Effects of MC-LR on ROS level in human bronchial epithelia cells and Chinese hamster ovary cells

Yang Li, Hui Huang, Lijian Xue, Donggang Zhuang, Xuemin Cheng, Liuxin Cui, Huizhen Zhang*

College of Public Health, Zhengzhou University, Zhengzhou, Henan 450001, China
Huizhen18@126.com

Abstract: Microcystins (MCs) are a family of cyclic heptapetide endotoxins that are mainly produced by cyanobacterial blooms in various eutrophic inland waters worldwide. More than 90 different structural analogues of MCs have been identified, of which microcystin-LR (MC-LR) is the most common variant. The effects of MC-LR on reactive oxygen species (ROS) were measured by flow cytometry in Chinese hamster ovary (CHO) cells and human bronchial epithelial (HBE) cells. The results showed that 2.5, 5, 10μg/ml MC-LR significantly increased the production of ROS in not only CHO) cells but also (HBE) cells, suggesting that ROS production involved in the procession of reproductive toxicity and respiratory toxicity induced by MC-LR. So a series of intracellular oxidative stress reactions induced by ROS lead to apoptosis need be further researched.
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1. Introduction

Cyanobacteria are natural occurring phytoplanktonic organisms in freshwater reservoirs. Under favorable conditions they reach high cell densities (blooms). Some cyanobacterial strains of diverse species produce toxins such as microcystins presenting a threat to animal/human health. Microcystins (MCs) are a family of cyclic heptapetide endotoxins that are mainly produced by cyanobacterial blooms in various eutrophic inland waters worldwide. More than 90 different structural analogues of MCs have been identified, of which microcystin-LR (MC-LR) is the most common endotoxic variant [1]. The major toxic effect of MC-LR is hepatotoxicity, MC-LR toxicity is primarily caused by inhibition of the serine/threonine protein phosphatases (PP1 and PP2A). Inhibition of the phosphatases induces accumulation of phospho-protein in the hepatocytes, consequently, intracellular signal transductions are disrupted [2,3]. Several PP1 and PP2A inhibitors such as okadaic acid, nodularin, and MC-LR are classified as tumor promoters [4].

The potential reproductive toxicity and toxicity on respiratory system have also been reported by several studies. Spermatogonia are particularly sensitive to the harmful substances in the environment, resulting in the death of damaged sperm, infertility [5]. There are evidences showed that MC-LR can accumulate in aquatic animals in a large number and pass on to the future generations, and affect the normal propagation growth, and reproductive of fish or mammals [6,7].

In 1989, there were people appeared the pneumonia by reason of direct contacting with water (such as swimming, boating) containing MCs in Britain [8,9]. MC could be detected in blood samples of people engaged in recreational activities that would generate aerosols on fresh water lakes during a microcystin-producing algal bloom.

There was a significant and rapid increase of reactive oxygen species (ROS) level and cells apoptosis in the hepatocytes treated with MC-LR, indicating ROS plays a critical role in MC-LR-induced apoptosis [11]. In this study, ROS were measured by Flow cytometry in human bronchial epithelial (HBE) cells and Chinese hamster ovary (CHO) cells to study the toxicity and potential toxicity mechanism of apoptosis induced by MC-LR.

2. Material and Methods

Chemicals

The chemical MC-LR with purity ⩾95% was obtained from Beijing Express Technology Co.,Ltd. RPMI-1640 medium and Trypsin were provided by Beijing Solarbio Science & Technology Co.,Ltd. ROS Assay Kit was obtained from Beyotime Institute of biotechnology. Other reagents were of analytical grade.

Cell culture

CHO cells were cultured in RPMI-1640 medium containing 10% fetal calf serum. When 80% of confluence was reached, cells were passaged. The culture solution was aspirated, cells was collected, washed with D-Hanks, added to 1ml 0.25% trypsin-EDTA for digesting 1~2min. Then RPMI-1640 medium supplemented with serum was added to suspend digestion. Cells were counted with Trypan blue staining, and then the cell concentrations were adjusted to 1×10^5cells/ml. The suspensions was seeded in 6-well plates, retained 1ml each well, and setted up three parallel samples each dose. The

medium was further cultured at 37℃ in a humidified incubator with 5% CO_2.

Measurement of ROS

ROS generation was analyzed by using the florescent probe 2-7-dichlorofluorescein diacetate (DCFH-DA) which is deacetylated to DCFH in the cells. ROS induces DCFH undergoes oxidation to the fluorescent product dichlorofluorescein (DCF). Briefly, after CHO cells and 16HBE cells exposed to 0, 2.5, 5, 10μg/ml MC-LR for 24h and 48h, cells were incubated with 10μmol/L DCFH-DA for 30mins at 37 ℃ in dark. The cells were harvested, washed with PBS and then ROS generation was measured by the fluorescence intensity on a FACS Calibur flow cytometer and was observed with a fluorescence microscope.

Statistical Analysis

At least three independent experiments were conducted for all analyzes. All calculations and statistical analyses were generated using SPSS for windows version 21.0 (SPSS Inc., Chicago, IL, USA). Values are expressed as means ± standard deviation. The one-way ANOVA and Tukey's multiple comparison tests were used to estimate statistical significance, and $P<0.05$ was considered statistically significant.

3. Results

3.1 The effect of MC-LR on ROS in CHO cells

ROS generation in CHO cells was assayed by DCF fluorescence intensity. **In table1**, the fluorescence intensity increased in 2.5, 5 and 10μg/ml MC-LR groups compared with the control group ($P<0.05$). After CHO cells was treated with MC-LR for 24h, percent of positive cell increased from 0.1% to 45.3%, and for 48h percent of DCF positive cell increased from 0.2% to 65.8% **(Figure 1**). The results indicated that when the exposure time is same, the relative level of ROS increased with the increase of MC-LR concentration; and when CHO cells were exposed to the the same concentration of MC-LR, the relative level of ROS increased with the increase of the exposure time.

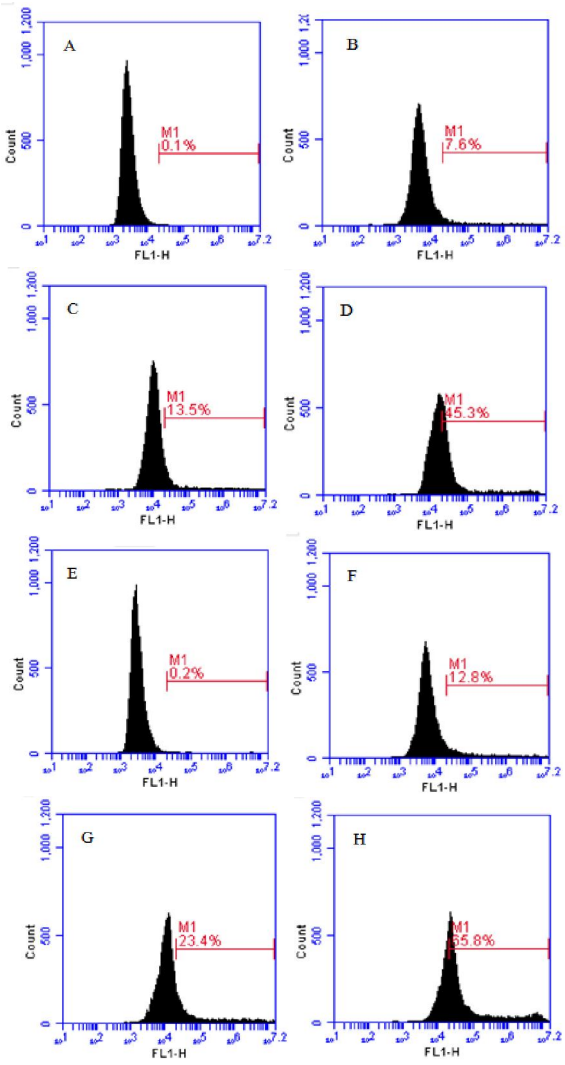

Figure 1. ROS generation in CHO cells treated with MC-LR for 24h and for 48h.

Notes: Concentrations of MC-LR. A.0μg/ml(24h); B.2.5μg/ml(24h); C.5μg/ml(24h); D.10μg/ml(24h); E.0μg/ml(48h); F. 2.5μg/ml(48h); G. 5μg/ml(48h); H. 10μg/ml (48h)

Table 1. The ROS levels in CHO cells exposed to MC-LR for 24h and 48h.

MC-LR (μg/ml)	Fluorescence Intensity (24h)	Fluorescence Intensity（48h）
0	3986.67±149.22	4120.00±449.37
2.5	95761.67±2620.78*	109095±9011.95*#
5	164396.33±4997.61*	181751.33±903.36*#
10	290262.67±2994.56*	354728.67±478.64*#

*** denotes a significantly different from the control group(0μg/ml)(*$P<0.05$) at the same time. # indicates a significantly different from the control group (24h) (*$P < 0.05$) at the same concentration.**

3.2 The effect of MC-LR on ROS in HBE cells

As shown in **Table 2**, after HBE cells exposed to MC-LR for 24h and 48h, the fluorescence intensity increased compared with the control group (P<0.05). After HBE cells was exposed MC-LR for 24h, percent of DCF positive cell increased from 0.0% to 63.2% (**Figure 2**). When the expoure time extended to 48h percent of positive cell increased from 1.0% to 79.4%. The results suggested when the treatment time was constant, the relative level of ROS in cells of each group increased with the increase of MC-LR concentration, comparing with the control group. When the MC-LR concentration was 5μg/ml or 10μg/ml, the fluorescence intensity values were increased with the increase of treatment time.

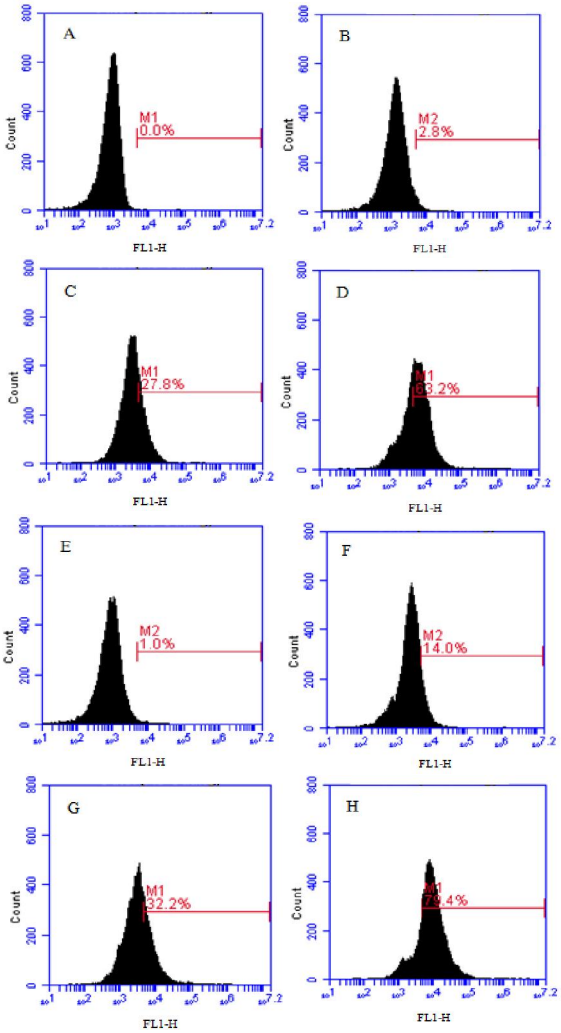

Figure 2. Flow cytometry analysis of ROS generation in HBE cells treated with various concentrations of MC-LR for 24h or 48h.

Notes: Concentrations of MC-LR. A.0μg/ml(24h); B.2.5μg/ml(24h); C.5μg/ml(24h); D.10μg/ml(24h) E.0μg/ml(48h); F. 2.5μg/ml(48h); G. 5μg/ml(48h); H. 10μg/ml (48h)

Table 2. The ROS levels in HBE cells exposed to MC-LR for 24h and 48h.

MC-LR (μg/ml)	Fluorescence Intensity (24h)	Fluorescence Intensity (48h)
0	975.34±152.84	1233.90±212.28
2.5	1735.00±259.02*	3095.80±257.47*#
5	4543.50±491.69*	6865.1±756.16*#
10	10819.00±847.90*	15336.00±461.29*#

*** denotes a significantly different from the control group (0μg/ml)(*P<0.05) at the same time. # indicates a significantly different from the control group (24h) (*P < 0.05) at the same concentration.**

4. Discussions

Extensive evidences have testified that mitochondria play a central role in the apoptotic death of many types of cells [12,13], and mitochondria are the major source of intracellular ROS production and also the primary target of ROS [14]. According to the oxidative stress hypothesis, ROS lead to a surge of detrimental biochemical reactions, including oxidation, peroxidation of membrane lipids, and apoptosis of cells [15]. Previous studies have demonstrated oxidative stress, where there is an imbalance between ROS and the cell's antioxidant capacity, is one of the classical mechanisms of apoptosis [16,17]. MC-LR could induced oxidative stress generation resulting from induction of ROS generation in Sertoli cells, and subsequently depressed cellular viability, increased expression of Caspase-3, and caused cells to undergo apoptosis, resulting in the reproductive toxicity in male rats[18].

At an early stage of apoptosis, oxidative stress can trigger the opening of the membrane permeability transition pores (MPTPs) [19,20]. As MPTPs becomes open, the permeability of the mitochondrial membrane would be increased, resulting in a decrease in MMP or even mitochondrial collapse and thus initiate cellular apoptosis [21]. The present study showed that the ROS generation increased with the increase of MC-LR concentration, and at the same exposure concentration, ROS increased with the increase of exposure time, namely there was a concentration- and time- dependent effect.

In conclusion, large amounts of ROS can be induced by MC-LR in CHO cells and 16HBE cells. ROS production could cause a series of intracellular oxidative stress reactions, leading to apoptosis, which may be the toxicity mechanisms of MC-LR in CHO cells and 16HBE cells, but the exact mechanism need be further research to confirm.

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***Corresponding Author:**
Dr. Huizhen Zhang.
College of Public Health, Zhengzhou University, Zhengzhou Henan, 450001 China
E-mail: huizhen18@126.com

References

1. Graham JL, Loftin KA, Meyer MT, Ziegler AC. Cyanotoxin mixtures and taste-and-odor compounds in cyanobacterial blooms from the Midwestern United States. Environ Sci Technol. 2010, 44 (19):7361-7368.
2. Komatsu M, Furukawa T, Ikeda R, Takumi S, Nong Q, Aoyama K, Akiyama S, Keppler D, Takeuchi T. Involvement of mitogen-activated protein kinase signaling pathways in microcystin-LR-induced apoptosis after its selective uptake mediated by OATP1B1 and OATP1B3. Toxicol Sci. 2007, 97(2):407-416.
3. Takumi S, Komatsu M, Furukawa T, Ikeda R, Sumizawa T, Akenaga H, Maeda Y,Aoyama K, Arizono K, Ando S, Takeuchi T. p53 Plays an important role in cell fate determination after exposure to microcystin-LR. Environ Health Perspect. 2010,118 (9):1292-1298.
4. Fujiki H, Suganuma M. Unique features of the okadaic acid activity class of tumor promoters. JCancer Res Clin Oncol. 1999,125 (3-4):150-155.
5. Zhou Y, Yuan J, Wu J, Han X. The toxic effects of microcystin-LR on rat spermatogonia in vitro. Toxicol Lett. 2012, 212(1):48-56.
6. Qiao Q, Liu W, Wu K, Song T, Hu J, Huang X, Wen J, Chen L, Zhang X. Female zebrafish (Danio rerio) are more vulnerable than males to microcystin-LR exposure, without exhibiting estrogenic effects. Aquat Toxicol. 2013, 142-143:272-82.
7. Wang X, Chen Y, Zuo X, Ding N, Zeng H, Zou X, Han X. Microcystin (-LR) induced testicular cell apoptosis via up-regulating apoptosis-related genes in vivo. Food Chem Toxicol. 2013,60:309-317.
8. Turner PC, Gammie AJ, Hollinrake K, Codd GA. Pneumonia associated with contact with cyanobacteria. BMJ.1990, 300(6737):1440-1441.
9. Duy TN, Lam PK, Shaw GR, Connell DW. Toxicology and risk assessment of freshwater cyanobacterial (blue-green algal) toxins in water. Rev Environ Contam Toxicol. 2000,163:113-185.
10. Backer LC, Carmichael W, Kirkpatrick B, Williams C, Irvin M, Zhou Y, JohnsonTB, Nierenberg K, Hill VR, Kieszak SM, Cheng YS. Recreational exposure to low concentrations of microcystins during an algal bloom in a small lake. Mar Drugs. 2008, 6(2):389-406.
11. Ding WX, Shen HM, Ong CN. Critical role of reactive oxygen species and mitochondrial permeability transition in microcystin-induced rapid apoptosis in rat hepatocytes. Hepatology. 2000, 32(3):547-555.
12. Orrenius S. Reactive oxygen species in mitochondria-mediated cell death. Drug Metab Rev. 2007,39(2-3):443-455.
13. Smith MA, Schnellmann RG. Calpains, mitochondria, and apoptosis. Cardiovasc Res. 2012, 96(1):32-37.
14. Rocha M, Hernandez-Mijares A, Garcia-Malpartida K, Bañuls C, Bellod L, Victor VM. Mitochondria-targeted antioxidant peptides. Curr Pharm Des. 2010, 16(28):3124-3131.
15. Csiszar A, Podlutsky A, Podlutskaya N, Sonntag WE, Merlin SZ, Philipp EE, Doyle K, Davila A, Recchia FA, Ballabh P, Pinto JT, Ungvari Z. Testing the oxidative stress hypothesis of aging in primate fibroblasts: is there a correlation between species longevity and cellular ROS production? J Gerontol A Biol Sci Med Sci. 2012, 67(8):841-852.
16. Piner P, Üner N. Oxidative stress and apoptosis was induced by bio-insecticide spinosad in the liver of Oreochromis niloticus. Environ Toxicol Pharmacol. 2013,36(3):956-963.
17. Sinha K, Das J, Pal PB, Sil PC. Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. Arch Toxicol. 2013,87(7):1157-1180.
18. Li Y, Han X. Microcystin-LR causes cytotoxicity effects in rat testicular Sertoli cells. Environ Toxicol Pharmacol. 2012, 33(2):318-326.
19. Sharma V, Anderson D, Dhawan A. Zinc oxide nanoparticles induce oxidative DNA damage and ROS-triggered mitochondria mediated apoptosis in human liver cells(HepG2). Apoptosis. 2012,17(8):852-870.
20. Miller TJ, Phelka AD, Tjalkens RB, Dethloff LA, Philbert MA. CI-1010 induced opening of the mitochondrial permeability transition pore precedes oxidative stress and apoptosis in SY5Y neuroblastoma cells. Brain Res. 2003, 963(1-2):43-56.
21. Huang J, Lv C, Hu M, Zhong G. The mitochondria-mediate apoptosis of Lepidopteran cells induced by azadirachtin. PLoS One. 2013,8 (3):e58499.

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