Effect of Microcystin-LR on cell viability and apoptosis-related proteins in primary cultured Sertoli cells for 48h

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Abstract: Microcystins (MCs) are a group of cyclic heptapeptide toxins produced by freshwater cyanobacteria threatened to human health, which have more than 90 identified analogues. Microcystin-LR(MC-LR), with a leucine and an arginine in its molecular structure, is the most toxic and extensively studied. Some recent studies have suggested that the gonad is the second most important target organ of MCs. However, the potential mechanism of reproductive toxicity has not been reported yet. The aim of this study was to investigate the effect on cell viability and apoptosis-related apoptosis in primary cultured Sertoli cells of rats after treated with different concentrations of MC-LR. The results of cell viability using MTT method and NR method indicated that the cell viability of Sertoli cell increased in low concentration groups (0.02~1μg/ml) and decreased in high concentration groups (10, 20μg/ml) after exposed to MC-LR for 48h. After cells were exposed to 1μg/ml MC-LR for 48h, the level of P53 protein increased but had no significant difference (P>0.05), the level of Bax protein significantly decreased (P<0.05), and the level of Bcl-2 protein significantly increased (P<0.05). When cells were exposed to 10μg/ml MC-LR, the level of P53 and Bax protein in sertoli cells significantly increased (P<0.05), and the level of Bcl-2 protein significantly decreased (P<0.05). After Sertoli cells were exposed to MC-LR for 48h, Caspase-3 activity significantly increased when cells were exposed 10μg/ml MC-LR (P<0.05).These results indicated low concentrations (0.02~1μg/ml) MC-LR had stimulation effect and enhanced cell viability, high concentrations (10, 20μg/ml) MC-LR had suppression effect on cell viability. MC-LR can induce apoptosis in primary cultured Sertoli cells. Apoptosis related-proteins P53, Bcl-2, Bax and Caspase-3 played a regulatory role in MC-LR-induced apoptosis of testicular Sertoli cells.
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1. Introduction

Microcystins(MCs) produced by Microcystis genus, Anabaena, Oscillatoria and Nostoc are a species of bacterial endotoxins in eutrophic water threatened to human health (Dittmann et al, 2013). Due to the various polypeptide of MCs composed by two kinds of amino acids with X and Y, there are 90 variants of MCs (Hoeger et al, 2005). However, MC-LR, MC-RR and MC-YR (L, R and Y represent the leucine, arginine and tyrosine, respectively) are the most widely distributed among these MCs in the nature, MC-LR is the most toxic MC.

MCs could strongly inhibit serine/threonine protein phosphatase1 and 2A, leading to protein phosphorylation and tumor formation (Yoshizawa et al, 1990; Nishiwaki-Matsushima et al, 1992). Liver is the main target organ of MCs. Studies have indicated that MCs could induce phosphorylation of cytoskeletal proteins, and destroy the structure of organisms such as microtubule (Alverca et al, 2009), microfilament, endoplasmic reticulum, mitochondria and lysosome in hepatocyte, resulting in necrosis and internal bleeding of liver (Bischoff, 2001). In addition, MCs can injury the functions of kidney stomach, intestine and pancreas (Berg et al, 2011; Towner et al, 2002). Some previous studies found the MCs affected macrophages and B lymphocyte, reducing the production of many cytokines (tumor necrosis factor, interleukin-1, interferon and other cytokines) (Chen et al, 2004) and the cellular immune function (Rymuszka et al, 2010). It has reported that MC-LR could induce apoptosis of plant cells (Yin et al, 2006).

According to investigation studies about accumulation of MCs, the gonad was considered as the second target organ of MCs (Prieto et al, 2007). Some studies suggested MC-LR had estrogen-like effect on fish (Rogers et al, 2011) and mammalian cells (Oziol and Bouaicha, 2010). Ding confirmed MCs can decrease the number and the activity of sperm, and induce the seminiferous tubules atrophy (Ding et al, 2006). Li also found MC-LR decreased the weight of testis, the concentration and the viability of sperm, and decreased the levels of serum testosterone, FSH and LH (Li et al, 2008). Chronic toxicity experiment showed low concentration MC-

LR could decline the sperm quality, decrease levels of serum testosterone, and injury to the testis (Chen et al, 2011). The literature results showed that studies about the toxic effects of MCs on the reproductive system were mainly related to reproductive organs, sperm quality and hormones, but studies about the cellular and molecular mechanism level were less.

Apoptosis is an orderly process of cell death, independently controlled by genes and cell autonomous, and also is known as programmed cell death, which plays an important role in tissue and organ formation, and is one of the important adjustments of body balance (Leist and Jaattela, 2001). Apoptosis may be induced by physiological factors, or external factors. Studies have found that MC-LR can induce significantly morphological changes of rat liver cells and epithelial cells, such as chromatin condensation, DNA fragmentation and cell shrinkage (McDermott et al, 1998). The occurrence of apoptosis is regulated by many genes, such as p53, bcl-2 family, caspase family, Fas and so on (Przemeck et al, 2007).

P53 gene is an important tumor suppressor gene, and is closely related to cell growth, differentiation and death, and also plays an important role in cell apoptosis, has called "molecular policeman". The product of p53 can activate WAF-1/CIP1 gene (tumor growth inhibitory factor), and its product is P21 protein. When p53 binds with cell cycle cyclin dependent kinase, p53 activity is inhibited, G1 phase is arrested or cell apoptosis is induced (el-Deiry et al, 1994). P53 can interact with a variety of oncogenes and growth inhibitory factor such as c-myc, bcl-2, IL-3 etc. Gupta showed that p53 expression have also increased in varying degrees with the increase of toxic concentrations (Gupta et al, 2003).

The bcl-2 protein family plays a critical role in the mitochondrial apoptosis pathway, which is divided into two categories. One category is the executioner apoptosis such as bcl-2, another category is the promoting apoptosis such as bax and bid. Pro-apoptotic proteins interacting with anti-apoptotic proteins of bcl-2 family in the mitochondrial membrane stimulate the release of cytochrome C and other apoptosis factor to adjust mitochondrial apoptosis pathway through regulating mitochondrial membrane permeability (Sharpe et al. 2004). A number of studies indicated the expression of bcl-2 decreased in the carcinogenesis and apoptosis, while the expression of bax increased significantly (Trask et al, 2002). Weng showed that MC-LR upregulated the expression of bax and bid, caused the mitochondrial membrane potential loss and hepatocyte apoptosis as well as liver injury (Weng et al, 2007).

Caspase is a family of specific aspartate cysteine proteases, which can discern specific n-terminal aspartic acid hydrolysis, and conduct enzymatic hydrolysis. Caspases enzymes play an important role in apoptosis, the Caspase family has 14 members, which are divided into initiator caspases (such as caspase-8) and executioner caspases (such as caspase-3,7) (Pop and Salvesen 2009). Caspase-3 is a key enzyme in downstream of apoptosis pathway and is the actuator of apoptosis (Li et al, 2013).

In this experiment, rat testicular Sertoli cells were incubated in vitro as a model to study the cell viability and morphological features of apoptotic cells, and expression of related gene and protein after treated with MC-LR, thus to elucidate and explore reproductive toxicity of MC-LR and mechanism of apoptosis. The study will provide theoretical basis for clarifying the molecular mechanism of apoptosis in Sertoli cells induced by MC-LR, and provide a new idea for further understanding mechanism of reproductive toxicity of MC-LR.

2. Materials and methods

Animals

Male Sprague-Dawley rats (18- to 20- day old) were purchased from the Experiments Animal Center of Henan Province (Zhengzhou, China). All experiments were approved by Animal Ethics Committee of Zhengzhou University, China, and were conducted according to the ethical guidelines 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 testicular Sertoli cells

The testicular Sertoli cells were isolated using the method of Qiu Zhiqun (Pop and Salvesen, 2009). The testis were isolated from Sprague-Dawley (SD) rats of 18-20 days and washed twice with D-Hanks. The testis were cut into 1~2mm^3 fragments and digested with 0.25% trypsin (Amresco, Solon, OH, USA) and 0.1% collagenase (type I, Invitrogen, Grand Island, NY, USA) for 20min at 37℃. The digested liquid was centrifuged at 800r/min for 5min. The testicular Sertoli cells were collected and incubated in DMEM (Invitrogen, Grand Island, NY, USA) media at 37℃. The testicular Sertoli cells were purified using 20mM Tris-HCl (pH=7.4) after they were cultured for 48h. The medium was changed every other day.

Cells treatment

The resuspended cells were stained using 0.25% trypsin. Then the cells were adjusted to 1×10^4~2×10^4/ml and incubated in 96-well plates. After 48h, the cells were exposed to low

concentrations (0, 0.02, 0.04, 0.1, 1μg/ml) and high concentrations (10, 20μg/ml) of MC-LR in 6 repetitive samples for each groups. Cells were inoculated with two 96-well plates, one for MTT, another for the NR experiment.

Cell viability were measured using MTT method and NR method

MTT solution (20μL, 5mg/ml) was added into each well. After cells were sequentially cultivated for 4h, the culture media with MTT was discarded. DMSO(150μL) was added into each well, and vibrated for 10min. Then the absorption wavelength was 492nm.

NR (200μL, 100mg/L) was added into each well. After cells were sequentially cultured for 3h, the cells were washed two times using phosphate buffer saline (PBS) solution. Acid alcohol (water: glacial acetic acid: alcohol=49:1:50) was added and vibrated for 10min. Then the absorption wavelength was 492nm. Relative cell viability (A value) = (experimental hole/control hole)*100%.

Western blot analysis for apoptosis related proteins

After treated with MC-LR, DMEM medium was abandoned, cells were digested with 0.25% trypsin, and cells were collected in centrifuge tube at 1000r/min for 5min. The supernatant was abandoned, D-Hanks was added to resuspend cells and the cells were collected in centrifuge tube at 1000r/min for 5min. RIPA protein lysis (100μl) was added in the centrifuge tube, shocked and mixed, and lysed on ice cells for 20~30min, and collected in 1.5ml centrifuge tube and centrifuged at 1000r/min for 5min. The expression level of proteins was measured by Western blot. In briefly, cellular pellets were lysed by lysis buffer. 30μg of total protein samples was separated by 12% SDS-PAGE and electrophoretically transferred to PVDF membranes. Membranes were blocked by 5% nonfat milk in TBST and incubated for 12h at 4℃ with primary antibody. Secondary antibody HRP-IgG was applied for 1h at room temperature. After three additional TBST washes, signals were detected by enhanced chemiluminescence (Amersham Bioscience).

The measurement of Caspase-3 activity

Cells were digested with trypsin, terminated digestion with DMEM medium containing fetal calf serum, stained with trypan blue, and then the cell concentration was adjusted to $4x10^4$~$5x10^4$/ml, added into in 6-well well containing the cover glass. After were cultivated for 48h, different concentration of MC-LR (0, 1, 10μg/ml) was added, respectively. The cells were used to detect Caspase-3 activity after cultivating 48h. The Caspase-3 activity was measured using the colorimetric ApoAlert Caspase-3 assay kit (ClonTech, Shanghai, China) according to the manufacturer's instructions.

Statistical analysis

All data are shown as mean ±SD. Significance was assessed by ANOVA, and mean values were compared by subsequent student-New-mean-Keuls (SNKs) using SPSS 21.0 software (SPSS Inc, Chicago, IL, USA). A difference at $P<0.05$ was considered statistically significant. All assays were performed three times.

3. Results

The effect of different concentrations on cell viability

When the cells were exposed to low concentrations MC-LR (0.02~1μg/ml) for 48h, the results tested by MTT and NR showed that cell viability increased but had no significantly change compared to the control group. The cell viability of 10μg/ml and 20μg/ml group were significantly decreased compared to the control group ($P<0.05$). (Figure1A and 1B).

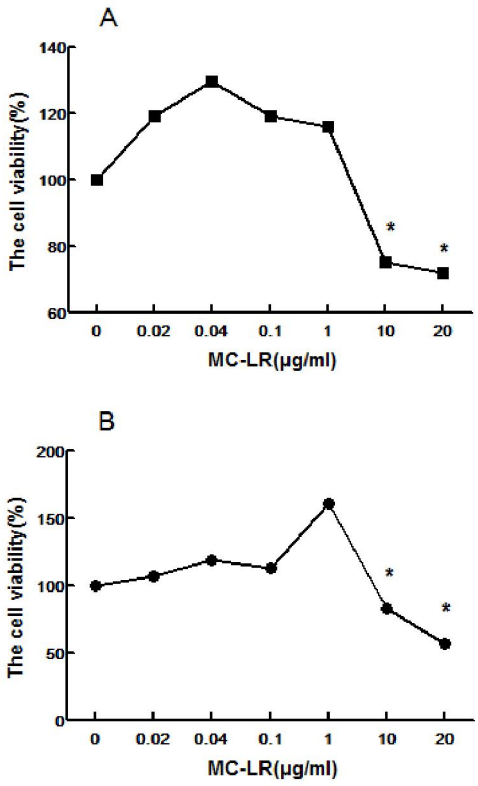

Figure 1. The cell viability after treated with MC-LR for 48h. **A** and **B** represent cell viability was measured by MTT method and NR method, resperctively. *Indicates a significant difference ($P<0.05$).

The relative expression levels of apoptosis related-proteins in testicular Sertoli cells after exposed to MC-LR for 48 h

The proteins expression levels in different groups after treated with MC-LR for 48h were shown in figure 2. The expression level of the P53 proteins increased in 1μg/ml MC-LR, but have no statistically significant differences, the expression level of the P53 and Bax proteins significantly reduced (P<0.05) and the expression level of the Bcl-2 proteins significantly increased (P<0.05) in 10μg/ml MC-LR.

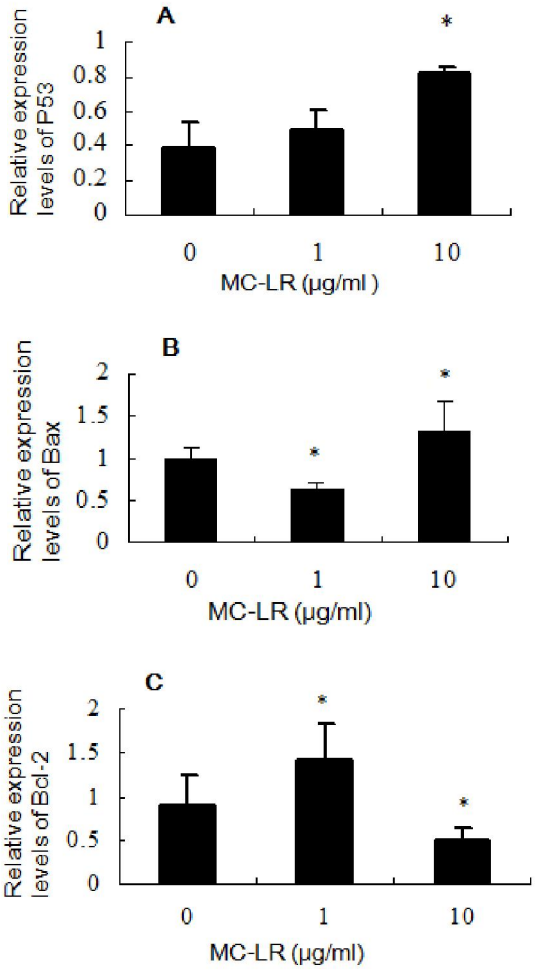

Figure 2. Relative expression levels of P53, Bax, and Bcl-2 proteins in Sertoli cells treated with MC-LR. *Indicates a significant difference compared to control group.

The effect of MC-LR on Caspase-3 activity

After testicular Sertoli cells exposed to MC-LR for 48h, Caspase-3 activity in 1μg/ml MC-LR group increase compared to the control group, but have no statistically significant differences, Caspase-3 activity significantly increase (P<0.05) in 10μg/ml MC-LR group compared to the control group (Figure 3).

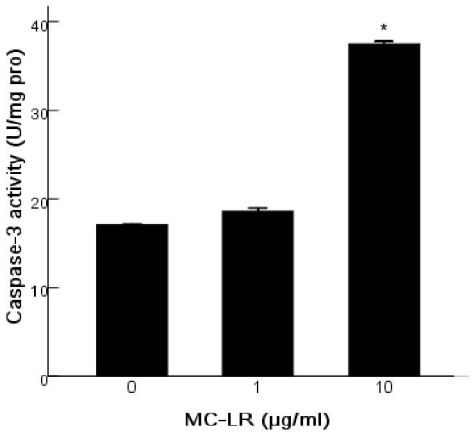

Figure 3. Caspase-3 activity in Sertoli cells treated with MC-LR for 48h. *Indicates a significant difference compared to control group.

4. Discussion

Establishment of primary cultured Sertoli cells from Sprague-Dawley as a model to study reproductive toxicity induced by MC-LR.

With the rapid development of industrialization and urbanization, more and more materials are harmful to reproductive system of animals and human, the study of reproductive toxicity becomes the hotspot of research. Testicular Sertoli cells can synthesize and secrete androgen binding protein, providing high concentration of androgen environment for sperm maturation. Testicular Sertoli cells compose the blood testis-barrier through tight junction, which have the protective effect on sperm production. Exogenous chemical can cause damage to Sertoli cell in male animal reproductive system. In this study primary cultured Sertoli cells from Sprague Dawley were selected as a model of reproductive toxicity induced by MC-LR.

The effect of MC-LR on primary cultured Sertoli cell viability

MTT and NR methods were used to detect the effect of MC-LR on cell viability, and the results showed there were no significant differences between the two methods. In this experiment, cell viability decreased with the prolonging of time when Sertoli cell exposed to high concentrations, which indicated the toxic effect of MC-LR on Sertoli cells also had time concentrations effect relationship (Alverca et al, 2009). Our results are in accordance with previous reports.

High concentrations of MC-LR were used to study the cell viability in some previous studies. While the low concentrations and high concentrations of MC-LR were selected to observe the effect on cell viability in this experimental, the results showed that

high concentrations of MC-LR inhibited cell viability, which were in accordance with previous reports about the effect of MC-LR on other types of cells. The effect of low concentrations of MC-LR on cell viability has not been reported yet, the results of this study showed that the low concentrations MC-LR had stimulation effect, and enhanced the cell viability. Nong's study showed that cell viability was inhibited when cells were exposed to MC-LR (1μg/ml) for 24h(Nong et al. 2008). Dias reported that when cells were exposed to MC-LR (25μg/ml and 22μg/ml) for 24h and 48h respectively, the cell viability was obviously inhibited(Dias et al, 2009). The study showed that activity of Sertoli cells were inhibited when cells were exposed to MC-LR (10μg/ ml and 20μg/ ml) for 48h.

The results of MTT and NR methods indicated the cell viability increased in low concentrations groups (0.02~1μg/ml) and decreased in high concentrations groups (10 and 20μg/ml) when testicular Sertoli cells were exposed to MC-LR for 48h. Thus the 1μg/ml and 10μg/ml MC-LR was used as the experimental concentrations in subsequent experiments.

The effect of MC-LR on the expression of apoptosis-related proteins P53, Bcl-2, Bax in the Sertoli cells

Reproductive cell apoptosis and proliferation are controlled by multiple genes. P53, an important apoptosis induced gene, plays an important role in the cell cycle regulation, DNA repair and cell differentiation. Over expression of p53 can promote cell apoptosis, and thus can inhibit the occurrence and growth of tumor. It have shown that p53 also played an important role in reproductive cell apoptosis (Gupta et al, 2012), the expression of p53 significantly increased when rat testis was exposed to X-ray (Sinha Hikim and Swerdloff, 1999). Research of Brzuzan showed that liver cells appeared apoptosis and mRNA expression level of p53 significantly increased when the fish was exposed to MC-LR (Brzuzan et al, 2009). In this study, Western blot method was used to detect the mRNA and protein expression level of p53 after testicular Sertoli cells treated with different concentrations of MC-LR. The experimental results showed the mRNA and protein expression level of p53 significantly increased when testicular Sertoli cells treated with MC-LR, thus the p53 played an important role in apoptosis induced by MC-LR in testicular Sertoli cells. P53 and related factors of apoptosis regulate cell apoptosis through regulating expression level.

The important feature of the Bcl-2 protein family is involved in regulation of mitochondrial apoptosis pathway. The sensitivity of cell death depended on dimerized process of the Bcl-2/Bax, and regulated cell apoptosis by regulating the Bcl-2 and Bax (Yang et al, 1995). This study showed the expression of Bcl-2 protein increased, Bax decreased and cell viability increased when testicular Sertoli cells was treated with 1μg/ml MC-LR for 48 h, which can be speculated that the increase of Bcl-2 expression protected the cells and inhibited testicular Sertoli cells apoptosis. When cells were exposed to MC-LR (10μg/ml) for 48h, the protein expression levels of Bcl-2 were significantly lower compared to the control group, Bax protein significantly increased at the same time. In conclusion, the Bcl-2 and Bax also played an important regulatory role in testicular Sertoli cells apoptosis.

MC-LR also effect activity of caspase-3 in testicular Sertoli cells

Caspase family forms the active molecule through self-cleavage resulting in cell apoptosis. In the execution of a cascade of apoptosis, Caspase-3 is an important public downstream protease in apoptotic execution, so that Caspase-3 is known as a key enzyme in apoptosis (Lakhani et al, 2006). Fladmark has shown that Caspase-3 also participated in apoptosis process induced by algal toxins (Fladmark et al, 1999). The results showed that low concentrations of MC-LR (1μg/ml) can slightly increased Caspase-3 activity, high concentrations of MC-LR (10μg/ml) can significantly increase the Caspase-3 activity, and indicated Caspase-3 activate apoptosis and promote cell apoptosis in high concentrations of MC-LR group.

5. Conclusion

This study showed that low concentrations (0.02~1μg/ml) MC-LR had stimulation effect on cells and enhanced cell viability, high concentrations (10, 20μg/ml) MC-LR had suppression effect on cell viability. MC-LR can induce apoptosis of testicular Sertoli cells, and P53, Bcl-2, Bax, and Caspase-3 played a regulatory role in apoptosis of primary cultured testicular Sertoli cells induced by MC-LR. These results will help to elucidate and explore reproductive toxicity of MC-LR and mechanism of apoptosis. And this study provided theoretical basis for clarifying the molecular mechanism of apoptosis in Sertoli cells induced by MC-LR, and also gave a new idea for further understanding mechanism of reproductive toxicity of MC-LR.

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