Effects of MC-LR exposure on inflammation factors and apoptosis-related proteins in small intestine of mice

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Abstract: Microcystin-LR, a monocyclic hepatotoxin can enter body via various routes such as skin contact, medical water, drinking water contaminated by MC-LR, eating MC-LR-contaminated aquatic products and uptake of crops that are irrigated by MC-LR-contaminated water. MC-LR can cause multiple organ toxicity to the body and the small intestine too, depending on the intake of MC-LR. The small intestine is the main digestive and absorptive organ, and the damage to the small intestine induced by MC-LR can pose a serious threat to human health. To observe the pathological damage and inflammatory reactions in the small intestine induced by MC-LR, C57BL/6 mice were intraperitoneal injected with 12.5 and 25 μg/kg MC-LR for 14 days. Results showed that there were no obvious pathological lesions observed in control groups. However, MC-LR induced dose-dependent pathological lesions to small intestine and no differences between male and female mice. MC-LR can inhibit the secretion of inflammatory cytokines (TNF-α, IL-1β, IL-6) with no obvious effect on anti-inflammatory factors TGF-β. Moreover, MC-LR can also promote the expression of bax, caspase-3 and caspase-9 in small intestine with exposure to MC-LR. Those results showed that MC-LR can induce the pathological damage in the small intestine and cause changes in the expression of inflammatory factors and apoptosis-related proteins. Our findings provide a foundation for exploring the potential mechanisms of intestinal damage MC-LR-induced.
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1. Introduction

Microcystin, a secondary metabolite, is mainly produced by freshwater alga microcystis aeruginosa [1]. Microcystin-LR (MC-LR) is the most virulent and studied among microcystins [2]. As a heptapeptide monocyclic hepatotoxin, it can strongly inhibit the activity of protein phosphatases and break the relative viability balance between intracellular protein kinases and protein phosphatases, which leads to the disordered regulation of protein kinases and protein phosphatases and triggers the hyper phosphorylation of proteins and ultimately leads to metabolic disorders in physiology and biochemistry of cells and the formation of tumors [3]. The harm of MC-LR to water environment and human health has been a global concern and one of the major environmental issues.

Epidemiological surveys have shown that MC-LR in drinking water sources is one of the leading causes for high incidence of primary liver cancer in some regions in southern of China [4]. Studies found that MC-LR causes multiple organs toxicity such as nephrotoxicity, reproductive toxicity, cardiotoxicity, and immunosuppressive effects [5-8].

Small intestine is the main site for digestion and absorption. The damage to the small intestine MC-LR-induced is undoubtedly related to our human health. Previous studies showed that MC-LR induced damage to intestine may be mediated by oxidative stress [9, 10], by decreasing the expression levels of the tight junction proteins in the small intestine [11]. Studies have shown that MC-LR can affect the integrity of small intestinal epithelial cells, leading to the destruction of intestinal villi [12]. Furthermore, it can also severely deform blood vessels and smooth muscle layer [13]. In addition, MC-LR also inhibits the secretion of intestinal digestive enzymes [14], influencing the expression of immune-related genes and the secretion of inflammatory factors [12], disturbing the balance of intestinal flora [15] and inhibits multidrug resistance proteins efflux activity in the small intestine [16]. However， the initial events underlying this dysfunction are not yet clear. Therefore， the present study aimed to analyze possible pathological injury and the regulation of inflammatory cytokines as well as pro-apoptosis proteins in small intestine of C57BL/6 mice submitted to MC-LR.

2. Materials and methods

Reagents

MC-LR was purchased from Beijing Express Technology Co. (Beijing, China) with a purity of > 96%. RevertAid First Strand cDNA Synthesis Kit

(Thermo, China); RT-PCR kit (TaKaRa Bio Inc., Japan). Antibody: caspase-3 monoclonal antibody (9665, CST, USA); caspase-9 polyclonal antibody (servicebio, China); bax polyclonal antibody (servicebio, China).

Animals and treatments

Eight-week-old C57BL/6 mice, purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., and raised in barrier (SPF animals), were randomly divided into three groups. Mice were treated daily with MC-LR intraperitoneal (i.p.) injection for 14 days and then killed under i.p. of 5% anaesthesia chloral hydrate at a dose of 10 ml/kg body weight (10ml/kg BW).

Histopathological observation in small intestine

Small intestine was dissected and then fixed in 4% paraformaldehyde solution to dehydrate and embed in paraffin to prepare the sample (4 μm thick) after exposure to MC-LR for 14 days. Staining with H & E and observed under light microscope and photographed (Microscope: NIKON Eclipse Ci, imaging system: NIKON digital sight DS-FI2, MADE IN JAPAN, film multiple: 400×).

RT-PCR assay

Trizol reagent (Ambion, Beijing, China) was used to isolate RNA from small intestine. Synthesis of cDNA was carried out in 20 μl reaction system using RevertAid first Strand cDNA Synthesis kit (Thermo, China) following manufacturer's instructions. The RT-PCR assay was performed on a QuantStudio 7 Flex real time PCR machine (life technologies, USA) with SYBR premix Ex Taq (TaKaRa Bio Inc., Japan) was used. 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')
TNF-α	AGCCGATGGGTTGTACCTTG	ATAGCAAATCGGCTGACGGT
IL-1β	ATGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT
IL-6	TGTTCTCTGGGAAATCGTGG	CAAGTGCATCATCGTTGTTCATAC
TGF-β	GCTAATGGTGGACCGCAAC	GCTTCCCGAATGTCTGACGTA
β-actin	TCAAGATCATTGCTCCTCCTGAG	ACATCTGCTGGAAGGTGGACA

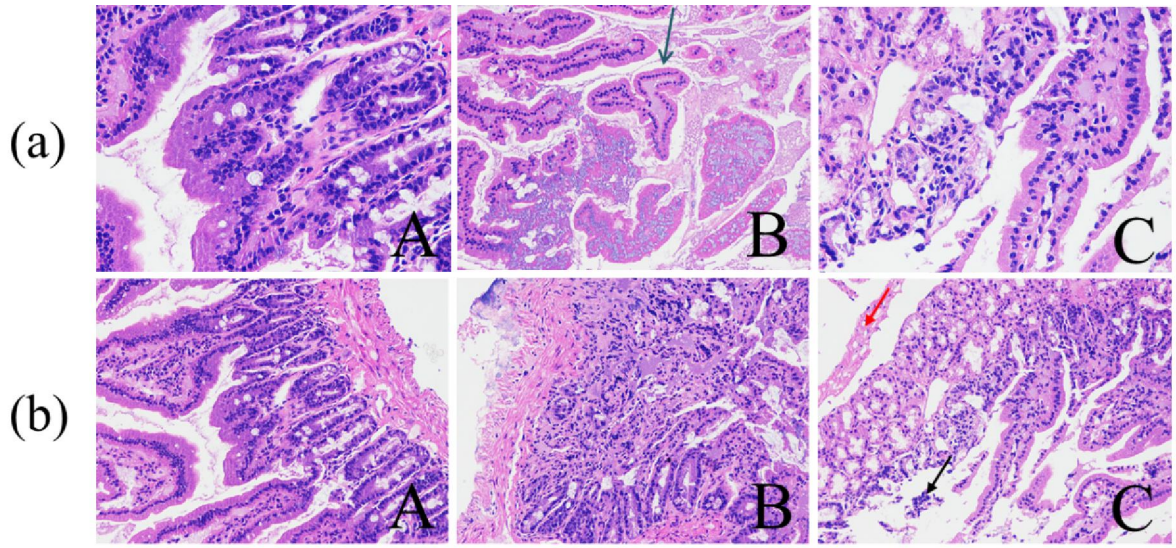

Figure 1 The histopathologic lesions in the small intestine with exposure to MC-LR. The small intestine stained with *H & E* for intestinal pathological observation in (a) male and (b) female C57BL/6 mice (A, B, and C: control groups, 12.5 μg/kg body weight MC-LR groups, and 25 μg/kg MC-LR body weight groups, respectively). Green arrow: The shedding or necrosis of the intestinal villi. Red arrow: The muscle fibers are loose and disordered. Black arrow: Shedding of mucosal.

Western blotting

Total proteins were isolated from intestine of C57BL/6 mice after exposed to MC-LR for 14 days. Electrophoresis and electric transfer were performed on Bole electrophoresis apparatus. The analysis of protein bands and its intensity were performed on

enhanced chemiluminescence detection kit (BeyoECL Star, Byotime, China) and Bio-Rad Quantity One software (Bio-Rad, California, USA), respectively.

Statistical analysis

GraghPad Prism6 software was used for statistical analysis and the results were expressed as mean ± stand deviation (SD). All groups were in respect to control group. Statistical significance was determined by One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test. *P<0.05 was regarded as statistically significant.

3. Results

MC-LR promotes intestinal histopathology lesions in C57BL/6 mice

MC-LR can induce different degree lesions in small intestine, much depending on the dose. As presented in Figure 1, results showed that there were no obvious pathological lesions observed in control groups. In 12.5 μg/kg MC-LR groups, the loss of intestinal villi were common in mucosal layer; liquefaction necrosis was found in part of the lamina-propria and small intestine glands with no invasion to submucosa; intestinal villi shedding was often seen in intestinal lumen (green arrow); muscle fiber arranged irregularly. There was no significant difference between male and female. In 25 μg/kg MC-LR groups, local liquefaction necrosis, shedding of villus, disappearance of crypts, accompanied by a small amount of inflammatory cell infiltration were observed in the mucous layer; muscle fibers arranged loosely and irregularly (black arrow); much intestinal fluff tissue with shedding or necrosis seen in intestinal lumen (black arrow).

Effects of MC-LR on inflammatory cytokines in small intestine

MC-LR induced dose-dependent downregulation of TNF-α (P<0.05), IL-1β, and IL-6 (P<0.05) with respect to control group. There was a higher level of IL-1β in 25 μg/kg MC-LR group than that in 12.5 μg/kg group, but no significant differences were observed. However, almost no IL-6 was detected in the 25 μg/kg MC-LR group. In addition, there were no obvious changes in TGF-β compared to control group and no significant was observed.

Table 2. The expression of inflammatory cytokines in small intestine.

Genes	N	Control	12.5μg/kg MC-LR	25μg/kg MC-LR
TNF-α	6	1.00±0.51	0.48±0.52	0.23±0.77
IL-1β	6	1.00±0.44	0.63±0.37	0.87±0.13*
IL-6	6	1.00±0.68	0.04±0.96*	-
TGF-β	6	1.00±0.20	1.00±0.40	1.14±0.14

-" represent on data. *$P < 0.05$ with respect to the control group.

MC-LR can induce the expression of pro-apoptosis proteins

In order to further explore the mechanism of the damage in intestine induced by MC-LR, the pro-apoptosis proteins bax, caspase-3, caspase-9 were detected. Results showed that the content of bax, and caspase-8 were increased in intestine compared with that in control group (P<0.05). MC-LR fails to change the expression of caspase-3 significantly.

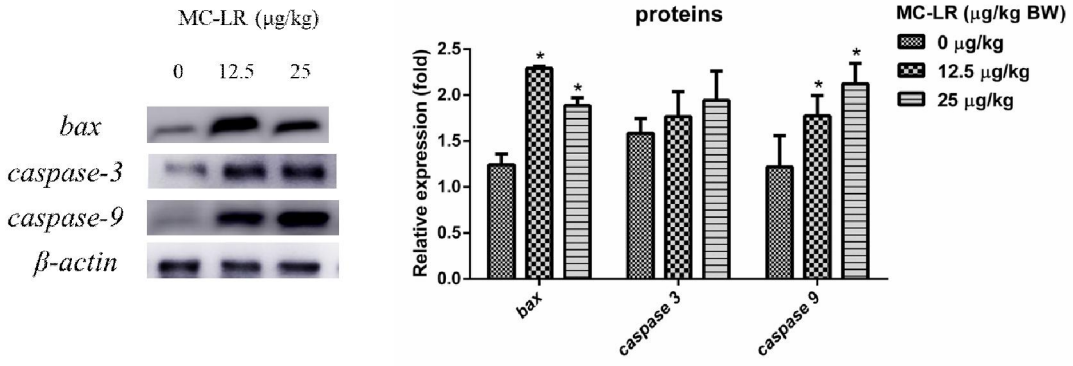

Figure 2 The expression of pro-apoptosis proteins in small intestine with exposure to MC-LR. The expression of *bax*, *caspase-3* and *capase-9* were detected by western blotting and normalized to *β-actin*. *$P<0.05$ with respect to control group.

4. Discussion

The small intestine determines the body's nutritional status as the main organ for human digestion and absorption. Studies have shown that MC-LR can across the small intestine barrier through organic anion transport polypeptide 3A1 and 4A1 (OATP3A1 and 4A1) in intestinal epithelial cells [17]. Therefore, MC-LR may cause serious pathological damage to the small intestine and pose a serious threat to human health.

In order to observe the pathological damage of MC-LR to the small intestine, H & E staining of the small intestine was performed to observe the changes of intestinal pathological lesions after exposure to different concentrations of MC-LR. Results showed that MC-LR induced dose-dependent lesions to the small intestine and no differences were observed between male and female, which is consistent with previous research results [12, 13].

The liver is the primary target organ of MC-LR, and MC-LR can induce various degrees of hepatic injury, which mainly depends on the intake of MC-LR. MC-LR can induce hepatocyte damage through multiple pathways such as oxidative stress, mitochondrial-dependent pathways, endoplasmic reticulum stress pathways, and caspase-dependent pathways. The overproduction of inflammatory factors would lead to the damage to the small intestine. To explore whether MC-LR induce inflammation of the small intestine, the expression of related inflammatory factors were examined.

TNF-α, IL-1β, IL-6 and IL-8 are the major pro-inflammatory factors, of which TNF-α produced by monocyte-macrophages which is a multi-function cytokine and activated first in the inflammatory cascade [18]. TNF-α has complex interactions with other cytokines and can induce the secretion of IL-1β, IL-6 and IL-8 [19]. IL-1 can induce the production of other cytokines and inflammatory mediators, directing the expression of immune molecules on the surface of antigen-presenting cells to provide a second messenger for activation of T lymphocytes and complement, which can enhance the damage to tissues during cellular immunity and humoral immunity [20]. IL-6 secreted by activated macrophages, lymphocytes and epithelial cells, plays an important role in maintaining the physiological balance of the body [18]. IL-8 has a significant role in chemotaxis and activation of neutrophils, causing damage to the vascular endothelial cells and accelerates the infiltration of inflammatory cell [21, 22].

TGF-β is secreted by many kinds of cells such as lymphocyte, platelets, and macrophages and the prominent effect is to promote the formation of collagen and extracellular matrix and inhibit the degradation of collagenase [23]. TGF-β1, a class of cytokine with many biological activities, participates in the regulation of cell growth, differentiation, and many other functions [24]. As a main inhibitor of the immune system, TGF-β1 is widely involved in the interaction between cells in the immune system. Furthermore, TGF-β can influence the proliferation and differentiation of thymocytes and NK cells and interfere the production and conversions of various immunoglobulins of B cells, and plays an immunosuppressive role by directly and indirectly affecting the production of IL-1, IL-2, and IFN-γ [25]. Our study found that MC-LR induced a dose-dependent decrease of inflammatory cytokines TNF-α, IL-1β, and IL-6 in C57BL/6 mice with no obvious changes in the inflammatory inhibitor TGF-β after intraperitoneal injection of MC-LR for two weeks. Furthermore, IL-8 was undetectable. Those results showed that the pathological damage to the small intestine induced by MC-LR was not mediate by inflammation.

In order to further explore the mechanism of the damage in small intestine induced by MC-LR, the expression of bax, caspase-3, caspase-9 in small intestine was detected. Bax, a pro-apoptosis protein determines the transition of mitochondrial membrane potential. The increase of mitochondrial membrane potential can lead to the release of Cyt-c, which would induce the occurrence of apoptosis. Caspase-3 and caspase-9 were the apoptotic executive protein in the downstream of caspase-dependent pathway and mitochondria-dependent pathway. The increase of bax, caspase-3 and caspase-9 may explain the damage of intestine induced by MC-LR.

5. Conclusion

MC-LR did induce the damage to the small intestine. Moreover, the degree of damage to the small intestine increased with the increase exposure dose of MC-LR. MC-LR can also cause changes in the expression of inflammatory factors and induce the expression of apoptosis-related proteins. The damage to the small intestine may relate to the activation of apoptosis-related proteins.

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