Oxdative stress on Sertoli cells of rats induced by microcystin-LR☆

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Abstract: Objective: To study the oxidative stress effects of microcystin-LR on rats Sertoli cells, and to explore the toxic mechanisms of microcystin-LR on reproductive system. **Methods:** Rat Sertoli cells were isolated and cultured. Purity of extracted Sertoli cells was identified by Feulgen staining method. Viability of primary rat Sertoli cells and the maximum dose of non-cytotoxicity of MC-LR on Sertoli cells were ascertained with MTT method after the cells were treated with the different concentrations of MC-LR for 24h. LDH, MDA, SOD, ROS in Sertoli cells were analyzed after Sertoli cells were cultured with different concentrations (0 μg/L, 0.15 μg/L, 1.5 μg/L, 15 μg/L) of MC-LR for 6, 12, 24h,. **Results:** The Sertoli cells model was obtained to study the toxic effects of MC-LR. The highest non-toxicity concentration of MC-LR was 15μg/L. The level of ROS in cells increased after exposed to the different concentrations of MC-LR, and there was a statistically significant difference when cells were exposed to 15g/L compared to control cells($P<0.05$). There was no statistically significant difference of MDA between control cells and cells exposed to MC-LR ($P>0.05$). The changes of lactate dehydrogenase (LDH) leakage amount were not significant after cells were cultured with the different concentration of MC-LR ($P>0.05$). The decreases of superoxide dismutase (SOD) were found to be dependent on the dose ($P<0.05$). **Conclusion:** MC-LR caused change of ROS in this study, but it had no effects on MDA. MC-LR had no effects on leakage rate of lactate dehydrogenase (LDH) but enhanced the activity of superoxide dismutase (SOD). The results suggested that MC-LR can induce oxidative stress in primary cultured rat Sertoli cell, but can not lead to lipid peroxidation.
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1. Introduction

Microcystins (MCs) are cyclic heptapeptides consisting of five common amino acids and two variable-amino acids, and their general structure is cyclo(-$_D$-Ala-L-X-erythro-X-methyl-$_D$-isoAsp-L-Y-Adda-$_D$-isoGlu-N-methylde-hydro-Ala), in which Adda(3-amino-9-methoxy-2-,6,8-trimethyl-10-phenyldeca-4, 6-dienoic acid), consisted of 20 special atomic carbons, is nearly related to the toxicity of Microcystins [1,2,3,4]

Microcystins, specific hepatotoxins produced by several cyanobacteria species in eutrophic surface waters, have received increasing worldwide concern due to their toxic potential in the past decades [1,5]. Microcystins are typically found only within the bacterial cells. However, when Microcystis cells die or are handled, Microcystins are released and can pollute drinking water sources [6,7,8]. To date, 67 kinds of Microcystin isoforms have been found [4], but Microcystin-LR (MC-LR) is the most common and the most toxic member. MC-LR has a LD_{50} value of 0.05mg/kg in mice by intraperitoneal injection [2].

MC-LR is a kind of intracellular toxin and has been documented to cause intoxication and lethalities in livestock, wildlife and even human [2]. Epidemiological investigations have indicated that Microcystins cause stomach and intestinal inflammation, disease of the spleen, and liver cancer in humans who drink water containing Microcystins. These effects correlate in part to the relative role of different organs (liver, kidney and lung) in the accumulation of Microcystins[13]. Lately, some studies have proved that Microcystins also were accumulated in the gonads, and the gonads were considered as the second target organ of Microcystins [9,10]. But so far, there have been few previous reports about the reproductive toxicity of Microcystins. For these reasons, the aim of the present study was to investigate the toxicity of MC-LR on the male rat reproductive system using primary cultured rat Sertoli cell.

2. Material and Methods

2.1. Chemicals

MC-LR was purchased from Institute of Hydrobiology (Wuhan, China). Dulbecco's modified Eagle's medium-Ham's F-12 medium(DMEM-F12 medium) were purchased from Sciencell Inc.

Penicillin-streptomycin antibody(SV30010.01)were obtained from BBI Inc. Trypsin (SH30042.01) and L-glutamine (SH30034.01) were purchased from HyClone Inc. Collagenase I were obtained from Beijing Solarbio Science & Technology Co.Ltd. Trypan blue and Tris were obtained from Sigma Inc. Dimethyl sulfoxide (DMSO) was obtained from Tianjin Damao Chemicals station. 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco Inc. Superoxide dismutase (SOD), Lactate dehydrogenase (LDH) and Maleic dialdehyde (MDA) test kits were purchased from Nanjing Jiancheng Bioengineering Inc(Nanjing, Jiangsu, China). Reactive oxygen species were purchased from Biyuntian Bioengineering Institutes. All other reagents were of analytical grade.

2.2. Animals

Healthy male Sprague-Dawley rats of 18-20 days weighing from 45 to 50 g were obtained from The Experimental Animal Center of Henan Province and kept in a well-ventilated room (23±1℃, 12h light/ 12h dark cycles, free access to water and standard pellet diet) for a week before used**.**

2.3. Treatment

A method was set up for obtaining a large number of viable Sertoli cells from Sprague-Dawley (SD) rats of 18-20 days. Whole testes minced from SD rats were sequentially treated with 0.25% pancreatin for 15-20 min and 0.1% collagenase for 20-25 min, and then Sertoli cells were washed and cultured in DMEM/F-12 media with 10% fetal bovine serum incubated at 37℃ in a humidified incubator with an atmosphere of 5% CO_2。After 4 days, the cells containing sperm were eliminated through lysing with a hypotonic solution of 20 mM Tris-HCL for 3 min. Feulgen staining was used to identify the purification of Sertoli cells. The viability of Sertoli cells was ascertained with MTT method after the cells were treated with the different concentrations of MC-LR for 24h.

Sertoli cells were treated with different concentrations (0μg/L, 0.15μg/L, 1.5μg/L, 15μg/L) of MC-LR. After 6, 12 and 24h, LDH, MDA, SOD, ROS of Sertoli cell line were analyzed.

2.4. Statistical analysis

The SPSS 12.0 statistical software was used for one-way ANOVA (analysis of variance, ANOVA), Bonfferoni pairwise comparison, Levene test of homogeneity of variance of data for statistical analysis (significance level $\alpha = 0.05$).

3. Results

3.1. Effect of MC-LR on the viability of Sertoli cells

Sertoli cells isolated and cultured in this study grew well, and the relative purity was more than 95%, which can be used in vitro experiments. Sertoli cells were cultured with different concentrations of MC-LR for 24h and it was found that optical density (OD) had no significant difference ($P>0.05$). That indicated MC-LR concentrations of up to 15μg/L hadn't obvious effect in cell survival rate (Table1). Based on this result, MC-LR of 0μg/L, 0.15μg/L, 1.5μg/L and 15μg/L was used in subsequent experiments.

3.2. LDH leakage rate

After treated with MC-LR (0.15, 1.5, 15μg/L) for 24h, LDH leakage rate had no statistically significant difference compared with the control group ($P>0.05$). Studying time-response relationship, LDH leakage rate of cells treated with the high dose (15μg/L) MC-LR showed a slight increase, but it was not statistically significant ($P>0.05$) (Table2).

3.3. Variation of SOD

Studying the dose-response relationship, SOD content in treated cells changed with MC-LR (0.15, 1.5, 15μg/L) after 24h. SOD activity in treated cells of 1.5μg/L MC-LR and 15μg/L MC-LR group had statistically significant difference compared with the control group($P<0.05$). When the time-response relationship was studied, SOD content in cells treated with the high-dose (15μg/L) MC-LR had no significant difference with the control group ($P> 0.05$) (Table3).

3.4. Variation of ROS

Compared with the control group, the amount of ROS in cells of each group increased. In the same group with different exposure time, the amount of ROS increased with the increase of the MC-LR concentration. In the same exposure concentration and exposure time, the amount of ROS was also increased. 6h and 24h after exposure, ROS in the treated group increased. However, only 15μg/L group had significant difference with the control group ($P <0.05$) (Table4).

3.5. MDA content in Sertoli cell

MDA content in cells with the different exposure time did not change significantly. In the different MC-LR concentration, MDA levels had no significant difference during the exposed time ($P>0.05$) (table5).

Table 1. OD value of rat Sertoli cells in different exposure concentration groups

Exposure concentration (μg/L)	n	OD value($\bar{x}\pm s$)
0	6	0.435±0.035
0.15	6	0.500±0.063
1.50	6	0.473±0.095
15.00	6	0.457±0.033

Table 2. LDH leakage rate of cells with different MC-LR exposure times and concentrations

Exposure time	LDH leakage rate(%)($\bar{x}\pm s$,n=6) with different MC-LR concentration(μg/L)			
	0	0.15	1.50	15.00
6h	56.57±1.77	56.39±0.33	57.04±1.1	58.1±0.91
12h	58.25±0.47	56.8±1.13	58.15±0.62	57.69±1.22
24h	58.35±0.35	58.52±0.79	57.92±0.17	57.54±0.64

Table 3. SOD content of cells with different MC-LR exposure times and concentrations

Exposure time	SOD content (*U/mgprot*) ($\bar{x}\pm s$,n=6)with different MC-LR concentration(μg/L)			
	0	0.15	1.50	15.00
6h	52.17±5.22	53.41±8.55	63.57±6.64	62.64±5.02
12h	61.2±10.6	68.33±10.49	67.57±18.6	67.99±17.85
24h	92.92±12	77.46±17.65	67.2±1.14 *	65.69±6.41 *

Note:*: groups exposed to MC-LR compared with control group, $p<0.05$

Table 4. OD value of ROS in cells with different MC-LR exposure times and concentrations

Exposure time	OD value of ROS($\bar{x}\pm s$,n=6) with different MC-LR concentration(μg/L)			
	0	0.15	1.50	15.00
6h	0.85±0.16	0.826±0.09	0.933±0.113	0.995±0.047
12h	1.073±0.121	1.26±0.28	1.48±0.295	1.715±0.132 *
24h	1.927±10.105	2.032±0.07	2.06±0.063	2.21±0.028 *

Note:*: groups exposed to MC-LR compared with control group,$p<0.05$

Table 5. MDA content of cells with different MC-LR exposure times and concentrations

Exposure time	MDA content (*U/mgprot*) ($\bar{x}\pm s$,n=6) with different MC-LR concentration(μg/L)			
	0	0.15	1.50	15.00
6h	0.071±0.005	0.069±0.006	0.073±0.007	0.061±0.003
12h	0.077±0.003	0.073±0.016	0.066±0.002	0.075±0.002
24h	0.089±0.003	0.073±0.006	0.075±0.001	0.075±0.005

4. Discussions

In the last decade, masses of cosmopolitan lethal animal poisonings and a plenty of cases of human illness caused by toxic cyanobacteria blooms have brought about the attention of World Health Organization and the public. Microcystins are a group of heptapeptide toxins produced by cyanobacteria in eutrophic freshwater, some of which are potent toxins, and Microcystin-LR is one of the most toxic and abundant variants in blooms. MCs form a health risk to wildlife, livestock and even humans.

The main mechanisms of Microcystins toxicity is through inhibiting the protein phosphatase of serine and threonine (PP1 and PP2A)[11] to increase the protein phosphorylation and cell toxicity of direct relevant tumor[12,13], and make the cytoskeleton proteins highly phosphorylated. Moreover, they regulate cell apoptosis by leading to important control protein phosphorylation[14]. Researches proved that exposure to low level of MC-LR can promote many cells apoptosis [15,16].

There are many kinds of statements about toxicology mechanisms of MCs. For example, some studies reported MCs could cause DNA-protein

crosslinks (DPC)[17]. But among these mechanisms, the oxidative stress is attracting more and more attention. It has been reported that MCs can cause oxidative damage in animals both in vitro[15,18]and in vivo experimental models[19,20,21]. Yan Li studied the toxic effect of MC-LR on male rats reproductive system in vitro and vivo[23]. They found MC-LR can cause oxidative stress on leydig's cell and produce cell toxicity. Besides, MC-LR played an important role in cell apoptosis, thus reducing ability of leydig's cell to secrete testosterone and produce the reproductive toxicity [22]. The damage mechanisms may be that MC-LR entered the body and caused the oxidative stress and testicular change, resulting in that testicular function was seriously damaged [23].

In this study, we have made clear that the changes of ROS, LDH, MDA and SOD were associated with MC-LR-induced reproductive system damage in vitro. Reactive oxygen species (ROS) were certain metabolites of oxygen and oxygen derivatives. Their chemical reactivity was more lively than oxygen. Under normal circumstances, ROS had very important physiological role in reproductive system. However, when the generation of ROS beyond the clearing abilities of antioxidant system, they could cause lipid peroxidation. In this experiment, we found that ROS levels of each MC-LR group were higher than that of the control group in different exposure time. This result was consistent with the result of Ding's, who reported the oxidative stress of MC-LR on liver cells [24]. This study showed that the ROS of high concentration MC-LR group had statistically significant difference with control group ($P<0.05$) after exposed for 12h and 24h. The study illustrated that the toxic effect of MC-LR on Sertoli cells was enhanced with the extension of exposure time and the increasing of exposure concentration.

LDH is a special kind of catalyzing enzymes, which is in cytoplasm and participated in glycolytic cycle. LDH leakage is generally accepted as a sign of necrosis. However, recent studies by Chong et al. [25] and Riss et al. demonstrate that LDH leakage is related cells apoptosis [26]. When cells cultured in vitro, LDH will leak out from cell to culture solution if cell membrane is damaged[27,28]. Therefore, cellular damage degree can be measured objectively by measuring LDH leakage rate. The result showed that significant difference of LDH leakage rate between each test group was not observed after Sertoli cells were treated by MC-LR for 6~24h ($P>0.05$). This illustrated that low doses of MC-LR didn't cause cell membrane damage obviously and cell permeability didn't change.

MDA is one of the final products of lipid peroxidation, and it was often used to evaluate the lipid peroxidation degree. This study found that MDA content of each MC-LR dose group hadn't significant change ($P>0.05$) compared with control group cells in different exposure time. The reason may be that the exposure dose was lesser. The results suggested that lipid peroxidation did not occur in Sertoli cells exposed to lesser dose of MC-LR.

SOD is a kind of important intracellular antioxidant enzymes. SOD can transform superoxide anion radical (O_2) into hydrogen peroxide (H_2O_2). With CAT was activated, H_2O_2 can be transformed into H_2O. Too much toxins may cause the excessive consumption of SOD activity [20]. Studies have shown that SOD is the most sensitive antioxidant enzymes after cells are exposed to the MCs. This study also found that the content of SOD in Sertoli cells was reduced with the increase of MC-LR dose. This experiment ascertained that oxidative stress strengthened along with the exposure concentration of MC-LR increasing. But SOD had no statistically significant difference between the different exposure time.

In conclusion, the results suggested that MC-LR can induce oxidative stress in primary cultured rat Sertoli cells, but it can not lead to lipid peroxidation. The present study revealed that MC-LR could exert a generally chronic toxicity on reproductive system of male rat, specially on the testes. Oxidative damage may underlie these pathological changes.

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