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.

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 mm²/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 mm²/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 Bel-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; Logan, UT, USA) at 37°C in an atmosphere containing 5% CO₂. 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.

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 1x10⁴ 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% CO₂. 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.

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 (3x10⁵ cells/ml) were seeded into 12-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/FITC assay. Annexin-V FITC / PI double staining assay was used to detect cell apoptosis, according to the manufacturer’s protocol. HBE cells (1x10⁵ 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 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). 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,
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, 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) when compared with the non-pretreatment group (0 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
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 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 mitochondria 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.
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References

1. Wood R: Acute animal and human poisonings from cyanotoxin exposure - a review of the literature. Environ Int 91: 276-282, 2016.
2. Chen DN, Zeng J, Wang F, Zheng W, Tu WW, Zhao JS and Xu J: Hyperphosphorylation of intermediate filament proteins is involved in microcystin-LR-induced toxicity in HL7702 cells. Toxicol Lett 214: 192-199, 2012.
3. Zhou Y, Chen Y, Yuan M, Xiang Z and Han X: In vivo study on the effects of microcystin-LR on the apoptosis, proliferation and differentiation of rat testicular spermatogenic cells of male rats induced with toxicants. Toxicol Sci 38: 666-674, 2003.
4. Christen V, Meili N and Fent K: Microcystin-LR induces endoplasmic reticulum stress and leads to induction of NFkB, interferon-alpha, and tumor necrosis factor-alpha.Environ Sci Technol 37: 3387-3395, 2003.
5. Carmichael WW, Azevedo SM, An JS, Molica RJ, Jochimsen EM, Lau S, Rinehart KL, Shaw GR and Eaglesham GK: Human fatalities from cyanobacteria: Chemical and biological evidence for cyanotoxins. Environ Health Perspect 109: 663-666, 2001.
6. Oliveira VR, Mancin VG, Pinto EF, Soares RM, Azevedo SM, Machado M, Carvalho AR and Zin WA: Repeated intranasal exposure to microcystin-LR affects lungs but not nasal epithelium in mice. Toxicon 104: 14-18, 2015.
7. Duy TN, Lam PK, Shaw GR and Connell DW: Toxicology and risk assessment of freshwater cyanobacterial (blue-green algae) toxin in water. Rev Environ Contam Toxicol 163: 113-185, 2000.
8. Giannuzzi L, Sedan D, Echenique R and Andrinolo D: An acute case of intoxication with cyanobacteria and cyanotoxins in recreational water in Salto Grande Dam, Argentina. Mar Drugs 9: 2164-2175, 2011.
9. Turner PC, Gammie AJ, Hollinrake K and Codg GA: Pneumonia associated with contact with cyanobacteria. BMJ 300: 1440-1441, 1990.
10. Backer LC, McNeel SV, Barber T, Kirkpatrick B, Williams C, Irvin M, Zhou Y, Johnson TB, Nierenberg K, Aubel M, et al: Recreational exposure to microcystins during algal blooms in two California lakes. Toxicon 55: 909-921, 2010.
11. Piloto LS, Douglas RM, Burch MD, Cameron S, Beers M, Rouch GJ, Robinson P, Kirk M, Cowie CT, Hardiman S, et al: Health effects of exposure to cyanobacteria (blue-green algae) during recreational water-related activities. Aust N Z J Public Health 21: 562-566, 1997.
12. Stewart I, Webb PM, Schluter PJ, Fleming LE, Burns JW Jr, Gantar M, Backer LC and Shaw GR: Epidemiology of recreational exposure to freshwater cyanobacteria - an international prospective cohort study. BMC Public Health 6: 93, 2006.
13. Backer LC, Carmichael W, Kirkpatrick B, Williams C, Irvin M, Zhou Y, Johnson TB, Nierenberg K, Hill VR, Kieszak SM and Cheng YS: Recreational exposure to microcystins during an algal bloom in a small lake. Mar Drugs 6: 389-406, 2008.
14. Schmelz E, Fischer G, Gross R, N’Guessan PD, Hocke AC, Hammerschmidt S, Mitchell TJ, Rosseau S, Suttrop N, and Hinnenstiel S: Streptococcus pneumoniae-induced caspase 6-dependent apoptosis in lung epithelium. Infect Immun 72: 4940-4947, 2004.
15. Walsh GM, Sexton DW and Blaylock MG: Corticosteroids, eosinophils and bronchial epithelial cells: New insights into the resolution of inflammation in asthma. J Endocrinol 178: 37-43, 2003.
16. Li L, Qiu P, Chen B, Lu Y, Wu K, Thakur C, Chang Q, Sun J and Chen F: Reactive oxygen species contribute to arsenic-induced EZH2 phosphorylation in human bronchial epithelial cells and lung cancer cells. Toxicol Appl Pharmacol 276: 165-170, 2014.
17. Myerburg MM, Latoche JD, McKenna EE, Stabile LP, Siegfried J, Feghali-Bostwick CA and Pilewski JM: Hepatocyte growth factor and other fibroblast secretions modulate the phenotype of human bronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol 292: L1352-L1367, 2007.
18. Gao W, Li L, Wang Y, Zhang S, Adcock IM, Barnes PJ, Huang M and Yao X: Bronchial epithelial cells: The key effector cells in the pathogenesis of chronic obstructive pulmonary disease? Respiratory 20: 722-729, 2012.
19. Chen D, Xu YM, Du JY, Huang DY and Lau AT: Cadmium induces cytotoxicity in human bronchial epithelial cells through upregulation of eIF5A1 and NF-kappaB. Biochem Biophys Res Comm 445: 95-99, 2014.
20. Yoon DH, Lim MH, Lee YR, Sung GH, Lee TH, Jeon BH, Cho JY, Song SO, Park H, Choi S and Kim TW: A novel synthetic analog of Militarin, MA-1 induces mitochondrial dependent apoptosis by ROS generation in human lung cancer cells. Toxicol Appl Pharmacol 273: 659-671, 2013.
21. Alverca E, Andrade M, Dias E, Sam Bento F, Batoré MC, Jordan P, Silva MJ and Pereira P: Morphological and ultrastructural effects of microcystin-LR from Microcystis aeruginosa extract on a kidney cell line. Toxicon 54: 283-294, 2009.
22. Huang X, Chen L, Liu W, Qiao Q, Wu K, Wen J, Huang C, Tang R and Zhang X: Involvement of oxidative stress and cytoskeletal disruption in microcystin-induced apoptosis in C6 cells. Aquat Toxicol 136: 355-365, 2013.
23. Li L, Xie P and Guo L: Antioxidant response in liver of the phytophilantivorous bighhead carp ( Aristichthys nobilis) intraperitoneally-injected with extracted microcystins. Fish Phys Biochem 36: 165-172, 2010.
24. Turrens JF: Mitochondrial formation of reactive oxygen species. J Physiol 552: 335-344, 2003.
25. Ding WX and Nam Ong C: Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity. FEMS Microbiol Lett 220: 1-7, 2003.
26. Zhao Y, Xie P, Tang R, Zhang X, Li L and Li D: In vivo studies on the toxic effects of microcystins on mitochondrial electron transport chain and ion regulation in liver and heart of rabbit. Comp Biochem Physiol C-Toxicol Pharmacol 148: 204-210, 2008.
27. Zhang HZ, Zhang FQ, Li CF, Yi D, Fu XL and Cui LX: A cyanobacterial toxin, microcystin-LR, induces apoptosis of sertoli cells by changing the expression levels of apoptosis-related proteins. Tohoku J Exp Med 224: 235-242, 2011.
28. Shah P, Dijsam R, Damulira H, Aganze A and Danquah M: Embelin inhibits proliferation, induces apoptosis and alters gene expression profiles in breast cancer cells. Pharmac Rep 68: 638-644, 2016.
29. Airt M, Frimer AA and Cohen Y: Active oxygen chemistry within the liposomal bilayer. Part V: Locating 2',7'-dichlorofluorescein (DCF), 2',7'-dichlorodihydrofluorescein (DCFH) and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) in the lipid bilayer. Chem Phys Lipids 131: 123-133, 2004.
30. Kopp A, Lee R, Durrant M and Ritter S: The Influence of C-Ions and X-rays on human umbilical vein endothelial cells. Front Oncol 6: 5, 2016.
31. MacKintosh C, Beattie KA, Klumpp S, Cohen P and Codd GA: Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. FEBS Lett 264: 187-192, 1990.
32. Kawamoto M, Fujiwara A and Yasunasho I: Changes in the activities of protein phosphatase type 1 and type 2A in sea urchin embryos during early development. Dev Growth Differ 42: 395-405, 2000.
33. Xue L, Li J, Li Y, Chu C, Xie G, Qin J, Yang M, Zhuang D, Cui L, Zhu H and Fu X: Nitric oxide and reactive oxygen species promote amnion stem cell chemotaxis and contribute to oxidative injury and apoptosis induced by microcystin-LR. Int J Clin Exp Med 8: 4911-4921, 2015.
34. Rymuszka A: Microcystin-LR induces cytotoxicity and affects carp immune cells by impairment of their phagocytosis and the organization of the cytoskeleton among other effects. Fish Phys Biochem 39: 1294-1302, 2013.
35. Sun Y, Meng GM, Guo ZL and Xu LH: Regulation of heat shock protein 27 phosphorylation during microcystin-LR-induced cytoskeletal reorganization in a human liver cell line. Toxicol Lett 207: 270-277, 2011.
36. Vyssokikh MY, Antonenko YN, Lyamzaev KG, Rokitskaya TI and Skulachev VP: Methodology for use of mitochondria-targeted cations in the field of oxidative stress-related research. Methods Mol Biol 1265: 149-159, 2015.
37. Chen L, Zhang X, Zhou W, Qiao Q, Liang H, Li G, Wang J and Cao M: The interaction of cytoskeleton disruption and mitochondria dysfunction lead to reproductive toxicity induced by microcystin-LR. PloS One 8: e53949, 2013.
38. Oh SH and Lim SC: A rapid and transient ROS generation by cadmium triggers apoptosis via caspase-dependent pathway in HepG2 cells and this is inhibited through N-acetylcysteine-mediated catalase upregulation. Toxicol Appl Pharmacol 212: 212-223, 2006.

39. Ding WX, Shen HM and Ong CN: Critical role of reactive oxygen species and mitochondrial permeability transition in microcystin-induced rapid apoptosis in rat hepatocytes. Hepatology 32: 547-555, 2000.

40. Zhao M, Zhang Y, Wang C, Fu Z, Liu W and Gan J: Induction of macrophage apoptosis by an organochlorine insecticide acetofenate. Chem Res Toxicol 22: 504-510, 2009.

41. Li G, Bush JA and Ho VC: p53-dependent apoptosis in melanoma cells after treatment with camptothecin. J Invest Dermatol 114: 514-519, 2000.

42. Ji YB, Ji CF and Yue L: Study on human promyelocytic leukemia HL-60 cells apoptosis induced by fucosterol. Biomed Mater Eng 24: 845-851, 2014.

43. Vander Heiden MG, Chandel NS, Williamson EK, Schumacker PT and Thompson CB: Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. Cell 91: 627-637, 1997.

44. Kim HG, Song H, Yoon DH, Song BW, Park SM, Sung GH, Cho JY, Park HI, Choi S, Song WO et al: Cordyceps pruninae extracts induce apoptosis of HeLa cells by a caspase dependent pathway. J Ethnopharmacol 128: 342-351, 2010.

45. Wiebe JP, Beausoleil M, Zhang G and Cialacu V: Opposing actions of the progesterone metabolites, 5alpha-dihydroprogesterone (5alphaP) and 3alpha-dihydroprogesterone (3alphaHP) on mitosis, apoptosis, and expression of Bcl-2, Bax and p21 in human breast cell lines. J Steroid Biochem Mol Biol 118: 125-132, 2010.

46. Zhang H, Cai C, Fang W, Wang J, Zhang Y, Liu J and Jia X: Oxidative damage and apoptosis induced by microcystin-LR in the liver of Rana nigromaculata in vivo. Aquat Toxicol 140-141: 11-18, 2013.

47. Spencer SL and Sorger PK: Measuring and modeling apoptosis in single cells. Cell 144: 926-939, 2011.

48. Ferri KF and Kroemer G: Mitochondria--the suicide organelles. BioEssays 23: 111-115, 2001.

49. Zhang S, Zhang Y, Zhuang Y, Wang J, Ye J, Zhang S, Wu J, Yu K and Han Y: Matrine induces apoptosis in human acute myeloid leukemia cells via the mitochondrial pathway and Akt inactivation. PLoS One 7: e46853, 2012.

50. Xiong Q, Xie P, Li H, Hao L, Li G, Qiu T and Liu Y: Involvement of Fas/FasL system in apoptotic signaling in testicular germ cells of male Wistar rats injected i.v. with microcystins. Toxicol 54: 1-7, 2009.

51. Fladmark KE, Brustugun OT, Hovland R, Boe R, Gjertsen BT, Zhivotovsky B and Døskeland SO: Ultrarapid caspase-3 dependent apoptosis induction by serine/threonine phosphatase inhibitors. Cell Death Differ 6: 1099-1108, 1999.

52. Hirata H, Takahashi A, Kobayashi S, Yonehara S, Sawai H, Okazaki T, Yamamoto K and Sasada M: Caspases are activated in a branched protease cascade and control distinct downstream processes in Fas-induced apoptosis. J Exp Med 187: 587-600, 1998.

53. Jänicke RU, Sprengart ML, Wati MR and Porter AG: Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J Biol Chem 273: 9357-9360, 1998.

54. Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kagi D, Hakem A, McCurrach M, Khoo W, Kaufman SA, et al: Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev 12: 806-819, 1998.