The mechanisms of reproductive toxicity induced by MCs

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Abstract: Microcystins (MCs) are the most common cyanobacteria toxins and endocrine disruptors that can cause hormonal disorders and affect the normal reproduction of humans, fish and mammals. In this review, we summarized the possible toxicity mechanisms of MCs in reproductive system. MCs could inhibit activity of protein phosphatase 2A（PP2A） and induce a great quantity of reactive oxygen species (ROS), which cause reproductive toxicity via apoptosis, autophagy, cytoskeletal destruction, reproductive tumors and endocrine disrupting. At the same time, we proposed that the mechanisms of absorption, transport pathways, distribution and toxic effects of MCs in the gonad need to be studied in detail.
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Introduction

With the increase of environmental pollution, the relationship between decline of human fertility and environmental exposure has attracted worldwide attention. Microcystins (MCs) are the most common cyanobacteria toxins and are endocrine disruptors that can cause hormonal disorders in humans and affect the normal reproduction of humans, fish and mammals. The toxic effects and the molecular mechanisms of MCs on reproductive system are a hot topic in environmental toxicology.

MCs, produced by cyanobacteria in eutrophic water, are a class of cyclic heptapeptide intracellular toxins, with more than 100 different congeners [1,2]. Microcystin-LR (MC-LR) is one kind of analogue of MCs [3]. Existing treatment methods of drinking water can't effectively remove MCs [4,5]. MCs are widely presented in freshwater. Humans and animals can contact to MCs in a variety of ways. MCs can pass through the digestive tract (diet, drinking water), respiratory tract (MCs with water vapor in the air), the skin (exposure to the skin of the air, bath skin contact) and other ways into the human and animal body. People and animals are difficult to avoid the harm caused by MCs [6,7]. To reduce risks caused by MCs, the World Health Organization (WHO) has set a provisional guideline of 1μg/L MCs in water destined as human drinking water standard.

MCs can accumulate in muscle, liver, gonads, brain and other tissues [8-10]. Recent studies have found that plants and groundwater also can accumulate MCs by irrigating water contaminated by microcystins, which threaten human health [11,12]. Zhao et al. found that the content of MCs in serum of Taihu fishermen in China was 0.10 to 0.64 μg/L [13]. MCs are a kind of recognized liver toxin. Liver can accumulate a large number of MCs. It is the first target organ of MCs [14]. MCs can instantaneously inhibit the activity of protein phosphatase 2A （PP2A）and protein phosphatase 1 (PPl), causing protein hyperphosphorylation, hematopoietic breakdown of cells, cell rupture and even liver hemorrhage [15]. MCs can cause ROS level and malondialdehyde (MDA) in hepatocytes increase and affect the activity of glutathione peroxidase, which leads to the apoptosis of hepatocytes [16]. MCs can accumulate in gonad of animals and be transferred from the mother to the offspring, so gonads are considered the second target organ [6,9,10]. Vivo experiments demonstrated MCs can cause substantial damage to reproductive system. MCs caused germ cell apoptosis [17]. MCs also cause destruction of germline cytoskeleton by inhibiting the activity of PP2A and PP1 and promoting the production of ROS, which in turn causes gonadal tissue destruction [18].

Our team has been working on the studies about reproductive toxicity of MCs and the possible molecular mechanism. Therefore, the aim of this review was to provide the most current information covering the research of other scholars and our previous studies to better understand the reproductive toxicity mechanism induced by MCs.

1. Biological effects

After male Sprague Dawley (SD) rats were administered an intraperitoneal injection with 0, 50 μg/kg body weight (1/3 lethal dose 50, LD50) and 100 μg/kg body weight (2/3 LD50) of MC-LR for one

week， the relative weight of the testis and the diameter of the seminiferous tubules were significantly reduced at 100μg/kg MC-LR exposure group. HE staining showed that seminiferous tubules were blocked; spermatogenic cells were sparse, disorder and dissolved. TUNEL staining showed that apoptotic cells were increased [19]. Wu et al. also found that after female mice were orally given 0, 1, 10 and 40 μg/L MC-LR for 3 months or 6 months, the gonad somatic index (GSI) dropped significantly when the dose of MC-LR was given 40 μg/L. In 6-month exposure group, follicular atresia and concentration of MC-LR showed a dose-response relationship. The loss of primitive, primary, secondary follicle increased significantly at dose of 40 μg/L. And serum progesterone increased significantly in high-dose group, while estradiol significantly reduced [20]. Male zebrafish were exposed with 0, 0.3, 3 and 30 μg/L MC-LR for 90 days, the length and weight of zebrafish decreased significantly compared with the control group and showed a dose-response relationship. In exposure groups, histological observation revealed that the cell density was reduced, the seminiferous tubules were disordered and their epithelial cells were dissolved, resulting in a widening of the gap between cells and cells [21]. Chen et al. put forward that male Wistar rats were intraperitoneally injected with 1 and 10 μg/kg body weight of MC-LR daily for 50 days. Dose of 10μg/kg MC-LR induced cytoplasmic contraction, mitochondrial swelling and cytoskeleton destruction. And ultrastructural observation showed testicular index decreased significantly [22].

In vitro experiments, Zhou et al. found that the cell viability of SD rat spermatogonia was significantly decreased after exposure to MC-LR. FDA and PI staining showed that the ratio of apoptotic cells of spermatogonia was significantly increased, ROS production increased and antioxidant capacity decreased with the increase of exposure concentration [23]. Our previous studies also found that the activity of sertoli cells significantly decreased and the morphology of the nucleolus changed when sertoli cells were cultured with MC-LR for 24 h [24]. The level of ROS and the apoptosis rate of Chinese hamster ovary (CHO) cells was significantly increased when CHO cells were exposed to MC-LR [25].

In conclusion, MCs can induce apoptosis of germ cells, induce morphological changes of gonad tissues, destroy cytoskeleton and produce ROS. Undoubtedly, there are some differences in toxic effects of different variants of MCs on reproductive system due to different cells and test animals used in studies, which need further study.

2. The mechanisms of apoptosis induced by MCs in germ cells

MCs can induce apoptosis of germ cells and cause some other biological effects. MCs should have enough doses to enter the reproductive system through the blood system to exert its reproductive toxicity. However, the higher molecular weight of MCs determines that it cannot enter cells through passive transport and must be transported by means of active transport [1]. Organic anion transporting polypeptide (OATP) is an important trans-membrane transporter that can participate in a variety of toxin transport [26]. Zhou et al. found that the expression of Oatp3a1 in MC-LR exposed group was significantly increased compared with the control group in primary cultured spermatogonia of male SD rat [23]. Faltermann et al. also found that the expression of Oatp1d1 was significantly increased in CHO cells exposed to MC-LR [27]. Different concentrations of MC-LR led to different damage to zebrafish embryo. MC-LR with concentration less than 0.04 mmol/L was mainly adsorbed on outer membrane of embryo when the MC-LR. While concentration was more than 0.50 mmol/L, MC-LR can directly enter the cytoplasm, causing thinning and rupture of the membrane [28]. Above evidences show that MCs can enter the reproductive system with the help of OATP.

Substantial studies have shown that MCs can combine with protein phosphatase in liver cells, inhibit the activity of protein phosphatase and cause protein hyperphosphorylation [15,29]. In reproductive system, Liang et al. observed the binding of MC-LR to PP2A by western blot (WB) and immunofluorescence staining when human amniotic epithelial cells exposed to MC-LR. At low concentration, the activity of PP2A increased and the activity of PP2A was inhibited at high concentration. The acetylation level of tubulin also changes with PP2A activity [30]. PP2A could regulate the phosphorylation and de-phosphorylation in cells to achieve dynamic balance and maintain the normal growth and reproduction of cells, which play an important role in the process of cell signal transduction [31,32]. Therefore, MCs could break dynamic balance of phosphorylation and de-phosphorylation in cells to play its reproductive toxicity by inhibiting the activity of PP2A. MC-LR can cause phosphorylation of some proteins in the mitogen activated protein kinases (MAPK) signaling pathway by altering the activity of PP2A. And thus cause liver cells, kidney cells and other cytoskeleton destruction and apoptosis [33]. In reproductive system, Wang et al. found that the P53 and Bcl-2 families have a significantly higher phosphorylation level [34].

2.1 MCs induce apoptosis in germ cell via mitochondrial pathways

MCs could cause a significant increase in ROS and MDA, mitochondrial swelling, mitochondrial membrane damage and cytochrome c (Cyt-c) release in testes of frogs. And the protein expression levels of Caspase-3 and Caspase-9 were significantly increased [35]. Our previous study found that MC-LR can induce sertoli cell apoptosis and intracellular ROS significantly increased, mitochondrial membrane potential significantly decreased. Cyt-c, activated Caspase-9 and activated Caspase-3 was significantly increased with the increase of exposure concentration. The apoptosis rate decreased after adding N-acetyl-L-cysteine (NAC) [36]. The content of MDA in zebrafish ovary exposed to MC-LR was significantly increased, suggesting that the level of reactive oxygen species was significantly increased. And MCs activated the antioxidant system, the antioxidant enzyme (catalase (CAT), superoxide dismutase (SOD), glutathion peroxidase (GPx)) activity and transcription level were significantly increased. While the level of glutathione significantly decreased, suggesting that GPx via glutathione to play a role in detoxification [37]. Li et al. found that MC-LR could induce the protein expression of P53 and Bax and decrease the expression of Bcl-2 protein [38]. When Bcl-2 expression reduced, the mitochondrial PT pore will open, which lead to the decrease of mitochondrial membrane potential and the release of Cyt-c [39].

Cyt-c released into cytoplasm will bind to apoptosis associated factor -1 (Apaf-1) to form a multimeric body [40]. And it is able to promote the combination of Caspase-9 with the multimeric body to form apoptotic bodies. Cyt-c is an important carrier for the transmission of electrons in respiratory chain. Cyt-c level decrease in mitochondria when cyt-c is released from the mitochondria into the cytoplasm. And it will lead to the escape of electrons in respiratory chain, resulting in a large number of ROS production [41]. ROS can cause lipid peroxidation, destroy the mitochondrial membrane and thus form a vicious cycle.

2.2 MCs induces apoptosis in germ cells via death receptor pathway

MCs could increase the expression levels of Caspase-3 and Caspase-8 in sertoli cell [34]. Caspase-8 is a significant protein backward position in death receptor pathway. MCs also could induce the expression of Fas and Fas ligand (FasL) and the expression of Apaf-1, Fas-associated death domain-containing protein (FADD), Bid. Casepase-3, -8 proteins was also significantly increased [42].

Therefore, MCs could increase the expression of Fas and FasL and then induced Caspase-8 self-cleavage by FADD precursor. Cleaved Caspase-8 directly activates the downstream effector Caspase-3, -9, which causes germ cell apoptosis in turn. Moreover, the cleaved Caspase-8 can cleave Bid in Bcl-2 family. And then cause Bid to transfer to the mitochondrial membrane, which leads to a decrease in mitochondrial membrane permeability and release of cyt-c, leading to germ cells apoptosis via the mitochondrial pathway [43].

2.3 MCs induces apoptosis in germ cells via endoplasmic reticulum pathway

MC-LR could induce expression of C/EBP-homologous protein (CHOP) and Caspase-12 and induce liver injury through the endoplasmic reticulum pathway [44]. In current study, MC-LR could induce reproductive toxicity of zebrafish larvae through endoplasmic reticulum stress. In experimental group, the expressions of gene related to endoplasmic reticulum stress were significantly increased in mRNA level, such as eif2s-1, atf4b1, atf6, mapk8 and chop. Expression of Caspase8 and Caspase3 was significantly increased not only in mRNA level and but also in protein level. Acridine orange (AO) staining showed that the proportion of apoptotic cells was significantly lower than control group after the addition of endoplasmic reticulum stress inhibitor TUDCA [45]. Our preliminary study also showed that the expression of glucose-regulatedprotein78 (GRP78), activating transcription factor 6 (ATF-6）, PKR-like ER kinase (PERK ）, Inositol-requiring enzyme 1 (IRE1), CHOP was significantly higher in CHO cells exposed to MC-LR compared than that in control group. And fluorescence intensity of intracellular Ca^{2+} increased gradually with the concentration of exposure [25]. The results suggest MCs could induce ERs and further cause apoptosis in germ cells via activating GRP78, ATF-6, IRE1 and PERK.

2.4 The role of P53 and MAPK in regulating apoptosis

P53 is an important factor in regulation of apoptosis and plays an vital role in process of apoptosis. Studies have showed that p53 target genes such as Fax, Apaf-1 and Bcl-2 families also play an important role in the process of apoptosis [46]. In reproductive system, the phosphorylation levels of P53 and Bcl-2 families significantly increased after exposed to MC-LR [34]. Meng et al. found that the phosphorylation levels of spermatogonia in mouse testes significantly increased after exposed with MC-LR. In these study, miR-541 transcription levels increased and p15 transcription levels decreased, suggested that increased miR-541 led to reduced expression of p15 and then led to activation of p53 to induce apoptosis [47]. MCs could increase phosphorylation of P53 and Bcl-2 and increased levels of protein expression in Caspase-3 and Caspase-8[34,38]. Germ cell apoptotic rate of P53 related gene knockout

caenorhabditis elegances significantly decreased compared with the MC-LR group [40]. Xiong et al. found expression levels of Fas and FasL protein were significantly increased [42], while Fas was one of the many pro-apoptotic proteins encoded by p53. These studies suggest that p53 plays an important role in MCs-induced germ cell apoptosis. It is likely that MCs inhibit PP2A activity to induce the phosphorylation of p53 and then induce apoptosis by means of mitochondria and death receptor pathways through regulating the Bcl-2 family.

Mitogen activated protein kinase (MAPK) plays an important role in process of intracellular signal transduction and regulates various physiological processes of cells by phosphorylated transcription factors, cytoskeletal proteins and enzymes. Apoptosis is induced mainly through the JUN amino-terminal kinase (JNK)/stress-activated protein kinases（SAPK）subfamily and the p38MAPK in MAPK signal pathway [48,49]. The germ cells apoptotic rate of MAPK related gene knockout caenorhabditis elegances was significantly decreased compared with the MC-LR groups [40]. Liu et al. also found that the phosphorylation level of JNK and P38MAPK was increased by inhibiting the activity of PP2A in the liver of MC-LR-exposed mice [29]. In reproductive system, MC-LR can activate MAPK, which in turn activates Bax/Bcl-2 and Caspase-dependent apoptosis pathways to cause reproductive toxicity of zebrafish [44]. Chen et al. found MC-LR induced expression of miR-758 and miR-98-5p decrease in MC-LR-exposed male SD rats and in vitro cultured sertoli cell, which increased expression of p38MARK protein phosphorylation. And phosphorylated p38MAPK could induce the phosphorylation of ATF-2 protein and then activate tumor necrosis factor-α (TNF-α) and tumor necrosis factor receptor 1 (TNFR1) to induce sertoli cell apoptosis through death receptor pathway [50].

3. MCs induce destruction of the cytoskeleton

Cytoskeleton plays an major role in maintaining cell shape and maintaining cell motility and mainly consists of microfilaments, microtubules and intermediate fibers [51]. Mariann et al. found that actin and tubulin were reduced or disappeared in MC-LR-exposed CHO-K1 cells, leading to changes in microfilaments and microtubules [52]. The microfilament and microtubule structures were destroyed after exposure to MC-LR in embryos of New Zealand white rabbits cultured in vitro [53]. MCs also induced significant changes in cytoskeletal related genes in testis of male Wistar rats. And the transcriptional levels of beta-actin and beta-tubulin were significantly lower in the exposed group [22]. Therefore, MC-LR can cause the destruction of cytoskeleton. When cytoskeleton destroyed, it will further contribute to cell apoptosis due to cytoskeleton plays a vital role in maintaining normal cell morphology.

4. MCs promote the occurrence of reproductive tumors

A large number of previous studies suggested that MC-LR induced phosphorylation of MAPK and protein kinase B (Akt) signaling by inhibiting the activity of PP2A. Phosphorylated p38MAPK and JNK could enhance the expression of c-myc and activate c-Jun and c-fos. Phosphorylated Akt could further activate S6K1 and cause cell proliferation, thereby promoting tumor induced [29, 54,55]. In reproductive system, the expression of c-myc, c-jun and c-fos were significantly increased in mRNA levels in whitefish ovarian and BALB/c mice testes after exposed to MC-LR [34,56]. Chen et al. also found that expression of c-jun and c-fos in both mRNA and protein levels were significantly increased. And phosphorylation levels of p38MAPK were significantly higher in MC-LR exposed groups [50]. This suggests that MCs could induce p38MAPK phosphorylation by inhibiting the activity of PP2A, which in turn activates c-jun and c-fos to induce reproductive tumors. It is well known that the reproductive system is extremely important for breeding offspring and the toxic effect of toxicant on sperm or egg gene of the offspring is irreversible. MC-LR increased the number of apoptotic DNA fragments in both testis and cultured sertoli cell of SD rats [17]. MC-LR also induced mitochondrial DNA damage to testis of Wistar rats [22]. Lankoff et al. found MC-LR played its reproductive toxicity by interfering with nucleotide excision repair [57]. Therefore, MCs phosphorylate p38MAPK via inhibit the activity of PP2A, further induced c-myc, c-fos, c-jun activation to promote tumorigenesis. MCs not only cause DNA damage but also interfere nucleotide excision repair to play toxicity on the genes.

5. MCs cause endocrine disrupting and have estrogenic potential

MC-LR interfered with the endocrine function of female zebrafish and the development of eggs. The concentrations of estradiol (E2) and vitellogenin (VTG) were significantly increased compared with the control group in the 10ug/L exposure group. The concentrations of E2, VTG and testosterone were significantly decreased when exposed to a concentration of 50μg/L. And the mRNA expression level of related to hypothalamus-pituitary-gonadal axis (HPG) gene significantly changed, it corresponded not only to changes in hormone levels, but also to a dose-response relationship [58]. MC-LR significantly reduced the ratio of T/E2 in male

zebrafish testis and thus destructive steroid hormone balance. And the mRNA expression level of HPG-related genes was significantly changed to promote the transformation of T to E2 in circulating blood [21]. Li et al. found that MC-LR decreased serum testosterone levels, follicle stimulating hormone (FSH) and luteinizing hormone (LH) in SD male rats [17]. Hou et al. found that the testosterone concentration in ovary was decreased in female zebrafish exposed to MC-LR. However, the transcriptional level of FSH and LH in pituitary gland and estrogen receptor (ERα), FSH receptor (FSHR) and LH receptor (LHR) was significantly increased. It may be a positive feedback of the HPG axis due to the low exposure concentration [59]. Ding et al. also found that MC-LR transported to neurons secreted gonadotropin releasing hormone (GnRH) via Oatp1a5 and further interfered with male endocrine secretion due to testosterone production must depend on GnRH [60]. Therefore, MCs can interfere with the expression of HPG axis genes, not only directly damage the gonadal hormone synthesis of hormones, but also through the hypothalamus and pituitary indirect effects of endocrine function. The expression of vitellogenin (vtg) both in female zebrafish and immature zebrafish was significantly higher in exposure groups [21,61]. The activation of the luciferase gene in the receptor–reporter gene assay using transgenic human cells line MELN indicates clearly that MC-LR at low concentrations present estrogenic potential likely by indirect interaction with estrogen receptors [62], suggesting that MCs may have estrogenic potential.

6. The mechanisms of autophagy induced by MCs in germ cells

Chen et al. found that the expression of LC3 was significantly increased in MC-LR-exposed SD rat sertoli cells. The levels of cyt-c, Bcl-2 and Caspase-3 were also decreased compared with the single MC-LR exposure group after treated with autophagy inhibitor 3-methyladenine (3-MA). This suggested that MC-LR had toxic effects on Sertoli cells by inducing autophagy and apoptosis [63]. Our recent studies also found that the number of autophagosome increased gradually with the increase of exposure concentration in CHO cells exposed MC-LR. The expression of autophagy marker proteins Beclin1 and LC3II was elevated. The apoptosis rate and autophagy level were significantly lower than single MC-LR exposure group when added endoplasmic reticulum stress inhibitor. However, the apoptosis rate increased compared with the exposure alone group when mixed in autophagic inhibitors [25]. These results suggested that ERs and autophagy are involved in the MC-LR-induced apoptosis of CHO cells. Targeting ERs and autophagy could be a promising therapeutic strategy for protecting against MC-LR toxicity. However, autophagy and apoptosis may be interacted with each other under endoplasmic reticulum stress [64,65], which has not been reported in the field of MCs research and needs further study.

7. MCs induce cell cycle arrest and inflammation

Chen et al. found that MC-LR inhibits TIFA expression and blocks the sertoli cells cycle through p53 pathway [50]. Our study also found that the cell cycle was blocked in G2/M phase in MCs-exposed CHO cells. MC-LR can also induce the inflammatory response of sertoli cells by stimulating the expression of TNF-α. Another study found that MC-LR can inhibit the development of prostate progeny in the offspring of mice by inducing inflammation [66]. These showed that MCs can induce reproductive toxicity through not only the apoptosis, autophagy, endoplasmic reticulum stress and cytoskeleton destruction but also other pathways, which also requires further in-depth study.

Summary

In this review, we summarized the possible mechanisms of MCs toxicity on reproductive system. First of all, MCs can enter the reproductive system with the help of OATP and accumulate in the gonad. Secondly, MCs can inhibit the activity of PP2A to causes hyperphosphorylation of key control proteins that regulate apoptosis, cytoskeleton organization and ERs. Meanwhile, a great quantity of ROS generated in gonad. But it is not clear that how can large amounts of ROS are produced, which needs to be addressed by further studies. Thirdly, MCs induce germ cell apoptosis via mitochondrial pathways, death receptor pathway and endoplasmic reticulum pathway. Moreover, p53 and MAPK play an important role in MCs-induced germ cell apoptosis. The fourth, MCs induce cytoskeleton destruction, reproductive tumors, cell cycle arrest and inflammation in reproductive system due to PP2A inhibition and ROS generation. The last but not least, MCs induce autophagy via ER pathway. And autophagy and apoptosis may be interacted with each other under endoplasmic reticulum stress, which has not been reported in the field of MCs research and needs further study.

In summary, studies have shown that MCs induce reproductive toxicity. But there are still many gaps or problems that need to be solved: (1) The role of ROS as a mechanism of reproductive toxicity induced by MCs. (2) The molecular mechanisms participating in autophagy and apoptosis by ER pathway in MCs induced-reproductive toxicity. (3) More studies on toxicity induced by MCs in female reproductive system should be conducted in further. (4) Whether the decline in human fertility in recent

decades is associated with MCs pollution? This requires crowd cohort data to study. (5) More effective detection methods and removal methods of MCs are limited. (6) The toxicity mechanism of MCs on reproductive system should be explored in further. The combined effects of different MCs variant or MCs with other pollutants in water also should be explored.

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