Microcystin-LR induces mitochondria-mediated apoptosis in human bronchial epithelial cells

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Abstract. The present study aimed to investigate the toxicity of microcystin-LR (MC-LR) and to explore the mechanism of MC-LR-induced apoptosis in human bronchial epithelial (HBE) cells. HBE cells were treated with MC-LR (1, 10, 20, 30 and 40 μg/ml) alone or with MC-LR (0, 2.5, 5 and 10 μg/ml) and Z-VAD-FMK (0, 10, 20, 40, 60, 80, 100, 120 and 140 μM), which is a caspase inhibitor, for 24 and 48 h. Cell viability was assessed via an MTT assay and the half maximal effective concentration of MC-LR was determined. The optimal concentration of Z-VAD-FMK was established as 50 μm, which was then used in the subsequent experiments. MC-LR significantly inhibited cell viability and induced apoptosis of HBE cells in a dose-dependent manner, as detected by an Annexin V/propidium iodide assay. MC-LR induced cell apoptosis, excess reactive oxygen species production and mitochondrial membrane potential collapse, upregulated Bax expression and downregulated B-cell lymphoma-2 expression in HBE cells. Moreover, western blot analysis demonstrated that MC-LR increased the activity levels of caspase-3 and caspase-9 and induced cytochrome c release into the cytoplasm, suggesting that MC-LR-induced apoptosis is associated with the mitochondrial pathway. Furthermore, pretreatment with Z-VAD-FMK reduced MC-LR-induced apoptosis by blocking caspase activation in HBE cells. Therefore, the results of the present study suggested that MC-LR is capable of significantly inhibiting the viability of HBE cells by inducing apoptosis in a mitochondria-dependent manner. The present study provides a foundation for further understanding the mechanism underlying the toxicity of MC-LR in the respiratory system.

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Introduction

Cyanobacterial blooms remain a global burden, due to the production of cyanotoxins (1). An outbreak of cyanobacterial blooms induces the release of microcystins (MCs) into water, and is a serious threat to aquatic organisms, wildlife and humans that ingest the toxins from cyanobacteria or water aquatic ecosystems (2). MCs are a group of >100 cyanobacterial toxin variants, of which MC-LR is the most common variant and the most potently toxic peptide (3). Furthermore, it has previously been reported that MC-LR is highly hepatotoxic and is a liver tumor-specific promoter (4).

MCs are a group of highly stable environmental pollutants that are not readily hydrolyzed or oxidized at normal pH, thus, they may survive for months to several years (5). Toxins released into the water from broken algal cells are a threat to human health through skin contact, inhalation, hemodialyses and oral ingestion. It has been reported that MCs may cause damage to the respiratory system (6); however, the associated mechanism has yet to be elucidated. Incidents involving poisoning of the respiratory system have been reported in several countries and regions as a result of contact with poisonous algae since the 20th century (7-9). In 1916, respiratory system symptoms were reported in patients following algal poisoning on the West Coast of Florida in the United States (8). Furthermore, in Britain in 1989, pneumonia was detected in patients after direct contact with MCs-contaminated water as a result of swimming or boating (7,9). Toxic cyanobacteria present in water entertainment parks can also generate atomized microcystins that enter the respiratory tract, which is the predominant route leading to disease of the respiratory system (10). Pilotto *et al* (11) reported that participants exposed to >5,000 cyanobacteria cells/ml for >1 h had a significant increase in flu-like symptoms, such as fever and skin rashes, as compared with unexposed participants over the course of 7 days (11). In lakes with a high concentration of cyanobacteria (cell surface area >12.0 mm^2/ml), the probability of individuals developing

respiratory symptoms is 2.1 times that of individuals who are exposed to a low concentration of cyanobacteria (cell surface area <2.4 mm^2/ml) (12). Water-based recreational activities can expose participants to low concentrations of microcystins via the aerosol; Backer *et al* (13) recruited 104 participants planning recreational activities in a lake containing cyanobacteria, as well as a nearby cyanobacteria-free lake, and demonstrated that low levels of microcystins were detected in the blood of all participants (13).

Apoptosis is a key pathophysiological mechanism associated with pneumonia. When pneumonia occurs, pneumococci induce the apoptosis of human alveolar and bronchial epithelial cells (14). Bronchial epithelial cells are the first-line defense and are therefore the first cells to be damaged (15). The damage and proliferation of bronchial epithelial cells has an important role in the repair and regeneration of lung tissues, pulmonary fibrosis and cancer (16-18). When bronchial epithelial cells are exposed to adverse factors, molecular events may occur, including oxidative stress, damage of genes, activation of proto-oncogenes or the inhibition of tumor suppressor genes in cells. These events may subsequently alter the expression levels of apoptosis-regulatory genes, leading to proliferation or damage and malignant transformation of alveolar epithelial cells, culminating in their development into lung cancer cells (19,20).

Several studies have proposed that MC-LR induces apoptosis (21,22), and it has been demonstrated that oxidative stress is an important mechanism of MCs toxicity (23). Oxidative stress may be induced by the imbalance between reactive oxygen species (ROS) formation and antioxidants (24). MC-LR may cause oxidative stress by increasing intracellular ROS production and diminishing glutathione in mouse hepatocytes (25). Furthermore, it has also been reported that MC-LR is capable of inducing mitochondrial damage (26) and MC-LR has been shown to persistently decrease B-cell lymphoma-2 (Bcl-2) expression levels and increase the expression levels of p53, Bcl-2-associated X protein (Bax) and caspase-3 (23,27). These findings indicated that oxidative stress and mitochondrial damage have an important role in MC-LR-induced apoptosis.

In the present study, human bronchial epithelial (HBE)cells were used to assess MC-LR-induced toxicity and its potential mechanisms. Cell viability, ROS, mitochondrial membrane potential (MMP), apoptosis rate, and protein expression levels of caspase-3, caspase-9, cytochrome *c* (Cyt *c*), Bax and Bcl-2 were determined to investigate MC-LR toxicity, and to explore the role of the mitochondrial pathway in MC-LR-induced apoptosis of HBE cells. The present study aimed to investigate the toxicity of MC-LR on the respiratory system.

Materials and methods

Cell culture. HBE cells were kindly provided by Dr. XiuliAn in the New York Blood Center (New York, NY, USA). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C in an atmosphere containing 5% CO$_2$. When the cells reached >90% confluence, they were trypsinized (Beyotime Institute of Biotechnology, Inc., Haimen, China) and subcultured. The cells were generally used between passages 20-30 to avoid variation.

Chemicals and reagents. MC-LR with purity of ≥95% was obtained from Beijing Express Technology Co., Ltd., (Beijing, China). RPMI-1640 medium, Annexin V-fluorescein isothiocyanate (FITC) /propidium iodide (PI) assay kit and trypsin were purchased from Beijing Solarbio Science & Technology Co., Ltd., (Beijing, China). ROS Assay kit and Mitochondrial Membrane Potential Assay kit were purchased from Beyotime Institute of Biotechnology and an MTT assay kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). Furthermore, rabbit anti-human caspase-3 (cat. no. KGYT0656-7), rabbit anti-human caspase-9 (cat. no. KGYT0661-7), goat anti-rabbit Cyt *c* (cat. no. KG22230-2), rabbit anti-human Bax (cat. no. KGYT0459-7) and rabbit anti-human Bcl-2 (cat. no. KGYT0469-7) polyclonal antibodies, goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (cat. no. KGAA35; all Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) and fetal calf serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China) were used in the present study. HBE cells were maintained in RPMI-1640 medium supplemented with 10% FCS at 37°C in an atmosphere containing 5% CO$_2$. When the cells reached >90% confluence, they were trypsinized (Beyotime Institute of Biotechnology, Inc.) and subcultured. The cells were generally used between passages 20-30 to avoid the generation of variation. All other reagents were of analytical grade.

Cell viability assay. Cell viability was assessed by MTT assay as described previously (28). Briefly, HBE cells were seeded into 96-well plates at a density of 1x10^4 cells/ml and, after 24 h, cells were treated with various concentrations of MC-LR (1, 10, 20, 30 and 40 μg/ml) for 24 h. The cells were maintained in RPMI-1640 medium supplemented with 10% FCS at 37°C in an atmosphere containing 5% CO$_2$. Following this, each well was supplemented with 20 μl MTT solution (500 μg/ml) and incubated at 37°C for 4 h, prior to incubation with 150 μl dimethyl sulfoxide at 37°C for 10 min. Absorbance was measured at 490 nm using a microplate reader (Multiskan MK3; Thermo Electric (Shanghai) Technology Instrument Co., Ltd., Shanghai, China). EC$_{50}$ was defined as the concentration of MC-LR at which 50% of cell growth was inhibited when compared with the control group.

Cell viability was also assessed following pretreatment with Z-VAD-FMK. Briefly, HBE cells were seeded into 96-well plates at a density of 1x10^4 cells/ml. The cells were grown for 24 h prior to treatment. After 24 h, cells were pretreated with Z-VAD-FMK at various concentrations (0, 10, 20, 40, 60, 80, 100, 120 and 140 μM) for 30 min prior to treatment with various concentrations of MC-LR (2.5, 5 and 10 μg/ml) for 24 h. An MTT assay was performed to detect cell viability, as described for MC-LR alone.

Detection of ROS and MMP. 2',7'-dichlorofluorescein diacetate (DCFH-DA; Beyotime Institute of Biotechnology, Inc.) was used for the detection of intracellular ROS. DCFH-DA is deacetylated to DCFH, and ROS then converts DCFH into oxidized DCF, which fluoresces (29). Fluorescence intensity is proportional to oxidant production (29). JC-1 staining (Beyotime Institute of Biotechnology, Inc.) was used to detect MMP, according to the previously reported

protocol (30). ROS and MMP levels were determined by flow cytometry (BD Accuri™ C⁶ Flow Cytometer; BD Biosciences, Franklin Lakes, NJ, USA). HBE cells (3×10^5 cells/ml) were seeded into 6-well plates. After 24 h the cells were treated with various concentrations of MC-LR (2.5, 5 and 10 μg/ml) for 24 and 48 h, washed twice with phosphate buffered saline (PBS) and stained with DCFH-DA and JC-1 for 20 min at 37°C in darkness prior to washing twice with PBS.

Detection of cell apoptosis via Annexin V/PI assay. Annexin-V FITC / PI double staining assay was used to detect cell apoptosis, according to the manufacturer's protocol. HBE cells (1×10^5 cells/ml) were seeded into 12-well plates and treated with various concentrations of MC-LR (2.5, 5 and 10 μg/ml) for 24 and 48 h. In addition, the cells were pretreated with 50 μM zVADfmk for 1 h prior to the addition of 10 μg/ml MC-LR. Cells were then washed twice with PBS and, after re-suspension in 500 μl binding buffer (Nanjing KeyGen Biotech Co., Ltd.), were incubated with 5 μl Annexin V-FITC and 5 μl PI for 15 min at room temperature in darkness. The percentage of apoptotic cells was determined by flow cytometry. All experiments were repeated three times.

Western blot assay. MC-LR-treated cells were washed with PBS, lysed in lysis buffer (Beyotime Institute of Biotechnology, Inc.) for 30 min on ice for protein extraction, centrifuged at 12,000 x g at 4°C for 5 min and the supernatants were collected. Protein concentrations were determined using the BCA method and ~40 μg of extracted protein from each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred onto polyvinylidene difluoride membranes (Beyotime Institute of Biotechnology, Inc.) using electroblotting apparatus. Membranes were subsequently blocked in Tris-buffered saline and Tween 20 (TBS-T; Beijing ComWin Biotech Co., Ltd. Beijing, China) supplemented with 5% non-fat milk for 2 h at room temperature, prior to incubation with anti-caspase-3 (1:200), anti-caspase-9 (1:200), anti-Cyt *c* (1:200), anti-Bax (1:200) anti-Bcl-2 (1:200) and rabbit anti-β-actin (1:500; cat. no. KGAA006-2; Nanjing KeyGen Biotech Co., Ltd.) antibodies, respectively, at 4°C overnight. Membranes were then washed three times in TBS-T (5 min each) and incubated with HRP-conjugated secondary antibody (1:5,000) for 1 h at room temperature. Membranes were washed three times with TBS-T for 15 min and visualized with chemiluminescent substrates (Beijing ComWin Biotech Co., Ltd.). The immunoreactive protein was visualized using electrochemiluminescence reagents kit (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and Image J version 1.49 was used to analyze the immunoblots.

Statistical analysis. Data were obtained from three independent experiments and are presented as the mean ± standard deviation. Comparisons were performed using one-way analysis of variance following the appropriate transformation to achieve a normal distribution and equalized variance where necessary. Further to this, a Student-Newman-Keuls test was used for multiple comparison of variances with homogeneity and a Games-Howell test in variances with no homogeneity. SPSS 21.0 (SPSS, Inc., Chicago, IL, USA) was used for data
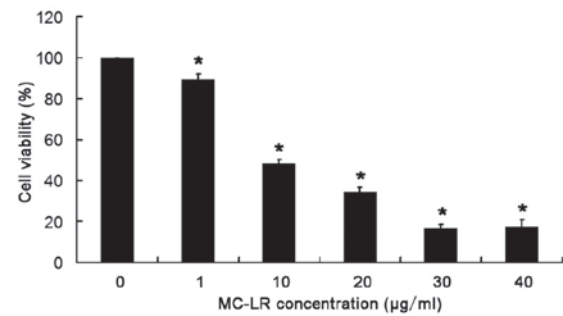

Figure 1. Human bronchial epithelial cells were exposed to various concentrations of MC-LR (0, 1, 10, 20, 30 and 40 μg/ml) for 24 h, and cell viability was determined using an MTT assay. Mean ± standard deviation (n=5). *P<0.05 vs. control group (0 μg/ml). MC-LR, microcystin-LR.
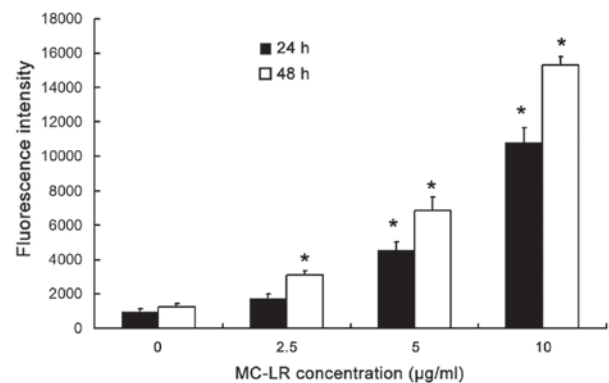

Figure 2. Flow cytometry of reactive oxygen species. Human bronchial epithelial cells were treated with various concentrations of MC-LR (0, 2.5, 5 and 10 μg/ml) for 24 and 48 h. *P<0.05 vs. control group (0 μg/ml). MC-LR, microcystin-LR.

analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

MC-LR treatment significantly decreases the viability of HBE cells in a concentration-dependent manner. An MTT assay was used to detect the viability of HBE cells following MC-LR treatment. As displayed in Fig. 1, cell viability reduced with the increase in MC-LR concentration, and a significant reduction in cell viability (P<0.05) was observed in cells after MC-LR treatment (1, 10, 20, 30 and 40 μg/ml), when compared with the control group (0 μg/ml). The EC50 value of MC-LR was 10 μg/ml in HBE cells following 24 h treatment. Thus, the viability of HBE cells significantly decreases with the increase in MC-LR concentration.

MC-LR treatment increases ROS production in a concentration- and time-dependent manner. ROS in HBE cells were assessed by DCF assay. As displayed in Fig. 2, when the treatment time was constant at 24 or 48 h, ROS levels increased with the increase in MC-LR concentration, when compared with the control group (0 μg/ml MC-LR). An MC-LR concentration of 5 or 10 μg/ml resulted in a significant increase in the fluorescence intensity in association with the increase in treatment time (P<0.05). As compared with the control,
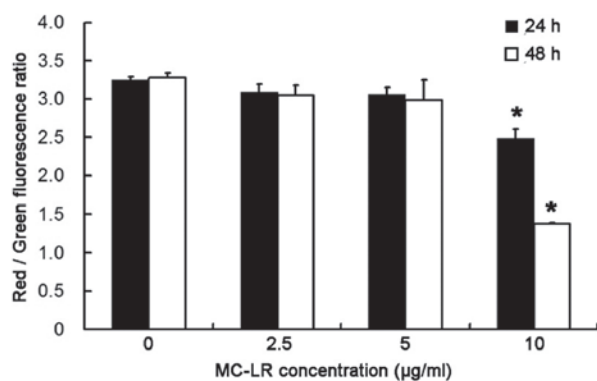
Figure 3. Flow cytometry of mitochondrial membrane potential. Human bronchial epithelial cells were treated with with various concentrations of MC-LR (0, 2.5, 5 and 10 µg/ml) for 24 and 48 h. Mean ± standard deviation (n=3). *P<0.05 vs. control group (0 µg/ml) at the same time.
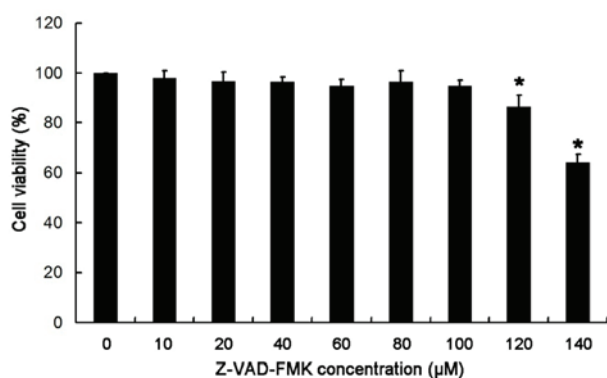

Figure 4. Human bronchial epithelial cells were pre-treated with various concentrations of Z-VAD-FMK (0, 10, 20, 40, 60, 80, 100, 120 and 140 µM) for 24 h, and cell viability was determined using an MTT assay. Mean ± standard deviation (n=5). *P<0.05 vs. control group (0 µM).

ROS increased significantly (P<0.05) in cells treated with 2.5 µg/ml MC-LR for 48 h, whereas no significant change in ROS was observed following MC-LR treatment for 24 h. Therefore, MC-LR may increase ROS production in a concentration- and time-dependent manner.

Treatment with 10 µg/ml MC-LR significantly decreases MMP in a time-dependent manner. To investigate the alterations in MMP following MC-LR treatment, the ratio of red:green fluorescence was determined following staining with JC-1. As demonstrated in Fig. 3, after exposure to 10 µg/ml MC-LR for 24 and 48 h, a significant decrease in MMP was observed in HBE cells in a time-dependent manner (P<0.05). Therefore, MMP in HBE cells decreases with the increase of the treatment time.

Treatment with >100 µM Z-VAD-FMK significantly reduces the viability of HBE cells. As demonstrated in Fig. 4, after treatment with Z-VAD-FMK at various concentrations (0, 10, 20, 40, 60, 80, 100, 120 and 140 µM) for 24 h, no significant difference in cell viability was observed when Z-VAD-FMK concentration were ≤100 µM; however, cell viability significantly reduced when treated with >100 µM Z-VAD-FMK (P<0.05) when compared with the control group (0 µM). Thus,
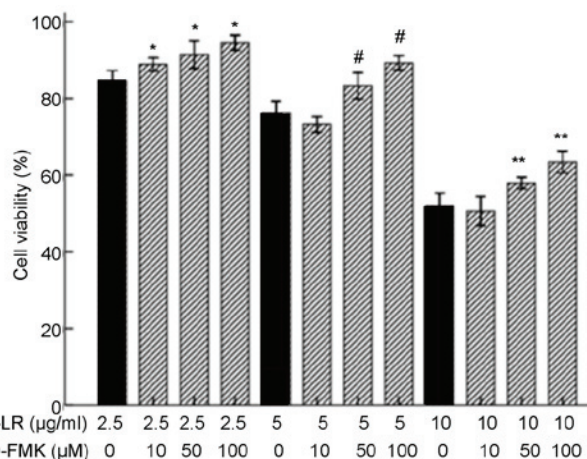

Figure 5. Human bronchial epithelial cells were treated with MC-LR (0, 2.5, 5 and 10 µg/ml) for 24 h after pretreatment with Z-VAD-FMK (10, 50 and 100 µM) for 30 min. Cell viability was determined using an MTT assay. Mean ± standard deviation (n=5). *P<0.05 vs. the respective control group at the same concentration of MC-LR. #P<0.05 vs. the respective control group at the same concentration of MC-LR. **P<0.05 vs. the respective control group at the same concentration of MC-LR. MC-LR, microcystin-LR.
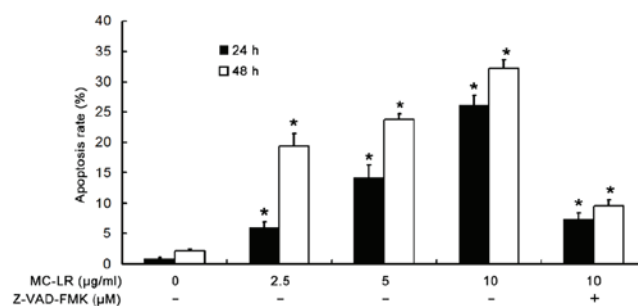

Figure 6. Cell apoptosis rate was determined by flow cytometry following Annexin-V-fluorescein isothiocyanate/propidium iodide staining. Human bronchial epithelial cells were treated with MC-LR at various concentrations for 24 h and 48 h in the presence or absence of Z-VAD-FMK pretreatment for 30 min. Mean ± standard deviation *P<0.05 vs. control group (0 µM). MC-LR, microcystin-LR.

Z-VAD-FMK concentrations ≤100 µM (2.5, 5, 10, 50 and 100 µM) were selected for all subsequent experiments. When the concentration of Z-VAD-FMK >100 µM, the viability of HBE cells was significantly reduced.

Treatment with 50 or 100 µM Z-VAD-FMK inhibits the effect of MC-LR on the viability of HBE cells. The viability of HBE cells was determined by MTT assay. As demonstrated in Fig. 5, treatment with 2.5 µg/ml MC-LR induced significantly increased cell viability following pretreatment with 10, 50 and 100 µM Z-VAD-FMK (P<0.05) when compared with the non-pretreatment group. Treatment with 5 or 10 µg/ml MC-LR significantly increased cell viability following pretreatment with 50 and 100 µM Z-VAD-FMK (P<0.05), whereas a decrease was detected following pretreatment with 10 µM Z-VAD-FMK when compared with the non-pretreatment group (0 µM Z-VAD-FMK). Therefore, 10 µM Z-VAD-FMK was not selected for use in subsequent experiments. As concentrations of 50 or 100 µM Z-VAD-FMK inhibited the effect of MC-LR on the viability of HBE cells, 50 µM Z-VAD-FMK
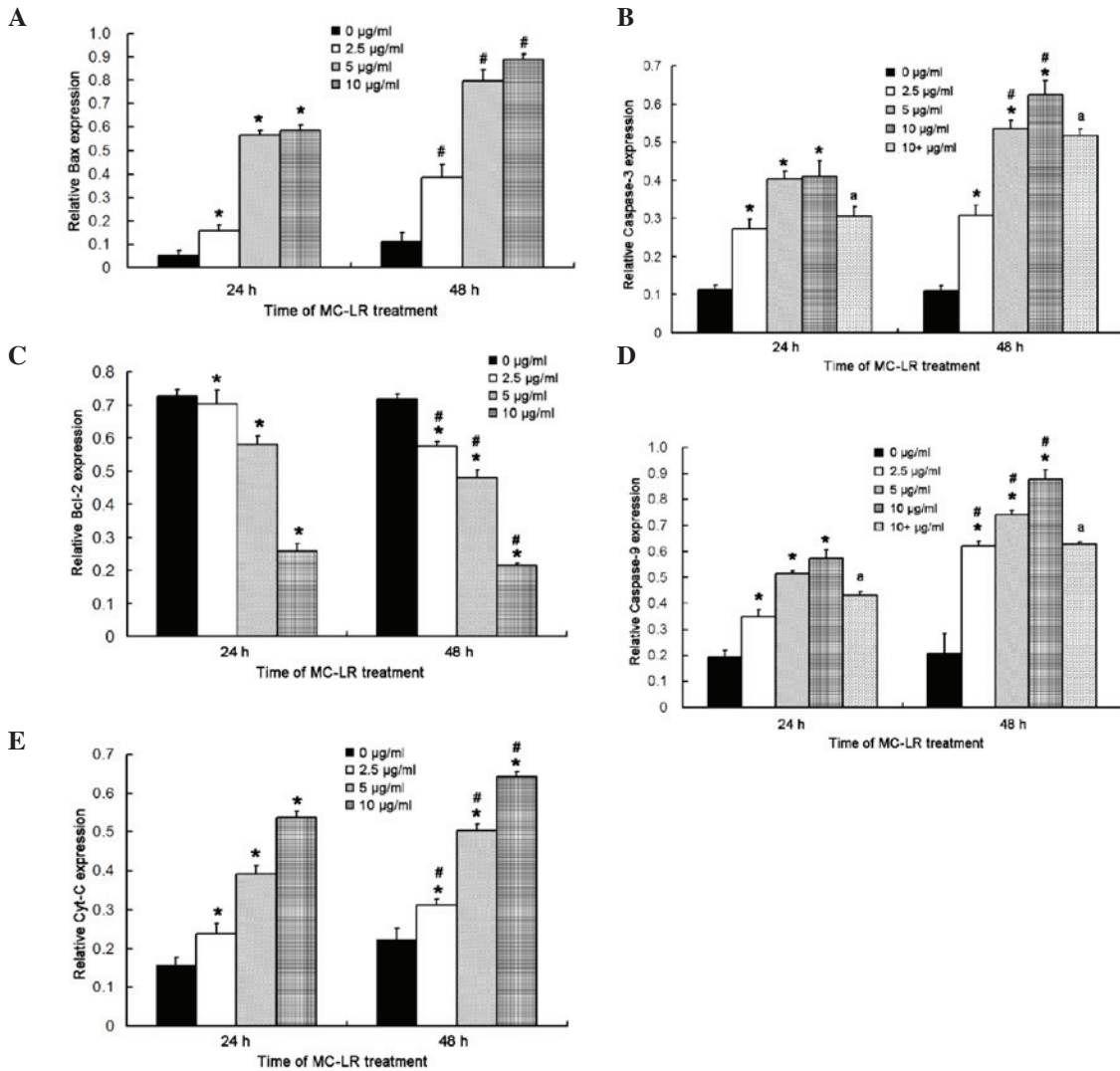
Figure 7. Effect of MC-LR and Z-VAD-FMK on the protein expression of mitochondrion-related proteins. Western blotting analysis was used to determine (A) Bax, (B) caspase-3, (C) Bcl-2, (D) caspase-9 and (E) cyt-c protein expression levels. Mean ± standard deviation (n=3). *P<0.05 vs. control group (0 μg/ml) at the same time. #P<0.05 at 48 h vs. 24 h group at the same concentration. aP<0.05 vs. control group (10 μg/ml) at the same time. 10+ μg/ml, 10 μg/ml MC-LR group after pretreatment with 50 μM Z-VAD-FMK. MC-LR, microcystin-LR; Bcl-2, B-cell lymphoma-2; cyt-c, cytochrome-*c*.

was selected for subsequent experiments. The viability of HBE cells exposed to MC-LR significantly increased when treated with 100 μM Z-VAD-FMK and when the concentration of Z-VAD-FMK >100 μM, whereas the viability of HBE cells unexposed to MC-LR was significantly reduced.

MC-LR treatment significantly increases the apoptotic rate of HBE cells in a time- and dose-dependent manner. To determine the apoptotic rate of HBE cells, flow cytometry was performed following Annexin V FITC and PI double staining. As demonstrated in Fig. 6, MC-LR significantly increased the apoptotic rate of HBE cells in a time- and dose-dependent manner (P<0.05). In addition, when MC-LR was administered at a concentration of 10 μg/ml, the apoptotic rate was significantly inhibited by pretreatment with Z-VAD-FMK (P<0.05) when compared with the non-pretreatment group.

MC-LR treatment significantly increases the expression levels of caspase-3, caspase-9, Cyt c and Bax and reduces Bcl-2 expression. Quantification of the western blot assay indicated

that the expression levels of caspase-3, caspase-9, Cyt c and Bax significantly increased in HBE cells after exposure to MC-LR for 24 h (P<0.05), whereas Bcl-2 expression levels significantly decreased (P<0.05), when compared with the control group (Fig. 7). Expression level trends of the aforementioned proteins were consistent with those at 24 h when the duration of MC-LR exposure was increased to 48 h. In addition, when HBE cells were exposed to MC-LR at the same concentration, the expression levels of caspase-3, caspase-9, Cyt *c* and Bax increased over time, however, those of Bcl-2 decreased, indicating a time- and concentration-dependent association. When HBE cells were exposed to MC-LR for 48 h at the same concentration, the expression levels of caspase-3, caspase-9, Cyt *c* and Bax increased; however, the expression levels of Bcl-2 decreased, as compare with those of HBE cells exposed to MC-LR for 24 h, indicating a time- and concentration-dependent association. Furthermore, pretreatment with Z-VAD-FMK significantly inhibited the expression levels of caspase-3 and caspase-9 at 24 and 48 h after (P<0.05), when compared with the non-pretreatment group (Fig. 7B and D, respectively).

Discussion

MC-LR is a potent inhibitor of protein phosphatase 1 and protein phosphatase 2A (31). The aforementioned phosphatases are critical regulators of embryonic development (32). Our previous *in vitro* studies revealed that MC-LR exerts toxic effects on Sertoli and CHO cells, which are associated with the reproductive system (27,33). In recent years, the toxicity of MCs has been investigated, and several studies have assessed the effect of MC-LR on the apoptosis of liver cells, kidney cells, cells in the lymph nodes and germ cells, in addition to the potential mechanisms of MC-LR-induced toxicity (27,34,35).

It has been reported that cyanobacterial toxins may be inhaled into the body via spindrifts produced in water, which may induce respiratory diseases (26). Under these conditions, patients typically present with symptoms of the respiratory system including polypnea, cyanosis, asphyxia, which may even be fatal (8). Although previous studies have reported that MCs are capable of inducing damage to the respiratory system, little is known about the mechanism of MC toxicity to the respiratory system (8,26). In the present study, HBE cells were used to investigate MC toxicity and its potential mechanism.

It is well-established that ROS and oxidative stress may trigger an apoptotic cascade (36). Previous studies have suggested that MC-LR may induce excessive ROS production (37,38). For example, Chen *et al* (37) demonstrated that MC-LR is able to damage mitochondrial respiratory chains and oxidative phosphorylation systems by inducing ROS formation and oxidative stress. The effects of MC-LR on ROS generation are dependent on time and concentration, and N-acetylcysteine significantly decreases MC-LR-induced ROS generation (38). Ding *et al* (39) reported significant and rapid increases in ROS production and the apoptosis of hepatocytes following MC-LR treatment, indicating that ROS have a critical role in MC-LR-induced apoptosis. The results of the present study demonstrated that ROS production increased with increasing MC-LR concentration, and that when the MC-LR concentration was constant, ROS increased over time, suggesting a concentration and time-dependent association. These findings indicated that MC-LR may induce ROS generation and oxidative stress in HBE cells, resulting in their apoptosis.

As a key process for eliminating unwanted or defective cells, apoptosis is an orderly process of cellular disintegration which is critical for the development and homeostasis of normal tissues. The majority of apoptotic signaling processes are associated with the alteration of apoptosis-related molecules, including Bcl-2/Bax and Cyt *c* (40). Bax is a pro-apoptotic member of the Bcl-2 family which is located in the outer membrane of mitochondria (41); whereas Bcl-2 is an anti-apoptotic member of the Bcl-2 family that is present in the outer mitochondrial membrane, where it is able to suppress apoptosis via blocking Cyt *c* release and binding to apoptotic protease-activating factor 1 (42,43). Furthermore, previous studies have indicated that Bax expression is upregulated and Bcl-2 expression is downregulated following prolonged exposure to MC-LR, and a decrease in the Bcl-2/Bax ratio has been revealed to be associated with apoptosis or cell death (44,45). In addition, it has been demonstrated that proteins of the Bcl-2 family are able to regulate the mitochondrial apoptotic pathway (46). The results of the present study indicated that, after exposure to MC-LR for 24 h, the expression levels of Bax significantly increased and those of Bcl-2 significantly decreased. Following exposure to MC-LR for 48 h, a similar change in the expression levels of the aforementioned proteins was observed. In addition, upon exposure to MC-LR at specific concentrations for 24 and 48 h in HBE cells, Bax expression levels increased over time whereas those of Bcl-2 decreased. The aforementioned findings indicated that MC-LR administration may increase Bax expression and decreases Bcl-2 expression in a time- and concentration-dependent manner.

It is widely recognized that apoptosis is initiated by two pathways, the mitochondria-mediated intrinsic pathway and the death-receptor-induced extrinsic pathway (47). Mitochondria have a key role in apoptosis and have been recognized as a central executioner, releasing apoptotic factors including Cyt *c* and apoptosis-inducing factors (48). In cases of mitochondrial dysfunction, the mitochondrial permeability transition pores open and Cyt *c* is released from the mitochondria to the cytosol (49). The release of Cyt *c* from the mitochondrion has a crucial role in the apoptotic pathway, as Cyt *c* may stimulate the formation of apoptotic bodies and subsequently activate caspase-9 which activates caspase-3. Caspase-3 activation results in the destruction of target cells (50) and it has been demonstrated that caspase-3 also participates in the process of MC-induced apoptosis (51). Zhang *et al* (46) reported that MC-LR stimulated hepatocytes to release Cyt *c*, which subsequently increased the protein expression levels of Bax, caspase-3 and caspase-9 and inhibited Bcl-2 expression over time via the mitochondrial pathway. Previous studies have demonstrated that caspase-3 is closely associated with apoptosis due to its ability to induce morphological changes in several types of cells (52-54). The results of the present study demonstrated that the expression levels of caspase-3, caspase-9 and Cyt *c* increased after exposure to MC-LR for 24 h. Following exposure to MC-LR for 48 h, similar proteins expression trends were observed. In addition, at specific concentrations of MC-LR, the expression of caspase-3, caspase-9 and Cyt *c* increased over time. The aforementioned findings indicated that MC-LR is capable of increasing the expression levels of caspase-3, caspase-9 and Cyt *c* in a time- and concentration-dependent manner. Furthermore, the present study also demonstrated the apoptotic rate of HBE cells and the expression levels of caspase-3 and caspase-9 were inhibited following MC-LR treatment when cells underwent pretreatment with Z-VAD-FMK.

In conclusion, the present study investigated the effects of MC-LR on HBE cells and explored the potential mechanism underlying MC-LR-induced apoptosis. The results suggested that MC-LR inhibits proliferation, increases ROS generation, reduces membrane potential and induces apoptosis of HBE cells in a dose- and time-dependent manner. In addition, it was demonstrated that the MC-LR-induced apoptosis of HBE cells may be associated with the mitochondria-dependent apoptotic pathway. Notably, the present study suggested that pretreatment with Z-VAD-FMK may attenuate the adverse effects of MC-LR in HBE cells, although further studies are required in order to fully elucidate the underlying mechanism. A further understanding of the effects of Cyt *c* and Bcl-2/Bax in caspase activation pathways is required in order to fully elucidate the mechanism underlying respiratory toxicity induced by MC-LR.

Acknowledgements

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