Life Science Journal

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MC-LR induces apoptosis of KK-1 cells via endoplasmic reticulum stress

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Abstract: Microcystin-LR (MC-LR) is a cyclic heptapeptide toxin produced by cyanobacteria, which can damage the reproductive system through multiple pathways. However, previous studies were focused on the male reproductive system, and little is known on MC-LR-induced female reproductive toxicity. Therefore, mouse ovarian granulosa (KK-1) cells were used to explore MC-LR-induced apoptosis in the present study. The expressions of pro-apoptotic factors and endoplasmic reticulum stress (ERs)-related factors in KK-1 cells were detected on protein and gene levels after exposed to different concentrations of MC-LR (0, 4.25, 8.5, 17 μg/mL). The results demonstrated that MC-LR could significantly increase the expressions of pro-apoptotic factors (caspase-3 and caspase-9) and ERs-related factors (CHOP and GRP78) in KK-1 cells, suggesting that MC-LR could induce apoptosis of KK-1 cell. This study provides a basis for exploring the potential mechanisms of MC-LR-induced apoptosis in female germ cells.
[Du XD, Liu HH, Yuan L, Wang YQ, Wang R, Ma Y, Zhang HZ. **MC-LR induces apoptosis of KK-1 cells via Endoplasmic Reticulum stress.** *Life Sci J* 2019;16(9):62-66]. ISSN: 1097-8135 (Print) / ISSN: 2372-613X (Online). http://www.lifesciencesite.com. 8. doi:10.7537/marslsj160919.08.

Keywords: Microcystin-LR; KK-1; Endoplasmic reticulum stress; Apoptosis

1. Introduction

Microcystins (MCs) are the most widely distributed freshwater cyanobacterial toxin in the world [1], which are produced by cyanobacteria in eutrophic water and have a cyclic heptapeptide structure. The stability of its structure determines that it is not easy to degrade in the environment. To date, more than 240 MC variants have been found [2]. Microcystin-LR (MC-LR) is one of the most frequent and toxic variants, which has been classified as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer (IARC) in 2010[3]. WHO has established a provisional guideline of 1 μg/L MCs for human drinking water.

MC-LR is a potent inhibitor of protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1). The liver is the most affected organ in humans, followed by the gonads [4]. MC-LR can also accumulate in animal gonads and be transmitted to the offspring [5]. MCs could induce a great quantity of reactive oxygen species (ROS) to cause reproductive toxicity. MC-LR induced oxidative stress in the male reproductive system *in vivo*, resulting in the decrease of serum testosterone level and testicular damage. MC-LR also could induce apoptosis in Leydig cells and Sertolicells in *vitro* [6]. In a previous study conducted by the investigators, it was demonstrated that MC-LR induced apoptosis in rat testicular Sertolicells via the caspase-dependent pathway [7]. With the deepening of the research, some attentions have been paid to MC-LR-induced female reproductive toxicity. The toxic effects of MCs on Chinese hamster ovary (CHO) cells showed that some female reproductive toxicity could be induced by MC-LR. MC-LR effectively inhibited the cell cycle of CHO cells [8]. In addition, it was also revealed that endoplasmic reticulum stress (ERs) and autophagy may play a vital role in CHO cell toxicity after MC-LR treatment. However, the specific mechanism is not clear.

The ovary is vulnerable to oxidative damage, because it is rich in unsaturated lipids. Previous studies have confirmed that MC-LR induced oxidative stress in the female reproductive system [9]. Excessive accumulation of ROS can aggravate oxidative stress and induce damage of endoplasmic reticulum function, eventually leading to the accumulation of a large number of unfolded proteins or misfolded proteins, which causes ERs. ERs further induces the unfolded protein response (UPR) [10]. UPR is regulated by PERK, IRE1 and ATF6, which could combine glucose-regulated protein 78 (GRP78), locating on ER in normal physiological situations [11,12]. In addition, CHOP, the downstream factor of PERK, IRE1 and ATF6, is also activated to induce apoptosis [13]. However, whether MC-LR can induce apoptosis in KK-1 cells and whether its apoptosis process is caused

by ERs remain unclear. Hence, the aim of the present study was to investigate the expressions of pro-apoptotic factors (caspase-3 and caspase-9) and ERs-related factors (CHOP and GRP78) in KK-1 cells and explore the mechanism of apoptosis.

2. Materials and methods

2.1 Reagents

MC-LR (Beijing Express Technology Co., Beijing, China); Trizol reagent (Ambion, Beijing, China); RevertAid First Strand cDNA Synthesis Kit (Thermo, China); RT-PCR kit (TaKaRa Bio Inc., Japan); RIPA Lysis Buffer (CWBIO, China); GRP78 antibody (3183s, Cell Signaling Technology, USA); CHOP antibody (WL00880, Wanleibio, China); caspase-3 antibody (8610, Cell Signaling Technology, USA); caspase-9 antibody (abm40077, Abbkine, China).

nd Treatments

KK-1 cells were grown in DMEM/high-glucose enriched with 10% FBS, 4.0 mM of L-glutamine, 4,500 mg/L of glucose, and 100 U/mL of penicillin/streptomycin. Cells were cultured in a humidified CO_2 chamber at 37°C under normal cell culture conditions. MC-LR was dissolved in PBS to generate 1mg/mL of stock solution and further diluted with culture medium to the desired concentrations, prior to incubation with KK-1 cells for 24 h.

2.3 Western blotting

Total protein was isolated from KK-1 cells exposed to various concentrations of MC-LR (0, 4.25, 8.5, 17 μg/mL). The protein content was measured by the BCA Protein Assay Kit (Beyotime, China). Samples containing 30 μg of protein were separated by SDS-PAGE and transferred on a polyvinylidenefluoride (PVDF) membrane (Millipore, USA). The membranes were blocked in TBST containing 5% BSA at room temperature for 2 h and then incubated with primary antibody. Finally, the membranes were treated with the secondary antibodies (1:5000 dilution) for 90min. Protein bands were analyzed with the enhanced chemiluminescence detection kit (CWBIO, China). The intensity of bands was quantified with the Bio-Rad Quantity One software (Bio-Rad, USA).

2.4 RT-qPCR assay

Total RNA was isolated from KK-1 cells exposed to various concentrations of MC-LR (0, 4.25, 8.5, 17μg/mL) by Trizol reagent. RevertAid first Strand cDNA Synthesis kit was used to synthesize cDNA in 20 μl reaction system following manufacturer's instructions. SYBR premix Ex Taq was used to prepare 10 μl system, and QuantStudio 7 Flex real time PCR system (Life Technologies, USA) was used to perform RT-PCR. All samples were assayed in triplicate and the expression levels were normalized to the gene of β-actin. The PCR primer sequences were presented in table 1.

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

genes	Forward primers (5'-3')	Reverse primers (5'-3')
CHOP	TACTCTTGACCCTGCGTCCCT	GGCCATAGAACTCTGACTGGAATC
GRP78	TTCATTAGCAGTTGCTCACATGTCT	GTTCCTACACCACATGTGCATGAC
caspase9	GTACATCGAGACCTTGGAT	GAGAATAATGAGGCAGAGAG
caspase3	GGAGAACAACAAAACCTCAGTGG	TGACTTGCTCCCATGTATGGTCT
β-actin	TCAAGATCATTGCTCCTCCTGAG	ACATCTGCTGGAAGGTGGACA

2.5 Statistical analysis

Data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to analyze the significant differences between groups, Student-Newman-Keuls test (SNK) was used for multiple comparisons in variances with homogeneity, and Dunnett T3 test was used for variances without homogeneity. $P<0.05$ was considered statistically significant. All statistical analyses were carried out using SPSS 21.0 (Armonk, NY, USA, 2012).

3. Results

3.1 MC-LR increases the expressions of pro-apoptotic proteins and genes in KK-1 cells.

MC-LR induced the increasing expressions of pro-apoptotic factors (caspase-3 and caspase-9) in KK-1 cells at the protein and gene levels. As shown in Figure 1A, the expressions of pro-apoptotic proteins caspase3 and caspase-9 had a dose-dependent increase after exposure to MC-LR. Compared with the control group, the expression level of caspase-3 was statistically significant in the high dose group ($P<0.05$) and caspase-9 was statistically significant in the middle and high dose groups ($P<0.05$). Figure 1B

showed that the expression levels of the pro-apoptotic genes *caspase-3* and *caspase-9* were significantly increased after MC-LR treatment (17 μg/mL). These results indicated that MC-LR could induce kk-1 cells apoptosis.

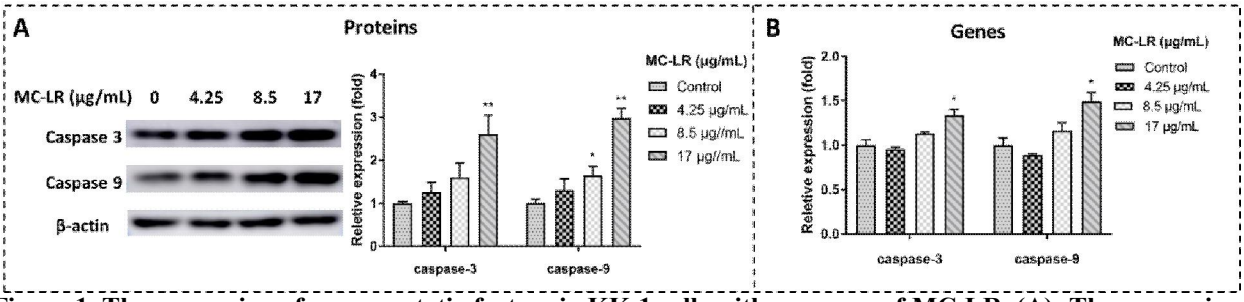

Figure 1. The expression of pro-apoptotic factors in KK-1 cells with exposure of MC-LR. (A): The expression of pro-apoptotic proteins caspase-3 and capase-9 in KK-1 cells after exposure of MC-LR. (B): The expression of pro-apoptosis genes caspase-3 and capase-9 in KK-1 cells after exposure of MC-LR. **P*<0.05 *vs*. the control group, ***P* < 0.01 vs. the control group.

3.2 MC-LR increases the expressions of ERs-related proteins and genes in KK-1 cells.

In order to explore the mechanism of apoptosis induced by MC-LR, the expression of ERs-related factors CHOP and GRP87 was detected at the protein and gene levels in KK-1 cells. As showed in Figure 1A, the protein expression levels of CHOP and GRP78 were increased after exposed to MC-LR (17 μg/mL) (*P*<0.01). Figure 1B showed that the expression levels of the genes *CHOP* and *GRP78* were significantly increased after MC-LR treatment (17 μg/mL). These results indicated that MC-LR could cause apoptosis by via ERs.

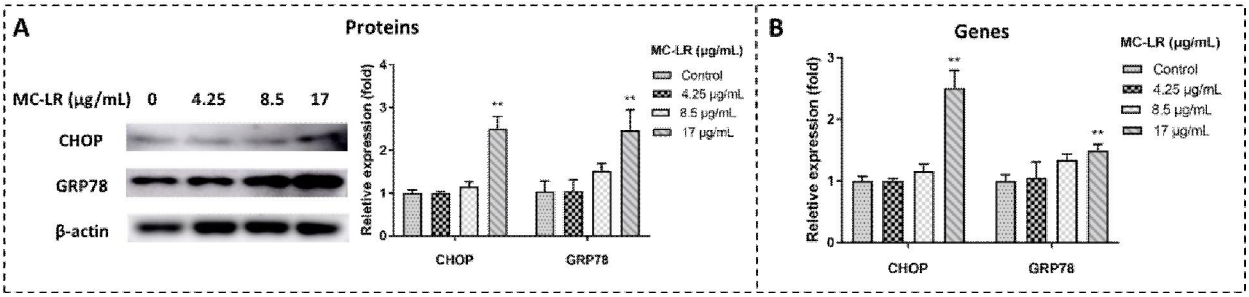

Figure 2. The expression of ERs-related factors in KK-1 cells exposure to MC-LR. (A): The expression of ERs-related proteins CHOP and GRP78 in KK-1 cells exposed to MC-LR. (B): The expression of ERs-related genes caspase-3 and capase-9 in KK-1 cells exposed to MC-LR. **P*<0.05 *vs*. the control group, ***P* < 0.01 vs. the control group.

4. Discussion

More and more studies have shown that MCs is closely related to the reproduction toxicity [14]. Although most studies were focused on the male reproductive system, MC-LR also could cause female reproductive toxicity [15]. Studies have shown that activation of ERs is associated with MC-LR-induced apoptosis in CHO cells [8].

Ovarian granulosa cells are one of the main functional cells in the follicle. They can maintain a microenvironment contributing to oocyte growth and maturation, and play an important role in the ovarian local microenvironment regulation system [16]. Apoptosis of ovarian granulosa cells is important for germ cell reduction and ovarian occlusion. Therefore, ovarian granulosa cells are an excellent model for studying the female reproductive toxicity of MC-LR.

The gonad is the second target organ after the liver. Many studies have confirmed that MC-LR could induce oxidative stress in the reproductive system [5]. It was also found that MC-LR could induce oxidative stress in zebrafish ovarian tissue causing reproductive

system damage and endocrine dysfunction [17].

Caspase-dependent pathways are the most important pathways for apoptosis [18]. Caspase-3 and caspase-9 were the apoptotic executive protein in caspase-dependent pathway [19]. MC-LR induced hepatocyte damage through caspase-dependent pathways [20]. In the present study, we found that MC-LR increased expression of pro-apoptotic factors (caspase-3 and caspase-9) in KK-1 cells, and it is consistent with the process of MC-LR-induced hepatocyte apoptosis.

To further investigate whether ERs is responsible for the apoptosis of KK-1 cells induced by MC-LR, the expressions of ERs-related factors CHOP and GRP78 were detected. CHOP is a specific transcription factor of ERs. In general, the expression of CHOP is low in cells. However, when cells are stimulated by stress, IRE1, PERK, and ATF6 can induce the activation of CHOP, which promotes the up-regulation of CHOP expression. The activation of CHOP is an important signal to induce apoptosis [21]. GRP78 is a key factor in the endoplasmic reticulum and plays an important role in maintaining the normal activities of cells [22]. Up-regulation of GRP78 can be considered as an important indicator of endoplasmic reticulum stress.

In the present study, the pro-apoptotic factors (caspase-3 and caspase-9) and ERs-related factors (CHOP and GRP78) were increased significantly after MC-LR exposure. These results indicated that ERs may play an important role in MC-LR-induced apoptosis of KK-1 cells.

5. Conclusion

Exposure to MC-LR can induce apoptosis of mouse ovarian granulosa cells, which may be mediated by ERs.

Acknowledgements:

This research was funded by The National Nature Science Foundation of China (grant number 81773384 and 81472948).

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9/14/2019