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Chronic exposure to microcystin-LR causes autophagy in male testicular cells through DNA damage

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Abstract: Microcystins are a new type of environmental pollutant, posing a significant risk to human health. Previous studies have found that microcystin-LR (MC-LR) can cause autophagy in testicular cells, but the specific mechanism is still unclear. Therefore, this study explored whether chronic exposure to MC-LR can cause autophagy in mouse testicular cells and its mechanism. Male mice were exposed to different MC-LR concentrations (0, 1, 30, 60, 90, 120 μg/L) with free drinking water for 15 months. Detect the expression of autophagy-related proteins and DNA damage-related factors in the testes of mice to explore the role of DNA damage in autophagy induced by chronic exposure to MC-LR. The results showed that MC-LR significantly up-regulated the expression of autophagy-associated proteins ATG5 and BECLIN1 in testicular cells. Furthermore, MC-LR may cause autophagy through DNA damage, manifested in the up-regulation DNA damage genes *ATM*, *ATR*, and *XRCC4* in testicular cells. In summary, our results suggest that chronic exposure to MC-LR triggers autophagy through DNA damage in the testes of mice. This study reveals the molecular mechanism of autophagy induced by chronic exposure to MC-LR and provides a new perspective for studying the chronic reproductive toxicity of MC-LR.
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1. Introduction

Climate change and industrial development have exacerbated environmental pollution. Water blooms caused by eutrophication of water bodies frequently occur. In the eutrophic water body, the algae grow wildly and emit a peculiar smell, which reduces the water quality. Some dominant algae can release toxins into the water body after death. Microcystins (MCs) produced by microcystis aeruginosa are one of the most harmful toxins. MCs are cyclic heptapeptide toxin, and more than 200 isomers have been reported[1]. Among them, microcystin-LR (MC-LR) is the most toxic and most studied isomer[2]. The chemical properties of MC-LR are stable, and the natural degradation process is also very prolonged. It can exist for months or even years in a suitable water environment[3, 4]. Humans and animals can be exposed to MC-LR in a variety of ways, such as ingestion of contaminated water or food, inhalation and skin contact. The stability, persistence and diversity of exposure pathways of MC-LR have brought considerable challenges to human health.

Epidemiological studies have proved that acute exposure to MC-LR can cause poisoning and even death[5, 6]. Chronic exposure to MC-LR can damage the liver, gonads, kidneys and other organs, and promote tumors and carcinogenesis[7]. The gonads are significant target organs for MC-LR. Studies have found that exposure of CHO cells to MC-LR triggered a large accumulation of autophagy vesicles, and the expression of autophagy-related proteins (BECLIN1 and LC3II) increases, indicating that MC-LR induces autophagy in CHO cells[8]. Acute toxicity studies found that MC-LR induced autophagy in mouse ovarian tissue[9]. However, whether chronic exposure to MC-LR can induce autophagy in the testes of male animals and its mechanism are unclear.

DNA damage sensors (such as ATM and ATR) are important autophagy regulators, and play a role in autophagy. ATM and ATR are two serine/threonine kinases that control multiple processes such as DNA replication, transcription, metabolic signaling, and DNA splicing[10]. It has been shown that ATM is involved in the process of autophagy. ATM activates the downstream molecule XRCC4, thereby driving the nucleation and formation of autophagosome membranes[11]. DNA damage seems to play an essential role in autophagy. However, whether chronic exposure to MC-LR can induce autophagy in mouse testicular cells, and whether DNA damage is involved in MC-LR-induced autophagy remains unclear. Therefore, the purpose of this study is to study the expression of

autophagy-associated proteins (BECLIN1 and ATG5) and DNA damage-related genes (*ATM*, *ATR* and *XRCC4*) in the testes of mice, and to explore autophagy mechanisms.

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); BECLIN1 antibody (ab62557, Abcam, USA); ATG5 antibody (ab108327, Abcam, USA).

2.2 Animal treatment

SPF male C57BL/6 mice aged six weeks were obtained from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China), which were littermates and randomly divided into six groups. Mice were treated daily with different concentrations of MC-LR (0, 1, 30, 60, 90, 120 μg/L) with free drinking water for MC-LR. Testicular tissues were extracted at 15 months after exposure for subsequent experimental analysis. All animals are raised in SPF-level laboratories, placed at appropriate temperature and humidity, and given standard rodent pellets, free drinking water, and a 12-hour light/dark cycle.

2.3 Western blotting

Remove the spare testicular tissue from -80 ºC to extract the total protein (Each group contains 3 replicate samples). 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 polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The membranes were blocked in TBST containing 5% BSA at room temperature for 2 hours and immunoblotted using primary anti-ATG5 and anti-BECLIN1. 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 mice testicular cells exposed to various concentrations of MC-LR (0, 1, 30, 60, 90, 120 μg/L) by Trizol reagent. RevertAid first Strand cDNA Synthesis kit was used to synthesize cDNA in a 20 μl reaction system following the manufacturer's instructions. SYBR premix Ex Taq was used to prepare a 10 μl system, and QuantStudio 7 Flex real-time PCR system (Life Technologies, USA) was used to perform RT - qPCR. All samples were performed in triplicate. 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')
ATM	CCGTGATGACCTGAGGCAAGATG	AGAACACCGCTTCGCTGAGAAAGGAATC
ATR	GCCACCATCAGACAGCCTACAAT	GCCACTTTGCCCTCTCCACA
XRCC4	ACAGGAGTTGCCGCTACAGGAG	GGGCTGCTGTTTCTCAGGG
β-actin	TCAAGATCATTGCTCCTCCTGAG	ACATCTGCTGGAAGGTGGA

2.5 Statistical analysis

Data were expressed as mean ± standard deviation (SD). The three data in the control group were averaged, and then the statistical analysis was performed after normalizing the data in each group to the geometric mean of the control group. 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 Chronic exposure to MC-LR causes tissue damage in the mice testis.

H&E staining can detect the pathological damage of testis tissue induced by chronic exposure to MC-LR. The results showed that the testicular tissue of the control group had a normal and intact seminiferous tube structure. The morphology of Sertoli cells and spermatogonia are normal and regular, and there are no other significant changes in the control group (Figure 1A). Increasing the exposure concentration of MC-LR will aggravate the damage to the testicular tissues, with varying degrees of cell arrangement

disorder (blue arrow), gaps between cells, cell loosening (red arrow), and even cell shedding (yellow arrow) (Figure 1B-F).

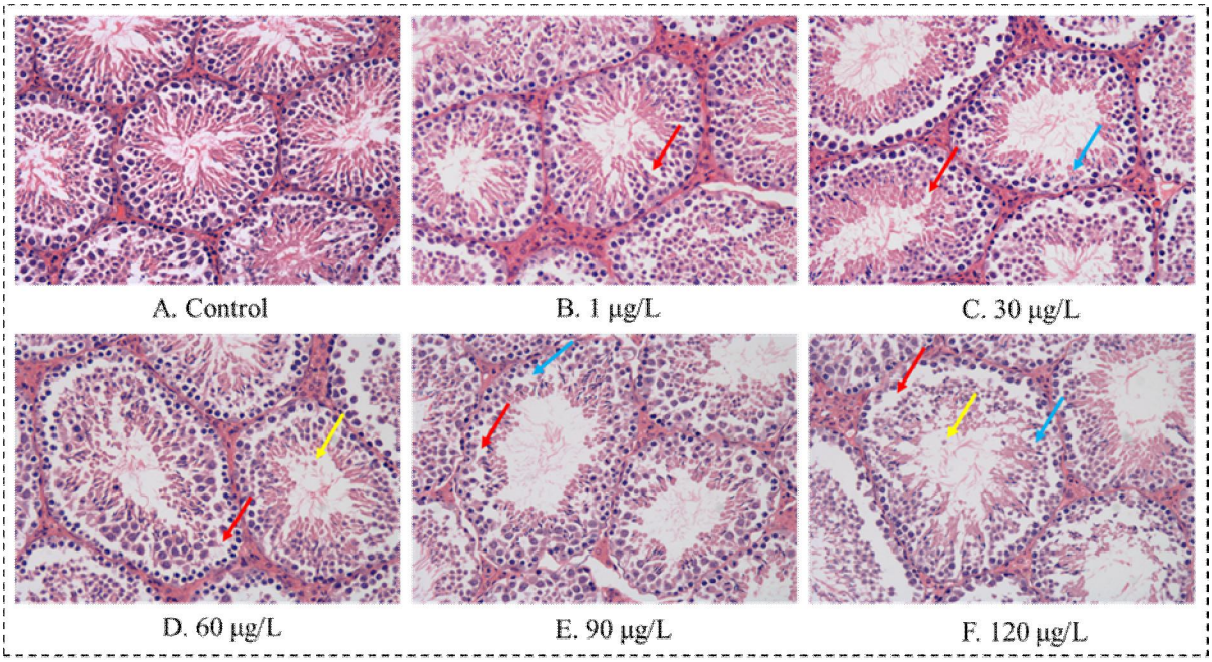

Figure 1. The results of hematoxylin and eosin (H&E) staining in testicular tissue of mice (×200). Red arrow: gaps in the testis with loose cells; Blue arrow: cell disorder; Yellow arrow: cell shedding.

3.2 MC-LR increases the expressions of autophagy associated proteins in mice testis.

MC-LR induced the increasing expressions of autophagy-associated proteins (ATG5 and BECLIN1) in mice testis. As shown in Figure 2 A and B, the protein expression of ATG5 had an increase after exposure to MC-LR. Compared with the control group, the expression level of ATG5 was statistically significant at a concentration of 30 μg/L, 60 μg/L, 90 μg/L and 120 μg/L (P<0.05). Figure 2C showed that the protein expression level of the BECLIN1 was significantly elevated after MC-LR treatment (90 μg/L and 120 μg/L). ATG5 and BECLIN1 are essential proteins for cellular autophagy, indicating that MC-LR could induce autophagy in mice testis.

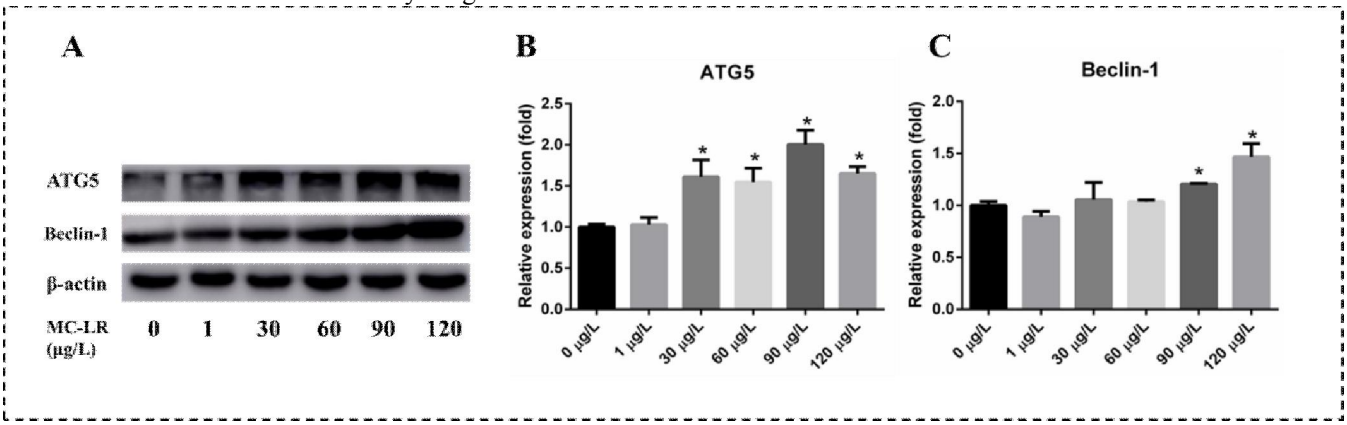

Figure 2. Effect of chronic MC-LR exposure on autophagy-related protein expression in mouse testis. (A): The expression of ATG5 and BECLIN1 in mice testis after chronic exposure of MC-LR. (B): Statistical analysis chart of ATG5 expression in mouse testis after chronic exposure to MC-LR. (C): Statistical analysis chart of BECLIN1 expression in mouse testis after chronic exposure to MC-LR. *P<0.05 vs. the control group.

3.3 MC-LR increases the expressions of DNA damage related genes in mice testis.

To explore the mechanism of autophagy induced by MC-LR, we tested the DNA damage sensors ATM, ATR and XRCC4 that regulate autophagy in mice testes. In Figure 3 A and B, compared with the control group, the gene expression levels of *ATM* and *ATR* were increased after exposed to MC-LR (60 μg/L, 90 μg/L and 120 μg/L) (P<0.05). Figure 3C showed that the gene *XRCC4* expression levels were significantly increased after MC-LR treatment (30 μg/L, 60 μg/L, 90 μg/L and 120 μg/L) (P<0.05). These results indicated that DNA damage might be related to MC-LR-induced autophagy.

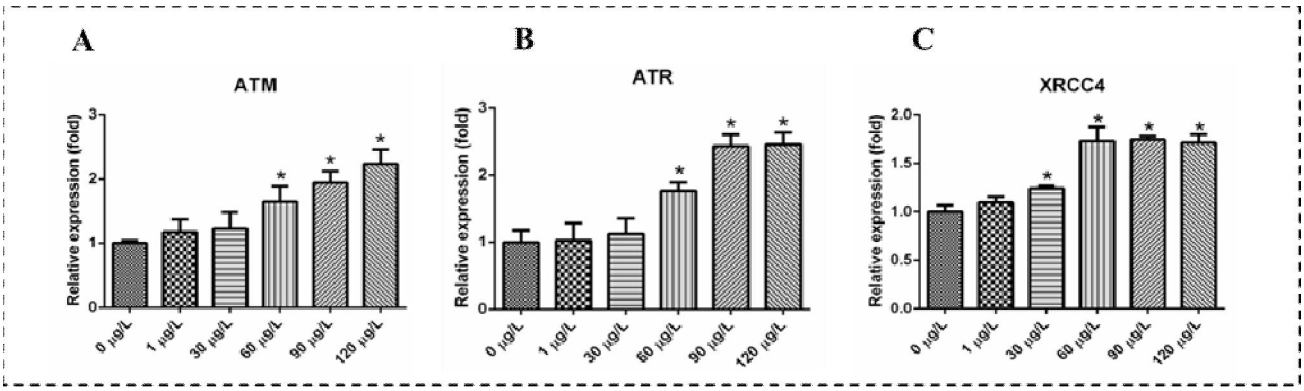

Figure 3. Effect of chronic MC-LR exposure on DNA damage-related gene expression in mouse testis. (A): The *ATM* gene expression in mice testis after exposure of MC-LR. (B): The *ATG* gene expression in mice testis after exposure of MC-LR. (C): The *XRCC4* gene expression in mice testis after exposure of MC-LR. *P<0.05 vs. the control group.

4. Discussion

Studies have shown that MC-LR has a toxic effect on the reproductive system[12, 13]. It can accumulate in the testes and ovaries causing structural damage and dysfunction of the gonadal glands. Acute toxicity studies have found that MC-LR induces autophagy in mice ovaries[9]. Whether MC-LR can induce autophagy in the testis is unclear, and whether chronic exposure to MC-LR can cause autophagy in the gonadal is still unknown.

Autophagy is a process of cell self-degradation. Cells use autophagy to remove damaged or excess proteins, organelles and foreign pathogens. However, excessive autophagy can cause cell death. More than 30 autophagy-related (ATG) proteins control autophagy activation[14]. ATG5 is an autophagy protein that fuses autophagosomes and lysosomes to form autolysosomes[15]. Therefore, ATG5 is an essential protein for autophagy. BECLIN1 is the first described mammalian autophagy protein and the first identified mammalian homologue of the essential yeast autophagy gene *ATG6*[16]. BECLIN1 is the core component of class III phosphatidylinositol 3-kinase (PI3K-III) complex. This complex plays an important role in membrane transport and reorganization involving autophagy. We detected the expression of autophagy-related proteins ATG5 and BECLIN1, and found that the protein expression levels of ATG and BECLIN1 in mice testes increased significantly under long-term exposure to MC-LR. This result suggests that chronic exposure to MC-LR can cause autophagy in mice testes.

Autophagy can be activated in response to different cellular stress factors, such as damaged organelles, accumulation of misfolded or unfolded proteins, endoplasmic reticulum stress, reactive oxygen species, and DNA damage. Certain DNA damage sensors (such as ATM and ATR) are known to be important autophagy regulators, so autophagy seems to play a role in the DNA damage response. ATM and ATR control multiple processes such as DNA replication, transcription, metabolic signal transduction, and DNA splicing[11]. Increased ATM gene expression suggests that there may be cellular DNA damage in the cells. XRCC4 is a structurally related protein that plays a role in DNA double-strand break repair[17]. To further study whether DNA damage is involved in the autophagy of testicular cells induced by MC-LR, we tested the gene expression of DNA-related factors *ATM*, *ATR* and *XRCC4* in the testis. The results showed that chronic exposure to MC-LR could induce the increase of *ATM*, *ATR* and *XRCC4* gene expression, suggesting that MC-LR could induce autophagy via DNA damage.

Our study found that chronic exposure to MC-LR can cause autophagy in mouse testes. This provides ideas for further research on the chronic toxicity caused by MC-LR. Besides, we found that DNA damage may play an important role in the autophagy mechanism of MC-LR. This provides novel insights for understanding the molecular mechanism of autophagy induced by long-term exposure to MC-LR.

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