Microcystin-LR induces oxidative stress and apoptosis in Chinese hamster ovary cells

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Abstract: Microcystin-LR (MC-LR) is the most common microcystins (MCs) due to its ubiquity, abundance and toxicity, and can cause oxidative stress in various organs, including the reproduction system. The aim of this study was to investigate the effect of MC-LR on oxidative stress and apoptosis of Chinese hamster ovary (CHO) cells. The viability of CHO cells was decreased after treatment with MC-LR at 5, 10, 15μg/ml, and the proliferation of CHO cells was declined at 2.5, 5, 10μg/ml MC-LR. MC-LR increased the content of MDA in CHO cells, indicating that MC-LR accelerated lipid peroxidation of CHO cells. MC-LR decreased the activity of CAT in CHO cells, indicating that MC-LR decreased the antioxidant ability of CHO cells. Moreover, the MC-LR also induced generation of reactive oxygen species (ROS), mitochondrial membrane potential loss, and cells apoptosis. Real-time PCR (qPCR) analysis of p53, bax, and bcl-2 genes revealed p53 and bax were up-regulated for about 1.5 fold, and bcl-2 was down-regulated in CHO cells after MC-LR treatment. Therefore, the acute exposure to MC-LR could promote generation of oxidative stress, depress cellular viability and cause cells apoptosis and the changes of apoptosis-related genes in CHO cells.

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Introduction

Microcystins (MCs) are a family of cyclic heptapeptide hepatotoxins, mainly produced by the various cyanobacterial blooms in eutrophic freshwater worldwide (1). Cyanobacterial blooms and their toxins seriously threaten both animals and humans health (2-6). So far, there have been more than 80 different structural analogues of MCs to be identified. Of these, MC-LR is considered as the most common and toxic MCs (7-8). MCs-treated whitefish had a massive weight loss and poor overall fitness, suggesting that health problems can be caused by a low-dose and long-term exposure to MCs (9).

MCs accumulated in multiple organs including kidney, gill and intestine, and cause damages to these organs (10-15). It had been proved that MCs could lead to severe liver damage, including massive intrahepatic hemorrhage, liver swelling, necrosis, cancer and even animal death (16-18). MCs caused the disorganization of cytoplasmic microtubules, cytokeratin intermediate filaments and actin microfilaments, sequentially resulted in cytoskeleton disruption in liver, and finally leaded to liver damage (19).

Phosphatases (PP1 and PP2A) inhibition is a well recognized toxic effect of MC-LR (1, 8, 20). However, the exact mechanisms of MC-LR toxicity have not been fully elucidated. Recently, many studies have indicated that the production of ROS may play a vital role in toxic effects of MC-LR, followed by oxidative stress and apoptosis (21-23).

In addition, the toxic effects of MCs on reproductive system have been reported (24). The distribution and accumulation of MC-LR in various organs of freshwater snails (Sinotaia histrica) were investigated in a temperate eutrophic lake (Lake Suwa) in Japan (25). There was marked temporal variation in the MC content of various organs of the snail, and the gonad was the second highest content of MCs. In our previous work, the effects of MC-LR on sertoli cells were analyzed, including oxidation stress and the expression of apoptosis-related genes (19). MC-LR had toxic effects on reproductive system of adult male rats. It had been reported that MC-LR decreased sperm motility, testis weight and sperm concentration (13, 24).

The sufficient evidence had been provided for testifying the noxious effects of MC-LR on various kinds of cells (16-17), including ROS generation, lipid peroxidation, antioxidative enzyme activity, mitochondrial membrane potential (3, 26), and the rate of cell apoptosis (16-17). However, less is known about the effect of MC-LR on the reproductive system, especially female reproductive toxicity. Based on the reasons, the aim of our present study is to explore the oxidation stress and apoptosis induced by MC-LR in vitro cultured CHO cells.

Material and Methods

Chemicals

The chemical MC-LR with purity≥95% was obtained from Beijing Express Technology Co., Ltd. RPMI-1640 medium and Trypsin (SH30042.01) were provided by Beijing Solarbio Science & Technology Co., Ltd. Coomassie brilliant blue G-250 and maleic dialdehyde (MDA), catalase (CAT) test kits were purchased from Nanjing Jiancheng Bioengineering Inc. Dimethyl sulfoxide (DMSO) was obtained from Tianjing Damao Chemical Reagent Factory. Trypan blue and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich Inc. (USA). Fetal bovine serum （FBS）was supplied by Hangzhou Sijiqing Biological Engineering Materials Co.,Ltd. Other reagents were of analytical grade.

Cell culture

CHO cells were cultured in RPMI-1640 medium containing 10% fetal calf serum at 37 ℃ in a humidified incubator with an atmosphere of 5% CO_2. When 80% of confluence was reached, cells were passaged. The culture solution was aspirated, cells was collected, washed with D-Hanks, added to 1 mL 0.25% trypsin for digesting 3~5 min. Then RPMI-1640 medium supplemented with serum was added to suspend digestion. Cells were counted with Trypan blue staining, and then the cell concentrations were adjusted to 1×10^5 cells/mL. The suspensions was seeded in 96-well plates, retained 200μL each well, and setted up five parallel samples each dose. The medium was further cultured at 37 ℃ in a humidified incubator with 5% CO_2.

Measurement of cell viability

The viability of CHO cells were measured with the MTT assay as described previously (27). Briefly, CHO cells were seeded in a 96-well plate and incubated for 24 h, followed by treatment with various concentrations of (0, 1, 5, 10, 15 μg/mL) MC-LR for 24 h. At the end of incubation period, 20 μL of MTT stock solution (5 mg/ml) was added to each well and the plates were further incubated for 3~4 h at 37 ℃. After the MTT was aspirated, 150 μL DMSO was added to each well, and gently swirled for 10min. The optical density was measured at 492 nm with a Sunrise Remote microplate reader (Thermo). Data were expressed as a percentage of control measured in the absence of MC-LR. The EC_{50} value, i.e., 50% of the cell death compared with control, was calculated for sequential experiments.

Colony forming assay

The effects of MC-LR on cell survival were evaluated with colony forming assay as described previously (28). After incubation for 24 h, cells were treated with various concentrations of MC-LR (0, $EC_{50/4}$, $EC_{50/2}$, EC_{50}) for another 24 h. After treatments, MC-LR was aspirated and replaced with fresh media, and then the cells were further cultured for 7~10 days. Thereafter, colony growth of CHO cells was evaluated with microscope after staining with 10% Giemsa solution for 15 min. Viable colonies (>20 cells) were counted and the relative colony forming efficiency was calculated.

The determination of CAT activity

CAT activity was assayed using the Catalase Assay Kit, following the manufacturer's instructions. CHO cells were seeded in a 6-well plate at a density of 1×10^6 cells /ml and incubated at 37 ℃ in 5% CO_2 for 24 h. The culture medium was aspirated. Various concentrations of MC-LR (0, $EC_{50/4}$, $EC_{50/2}$, EC_{50}) were added to each well and the plates were further incubated for 24 h. The CAT activity was expressed as U/mg protein, and the protein was determined with the Bradford method using bovine serum albumin as the standard.

MDA content assay

MDA, one of the most common markers for lipid peroxidation, is an indicator of oxidative stress (29). Lipid peroxidation products were quantified with the thiobarbituric acid (TBA) method (24). After CHO cells was treated with various concentrations of MC-LR (0, $EC_{50/4}$, $EC_{50/2}$, EC_{50}) for 24 h, MDA content were assayed using the MDA Assay Kit, following the manufacturer's instructions. MDA content was expressed as nmol/mg protein. The protein content was determined with Bradford method using bovine serum albumin as the standard.

ROS measurement

Intracellular ROS generation was analyzed using the florescent probe 2,7-dichlorofluorescein diacetate (DCFH-DA) which is deacetylated to DCFH inside the cell. ROS promotes DCFH oxidation to the fluorescent product dichlorofluorescein (DCF) (30). After exposed to MC-LR for 24 h, cells were incubated with 10 μmol/L DCFH-DA at 37 ℃ in dark for 30 min. The cells were harvested, washed and resuspended in phosphate buffered saline (PBS) and ROS generation was measured by the fluorescence intensity on a fluophotometer. The different morphology of CHO cells was observed with a fluorescence microscope.

The measurement of the mitochondrial membrane potential

The mitochondrial membrane potential (MMP) of CHO cells was measured as described previously using Mitochondrial Membrane Potential Assay Kit (31). CHO cells (1×10^6) treated with MC-LR were incubated with 1.0 μg/mL JC-1 for 15 min at 37 ℃ and analyzed immediately with a fluophotometer.

The cells fluorescence was surveyed by a fluorescence microscope.

Apoptosis detection

CHO cells apoptosis was tested by apoptosis detection kit and stained with either annexin-V-FITC alone or in combination with Propidium iodide (PI) according to manufacturer's recommendation as described previously (32). Cells were cultured in a 6-well plate at a density of 1×10^6 cells/mL and treated with various concentrations of MC-LR for 24 h. After incubation, the cells were washed, collected and resuspended in 500 µL of binding buffer. And then 5 µL of annexin V-FITC and 5 µL of propidium iodide were added to each sample, and the samples were incubated at room temperature for 15 min in the dark. The stained cells were analyzed by a FACS Calibur flowcytometer (Becton Dickinson, San Jose, CA, USA).

Statistical analysis

Results are presented as the mean ±S.D. and analyzed with the SPSS statistical package 12.0 (SPSS Inc., Chicago, IL, USA). Multiple comparisons were analyzed using the one-way ANOVA following appropriate transformation to normalized data and equalized variance where necessary. Further, the pairwise comparisons that equalized variance were analyzed using the Student-Newman-Keuls test (SNK), and unequal variances comparisons were analyzed using Games-Howell test. A difference at $P < 0.05$ was considered statistically significant.

Quantitative real-time PCR

Quantitative real-time PCR was conducted using SYBR Green dye (SYBR® Green Realtime PCR Master Mix-Plus-, Code No. QPK-212). PCR was conducted according to the manual. Parallel reactions using actin (U20114.1) genes were performed to normalize the amount of template cDNA. The protocol of real-time PCR was as follows: initiation with a 2 min denaturation at 94 ℃, followed by 45 cycles of amplification with 10 s of denaturation at 94 ℃, 15 s of annealing at 60 ℃, 30 s of extension at 72 ℃ and reading the plate for fluorescence data collection at 75 ℃. A melting curve was performed from 75 to 95 ℃ to check the specificity of the amplified product. Primer sequences for the real-time PCR assay are listed in Table 1. Three PCR replicates were performed for each RNA sample.

Results

The effects of MC-LR on CHO cells viability and colony forming

CHO cells were treated with various concentrations of 0, 1, 5, 10, or 15 µg/mL MC-LR for 24 h, respectively, and the viability of CHO cells was tested using MTT assay. As shown in Figure 1, the viability of CHO cells was significantly reduced after treatment with 5, 10 or 15µg/mL MC-LR. The viability of CHO cells treated with 10 µg/mL MC-LR were about 50% of control cells. So, the EC_{50} value was considered as 10 µg/mL. Based on this result, 0, 2.5, 5 and 10 µg/mL MC-LR was respectively used in subsequent experiments. After 24 h of exposure at concentrations of 2.5, 5 and 10 µg/mL MC-LR, the colony forming efficiency of CHO cells was in a dose-related pattern, and significantly decreased compared with the control group (Fig. 2).

Table 1. Sequences of the primers used for real-time PCR

Gene	Primer sequence
bax	F-5′-CCCCACCTGGCAGACAGTGA-3′
	R-5′-ATAGTCCAAGACGGCGGGCA-3′
p53	F-5′-CTCTGCACCAGCCACTCCCT-3′
	R-5′-TGCAGGAAGCCCAGACGGAA-3′
bcl2	F-5′-TCGCCCTGTGGATGACCGAG-3′
	R-5′-CCTCACACTGGGGCCGTACA-3′
actin	F-5′-GACCTTCAACACCCCAGCCA-3′
	R-5′-GCATGAGGGAGAGCGTAGCC-3′

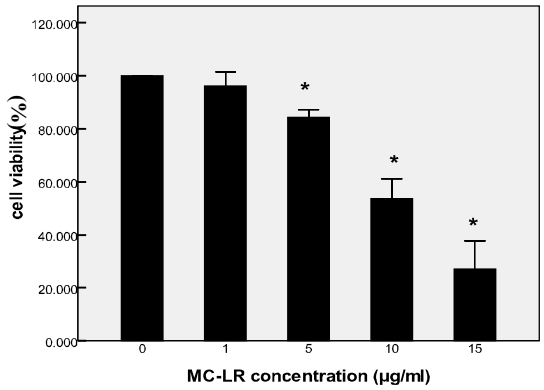


Figure 1. The effects of MC-LR on CHO cell viability. CHO cells exposed to different concentrations of MC-LR (0, 1, 5, 10, 15 µg/ml) for 24 h were incubated with MTT stock solution. Bars represent mean±S.D. (n=5). Asterisk denotes a response that is significantly different from the control (*$P < 0.05$).

The effects of MC-LR on CAT activity, MDA content and ROS generation

CAT activity in CHO cells with the different exposure concentrations (2.5, 5 and 10 µg/mL) was significantly lower than that in the control group ($P<0.05$) **(Fig. 3A)**. MDA content in CHO cells with the different exposure concentrations (2.5, 5 and 10 µg/mL) was significantly higher than that in the control group ($P<0.05$) **(Fig. 3B)**.

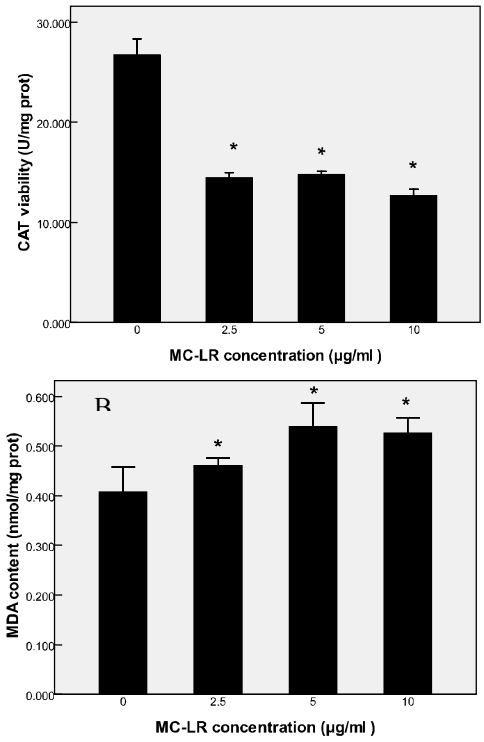


Figure 2. The effects of MC-LR on the colony forming of CHO cells.
Asterisk denotes a response that is significantly different from the control (*P < 0.05).

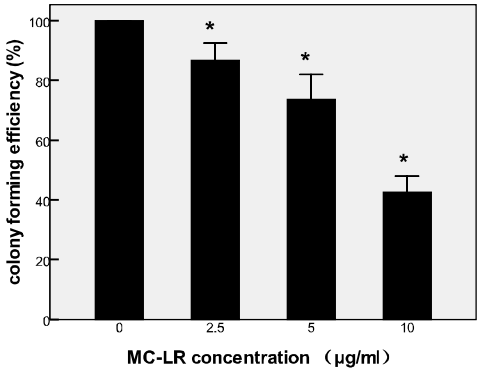
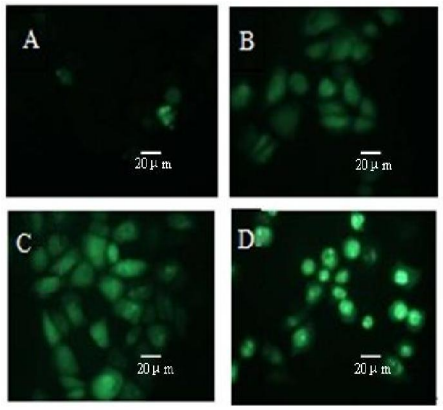


Figure 3. The effect of MC-LR on CAT activity and MDA content in CHO cells.
Asterisk denotes a response that is significantly different from the control (*P < 0.05).

ROS generation in cultured CHO cells was assayed by DCF fluorescence intensity. As shown in **Fig. 4A**, there was hardly any ROS generation in the control group. However, the amount of ROS in CHO cells with the different exposure concentrations (2.5, 5 and 10 μg/mL) significantly increased compared with the control group（**Fig. 4B, 4C, 4D**）. And the effects of 10μg/ml MC-LR exposure on the cells morphology were significant. The fluorescence microscope assessment of ROS generation was further confirmed by fluorophotometer methods, which provided the similar results. The intracellular ROS was in a dose-related pattern, and ROS generation in the treatment groups was significantly higher than that in the control group **(Fig.4E)**.



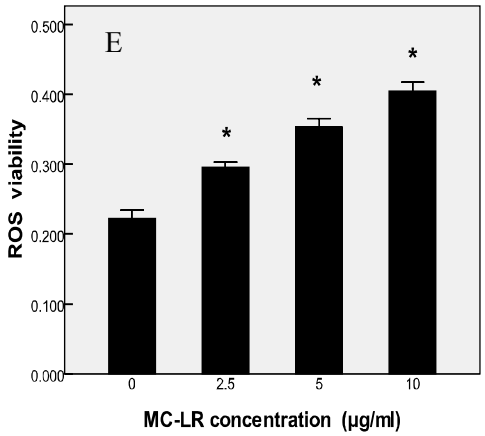


Figure 4. The effects of MC-LR on ROS levels in CHO cells.
Cells exposed to different concentrations of MC-LR (0, 2.5, 5, 10 μg/ml) for 24 h were stained with DCFH-DA. (**A, B, C, D):** The stained cells were visualized under an inverted fluorescence microscope. **E:** The data was obtained by fluorophotometer methods. Asterisk denotes a response that is significantly different from the control (*P < 0.05).

The effects of MC-LR on MMP

In this study, the CHO cells were stained with a fluorogenic probe JC-1 to detect changes of their mitochondrial membrane potential (MMP) after the treatment with MC-LR. The changes in the ratio of orange/green fluorescence were utilized to reflect the

variation of MMP. As shown in **Fig.5 A**, the orange fluorescence was observed in the mitochondria in the control group. The ratio of orange/green fluorescence decreased in 2.5, 5 and 10 μg/mL MC-LR treated groups **(Fig.5B, 5C, 5D)**. In the experiments, fluorophotometer assessment was used to further confirm mitochondrial membrane potential loss when the green fluorescence intensity was increased **(Fig.5E)**.

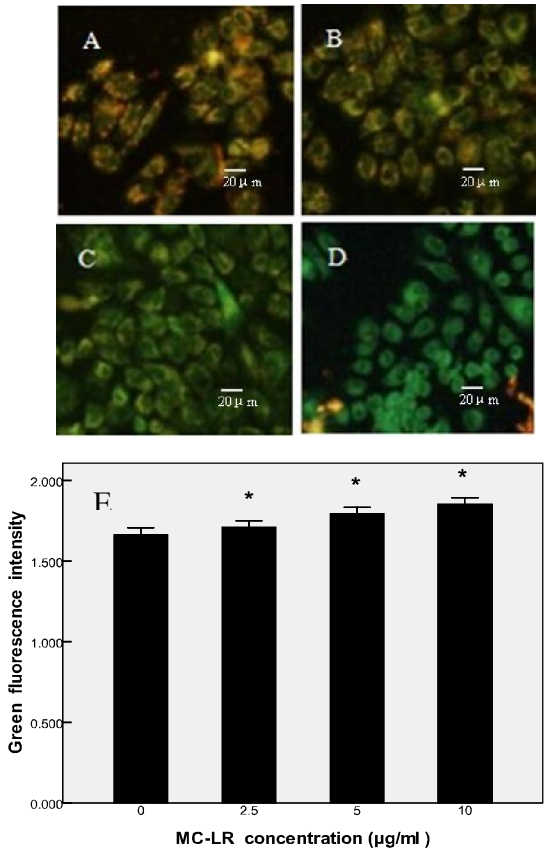


Figure 5. The effects of MC-LR on MMP of CHO cells.
After CHO cells were treated by various concentrations of MC-LR (A: 0 μg/ml, B: 2.5 μg/ml, C:5 μg/ml, D:10 μg/ml), the JC-1 stained cells were visualized under an inverted fluorescence microscope **(Fig. A, B, C, D)**. In the experiments mitochondria depolarization is indicated by an increase of green fluorescence intensity (**Fig. 5E)** obtained by fluorophotometer methods. Asterisk denotes a response that is significantly different from the control (*P < 0.05).

The effects of MC-LR on FCM

To determine MC-LR-induced CHO cells apoptosis, flow cytometric analysis with annexin-V FITC and PI double staining was performed. As shown in **Fig. 6**, MC-LR can induce cell apoptosis in a dose-dependent manner. Following the increase of MC-LR used in the treatments, the amount of live cells decreased from 99.9% to 56.5%, the amount of primary apoptotic cells increased from 0% to 27.3%, and the amount of secondary apoptotic cells increased from 0.1% to 15.1%. Moreover, the amount of apoptosis reached a peak value of 42.4% (primary apoptotic cells: 27.3%, secondary apoptotic cells: 15.1%) in the groups with 10 μg/mL MC-LR (Fig. 6D). The results suggest that MC-LR can induce CHO cells apoptosis.

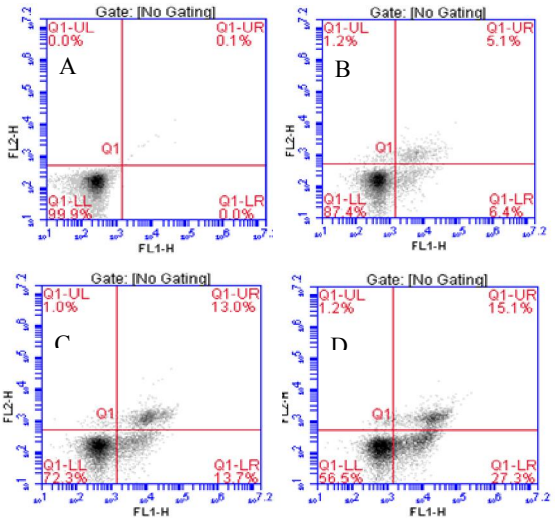


Figure 6. Apoptotic cells were detected by flow cytometry with annexinV-FITC in combination with PI staining. CHO cells were treated with various concentrations of MC-LR (A: 0 μg/ml, B: 2.5 μg/ml, C: 5 μg/ml, D: 10 μg/ml) for 24 h. In the graph, the different area represents the different situation of apoptotic. LL: live cells (Annexin V-FITC −/PI−), LR: early/primary apoptotic cells (Annexin V-FITC +/PI−), UR: late/secondary apoptotic cells (Annexin V-FITC +/PI+), and UL: necrotic cells (Annexin V-FITC −/PI+).

The effects of MC-LR on the expression of genes related to apoptosis

Regulators of apoptosis may be cell specific, and the first antiapoptotic gene identified was bcl-2. The tumor suppressor gene p53 is involved in the control of cell proliferation, particularly in stressed cells. Bax, a proapoptotic gene, can be induced by p53. Studies have also shown that the ration of Bax to Bcl-2 proteins increases during apoptosis (33). In the present study, p53 and bax were up-regulated for about 1.5 fold, and bcl-2 was down-regulated in CHO cells after MC-LR treatment (Fig 7), suggesting that the ration of Bax to Bcl-2 proteins in CHO cells can

be increased after MC-LR treatment. Therefore, CHO cells apoptosis can be induced by MC-LR.

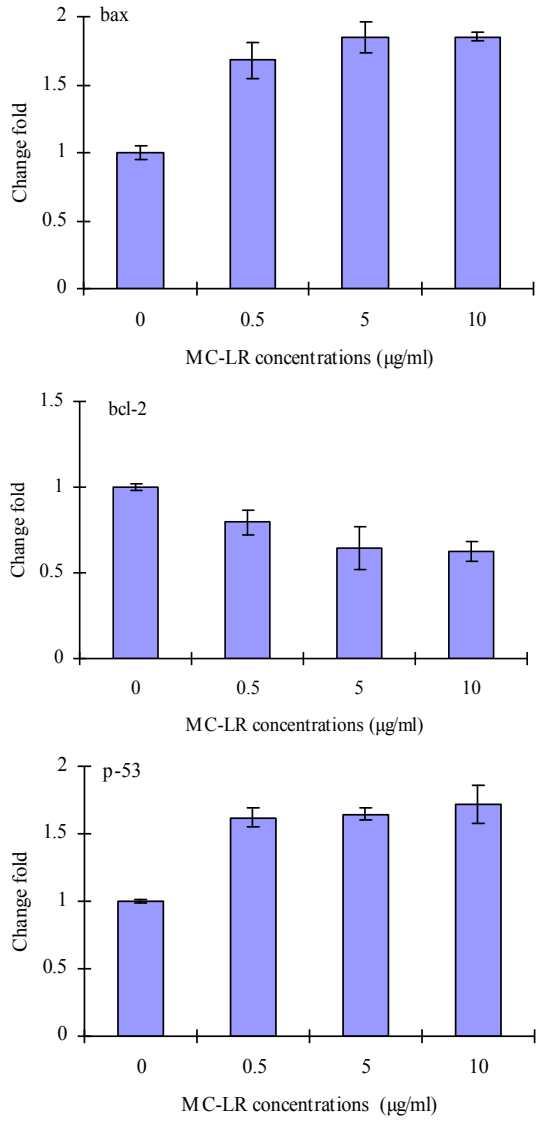


Figure 7. The effects of MC-LR on the expression of three genes related to apoptosis
Fold change was determined using the 2-△△Ct method and error bars represent the standard deviation of the mean (SD).

Discussion

In recent years, the harmful effect of MCs has drawn increasing attention as a worldwide public health concern (34-35). MC-LR, as the most toxic and abundant MCs, has been extensively studied during the past decades, and has been found to be harmful to wildlife, livestock and even humans (18, 36). For example, there were numerous worldwide lethal animal poisonings and cases of human illness resulted from toxic cyanobacteria blooms (37-38). Many studies have focused on the toxic effects of MC-LR, and it has been elucidated that exposure to low level MC-LR can enhance apoptosis in many types of cells (16).

In the present study, MC-LR decreased CHO cells viability **(Fig. 1)**, and the effective concentration of MC-LR-induced the half of death cells（EC_{50}）was 10 μg/mL. So the concentrations of $EC_{50/4}$, $EC_{50/2}$ and EC_{50} (namely 2.5, 5 and 10 μg/mL MC-LR) were used in the sequential experiments. The colony forming test was used to assay the effect of MC-LR on the colony forming efficiency of CHO cells and further evaluate the proliferation of CHO cells. The colony forming efficiency of CHO cells was significantly decreased compared with the control group and in a dose-related pattern **(Fig. 2)**.

The harmful effects of ROS on some kinds of cells have been well known, which ultimately leads to cell damage (21, 22). Under normal conditions, ROS is mainly generated in mitochondria, and is rapidly scavenged by cellular antioxidant enzymes. Antioxidants are considered to be a primary defense that prevents biological macromolecules from oxidative damage. CAT plays a major role in scavenging hydrogen peroxide and protecting cells against oxidative damage (39). Therefore, we examined whether MC-LR promoted the production of ROS in CHO cells. Our results showed that MC-LR could interfere with normal metabolism of oxygen, and then caused changes in oxidative stress in CHO cells, such as the increase of ROS and the decrease of the CAT activity **(Fig. 3A and Fig. 4)**. ROS can initiate lipid peroxidation and injure cells membrane integrality. MDA is often considered as a marker of the lipid peroxidation, and indirectly reflects cells damage degree (18). In this study, MC-LR had a dose-dependent effect on the MDA content **(Fig. 3B)**. It was suggested MC-LR can induce CHO cells lipid peroxidation, sequentially result in oxidation damage.

Apoptosis exerts a critical role in the development and homeostasis of normal tissue, and is a key process to eliminate unwanted or defective cells through an orderly process of cellular disintegration. However, many agents could destroy the cells morphology and structure, and ultimately trigger cells apoptosis. In the present study, there was dose-dependent relationship between the rate of cells apoptosis and the concentration of MC-LR treatment **(Fig. 6)**, suggesting that MC-LR can trigger the CHO cells apoptosis.

Extensive evidence has testified that mitochondria play a central role in the apoptotic death of many types of cells (40). Therefore, in the present study, the mitochondrial membrane potential was tested in

CHO cells. The results showed that MC-LR decreased mitochondrial membrane potential of the CHO cells, consequently lead to mitochondria function disorder and cells apoptosis **(Fig. 5)**.

The present study provided direct evidence for the effects of MC-LR exposure on CHO cells, including the decrease of cells viability and CAT activity, the increase of ROS and MDA, the decline of mitochondrial membrane potential, and the up-regulated expression of apoptosis related genes. Further work will focus on the toxicological mechanism of MC-LR exposure on CHO cells in apoptosis-related signaling pathways.

Conclusion

In the present study, the activity of CAT, the viability and proliferation of CHO cells was decreased after treatment with MC-LR. MC-LR increased the content of MDA in CHO cells, indicating that MC-LR accelerated lipid peroxidation of CHO cells. Moreover, the MC-LR also induced generation of ROS, mitochondrial membrane potential loss and cells apoptosis. Therefore, the acute exposure to MC-LR could promote generation of oxidative stress, depress cellular viability and cause cells apoptosis in CHO cells.

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