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A review of reproductive toxicity of microcystins

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HIGHLIGHTS

- Reproductive toxicity of MCs on mammals, fishes, amphibians, and birds is reviewed.
- PP1/2A inhibition and oxidative stress are important toxic mechanisms of MCs.
- Reproductive toxicity of MCs may be closely related to endocrine-disrupting effects.
- The trans-generational toxicity of microcystins is a matter of concern.
- Data concerning female reproductive and sex-specific effects of MCs are lacking.

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ABSTRACT

Animal studies provide strong evidence of positive associations between microcystins (MCs) exposure and reproductive toxicity, representing a threat to human reproductive health and the biodiversity of wild life. This paper reviews current knowledge of the reproductive toxicity of MCs, with regard to mammals, fishes, amphibians, and birds, mostly in males. Toxicity of MCs is primarily governed by the inhibition of protein phosphatases 1 and 2A (PP1 and PP2A) and disturbance of cellular phosphorylation balance. MCs exposure is related to excessive production of reactive oxygen species (ROS) and oxidative stress, leading to cytoskeleton disruption, mitochondria dysfunction, endoplasmic reticulum (ER) stress, and DNA damage. MCs induce cell apoptosis mediated by the mitochondrial and ROS and ER pathways. Through PP1/2A inhibition and oxidative stress, MCs lead to differential expression/activity of transcriptional factors and proteins involved in the pathways of cellular differentiation, proliferation, and tumor promotion. MC-induced DNA damage is also involved in carcinogenicity. Apart from a direct effect on testes and ovaries, MCs indirectly affect sex hormones by damaging the hypothalamic-pituitary-gonad (HPG) axis and liver. Parental exposure to MCs may result in hepatotoxicity and neurotoxicity of offspring. We also summarize the current research gaps which should be addressed by further studies.

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1. Introduction

Cyanobacterial blooms due to eutrophication in aquatic environments and associated cyanotoxin contamination are increasingly reported worldwide (Fig. 1) [1,2]. Besides other adverse effects such as the disruption of hydrochemistry or sunlight in the water column, production of an unpleasant odour and taste, cyanobacteria are also known to produce a wide variety of potent toxins, *i.e.*, cyanotoxins. Cyanotoxins can be accumulated in aquatic organisms and transferred to higher trophic levels through the food chain, representing a health hazard to animals and humans (Fig. 2) [3–5].

Among all the cyanotoxins, microcystins (MCs) are the most frequently studied because of their wide distribution and high toxicity [6–7]. MCs, a family of cyclic heptapeptide cyanotoxins (Fig. 3), are the secondary metabolites produced by several genera of blue–green algae, including *Microcystis*, *Anabaena*, *Anabaenopsis*, *Oscillatoria*, and *Nostoc*. [6–9]. The general structure of MCs is cyclo-(-D-Ala^1-L-X^2-D-isoMeAsp^3-L-Z^4-Adda^5-D-isoGlu^6-Mdha^7), where D-isoMeAsp is D-erythro-β-methyl-aspartic acid, Mdha is *N*-methyl-dehydro-alanine, Adda is (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4E,6E -dienoic

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Fig. 1. *Microcystis aeruginosa* bloom near the reeds in the north shore of the Gonghu Bay in Lake Taihu, China. (Photographed by Dr. Dezhao Yu).

acid, and X and Z in positions two and four are highly variable L-amino acids that determine the suffix in the nomenclature of MCs [10]. The cyclic structure and novel amino acids of MCs enhance their chemical stability and thus MCs can persist for several months or even years in natural water [11]. More than 100 different MC congeners have been identified, mostly due to substitutions of the variable L-amino acids X and Z, although modifications have been reported for all of the amino acids [7]. Microcystin-LR (MC-LR) is found to be the most common and potent variant, followed by microcystin-RR (MC-RR) and microcystin-YR (MC-YR) [12]. The most severe human toxicity event happened in February 1996 in Brazil, where 100 of 131 patients developed acute liver failure due to MCs contamination of the water used for hemodialysis and 52 patients died [13–15]. To reduce risks caused by MCs, the World Health Organization (WHO) has set a provisional guideline of 1 μg/L MCs in water destined for human drinking water.

MCs are known to be highly potent and specific inhibitors of eukaryotic protein serine/threonine phosphatases 1 and 2A (PP1 and PP2A) [16–19], which in turn causes hyperphosphorylation of key control proteins that regulate cytoskeleton organization and apoptosis [20–23]. But there is also substantial evidence that MC-dependent damage is accompanied by oxidative stress in the liver [24–29], kidney [25,29,30], testis [29,31–35], brain [36], and intestine [37].

Aquatic animals such as fishes and amphibians always or partly live in the water, thus, toxic cyanobacterial blooms and MCs may seriously threaten their survival [34,38,39]. Apart from direct contact with toxins over the body surface, fish can be exposed to MCs when the toxins pass through gills during breathing. Some phytoplanktivorous (*silver carp*) and omnivorous species (*nile tilapia*) can ingest cyanobacterial cells as food (Fig. 2). Bioaccumulation of MCs in zooplankton, shellfish, shrimps, fishes, frogs, and turtles have been documented [40–44]. Although there is no evidence for MCs biomagnification, the transfer of MCs within the aquatic food web takes place, suggesting that another route of exposure to the toxin, for aquatic species, is the consumption of aquatic organisms which have previously accumulated MCs in their tissues [44]. Terrestrial animals, including human beings, are also at risk of MCs exposure, for the use of untreated or inadequately contaminated water as the source of drinking water [45]. Consumption of contaminated aquatic products has also been postulated as a key exposure route to MCs. In two recent studies [3,4], MCs were also identified in human serum after chronical exposure via contaminated drinking water and aquatic products.

The decline in fertility of animals and humans over the last few decades that is potentially linked to environmental exposure has drawn global attention [46,47]. Both field and laboratory studies showed that high concentrations of MCs not only accumulated in the hepatopancreas/liver but also in the gonad [32,40–44,48,49], suggesting that the reproductive system is the second most important target organ of MCs [40]. In addition, the study of Chen and Xie [40] has raised questions and great concerns about the probable reproductive toxicity of MCs. Recent studies have verified that MCs accumulate in testis [32,50,51] and ovary [44,52], and exert toxic effects on the reproductive system [53,54].

The aim of this review is to make a compilation of existing data involving reproductive toxicity of microcystins, with regard to mammals, fishes, amphibians and birds. We also discuss the possible modes of action of MCs, as well as summarize the current research gaps which should be addressed by further studies.

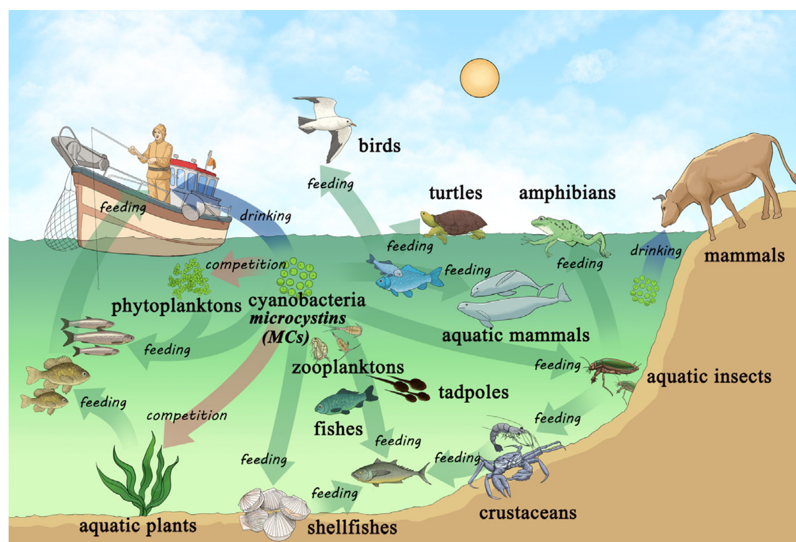


Fig. 2. Microcystins (MCs) in the aquatic environment. The cyanobacteria compete with other phytoplankton (algae) and aquatic plants, and the MCs produced and released into water by toxic cyanobacteria are harmful to other aquatic organisms including zooplankton, shellfishes, crustaceans, fishes, and turtles. MCs can also be accumulated in aquatic organisms and transferred to higher trophic levels through the food chain. The presence of MCs in drinking water and aquatic products may pose a threat for humans.

2. Reproductive toxicity of MCs on mammals

2.1. In vivo *studies*

2.1.1. *Cyanobacterial crude extracts (CCEs) (Table 1)*

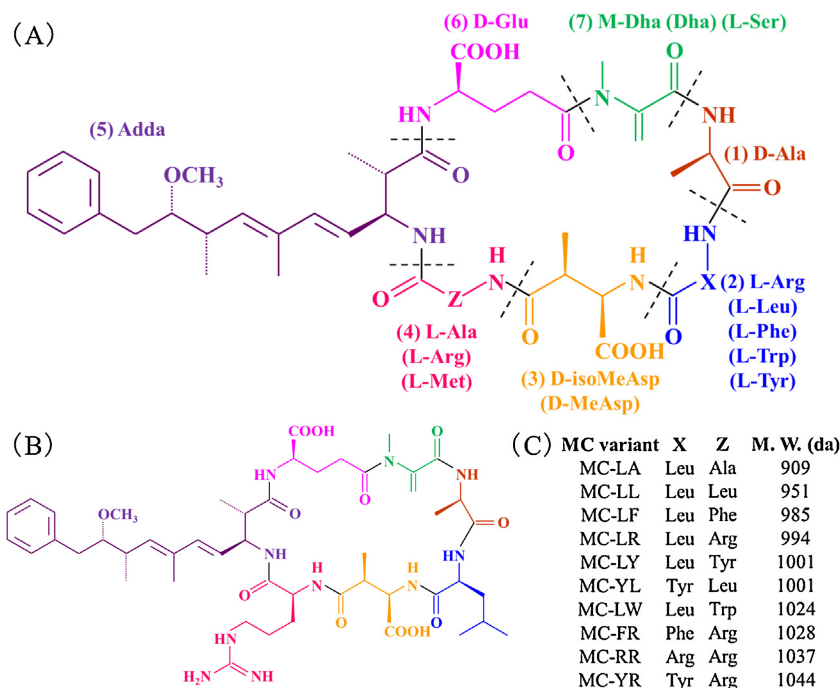
Male KM mice were administered an intraperitoneal (i.p.) injection of *Microcystis* cell extracts daily for 14 days at a dose of 3.33 or 6.67 μg MCs/kg body weight (bw) [55]. Mice had decreased mean body weight, and the mean absolute weight of the testes and epididymides was decreased. However, the mean relative weight of the testes increased. In addition, histological examination indicated that the testes of MC-treated mice were damaged and the space between the seminiferous tubules was more pronounced. The motility and viability of the sperm were also reduced in treated mice.

MCs increased the mRNA and protein levels of c-fos, c-jun and c-myc in testis of Wistar rats intravenously (i.v.) injected with lethal dose 50 (LD_{50}) of CCEs containing 86.7 μg MC-LR eq/kg bw (MC-LR eq, MC-LR equivalents) [56]. It's known that c-jun is a positive regulator of proliferation and induces positive regulators of cell cycle progression, while c-fos has oncogenic activity with frequent over-expression in tumor cells. C-myc gene is the best studied member of myc oncogene family, which encodes a transcription factor involved in cell proliferation and carcinogenesis. Therefore, the over-expression of c-myc, c-jun and c-fos in testis might be one of the molecular events related to the tumor promotion activity of MCs. In contrast, Xiong et al. [57] reported enhanced apoptosis of germ cells (spermatogonia, spermatocytes, and round spermatids) in the testis of Wistar rats after i.v. injection of CCEs. The apoptosis of germ cells was associated with up-regulation of the Fas/FasL system genes, including Fas, FasL, FADD, Apaf-1, casepase-3, -8, and -9, at both mRNA and protein level. Because Sertoli cells are the main cells expressing FasL in seminiferous tubules and Fas is localized to specific germ cell subtypes, the authors believed that MCs can cause damage directly to Sertoli cells. Li et al. [58] also confirmed that MCs could induce apoptosis in testis of Wistar rats that received i.v. injection of CCEs, and p53, Bax, and Bcl-2 might contribute to the mitochondria-dependent apoptotic pathway.

In another acute study, male Japanese White Rabbits were administered an i.p. injection of crude MC extracts at a dose of 12.5 μg MC-LR eq/kg bw [31]. MCs resulted in widened intercellular junctions, distention of mitochondria, endoplasmic reticulum, and Golgi apparatus in spermatogonia and Sertoli cells. The concentrations of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) increased significantly, indicating that MCs induced oxidative stress. Meanwhile, antioxidant and detoxification system, including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione *S*-transferase (GST), and antioxidants (GSH), effectively started to scavenge the reactive oxygen species (ROS) and xenobiotic, and finally succeeded in protecting the testis against damage indicated by gradual recovery of biochemical index and ultrastructure. Xiong et al. [29] also provided a new interpretation of the possible role of antioxidant enzymes in the toxicological mechanisms of MCs at the transcription level. After i.v. injection with 80 μg MC-LR eq/kg bw, the transcription abundance of CAT, GPX, Mn-SOD, Cu,Zn-SOD, glutathione reductase (GR), and gamma-glutamylcysteine synthetase (γ-GCS) were modulated in testis of Wistar rats. The results suggested an adaptative response to combat oxidative injury, confirming that oxidative stress was involved in the damage induced by MCs. Li et al. [59] also analyzed the mRNA abundance of 14 GST isoforms in the testis of Wistar rats that received i.v. injection of LD_{50} of CCEs containing MCs at 87 μg MC-LR eq/kg bw. Generally, the expression of most GSTs was suppressed. It is suggested that the transcription of GST isoforms varied in different ways exposed to MCs.

Table 1
Summary of reproductive toxicity of cyanobacterial crude extracts in mammalian studies *in vivo*.

Test organism/system	Toxicant	Exposure	Dose	Time points	Toxic effects	Reference
Male KM mice	Microcystis cell extracts	i.p.	3.33, 6.67 μg/kg MCs	14 d	Testis absolute weight ↓, testis relative weight ↑, epididymidis absolute and relative weight ↓, sperm motility and viability↓, microstructural damage	[55]
Male Japanese White Rabbits	Microcystin extracts	i.p.	12.5 μg MC-LR eq/kg	1, 3, 12, 24, 48 h	Ultrastructural damage, MDA ↑, H_2O_2 ↑, CAT ↑, SOD ↑, GPX ↑, GST ↑, GSH ↑	[31]
Male Wistar rats	Cyanobacterial crude extracts	i.v.	86.7 μg MC-LR eq/kg	1, 2, 4, 6, 12, 24 h	c-fos ↑, c-jun ↑, c-myc ↑	[56]
Male Wistar rats	Cyanobacterial crude extracts	i.v.	80.5 μg MC-LR eq/kg	1, 2, 4, 6, 12, 24 h	Apoptosis, induction of Fas/FasL system	[57]
Male Wistar rats	Cyanobacterial crude extracts	i.v.	87 μg MC-LR eq/kg	1, 2, 4, 6, 12, 24 h	p53 ↑, Bax ↑, Bcl-2 ↓	[58]
Male Wistar rats	Cyanobacterial crude extracts	i.v.	80 μg MC-LR eq/kg	1, 2, 4, 6, 12, 24 h	Modulation of CAT, Mn-SOD, Cu,Zn-SOD, GR, γ-GCS, GPX4 transcription	[29]
Male Wistar rats	Cyanobacterial crude extracts	i.v.	87 μg MC-LR eq/kg	1, 2, 4, 6, 12, 24 h	Modulation of 14 GSTs transcription	[59]
Male Kunming mice	Cyanobacterial bloom extracts	i.p.	225, 300, 375, 450 mg lyophilized algae cells/kg	5 d	Ultrastructural damage	[60]
Male Kunming mice	Cyanobacterial bloom extracts	i.p.	3, 6, 12 mg lyophilized algae cells/kg	21 d	MDA ↑, CAT ↓, olive tail moment values ↑	[60]



MC variant	X	Z	M. W. (da)
MC-LA	Leu	Ala	909
MC-LL	Leu	Leu	951
MC-LF	Leu	Phe	985
MC-LR	Leu	Arg	994
MC-LY	Leu	Tyr	1001
MC-YL	Tyr	Leu	1001
MC-LW	Leu	Trp	1024
MC-FR	Phe	Arg	1028
MC-RR	Arg	Arg	1037
MC-YR	Tyr	Arg	1044

Fig. 3. Chemical structure and molecular weight of microcystins (MCs). (A) Generic structure of the MCs. X and Z in positions two and four are highly variable L-amino acids that determine the suffix in the nomenclature of MCs. (B) Microcystin-LR (MC-LR), with the amino acid leucine (L) and arginine (R) in the variable positions two (X) and four (Z), respectively. (C) Molecular weight of some of the most frequent MCs.

Wang et al. [50] found almost invariable amounts of MCs (-LR + -RR, about 0.03 μg/g dry weight) were detected in the testis of Wister rats after i.v. injection of CCEs containing 80 μg MC-LR eq/kg bw, using liquid chromatography-mass spectrometry (LC-MS). Even at 24 h, a certain amount of MCs were still present in the gonad, suggesting the difficulty of elimination of MCs from the gonad. Hence, long-term exposure to MCs may cause chronic toxicity in the reproductive system. Li et al. [60] also reported the acute and sub-chronic reproductive toxicities of cyanobacterial bloom extract (CBE) on Kunming mice via i.p. injection. In the acute test, nuclear structures of the testis cells from the CBE treated groups were found with deformations; organelles (*e.g.*, mitochondria, Golgi bodies) showed a differential extent of dilation as well as decreased numbers; vacuoles were formed as a result of cytoplasm loss and portions of the nuclear membrane and cell membrane structures were damaged. The sub-chronic test suggested a dose-dependent relationship between DNA damage (shown by olive tail moment) in testicular cells and the amount of bloom extract administered to the mice, indicating considerable genotoxicity of CBE to germinal cells.

2.1.2. Pure microcystins (-LR) (Table 2)

Male KM mice were i.p. injected with 3, 6 or 12 μg/kg bw MC-LR per day for 7 and 14 days [61]. A dose of 3 μg/kg MC-LR could not significantly increase the DNA-protein crosslinks (DPC) coefficient in mice testicle cells, but DPC formation significantly increased when the dose of MC-LR was increased to 6 and 12 μg/kg. Doses of 6 and 12 μg/kg MC-LR also induced chromosome damage in early stage sperm cells, indicated by the increased number of micronuclei. In another study, Male Sprague-Dawley (SD) rats were treated with MC-LR (i.p.) at a dose of 5, 10 or 15 μg/(kg day) for 28 days [62]. Exposure to 5 μg/kg MC-LR decreased sperm motility and increased sperm abnormality rate, and 15 μg/kg MC-LR led to the decrease of testis weight, sperm concentration, and the levels of serum testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH). The histological findings showed that the seminiferous tubules atrophied and were obstructed. Saad et al. [63] also showed that exposure to 34.5 μg/kg MC-LR decreased the alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and GST activities in mice testes, while MDA and protein carbonyl formation and SOD activities were significantly increased.

Following exposure to MC-LR, spermatogenic epithelium damage and dose-dependent and duration-dependent cell apoptosis were also observed in Balb/c mice [64]. Further studies showed that the expressions of Bax, caspase -3 and caspase -8 were up-regulated, and significant increases in the phosphorylation of both p53 (p-p53) and Bcl-2 (p-Bcl-2) were also identified. MC-LR also resulted in over-expression of c-myc, c-jun and c-fos, suggesting that MC-LR is potentially carcinogenic for testis. Zhou et al. [65] also demonstrated that MC-LR can alter the apoptosis, proliferation and differentiation of spermatogenic cells. MC-LR was intraperitoneally administered to male SD rats daily at 50 and 100 μg/kg bw for 7 days. Changes occurred in the structure of testes; the tubular diameter and the relative weight of the testis were significantly decreased following treatment with 100 μg/kg MC-LR. Major differences in apoptosis and proliferation of testicular cells were also observed. The mRNA levels of testis-specific histone 2B (TH2B) and transition protein 2 (TP2) were both significantly decreased. Meanwhile, the expression of stem cell factor receptor (c-kit) was increased.

Chronic low-dose exposure to MC-LR also induced testicular injury and resulted in substantial toxicity to male reproduction. MC-LR was orally administered to male mice at 1, 3.2 and 10 μg/L for 3 and 6 months [46]. In the 3-month group, sperm quality declined at doses of 3.2 and 10 μg/L, testosterone concentrations decreased at 10 μg/L, levels of LH and FSH increased, Leydig cells exhibited apoptosis, and the spermatogenic epithelium presented a slightly loosened appearance in its organization at 10 μg/L. Similar, but more pronounced effects were observed in 6-month group. Chen et al. [33] also demonstrated that i.p. injection of 1 and 10 μg/kg bw MC-LR for 50 days exerted a generally sub-chronic toxicity to the male rat reproductive system. MC-LR affected the

Table 2
Summary of reproductive toxicity of pure microcystin-LR in mammalian studies *in vivo*.

Test organism/system	Exposure	Dose	Time points	Toxic effects	Reference
Male KM mice	i.p.	3, 6, 12 μg/kg	7 d	DNA-protein crosslinks ↑, micronucleus number ↑	[61]
Male SD rat	i.p.	5, 10, 15 μg/kg	28 d	Testis absolute and relative weight ↓, sperm motility ↓, sperm abnormality ↑, sperm concentration ↓, microstructural damage, change of testosterone, FSH, and LH levels	[62]
Male Balb/c mice	i.p.	34.5 μg/kg	1 d	ALP ↓, GST ↓, LDH ↓, MDA ↑, protein carbonyl ↑, SOD ↑	[63]
Male Balb/c mice	i.p.	3.75, 7.5, 15, 30 μg/kg	1, 4 d	Microstructural damage, apoptosis, Bax ↑, c-myc ↑, c-jun ↑, c-fos ↑, caspase -3 ↑, caspase -8 ↑, p-p53 ↑, p-Bcl-2 ↑	[64]
Male SD rat	i.p.	50, 100 μg/kg	7 d	Microstructural damage, testis absolute and relative weight ↓, seminiferous tubular diameter ↓, apoptosis, spermatogenic cells proliferation ↓, c-kit ↑, TH2B ↑, TP2 ↑	[65]
Male mice	orally	1, 3.2, 10 μg/L	3, 6 months	Sperm quality ↓, testosterone ↓, FSH ↑, LH↑, microstructural damage, apoptosis	[46]
Male Wistar rats	i.p.	1, 10 μg/kg	50 d	Testis relative weight ↓, microstructural and ultrastructural damage, mitochondrial swelling and DNA damage, testosterone ↓, FSH ↑, LH ↑, ROS↑, modulation of cytoskeletal and mitochondrial genes transcription	[33]
Male Balb/c mice	i.p.	3.75, 7.5, 15, 30 μg/kg	1, 4, 7, 14 d	Modulation of Gnrh1, Fshβ, Lhβ transcription, change of testosterone, FSH, and LH levels	[66]
Male C57BL/6 mice	i.p.	3.75, 7.5, 15, 30 μg/kg	1, 4, 7, 14 d	Epididymal sperm ↓, testosterone ↓, LH ↓, LHβ and GnRH1 transcription ↓	[67]
Female Balb/c mice	i.p.	5, 20 μg/kg	28 d	Relative ovary weight ↓, primordial follicles ↓, abnormal estrous cycle, progesterone ↓	[52]

expression of cytoskeletal genes, causing possible dysfunction of cytoskeleton assembly and morphological changes. In MC-LR treatments, 8 mitochondrial genes related to electron transport chain (ETC) and oxidative phosphorylation (OXPHOS) system significantly increased in expression, which might be responsible for promoted ROS formation and oxidative stress and may lead to cytoskeletal disruption and hormone homeostasis. Mitochondria swelling and DNA damage were also determined in the 10 μg/kg group. Cytoskeleton disruption could interact with mitochondria dysfunction, ultimately leading to disruption of cellular structure, metabolism, and testis impairment after exposure to MC-LR.

In order to exert reproductive toxicity, sufficient concentrations of MCs must enter the reproductive system. To confirm whether or not MC-LR can be distributed to testes and in which parts of the testes the MC-LR is located, a sub-acute toxicity test was conducted [51]. Male SD rats were i.p. injected with MC-LR at a dose of 300 μg/kg per day for 6 days. MC-LR was observed to be transported to the testes using immunofluorescence detection. In particular, MC-LR was mostly distributed on the tubal wall of seminiferous tubules, in which spermatogonia and Sertoli cells are mainly located. However, MC-LR was seldom found in the mesenchymal tissue of testes, where most Leydig cells are located. In addition, MC-LR-protein phosphatase 1/2A (PP1/2A) adducts were found in testis extracts by Western blot analysis, offering further evidence that MC-LR can enter testicular cells. LC-MS analysis showed that MC-LR levels reached 0.0056 μg/g dry weight in testis. Therefore, spermatogonia and Sertoli cells are probably critical target cells of MC-LR in testis.

Apart from a direct effect on testis, Wang et al. [66] suggested that MC-LR can indirectly affect male mice serum hormones and mRNA expressions by damaging the hypothalamic-pituitary-gonad (HPG) axis. Male Balb/c mice were exposed to MC-LR by i.p. injection with different injection concentration (3.75, 7.5, 15, and 30 $\mu g\, kg\, bw^{-1}\, day^{-1}$) and injection durations (1, 4, 7, and 14 days). MC-LR decreased the GnRH expression in a dose- and duration-dependent manner. MC-LR exposure led to an initial increase and subsequent decrease in the expressions of Fshβ, Lhβ, and the secretion of FSH, LH, and testosterone. Similarly, Xiong et al. [67] also demonstrated that MC-LR impaired spermatogenesis possibly through the direct or indirect inhibition of GnRH synthesis at the hypothalamic level in C57BL/6 mice.

Only one study has reported on female mammalian reproductive toxicity of MCs [52]. Female Balb/c mice were treated with 5 and 20 μg/kg MC-LR by i.p. injection for 28 days. Relative ovary weight was significantly reduced in the 20 μg/kg MC-LR group and this reduction may be related to pathomorphological changes of the ovary. Histological evaluation of follicles showed that the numbers of primordial follicles decreased roughly in half at high dose level compared with the control. As MC-LR induced a decrease in concentration of progesterone but not of FSH or LH, disturbance to the estrus cycle (the duration of the proestrus and estrus stage decreased) seemed to result from direct effect on the ovary rather than indirect effects from hypothalamus or pituitary. Western blot analysis also showed that MC-LR could enter the ovary of SD rats exposed to 200 μg/kg MC-LR (i.p.) for 6 days [52].

2.2. In vitro *studies (Table 3)*

In mature testes, spermatogonia are the most immature spermatogenic cells in the process of spermatogenesis, which is highly organized and originates with spermatogonia proliferation, followed by spermatocyte meiosis and spermatid morphological alterations. Wang et al. [51] and Zhou et al. [68] demonstrated that MC-LR was transported into spermatogonia, which significantly decreased cell viability and total antioxidant capacity, whereas ROS production, mitochondrial membrane potential (MMP), intracellular free calcium (Ca^{2+}), and the ratio of apoptotic cells increased after MC-LR exposure. The expression of 5 multispecific organic anion-transporting polypeptides (OATPs, -1a5, -3a1, -6b1, -6c1, and -6d1) was affected by MC-LR [68], especially OATP3a1, and Oatps may transport MC-LR into spermatogonia, resulting in cytotoxicity to the male reproductive system. With microarray analysis [69], 101 miRNAs were identified to be significantly altered in spermatogonia (GC-1 cells) treated with MC-LR, revealing an important link between miRNA and MC-LR in inducing reproductive toxicity. Inhibition of miR-96 may be one of the mechanisms for MC-induced sperm abnormality through regulating the expression of deleted-in azoospermia-associated protein 2 (DAZAP2).

Recently, Sertoli cells have been speculated to be a potential target of MC-LR for male reproductive toxicity [51,70–74]. The primary function of testicular Sertoli cells is to support and provide necessary nutrition for spermatogenic cells to promote spermatogenesis. They also contribute to the construction of the blood-testis barrier (BTB), which serve as a 'gatekeeper' to prohibit toxic substances from reaching developing germ cells [75]. Thus, damage to Sertoli cells may lead to impairment of male reproduction. Recently, Chen et al. [73] and Wang et al. [51] reported that MC-LR can be observed entering primary cultured Sertoli cells by immunofluorescence detection and Western blotting, resulting in oxidative stress and cell injury [70–73]. MC-LR could induce autophagy and apoptosis in a dose-dependent manner [73]. Upon exposure to high dose of MC-LR, the accumulated autophagosomes promoted the apoptotic process by increasing the Bax/Bcl-2 ratio and activation of the apoptosis cascade caspase-3, resulting in reproductive toxicity in male rats. Zhou et al. [74] also demonstrated that the expression of miR-374b and miR-181a following the exposure to MC-LR was comparable to the levels in patients with non-obstructive azoospermia or asthenozoospermia, supporting the evidence on MC-induced reproductive system toxicity.

Under the regulation of luteinizing hormone (LH), Leydig cells synthesize and secrete the androgens (testosterone) into seminiferous to promote spermatogenesis, and as such chemical injury to Leydig cells may lead to impaired male fertility [62]. When primary cultured Leydig cells were exposed to MC-LR they exhibited decreased testosterone production. Further studies showed that MC-LR increased ROS and MDA production, while SOD activity was decreased. It was observed that MC-LR significantly increased apoptotic DNA fragmentation and the ratio of necrotic cells. These results showed that oxidative stress was responsible for apoptosis induced by MC-LR, and the reduced production of testosterone in Leydig cells could result in reproductive toxicity [62]. However, two studies later by the same group [51,66] and a further study [67] reported that MC-LR did not enter and had no cytotoxicity on Leydig cells. Because MC-LR was not observed to be distributed to Leydig cells *in vitro*, the authors inferred that in the acute/sub-acute experiment, MC-LR did not directly act on the testosterone synthesis pathway in Leydig cells. A possible explanation is cell membrane damage in Li et al.'s experiment [62,66]. Thus, the toxicity of MC-LR on Leydig cells and testosterone synthesis could be achieved in an indirect way, with a large probability that the dysfunction of Leydig cells is a secondary effect due to the damage of the hypothalamic-pituitary-gonad (HPG) axis caused by MC-LR [51,66,67]. The underlying mechanisms need further research.

The toxic effects of MCs on Chinese hamster ovary (CHO) cells *in vitro* could reflect the female reproductive toxicity, to some extent. Lankoff et al. [76] demonstrated that both pure MC-LR and cyanobacterial bloom extract effectively deregulated the cell cycle of CHO-K1 cells by damaging the mitotic spindle apparatus and induced both apoptosis and necrosis. Similarly, Gácsi et al. [77] also reported that MC-LR reduced the number of mitotic cells, pre-

Table 3
Summary of reproductive toxicity of microcystins in mammalian studies *in vitro*.

Test organism/system	Toxicant	Dose	Time points	Toxic effects	Reference
Rat spermatogonia	MC-LR	0.5, 5, 50, 500 nM	6 h	Cell viability ↓, apoptosis, total antioxidant capacity ↓, ROS ↑, MMP ↑, Ca^{2+} ↑, modulation of Oatps expression	[68]
Mouse spermatogonia (GC-1 cell)	MC-LR	0.5, 5, 50, 500, 1000, 10,000, 100,000 nM	6, 24 h	Cell viability ↓, alteration of 101 microRNAs including down-regulation of miR-96	[69]
Rat Sertoli cell	MC-LR	0.15, 1.5, 15 μg/L	6, 12, 24 h	ROS ↑, SOD ↓	[70]
Rat Sertoli cell	MC-LR	0.5, 1, 10, 20 μg/mL	24 h	Cell viability ↓, apoptosis, p53 ↑, Bax ↑, Bcl-2 ↓, caspase-3 activity ↑	[71]
Rat Sertoli cell	MC-LR	0.5, 5, 50, 500 nM	12, 24, 48 h	Cell viability ↓, MDA ↑, SOD ↓, ROS ↑, MMP ↓, apoptosis, caspase-3 ↑, caspase-9 ↑	[72]
Rat Sertoli cell	MC-LR	0.5, 5, 50, 500 nM	24, 48 h	Cell viability ↓, cell membrane integrity ↓, LC3 ↑, Bcl-2 ↓, cyt c↑, caspase-3 ↑	[73]
Mouse Sertoli cell	MC-LR	0.5, 5, 50, 500 nM	24 h	Cell viability ↓, alteration of 115 microRNAs and 2494 genes, MAPK11 ↑, tubulin ↓, ZO-1 ↓, occluding ↓	[74]
Rat Leydig cell	MC-LR	0.5, 5, 50, 500 nM	12, 24, 48 h	Cell viability ↓, apoptosis, testosterone ↓, ROS ↑, MDA ↑, SOD ↓	[62]
Mice Leydig cell	MC-LR	1, 10, 100, 250, 500, 750, 1000 nM	24 h	No cytotoxicity	[66]
Mice Leydig cell	MC-LR	1, 10, 100, 250, 500, 750, 1000 nM	48 h	No cytotoxicity	[67]
CHO-K1 cell	MC-LR	25, 50, 100 μM	14, 18, 22 h	Mitotic indices ↑, abnormal anaphases and mitotic spindles, polyploid cells, apoptosis and necrosis	[76]
CHO-K1 cell	Cyanobacterial bloom extracts	25, 50, 100 μM MCs	14, 18, 22 h	Mitotic indices ↑, abnormal anaphases and mitotic spindles, polyploid cells, apoptosis and necrosis	[76]
CHO-K1 cell	MC-LR	1, 2, 5, 10, 20, 50 μM	12, 18, 24, 48 h	Apoptosis and necrosis, mitotic cells ↓, disrupted chromosome formation, cytoskeletal damage	[77]
CHO-K1 cell	MC-LR	1, 10, 20 μg/mL	6 h	Decreased repair capacity of DNA damage and increased frequency of micronuclei induced by UV, apoptosis	[78]
CHO cell	MC-LR	2.5, 5, 10 μg/mL	24, 48 h	G_2/M phase arrest	[79]
CHO cell	MC-LR	0.5, 1, 2.5, 10, 15 μg/mL	24 h	Cell viability ↓, colony forming efficiency ↓, CAT ↓, MDA ↑, ROS ↑, MMP ↓, apoptosis, p53 ↑, Bax ↑, Bcl-2 ↓	[80]
CHO cell	MC-LR	2.5, 5, 10 μg/mL	24 h	Cell viability ↓, ROS ↑, MMP ↓, SOD ↓, MDA ↑, GR ↓, GPX ↓, caspase-3 ↑, apoptosis	[81]
HeLa cell	MC-RR	20, 40, 60, 80 μg/mL	4, 8, 12, 24, 48 h	Hormesis, cell proliferation, apoptosis, modulation of NF-κB and its downstream genes	[82]

vented chromosome formation and induced apoptosis and necrosis in CHO-K1 cells, associated with shortening and degradation of major cytoskeletal components such as microfilaments (MFs) and microtubules (MTs), resulting in reproductive toxicity. MC-LR was also observed to induce cycle arrest in G_2/M phase, which may be related to the apoptosis of CHO cells [79]. Recently studies showed that MC-LR induced generation of ROS and oxidative stress, MMP loss and cellular apoptosis in CHO cells [80,81]. Although MC-LR alone did not induce DNA damage, it could target the nucleotide excision repair (NER) mechanisms by interference with the incision/excision phase as well as the rejoining phase of NER and lead to an increased level of UV-induced cytogenetic DNA damage in CHO-K1 cells [78]. Our previous study also showed that MC-RR could regulate the activity of NF-κB and promote cell proliferation and apoptosis in HeLa cells, which might be associated with reproductive tumor [82].

3. Reproductive toxicity of MCs on fishes (Table 4)

The daily activity at the time of mating and spawning disappeared in female and male zebrafish exposed to 50 μg/L MC-LR for 6 days [83], indicating that MC-LR reduced the spawning activity of zebrafish. Along with this reduced spawning activity, no spawned eggs were found. If this response also occurs for native fish species, then changes in species composition in eutrophic waters may not only be attributed to losses in spawning sites but also to direct effects of cyanotoxins on fish reproduction.

In a recent study, female and male medaka fish were exposed (via balneation/immersion) to a low concentration of MC-LR (5 μg/L) for 30 days [84]. In the testis of treated fish, in addition to abnormalities of cellular division that sometimes occur during normal spermatogenesis, numerous lytic areas were detected in the seminiferous tubules, indicating an increase of abnormal cell divisions progressing towards death. MC-LR has also a major impact on the ovary, in particular a destruction of the gonado-somatic-tissue that reduces the area of gonads and a reduction of vitellus storage that could result from changes in the relationships between the follicular cells and the oocytes. Such effects following MC-LR treatment, particularly yolk decrease, are certainly associated with the significantly lower number of eggs spawned per treated female, compared to controls, since yolk platelets are necessary for embryo development. Marked histological lesions were also observed in the livers, ovaries and testes of zebrafish exposed to MC-LR by subchronic immersion in 1, 5 and 20 μg/L for 30 days [85]. Significant down-regulations of liver VTG1 mRNA were detected in all treatment groups; however, in the 5 and 20 μg/L groups, the whole body vitellogenin (VTG) levels significantly increased in females, while considerably decreasing in males, suggesting that MC-LR does not cause any estrogenic effects in adult zebrafish. Liver lesion caused by MC-LR exposure may be responsible for the down-regulation of VTG1 mRNA levels. The hatchability and the 17 beta-estradiol (E2) concentration in gonads significantly decreased in the 20 μg/L group. Apoptotic rate in the ovaries significantly increased. Significant down-regulation of Bcl-2 transcriptional level was found in the gonads of all MC-LR treated fish, while marked up-regulation of Bax transcription level was determined in the 20 μg/L female group but a significant down-regulation was observed in males. Although the transcriptional level of caspase-3 dropped in ovaries of the 5 and 20 μg/L groups, significant increases of caspase-3 activation in the ovaries and testes were detected. The findings indicate that MC-LR exposure exerts diverse reproductive toxicity in zebrafish with females exhibiting more sensitivity than males.

In an acute toxic experiment [86], female zebrafish were i.p. injected at doses of 50 and 200 μg/kg bw MC-LR. Pathological lesions of zebrafish ovary progressed in severity and extent with the increasing exposure time and dose within 12 h post injection (hpi). Concurrently, the increases in MDA contents as well as the enzymatic activities and transcriptional levels of antioxidant enzymes CAT, SOD, and GPX showed the occurrence of oxidative stress. The significant decrease of GSH content in zebrafish ovary suggested the importance of MC-LR detoxification by GST via GSH. In addition to the detoxification process, the increased utilization in antioxidant reactions could also cause the reduction of GSH content. The final recovery of histostructure and antioxidative indices indicated that ovarian efficient antioxidant defense system might be an important mechanism of zebrafish to counteract MC-LR.

In a sub-chronic study [87], female zebrafish were exposed to 2, 10, and 50 μg/L of MC-LR for 21 days, and its effects on oogenesis, sex hormones, transcription of genes on the hypothalamic-pituitary-gonad (HPG) axis, and reproduction were investigated. Egg production significantly declined at ≥10 μg/L MC-LR. MC-LR increased the concentrations of E2 and VTG at the 10 μg/L level, whereas concentrations of E2, VTG, and testosterone declined at 50 μg/L MC-LR. The transcriptions of steroidogenic pathway genes (cyp19a, cyp19b, 17βhsd, cyp17, and hmgra) changed as well after the exposure and corresponded well with the alterations of hormone levels. A number of intra- and extra-ovarian factors involved in oocyte growth, maturation and ovulation, such as gnrh3, gnrhr1, fshβ, fshr, lhr, bmp15, mprβ, ptgs2, and vtg1, were also significantly changed with a different dose-related effect. Moreover, MC-LR exposure to female zebrafish resulted in decreased fertilization and hatching rates, suggesting the possibility of trans-generational effects of MC-LR exposure. The results demonstrate that MC-LR could modulate endocrine function and oogenesis, eventually leading to disruption of reproductive performance in female zebrafish.

MC-RR, another variant of MCs, has not been studied extensively, despite of its wide distribution and abundance in the environment, possibly due to its lower toxicity than MC-LR. Zhao et al. [32] reported that MC-RR caused a noticeable damage to testicular ultrastructure in zebrafish i.p. injected with 0.5 LD_{50} (2000 μg/kg bw), showing widened intercellular junction and distention of mitochondria. The testis showed a rapid response of its defense systems to the oxidative stress caused by MC-RR. This's the first study using a proteomic approach to obtain an overview of the effects of MC-RR on zebrafish testis. The proteomic results revealed that MC-RR remarkably altered the abundance of 24 proteins that were involved in cytoskeleton assembly, oxidative stress, glycolysis metabolism, calcium ion binding, and other biological functions.

4. Reproductive toxicity of MCs on amphibians

Male frogs (*Rana nigromaculata*) were exposed to 1 μg/L MC-LR for 7 and 14 days [34]. ROS production and MDA content were positively correlated with exposure time, while reduced GSH level and GPX activity rapidly decreased, implying that the defense system of the testis induces oxidative damage. MC-LR significantly inhibited the protein expression of Bcl-2 in the testis, while induced the protein expressions of Bax and caspase-3, -8, and -9, and stimulated the release of cytochrome c (cyt c). Ultrastructural observations showed distention of the mitochondria and endoplasmic reticulum (ER) and deformation of the nucleolus. Moreover, prolonged exposure time strengthened and weakened the relative expression levels of C/EBP homologous protein (CHOP) and the 78-kDa glucose-regulated protein (GRP78), respectively. These results indicate that MC-LR-induced apoptosis of the testis in male frogs may occur through the mitochondrial and ER pathways.

In another study, male frogs were exposed to 0.1, 1, and 10 μg/L MC-LR for 7 and 14 days [38]. Sperm motility and sperm count were significantly and negatively correlated with exposure time and concentration. By contrast, the rate of abnormal sperm increased in a

Table 4
Summary of reproductive toxicity of microcystins in fishes, amphibians, and birds.

Test organism/system	Toxicant	Exposure	Dose	Time points	Toxic effects	Reference
Female, male zebrafish	MC-LR	Immersion	50 μg/L	6 d	The spawning activity and success ↓	[83]
Female, male medaka fish	MC-LR	Immersion	5 μg/L	30 d	Microstructural and ultrastructural damage, areas of ovaries ↓, spawned eggs ↓	[84]
Female, male zebrafish	MC-LR	Immersion	1, 5, 10, 20 μg/L	30 d	Hatchability ↓, microstructural damage, E2 ↓, change of VTG level and transcription, apoptosis	[85]
Female zebrafish	MC-LR	i.p.	50, 200 μg/kg	1, 3, 12, 24, 48, 168 h	Microstructural damage, MDA ↑, CAT ↑, GPX ↑, GST ↓, GSH ↓, modulation of CAT1, SOD1, GPX1a, GSTr1	[86]
Female zebrafish	MC-LR	Immersion	2, 10, 50 μg/L	21 d	Abnormal pre-vitellogenic oocytes growth and maturation, spawned eggs ↓, fertilization success ↓, hatchability ↓, change of E2, testosterone, and VTG, modulation of HPG axis genes transcription	[87]
Male zebrafish	MC-RR	i.p.	2000 μg/kg	2, 6, 24 h	MDA ↑, H2O2 ↑, SOD ↑, CAT ↑, GST ↑, GPX ↑, GSH ↑, ultrastructural damage, alteration of 100 proteins spots	[32]
Male frog	MC-LR	Immersion	1 μg/L	7, 14 d	Ultrastructural damage, ROS ↑, MDA ↑, GPX ↓, GSH ↓, cyt c ↑, Bax↑, Bcl-2 ↓, caspase -3 ↑, caspase -8 ↑, caspase -9 ↑, CHOP ↑, GRP 78 ↓	[34]
Male frog	MC-LR	Immersion	0.1, 1, 10 μg/L	7, 14 d	Sperm cells ↓, sperm motility ↓, sperm deformity ↑, ultrastructural damage, testosterone ↓, E2 ↑, P450 aromatase ↑, SF-1 ↑	[38]
Male frog testis	MC-LR	In vitro	0.1, 1, 10, 100 nM	6 h	Sperm cells ↓, sperm motility ↓, sperm deformity ↑, MDA ↑, ROS ↑, SOD ↓, CAT ↑, GSH ↑, GST ↑, ultrastructural damage, P450 aromatase ↑, SF-1 ↑	[35]
Male Japanese quail	Microcystis biomass	Orally	0.045, 0.459, 4.605, 46.044 μg MCs	30 d	Microstructural damage	[88]
Female, male Japanese quail	Cyanobacterial biomass	Orally	61.62 μg MCs	8 w	Eggs weight ↓	[89]
Male Japanese quail	Cyanobacterial biomass	Orally	61.62 μg MCs	8 w	CAT ↑, GPX ↓, LPO ↓	[90]

dose- and time-dependent manner. Ultrastructural observational results revealed abnormal sperm morphologies, vacuoles in spermatogenic cells, cell dispersion, incomplete cell structures, and deformed nucleoli. MC-LR exposure also significantly decreased serum testosterone content, but rapidly increased estradiol content in male frogs. Prolonged exposure and increased concentration enhanced the relative expression levels of P450 aromatase and steroidogenic factor 1 (SF-1), suggesting the endocrine function in frogs was disrupted.

Decreased sperm motility and number of sperm cells and increased the sperm abnormality rate were also observed in frog testes *in vitro* following MC-LR exposure [35]. Moreover, MC-LR increased ROS production and MDA content. At the same time, CAT and GST activity and reduced GSH content rapidly increased, whereas SOD activity significantly decreased, implying that the defense system of the testis quickly responds to oxidative stress. Ultrastructural observation showed distention of the mitochondria, ER and Golgi apparatus and changes in the mitochondrial matrix color, cristae number and morphology. Moreover, increased relative expressions of P450 aromatase and SF-1 genes were also observed.

5. Reproductive toxicity of MCs on birds

Vacuolar degeneration of the testicular germinative epithelium was found in male Japanese quails that daily ingested *Microcystis* biomass containing MCs for 30 days [88]. Effects of cyanobacterial biomass on avian reproduction were further evaluated in male and female Japanese quails exposed to a cyanobacterial biomass mixed with the feed [89]. The chronic exposure of parent birds lasted 8 weeks with the daily sum of 61.62 μg MCs including 26.54 μg MC-RR, 7.62 μg MC-YR and 27.39 μg MC-LR. Eggs laid by cyanobacterial biomass-exposed parental hens had lower weight than in controls. However, the lower weight was not reflected in their biological quality because reproductive parameters such as egg viability, hatchability, and the overall effect of hatching in cyanobacterial biomass-exposed birds were better than in the control group. Males exposed to cyanobacteria in the feed showed moderate to marked atrophy of the seminiferous tubular epithelium with only sparse numbers of the developmental stages of spermatozoa and Sertoli cells [90]. Biomass-exposed birds had elevated CAT activities but decreased GPX activities and surprisingly lower levels of lipid peroxides (LPO) in the testis. The cell defensive system protecting testicular tissue from damage seemed to be insufficient and partly depleted after the chronic exposure of birds to the biomass. It might be hypothesized that compounds of hormonal activity, or some biologically active compounds potentially stimulating reproduction could be present in the complex cyanobacterial biomass [89,90]. The underlying mechanisms need further research.

6. Potential mechanisms of action (Fig. 4)

6.1. Cellular uptake of MCs

In order to exert reproductive toxicity, sufficient concentrations of MCs must enter the reproductive system, *i.e.*, MCs must be transported into the cells involved in the process of spermatogenesis or oogenesis. Cellular uptake of MCs has been demonstrated to occur exclusively via active transport, whereas passive transmembrane diffusion can be excluded because of the high molecular weight and structure of MCs [91]. Consequently, pathological changes following MC intoxications are restricted to cells, tissues and organs capable of actively transporting MC from the blood into the cell. Indeed, active transmembrane transport of MCs is mediated by specific organic anion transporting polypeptides (OATP) [92]. Systemic distribution of MCs in the organs will be therefore dependent on the degree of blood perfusion and types and expression level of OATP carriers [6] .

In males, the blood-testis barrier (BTB) is created by adjacent Sertoli cells near the basement membrane, which serve as a 'gatekeeper' to prohibit toxic substances from reaching developing germ cells [75]. The BTB also anatomically divides the seminiferous epithelium into the basal and adluminal (apical) compartment so that post-meiotic germ cell development, namely spermiogenesis and spermiation, can take place in a specialized microenvironment in the apical compartment behind the BTB. The presence of MCs in the testis of fish [32] and rats [50,51] suggests that MCs are able to get across the BTB. MC-LR was observed to enter spermatogonia and Sertoli cells of rat testis, but not their Leydig cells [50], indicating the different expression levels of OATPs in testicular cells. Zhou et al. [68] demonstrated that 5 OATPs (OATP1a5, -3a1, -6b1, -6c1, and -6d1) are present in rat spermatogonia and some of these OATPs may transport MC-LR into spermatogonia, exerting reproductive toxicity. Wu et al. [52] also reported that MC-LR can be found in rat ovaries. The uptake mechanisms of MCs by reproductive systems need to be further investigated.

6.2. Modulation of PP1 and PP2A activities

MC-LR-protein phosphatase 1/2A (PP1/2A) adducts were found in extracts from testes [51] and ovaries [52] by Western blot analysis, offering evidence that MC-LR can enter testicular cells, bind to PP1/2A and exert reproductive toxicity. MCs were found to interact with PP catalytic subunits (PP1c and PP2Ac) by a two-step mechanism involving rapid binding and inactivation of the catalytic subunits, followed by a slower covalent interaction (within hours) [93]. Recent reports suggest that MCs modulate PPs activity not only by the direct inhibition of enzyme activity, but also through the regulation of protein expression [16–19,94–97]. PP1 and PP2A inhibition induces the disruption of the dynamic equilibrium of protein phosphorylation/dephosphorylation, leading to the damage of numerous cellular processes such as cytoskeleton organization, Wnt, Akt, p38, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase 1/2 (ERK1/2) of mitogen-activated protein kinase (MAPK) signaling pathways, metabolism and cell cycle [20,98–101] (Fig. 4). Wang et al. [102] showed that germline apoptosis and reproductive toxicity in loss-of-function members of ERK, JNK, and p38 MAPK signaling pathways reduced significantly under MC-LR exposure. Significant increases in the phosphorylation of both p53 (p-p53) and Bcl-2 (p-Bcl-2) in mice testes were identified after the administration of MC-LR, suggesting the involvement of protein phosphorylation in MC-mediated reproductive toxicity [64].

6.3. Oxidative stress

It's known that reproductive system is highly sensitive to oxidative stress and lipid peroxidation (LPO), significant increase of which will cause reproductive toxicity and infertility [31]. Oxidative stress can, in turn, lead to potential molecular damage of basic cellular constituents, lipids, proteins, and nucleic acids, including lipid peroxidation (LPO), protein oxidation and DNA strand breaks. Gonads are rich in unsaturated lipids, thus may be vulnerable to peroxidative damage. MCs induced oxidative stress in pre-pubertal rabbit testis; meanwhile antioxidant and detoxification systems effectively started to scavenge the reactive oxygen species (ROS) and xenobiotics, and finally succeeded in protecting the testis against damage, as indicated by gradual recovery of biochemical index [31]. Xiong et al. [29] also found that MCs affect the transcriptional activities of some antioxidant enzymes in the testes of male Wistar rats, suggesting an adaptive response

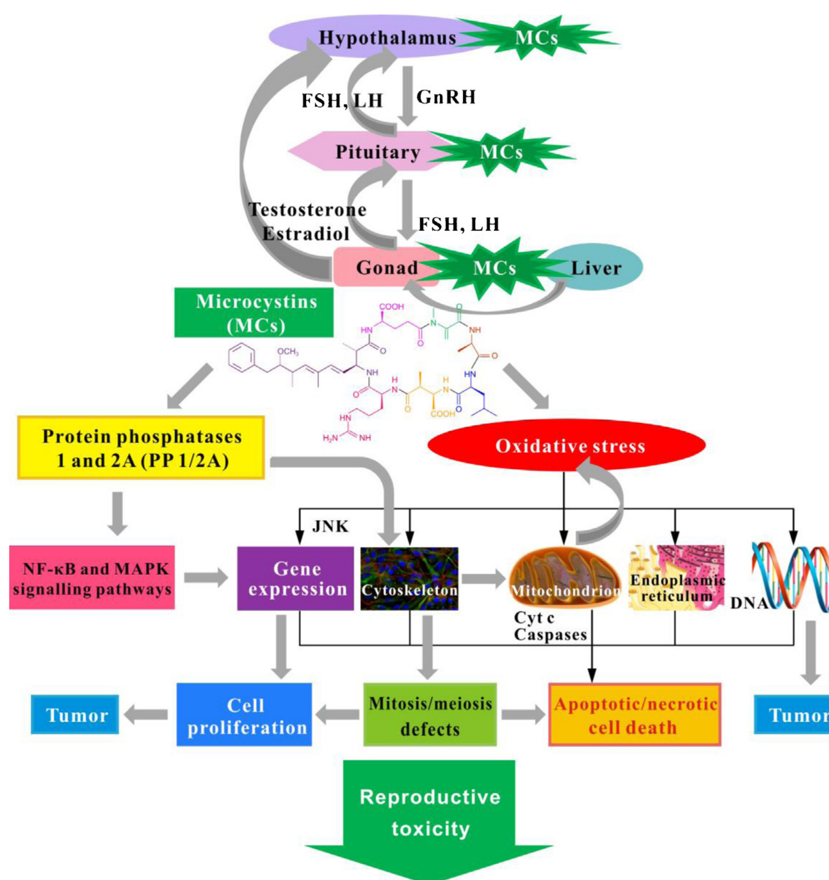


Fig. 4. A schematic review of reproductive toxicity of microcystins (MCs). Microcystins exert reproductive toxicity indirectly by affecting the hypothalamic-pituitary-gonad (HPG) axis and liver and directly by damaging testis and ovary. MCs are highly potent and specific inhibitors of eukaryotic protein serine/threonine phosphatases 1 and 2A (PP1 and PP2A), which causes phosphorylation/dephosphorylation imbalance of key control proteins that regulate cytoskeleton organization, cellular proliferation, apoptosis, and tumor development. MCs exposure is also related to the excessive production of reactive oxygen species (ROS) and oxidative stress, leading to cytoskeleton disruption, mitochondria dysfunction, endoplasmic reticulum (ER) stress, and DNA damage. MCs induce cell apoptosis mediated by the mitochondrial and ROS and ER pathways. Through PP1/2A inhibition and oxidative stress, MCs lead to the differential expression/activity of transcriptional factors and proteins involved in the pathways of cellular differentiation, proliferation and tumor promotion. DNA damage induced by MCs is also an important factor involved in the carcinogenicity.

to combat oxidative injury induced by MCs. GSTs play important roles in the detoxification of MCs and the suppression of many GST isoforms in the testis might be a key mode of MC toxicity [59]. After exposure to MC-RR, the levels of MDA and H_2O_2 increased remarkably in zebrafish testis, while the antioxidant defense systems such as SOD, CAT, GST, and GPX activities were also increased [32]. Chen et al. [33] also observed elevated ROS production in rat testis exposed to MC-LR. In the ovary of zebrafish injected with MC-LR [85], the increases in MDA contents as well as the enzymatic activities and transcriptional levels of antioxidant CAT, SOD and GPX also showed the occurrence of oxidative stress. The significant decrease of GSH content in zebrafish ovary suggested the importance of MC-LR detoxification by GST via GSH. The final recovery of histostructure and antioxidative indices indicated that ovarian efficient antioxidant defense system might be an important mechanism for zebrafish to counteract MC-LR [86]. Our research also showed that some antioxidant enzymes significantly changed in zebrafish testes and ovaries by sub-chronic immersion in 1, 5, and 20 μg/L MC-LR for 30 days (unpublished data). *In vitro* studies also showed that the oxidative stress induced by MC-LR might lead to cytotoxicity and reproductive toxicity. MC-LR may enhance the lipid peroxidation, decrease SOD activity in rat Leydig cells [62], Sertoli cells [72] and increase ROS production in Chinese hamster ovary (CHO) cells [81], rat spermatogonia [68] and frog Sertoli cells [35]. Saad et al. [63] demonstrated that pre-administration of antioxidants (anthocyanin and taurine) significantly decreased the level of oxidative stress induced by MC-LR in mice testes and afforded a protection against reproductive toxicity. *N*-acetylcysteine (NAC), a precursor of GSH and intracellular ROS scavenger, was also shown to protect CHO cells from oxidative injury and apoptosis induced by MC-LR [81].

However, it is not fully understood why and how MCs exposure can lead to excessive ROS formation that culminates in oxidative damage of reproductive system. MCs are incorporated into the cell via OATPs that use GSH as a driving force for the exchange with MCs [103]. In addition, it has been shown that MCs bind to GSH, forming conjugates (MC-GSH) via glutathione *S*-transferase (GST), as the first step in the detoxification process, followed by degradation to the cysteine conjugates (MC-Cys) [59,104–106]. As GSH is the first line of defense against ROS, its cell efflux should increase ROS generation. Moreover, mitochondria do not have the enzymatic pool associated with GSH synthesis and depends on cytoplasmatic GSH, so the depletion of cytosol GSH could reflect in a decreased GSH concentration inside mitochondria, a situation that favours ROS production and electron transport chain (ETC) disruption [33,103,107,108]. MCs can bind to the beta subunit of ATP-synthase [109], which could disturb oxidative phosphorylation (OXPHOS) system [33,108], reduce ATP synthesis and contribute to the intensification of the mitochondrial membrane depolarization, disruption of ETC and ROS generation. Mitochondrial disruption leads to an impairment of ATP production, affecting all cellular processes that depend on ATP, including GSH synthesis,

which will further contribute to the lowering of GSH inside the cell and elevate the level of oxidative stress. Some evidence also suggests a close connection between cellular hyperphosphorylation state and oxidative stress generation induced by MCs exposure [103].

6.4. DNA damage/genotoxicity

Li et al. [60] found a dose-dependent relationship between DNA damage in testicular cells and the amount of cyanobacterial bloom extracts administered to the mice, probably due to oxidative damage [110–112]. It should be noted that DNA damage that occurred in the germ cells may be transferred to the offspring. Also, MCs may interfere with DNA damage repair pathways [78,113]. Lankoff et al. [78] suggested that MC-LR targeted the nucleotide excision repair mechanisms and induced an increased level of UV-induced cytogenetic DNA damage in CHO-K1 cells. Hence, it is highly likely that the interference of MC-LR with the DNA repair process may be one of the mechanisms responsible for MC-promoted cancer development. Mitochondrial DNA (mtDNA) impairment was also observed in rat testis after MCs exposure, demonstrating the genotoxicity of MCs [33]. In fact, mtDNA was more sensitive than nuclear DNA (nDNA) to oxidative damage because the mitochondria are in close proximity to the free radical-producing electron transport chain (ETC) [33,112]. Chromatin alterations and abnormal anaphases were also reported in CHO-K1 cells exposed to pure microcystin-LR and *Microsystis* extracts, possibly associated with the shortening and degradation of cytoskeletal components [76,77]. If these changes occurred in spermatocyte and oocyte meiosis, the process of spermatogenesis or oogenesis would be affected, resulting in sperm and ovum abnormalities.

6.5. Cytoskeleton disruption

The cytoskeleton consists of three major structural elements: microfilaments (MFs), microtubules (MTs) and intermediate filaments (IFs) [114,115]. These elements exert a wide range of impacts on reproductive events including sperm maturation and motility, oocyte maturation, fertilization and early embryo development [116]. It has been documented that both protein phosphatase inhibition and oxidative stress induced by MCs may lead to cytoskeletal damage [117–122]. Gácsi et al. [77] showed that apoptotic shrinkage in Chinese hamster ovary (CHO-K1) cells was associated with the shortening and loss of MFs and with a concentration-dependent depolymerization of MTs. In our previous study, we found that subchronic MC-LR exposure significantly disrupted the transcriptional balance of several cytoskeletal genes including β-actin, β-tublin, vimentin, ezrin, radixin, moesin, and stathmin in rat testis [33]. It was assumed that MC-LR exposure caused cytoskeletal alterations in rat testis, thus leading to the morphological changes, and exerting prominent toxicity to the reproductive system. Another acute exposure study by our group also observed transcriptional alteration of nine cytoskeletal genes (β-actin, α-tubulin, vimentin, ezrin, radixin, moesin, MAP1b, tau, and stathmin) in testes of Wistar rats that received intravenous (i.v.) injections of lethal dose 50 (LD_{50}) of CCEs containing MCs at 80 μg MC-LR eq/kg bw (MC-LR eq, MC-LR equivalents) (unpublished data). In fact, MCs can significantly affect cytoskeleton organization, which could be either the cause or the result of altered expression of cytoskeletal genes [33,122]. The widening of cell junction and altered abundance of cytoskeletal proteins were also observed in zebrafish testis after MC-RR exposure, which might cause disruption of cytoskeleton organization in the testes and interrupt reproductive metabolism [32]. Recently, Zhou et al. [74] demonstrated that MC-LR could impair microtubule formation through the dysregulation of tubulin, thus damaging the tight junctions (TJs) between Sertoli cells and blood-testis barrier (BTB), which is critical for spermatogenesis. Disruption of microtubules by MC-LR might also affect normal movement of spermatogenic cells and arrest germ cell mitosis and meiosis, resulting in reproductive toxicity.

6.6. Apoptosis/necrosis

Recently, many studies showed that MCs induced the apoptosis of testicular cells and that the expression of several apoptosis-related proteins was significantly changed, suggesting the important role of apoptosis in MC-induced reproductive toxicity [33,57,58,64,71–73]. An apoptotic DNA ladder was detected in cultured rat Leydig cells by exposure to MC-LR [62]. The ultrastructural observation of rat testis exposed to MC-LR indicated some typical apoptotic features, including cell membrane blebbing, cytoplasmic shrinkage, swollen mitochondria, and deformation of the nucleus [33]. Using terminal deoxynucleotide transferase-mediated deoxy-UTP nick end labeling (TUNEL) staining, Xiong et al. [57] showed a remarkable increase in the number of apoptotic germ cells (spermatogonia, spermatocytes, and round spermatids) in the seminiferous tubule of rats treated with cyanobacterial crude extracts (CCEs). MC-LR exposure also induced TUNEL-positive testicular cells in rats following chronic, sub-chronic and acute exposure [46,64,65]. MC-LR-induced apoptosis/necrosis of rat Sertoli cells [71–73], spermatogonia [68], Chinese hamster ovary (CHO) cells [76,77,80,81], and ovary cells of zebrafish [85] were also observed. Apoptosis of HeLa cells was also observed following exposure to MC-RR [82].

There is no doubt that apoptosis/necrosis of cells involved in the process of spermatogenesis or oogenesis has a negative effect on reproductive system; however, the underling mechanism of apoptotic/necrotic cell death induced by MCs is largely unknown. Among the apoptotic pathways, mitochondria are recognized as the central executioner, and mitochondrial permeability transition (MPT) is implicated as a critical, rate-limiting event in apoptosis [117]. In normal conditions, Bcl-2 and Bcl-xL (pro-survival) form heterodimers with Bax and Bak (pro-apoptotic) to maintain the outer mitochondrial membrane (OMM) integrity and block the release of cytochrome c (cyt c) and other apoptotic factors such as apoptosis inducing factor (AIF) and Smac/DIABLO through the OMM, thus inhibiting apoptosis. The competition between Bax and Bcl-2 determines the cell fate in terms of execution of apoptosis or survival. However, MCs induced expression of p53 and the ratio of Bax to Bcl-2 [58], led to the opening of MPT pores, mitochondrial swelling (a common feature of mitochondrial membrane permeabilization) [33], loss of inner mitochondrial membrane potential (MMP), and mitochondrial release of cyt c [123]. Once cyt c is released from the intermembrane space of mitochondria to the cytoplasm, the cell is committed to die by activation of the apoptotic caspase cascade and nucleic DNA fragmentation, or necrosis due to collapse of the respiratory function [72]. Increased cyt c, caspase-3, caspase-8, and caspase-9 were observed after MCs exposure [57,64,72,73]. Xiong et al. [57] demonstrated that enhanced apoptosis of germ cells in the testis of MCs treated rats was associated with up-regulation of the Fas/FasL signaling system. The NF-κB pathway may be involved in the MC-induced apoptosis in HeLa cells [82]. Several recent studies demonstrate that MC-induced oxidative stress may induce cell apoptosis [33,34,68,72]. Increased ROS may trigger the dissociation of cyt c from the mitochondria [72]. Zhang et al. [34] reported endoplasmic reticulum (ER) stress as an event that occurs downstream of oxidative stress and found that MCs exposure may induce testis apoptosis involving both the mitochondrial and ER pathways. It was also confirmed that MC-LR-induced oxidative stress may stimulate sustained activation of JNK, which in turn induced apoptosis via AP-1 and Bid, two important substrates downstream of JNK [124]. Moreover, MCs could lead to cytoskeleton disruption and

the cytoskeleton may also play a key role in regulating apoptosis and necrosis in reproductive system (Fig. 4) [32,33,76,77].

6.7. Reproductive tumors

Li et al. [56] and Wang et al. [64] provided evidence that MCs may have tumor-promoting activity for testes. Proto-oncogenes especially c-myc, c-jun and c-fos, were significantly up-regulated in rat testis in response to MC-LR. Activation of c-fos and c-jun would result in the production of related proteins that could form the dimeric transcription factor AP-1. AP-1 has been speculated to be involved in the induction of tumorigenesis. At the same time, the c-myc gene is the best-studied member of the myc oncogene family, which encodes a transcriptional regulator involved in carcinogenesis. Even though the detailed mechanism of genital carcinoma-promoting activity of MCs has not been well elucidated, it has been suggested to arise from their inhibition of PP1/2A, which negatively regulated NF-κB and several mitogen-activated protein kinases (MAPK) [28,56,82,125,126]. Once activated, NF-κB and MAPK are transferred to the nucleus, where it could activate c-fos, c-jun, c-myc and other proto-oncogenes, which initiate transcription of genes necessary for growth and differentiation. Increased phosphorylation of p53 induced by MC-LR may be the reason for the up-regulation of c-fos, c-myc and c-jun [64]. In addition, NF-κB, c-fos and c-jun may be induced via oxidative mechanisms of MCs [28,56,82]. Chen et al. [82] also demonstrated that NF-κB and its downstream target genes including c-FLIP, cyclinD1, c-myc and c-IAP2 may be involved in the cellular proliferation and potential carcinogenicity induced by MCs. Lankoff et al. [78] suggested that MC-LR targets the nucleotide excision repair mechanisms and induces an increased level of UV-induced cytogenetic DNA damage in CHO-K1 cells. Hence, it is highly likely that the interference of MC-LR with the DNA repair process may be one of the mechanisms responsible for the MC-promoted reproductive tumors.

6.8. Sex hormones, HPG axis, liver and endocrine-disrupting effects

The hypothalamic-pituitary-gonad (HPG) axis is an important pathway for endocrine regulation and hypothalamic gonadotrophin releasing hormone (GnRH) stimulates the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary and plays a key role in the neurohormonal control of reproduction (Fig. 4). Treated with MC-LR, the serum testosterone level decreased; the levels of FSH and LH were increased in the 5 μg MC-LR/kg treatment group, but decreased in the 15 μg/kg group [62]. Chen et al. [33,46] also found that testosterone concentration decreased while levels of LH and FSH increased; as the secretion of FSH and LH is regulated by testosterone in a negative feedback manner. However, another study demonstrated that MC-LR exposure led to an initial increase and subsequent decrease in the secretion of FSH, LH and testosterone, and that mRNA expressions of Gnrh1 in hypothalamus and Fshβ and Lhβ in pituitary were also affected [66]. LH and FSH are synthesized by cells in the pituitary and are regulated by GnRH. GnRH, which is secreted by the hypothalamus, is regulated by Kisspeptin/GPR54. In addition, sex hormone changes were also reported in chronic liver disease [127], while MC-LR can damage liver and cause liver disease. The endocrine system is very complex and may be affected by many factors. The authors inferred that the changes of serum hormone levels induced by MC-LR may be combined effects of liver damage and hypothalamic-pituitary system damage, but not a direct effect on testis [66]. Similarly, Xiong et al. [67] also demonstrated that MC-LR impaired spermatogenesis possibly through the direct or indirect inhibition of GnRH synthesis at the hypothalamic level. However, in female mice exposed to MCs [52], serum progesterone was decreased, but there was no significant difference in FSH, LH, or estradiol serum levels compared to control animals. The results indicated that female reproductive toxicity seemed to result from direct impact on the ovary rather than indirectly from impact on the hypothalamus or pituitary.

In female zebrafish [87], MCs also disturb expression of steroidogenic genes and concentrations of hormones in the HPG axis, leading to inhibition of follicular development, oocyte maturation and ovulation. As recorded for mammals, liver function is also closely related to reproduction regulation of fish. For example, vitellogenin (VTG) is synthesized in the liver and is essential for vitellogenesis, oocyte maturation and yolk biosynthesis. Qiao et al. [85] suggested that liver lesions caused by MC-LR exposure were responsible for the down-regulation of VTG1 mRNA levels. Zhao et al. [87] also found that plasma VTG levels and liver vtg1 expression were increased in zebrafish exposed to 10 μg/L MC-LR, but decreased after 50 μg/L exposure.

Oziol and Bouaïcha [128] reported the estrogenic potential of MC-LR *in vitro* in a stably transfect cell line (MELN) with an estrogen-regulated luciferase gene at concentrations lower than those found in natural environments. MC-LR presents estrogenic potential likely by indirect interaction with estrogen receptors, possibly mediated by PP1/2A inhibition and oxidative stress. It seems that MCs may act as xenoestrogens at concentrations lower than those found in natural environments, providing some evidence of their reproductive toxicity. However, up-regulation of VTG was observed in *Microcystis*-exposed larvae but not in larvae exposed to MC-LR, while expression of VTG in male or juvenile fish is used as a biomarker of exposure to environmental estrogens [129]. Qiao et al. [85] also reported that both the whole body VTG levels and liver VTG1 mRNA levels were significantly reduced in 5 and 20 μg/L treatment groups in male zebrafish, but the whole body VTG levels increased in 5 and 20 μg/L groups in females, while liver VTG1 mRNA levels decreased. As mentioned above, Zhao et al. [87] also found that plasma VTG levels and liver vtg1 expression were increased in female zebrafish exposed to 10 μg/L MC-LR, but decreased after 50 μg/L exposure. Interestingly, MC-LR induced the expression of VTG 3 and significantly increased the abundance of VTG 1 in zebrafish brains [36]. These differences may be due to different developmental stages of fish, different MC exposures and different tissue samples used in each experiment.

Besides modulating the HPG axis, it has also been previously demonstrated that MCs alter the metabolism of cortisol and thyroid hormone, through activating the hypothalamic-pituitary-adrenal/interrenal (for fish) (HPA/HPI) and hypothalamic-pituitary-thyroid (HPT) axis [130–136]. As mentioned above, the endocrine system is very complex and affected by many factors, and previous studies have shown that liver damage may not only induce sex hormone changes [127], but also reduce hepatic thyroxine deiodination in animals [137], which in turn causes a decline in peripheral triiodothyronine production [130]. A previous study demonstrated that chemical-caused effects on one endocrine axis pathway will also indirectly affect the other endocrine axes in zebrafish [138]. Recently, it has been found that cholesterol, the precursor of all classes of steroid hormones, is significantly altered in zebrafish [139] and mice [136] after MCs exposure, suggesting that MCs are capable of disrupting the endocrine system and inducing reproductive toxicity [38,87]. Therefore, endocrine-disrupting effects and reproductive toxicity of MCs could have adverse impacts on aquatic ecosystems as a

whole, and also affect the terrestrial environment, including birds and mammals.

7. Current research gaps and future directions

7.1. Humans

Microcystins have been associated with numerous wildlife poisonings worldwide, but they are also a threat for human health. There are several possible routes of human exposure to MCs [10]. The first is through ingestion of drinking water from lakes, reservoirs, rivers and groundwater wells [1,45]. Another exposure route is possible through dermal contact and accidental inhalation/ingestion during recreational activities such as bathing and canoeing in waters subjected to a toxic bloom. Exposure through hemodialysis has also been reported [13–15]. Consumption of contaminated food has been postulated as a key exposure route to MCs. Several reports have described the accumulation of microcystins in edible parts (mainly muscle) of aquatic products (shellfish, crustaceans, fishes, frogs, turtles, water birds, etc.) [3,4,40–44]. There is also evidence that MCs may be transmitted to cultivated plants from contaminated irrigation water [140]. Another potential source of human exposure, that should not be neglected, is through dietary supplements sold as blue green algae supplements (BGAS) [141]. However, no data are available that describe the reproductive toxicity of MCs to humans. With the growing evidence of exposure to MCs and the identification of MCs in human serum [3,4], human epidemiological studies of reproductive effects are needed in order to understand toxicological mechanisms.

7.2. Female and sex differences

To date, there have been very limited studies on the effects of MCs and female reproductive toxicity [52,84–87]. Qiao et al. [85] reported that MC-LR exposure exerts diverse reproductive toxicity in zebrafish, with females exhibiting more sensitivity than males. However, Li et al. [142] demonstrated that male offspring are more susceptible to maternal exposure of MC-LR on memory function than females. Therefore, female reproductive effects of MCs should be strengthened in future studies. Moreover, the sex-specific effects of MCs also need to be studied. Whether MCs pollution may bias sex ratios of animal populations in the wild is as yet unknown.

7.3. Trans-generational toxicity

Chen and Xie [40] found that eggs of two freshwater shrimps, *Palaemon modestus* and *Macrobrachium nipponensis* from Lake Chaohu accumulated MC-LR and —RR. Zhang et al. [41] also reported the presence of MCs in the offspring of adult snails (*Bellamya aeruginosa*) and toxin content in the gonad and in the offspring correlated well. High MC levels were also found in egg yolk and egg white of birds, *Nycticorax nycticorax* and *Anas platyrhynchos* [43]. These studies indicated that MCs were likely transfered from adult females to their young with physiological connection [41]. Exposure of parental zebrafish to MC-LR led to decreases in egg production [84,87]. Lesser rates of fertilization and hatching success were also observed, suggesting the possibility of trans-generational effects of MC-LR exposure.

Recently, several studies have confirmed the trans-generational toxicity of MCs in zebrafish [143] and rats [142,144]. When adult zebrafish were continuously exposed to MC-LR (1, 5, and 20 μg/L) for 30 days, the growth (body weight and body length) of the F1 generation at 60 days post fertilization (dpf) was significantly inhibited even though the embryos and larvae were free of MC exposure [143]. Moreover, the transcription of some growth and immune-related genes and activities of some antioxidant enzymes of whole body fish were also modulated. Pathological changes in liver including vacuolar degeneration and pycnotic nuclei were observed.

Female SD rats were intragastrically administered with 1, 5, and 20 μg MC-LR/kg bw once every 48 h for 8 weeks [142]. Each female rat was mated with an unexposed adult male rat. Pups from the MC-LR-treated groups had significantly lower scores in the cliff avoidance test on postnatal day 7. Cognitive impairment shown by Morris water maze test was observed in adulthood (postnatal day 60, PD60) but not in the childhood (PD 28, after weaning) of rat offspring. The MDA level and SOD activity also significantly increased in MC-LR-exposed pups on postnatal day 60. Zhao et al. [144] also demonstrated that maternal exposure to MC-LR has adverse effects on neurodevelopment in rat offspring. Pregnant SD rats were exposed to MC-LR at 10 μg/kg bw/day via osmotic pumps from gestational day 8 (GD8) to PD15 of lactation. MC-LR enhanced toxin accumulation and MDA, but decreased GSH and the level of acetylcholine esterase (AChE) activity in the brains of rat offspring. MC-LR also caused changes to cerebrum ultrastructure, showing a sparse structure, distention of endoplasmic reticulum and swelling mitochondria. The proteomic results revealed that MC-LR remarkably altered the abundance of 49 proteins that were involved in neurodevelopment, oxidative phosphorylation, cytoskeleton, metabolism, protein folding and degradation.

It is not difficult to understand that the transfer of MC-LR from maternal rats to offspring (by penetrating the placental barrier and breast milk) exerts adverse short term effects, *i.e.*, on postnatal day 7 and 15 (PD7 and PD15), shown by the detection of MC-LR in the brains of rat pups [142,144]. The toxicity of MC-LR and MC-RR on human amnion epithelial FL cells are also documented [94,96,145–147]. However, neurobehavioral impairments were observed in adulthood (PD60) but not in the childhood (PD28) of rat offspring, indicating a potential long-term adverse effect of MC-LR on rat offspring [142]. We can imagine that the MCs contents in rat offspring on postnatal day 60 were very low according to the detoxification and dynamics of MCs [50,105,148]. Also, previous studies showed the recovery of liver, testis and ovary in a relative short time in both mammals and fish [31,86,148]. Thus, the trans-generational toxicity of MCs cannot be only attributed to the transfer of MCs from parents to offspring for the low MCs contents. More surprisingly, Liu et al. [143] found that MC-LR exposure in parent zebrafish leads to growth inhibition and immune suppression in F1 offspring even at 60 days post fertilization (dpf) while the embryos and larvae are free of MC exposure. We believe that there are other mechanisms, perhaps epigenetic changes (including DNA methylation), that lead to the trans-generational effects of MCs, which need to be further clarified.

7.4. Microcystin variants, cyanobacterial crude extracts, and cyanobacterial biomass

Several studies related to reproductive toxicities of MCs based on the use of cyanobacterial extracts have often been interpreted as if they were caused by individual MCs [55,149]. However, more than 100 different structural variants of MCs have been identified (Fig. 3) [7]. Different MC variants have varied biological affinities *i.e.*, their potential to enter a certain tissue would be different depending on the affinity of the cell transporters for that MC variant [150–152]. Moreover, distinct properties of MC variants in terms of PP1 and PP2A inhibition, cytotoxicity, biotransformation, and detoxification have also been reported [152–156]. However, at present, the toxicological database for MCs is almost exclusively limited to data on a single variant, MC-LR, while data on the other variants is very lacking. The exposure of several MC variants at the same time could lead to additive, synergistic or antagonistic toxic effects on the organisms. Also, MCs are certainly not the only toxic components present in cyanobacterial crude

extracts [157]. Cyanobacterial strains could also produce a wide range of other metabolites besides MCs, such as lipopolysaccharides, which may have an important contribution in the damage caused by MCs in cyanobacterial exposures [149]. Essa and Fathy [158] demonstrated that the nontoxic cyanobacterial species have the capability to produce some extracellular bioactive compounds into their surroundings that can disrupt mammalian reproductive hormones. This effect was attributed to the presence of sterol-like compounds in their filtrate that was confirmed with GC–MS and LC–MS/MS analyses. Consequently, cyanobacteria could potentially contribute to a hazardous effect on mammalian health via producing some endocrine disruptors with oestrogenic activity. Damkova et al. [89,90] also hypothesized that apart from MCs, compounds of hormonal activity, or some biologically active compounds potentially stimulating reproduction could be present in the complex cyanobacterial biomass. Quantification of the total toxicity caused by MC variants and other secondary metabolites is a major scientific challenge that needs to be addressed in future toxicity studies dealing with harmful algal blooms (HABs) [153].

7.5. Other environmental factors

Apart from MCs and other cyanotoxins, a number of other pollutants such as heavy metals, trace elements, persistent organic pollutants (POPs) and pesticide residues are present in the environment. Some of them are susceptible to degradation while others stay stable even in adverse conditions. These pollutants may either intensify the toxicity (synergy), or attenuate the toxicity of MCs (antagonism) [153]. Pavagadhi et al. [159] demonstrated that phosphate (PO_4^{3-}) and chloride (Cl^-) enhanced the toxic effects of MC-LR and MC-RR on zebrafish embryos while nitrate (NO_3^-) attenuated their toxic effects. Low oxygen, ammonium (NH_3) and nitrite (NO_2^-) stresses during the period of cyanobacterial bloom accumulation and decay might aggravate the adverse effect of MCs on aquatic organisms [160–162]. Moreover, physical conditions such as temperature could also play a crucial role in determining MCs toxicity [163,164]. Also, global warming due to climate change in conjunction with increased eutrophication may further complicate this environmental issue related to HABs [1]. Different components of climate change (e.g. temperature, hydrology and atmospheric composition) and resulting ecosystem changes (e.g. nutrient availability and food-web structure) would not only affect the occurrence, frequency and magnitude of HABs, but also toxin production and accumulation [153]. Therefore, to understand the toxicity potential of a particular MC and HABs in totality, all the above mentioned factors must be taken into account.

7.6. Application of high-throughput analytical techniques

Classic/conventional ecotoxicology has focused upon a bottom-up approach to understand stressor effects in which a few genes, proteins or biochemical reactions are studied at a time [165]. However, due to the complexity of organisms and the possible interactions and interrelationships of MCs with various environmental factors, classic techniques cannot meet the development of toxicology and therefore high-throughput analytical techniques are required. With the recent technological and analytical advancements, the scientific community is better equipped now to organize and conduct extensive toxicological studies involving MCs [153]. One such advancement is to take advantage of top-down 'omics' approaches, i.e. genomics, proteomics, and metabolomics (or metabonomics). Genomics refers to the study of the genome of an organism, either at the DNA or at the mRNA (transcriptomics) level [165]. Proteomics is the global profiling of the proteins of an organism, their structure, function and expression. Metabolomics, on the other hand, is often known as systematic investigation of low molecular weight (<1000 Da) metabolites that are the end products in various biological systems including cells, organs, tissues, biofluids, or even whole organisms [165].

For MCs, a number of studies have reported 'omics' analyses with multiple organisms, such as toxicogenomics (genomics applied to toxicology) [166–171], proteomics [32,36,144,145,171–174] and metabolomics [139,175–177]. These studies revealed that numerous genes and metabolic pathways involved in cytoskeleton assembly, oxidative stress, cell cycle regulation, and energetic metabolism were differentially regulated. Whilst most of these studies focused on the hepatotoxicity of MCs, only a few proteomic and microarray studies investigated the male reproductive toxicity in zebrafish testis [32], mouse spermatogonia [69] and Sertoli cells [74], respectively. Future work could better clarify the reproductive toxicological mechanism involved in MCs by the powerful 'omics' approaches.

However, presently, data of 'omics' often remain descriptive and are not yet fully understood; in other words, most of published studies provide descriptions only of how genes, proteins or metabolites are altered by stresses. Core questions are still not sufficiently answered such as "What do these alterations really mean in terms of adverse effects?" [178] Therefore, 'omics' cannot be meaningfully utilized for ecological risk assessment of environmental toxicants unless these technologies are combined and associated with toxicological and physiological outcomes (*e.g.*, functional analyses) and reduced ecological fitness (in particular, survival, reproduction and development). Also, some key baseline data are required in order that the effects (changes to the baseline) can be correctly identified and that the "omics" data can be meaningfully interpreted. Bioinformatics tools, such as a pathway analysis, will be important in providing functional insight into "omics" and physiological outcomes, and the integration of the above analysis (a tandem "top-down and bottom-up" approach) is termed "Systems toxicology". Systems analysis can be applied to many different levels, such as molecules, cells, organs, individuals, populations, and even ecosystems [165]. Systems biology uses a similar holistic approach first described by Aristotle in his work, *Metaphysics*: "The whole is more than the sum of its parts."

Research should be driven by scientific questions and hypotheses, and the question should dictate the study design, and what experimental techniques, methods and analyses are used in that study, not vice versa [178]. We should not do research in a particular way just because we can, whereas much of current research appears to originate rather from the availability of and even fascination for a new and sophisticated technique/tool. Just as the proverb goes, "every coin has two sides", and we should not forget that every technique has advantages as well as disadvantages/limitations.

8. Concluding remarks

In this review, we comprehensively summarize the current knowledge of the effects of microcystins (MCs) on the reproductive system. These studies show a strong association between reproductive toxicity and MCs exposure, at multiple doses and routes of exposures, and in various species. The possible mechanisms of reproductive toxicity of MCs are summarized in Fig. 4. After exposure, MCs are transferred into blood and then into the main organs and tissues. At this step, MCs preferentially accumulate in the liver but also in the gonad, brain, kidney and other organs. Having entered the testis and/or other organs, MCs can inhibit protein serine/threonine phosphatases 1 and 2A (PP1 and PP2A), which in turn causes hyperphosphorylation of key control proteins that regulate cytoskeleton organization, cellular proliferation, apoptosis, and tumor development. MCs exposure is also related to the excessive production of reactive oxygen species (ROS) and oxidative

stress, leading to cytoskeleton disruption, mitochondria dysfunction, endoplasmic reticulum (ER) stress, and DNA damage. MCs induce cell apoptosis mediated by the mitochondrial and ROS and ER pathways. Through PP1/2A inhibition and oxidative stress, MCs lead to the differential expression/activity of transcriptional factors and proteins involved in the pathways of cellular differentiation, proliferation and tumor promotion. DNA damage induced by MCs is also an important factor involved in carcinogenicity. Apart from a direct effect on testis and ovary, MCs also indirectly affect sex hormones by damaging the hypothalamic-pituitary-gonad (HPG) axis and liver. Parental exposure to MCs also exerts hepatotoxicity and neurotoxicity among offspring. We conclude that exposure to MCs poses a serious threat for decreased populations and biodiversity of wildlife, especially aquatic animals including fishes and amphibians. While no human data are available for the reproductive toxicity of MCs currently, we believe that reproductive toxicities resulting from MCs exposure could potentially be a threat to human health, particularly given the wide distribution and abundance of MCs in the environment. Gaps in our understanding of the reproductive toxicity of MCs need to be addressed by further studies.

Conflict of interest

The authors declare that there are no conflicts of interest.

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