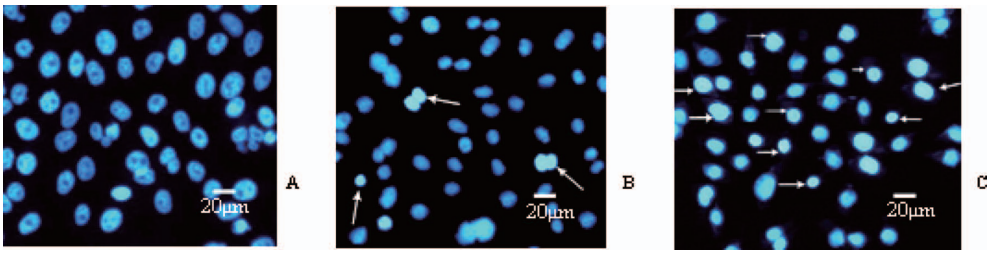

Fig. 2. Morphological changes in the apoptotic cells revealed by Hoechst 33258 staining (× 200). A: Untreated Sertoli cells; B: Sertoli cells treated with 1 μg/mL MC-LR for 24 h; C: Sertoli cells treated with 10 μg/mL MC-LR for 24 h. Note that arrows indicate apoptotic cells in B and C.

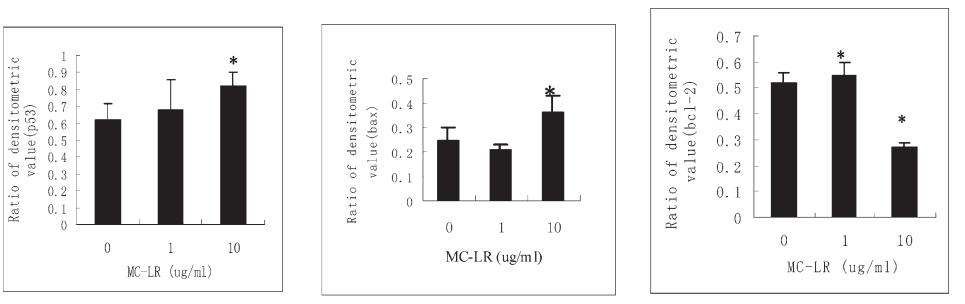

Fig. 3. Relative expression levels of p53, bax, and bcl-2 mRNAs in Sertoli cells treated with MC-LR. Bar graphs in A, B and C show the normalized expression levels of p53, bax, and bcl-2 mRNAs respectively. In each group ($n = 6$), error bar represents S.D. Statistical significance was analyzed using one-way ANOVA, * $p < 0.05$.

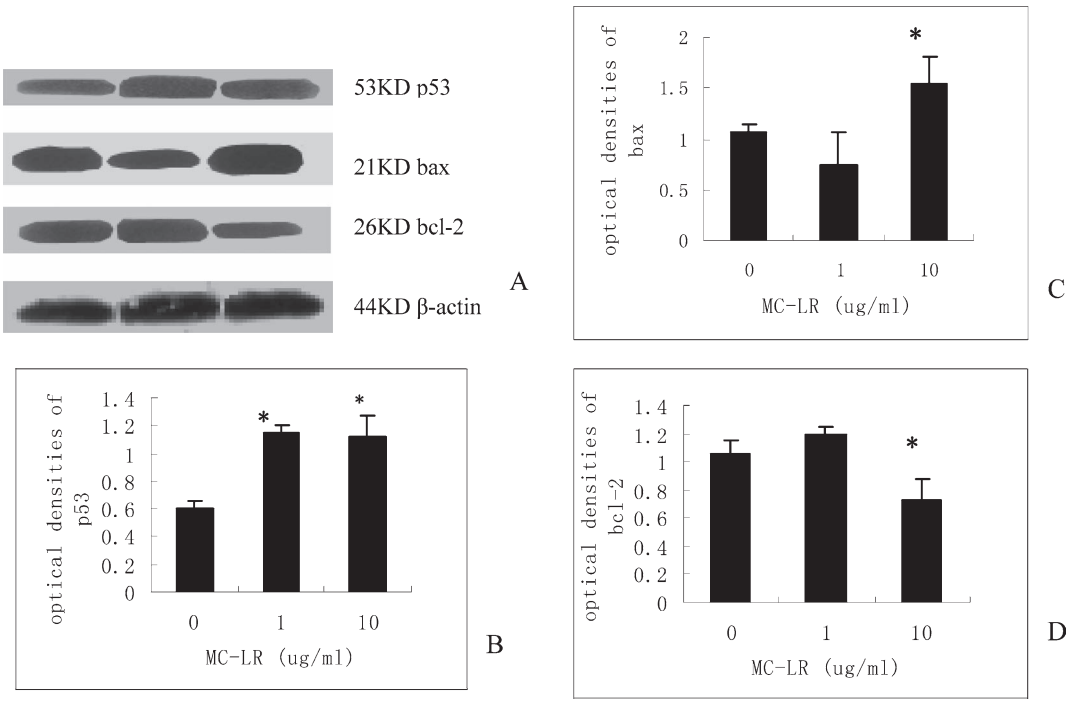

Fig. 4. Western blot analysis of p53, bax, and bcl-2 proteins in Sertoli cells treated with MC-LR
The Western blot analysis was performed using antibodies against p53, bax, bcl-2 or actin (A). Each blot corresponds to a representative experiment out of six experiments. Bar graphs in B, C and D show the normalized expression levels of p53, bax, and bcl-2 proteins, respectively. In each group ($n = 6$), error bar represents S.D. *$p < 0.05$.

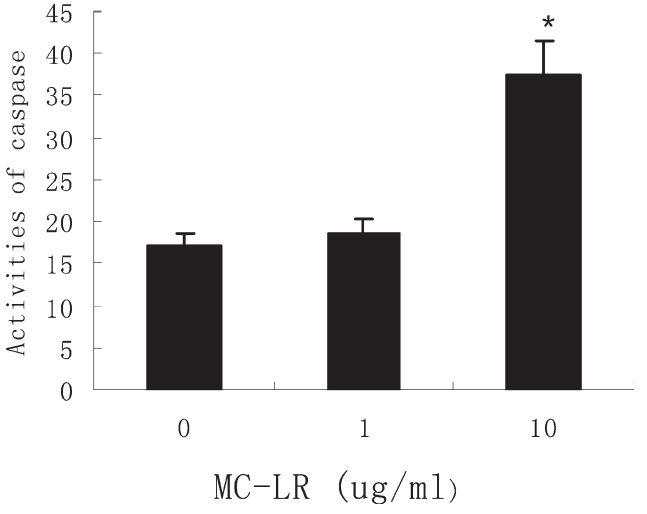

Fig. 5. Effects of MC-LR exposure on the caspase-3 activity in Sertoli cells.
In each group ($n = 6$), error bar represents S.D. * $p < 0.05$.

to wildlife, livestock and even humans. In recent years, numerous cosmopolitan lethal animal poisonings and cases of human illness caused by toxic cyanobacteria blooms have caused a great deal of attention of the public (Dziga et al. 2007; Jiang et al. 2008)

The main mechanism underlying the toxicity of microcystins is to inhibit the protein serine/threonine phosphatases PP1 and PP2A to enhance protein phosphorylation and cell toxicity in direct relevant tumors (Ackintosh et al. 1990; Yoshizawa et al. 1990; Carmichael 1994) and to make the cytoskeletal proteins highly phosphorylated. Moreover, microcystins regulate cell apoptosis by controlling protein phosphorylation (Fu et al. 2005). Researchers have proved that exposure to low level MC-LR can promote apoptosis in many types of cells (Botha et al. 2004; Zegura et al. 2008). A number of studies have focused on the deleterious effects of MC-LR (Li et al. 2010; Xiong et al. 2010; Zegura et al. 2011). It has been widely accepted that male reproductive organs are particularly susceptible to the deleterious effects of reactive oxygen species (ROS), which ultimately lead to impaired fertility (Williams et al. 1998). Our previous study reported that MC-LR exposure can cause changes in oxidative stress on rat Sertoli cells, such as decreased antioxidative enzyme activity and increased ROS activity (Yi et al. 2011). The production of ROS has been suggested to be involved in programmed cell death under many conditions including chemical injury (Buttke and Sandstrom 1994; Tan et al. 1998; Kannan and Jain 2000).

Apoptosis is an active process of cellular self-destruction that requires the expression of specific genes, including bax, bcl-2, p53, and caspase-3. Many apoptosis-inducing agents are either oxidants or stimulators of cellular oxidative metabolism. Conversely, many apoptosis inhibitors either have antioxidant activity or can enhance the cellular antioxidant defenses (Freeman and Crapo 1982). In the present study, we used rat Sertoli cells to study the reproductive toxicity of MC-LR. In addition to investigating the

morphological changes of Sretoli cells induced by exposure to MC-LR, we also analyzed the expression levels of three genes, bax, bcl-2, and p53, as well as the caspase-3 activity in rat Sertoli cells that were exposed to MC-LR.

p53 has been considered to be involved in the induction of apoptosis. It is a tumor suppressor gene which affects cell cycle through controlling the progression of the G1 stage (Van Gijssel et al. 1997). p53 appears to play an essential role in programmed cell death (PCD) since overexpression of p53 can induce apoptosis in a wide range of types of cells. In the present study, the expression level of p53 increased when Sertoli cells were exposed to MC-LR, suggesting that MC-LR induces apoptosis in Sertoli cells through modulating the expression of P53.

The Bcl-2 family of proteins includes pro-apoptotic and anti-apoptotic members (Nakazawa et al. 2003). When cell goes to apoptosis, pro-apoptotic protein Bax of the Bcl-2 family can translocate to the outer membrane of mitochondria, leading to the release of pro-apoptotic factors and inducing apoptosis. On the other hand, anti-apoptotic protein Bcl-2 of the Bcl-2 family can inhibit the release of pro-apoptotic factors and thus prevent apoptosis sequestering in mitochondria. When pro-apoptotic proteins interact with the anti-apoptotic proteins, the latter proteins lose their ability of inhibiting the release of pro-apoptotic factors, and apoptosis is subsequently promoted. Hence, altering the expression of anti-apoptotic and pro-apoptotic proteins of the Bcl-2 family could influence apoptosis (Yang et al. 2006). In the present study, MC-LR exposure resulted in an increase in the expression level of the pro-apoptotic protein bax but a decrease in the anti-apoptotic protein Bcl-2, suggesting that MC-LR induced apoptosis in Sertoli cells also through modulating the expression of the Bcl-2 family proteins.

Previous studies have demonstrated that caspase-3 is closely related to apoptosis induced cell morphological changes in several types of cells (Hirata et al. 1998; Janicke et al. 1998; Woo et al. 1998), suggesting that caspase-3 plays a central role in mediating nuclear apoptosis. Caspase-3 is essential for tissue development, and thus is probably important in some apoptotic scenarios in a remarkable tissue-, cell type- or death stimulus-specific manner. For instance, caspase-3-dependent signaling transduction mediates the morphological changes in nuclei during cell apoptosis (Porter and Janicke 1999). In the present study, the caspase-3 activity of Sertoli cells was increased in response to the exposure to MC-LR. Furthermore, condensed chromatin and fragmented nuclei, which are hallmark characteristics of apoptotic, were observed in a large number of MC-LR treated cells. These results indicated that caspase-3 is required for Sertoli cell apoptosis following MC-LR exposure, and is indispensable for apoptotic chromatin condensation and DNA fragmentation in Sertoli cells.

The present study provides direct evidence that MC-LR exposure can induce apoptosis relevant morphological changes, up-regulate the expression of p53, bax and caspase-3, and suppress the expression of bcl-2 in rat Sertoli cells. Hence, p53, bcl-2, bax and caspase-3 are probably involved in MC-LR-induced cell damage. Moreover, our study contributes to elucidate the toxicological mechanism underlying the effects of MC-LR on the productive system. Further work will focus on the toxicological role of MC-LR in apoptosis-related signaling pathways.

Acknowledgments

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Conflict of Interest

All authors have no conflict of interest.

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