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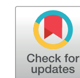
Estrogenic potency of MC-LR is induced via stimulating steroidogenesis: *In vitro* and *in vivo* evidence☆



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ABSTRACT

Waterborne microcystin-LR (MC-LR) has been reported to disrupt sex hormones, while its estrogenic potency remains controversial. We hypothesized that MC-LR could induce estrogenic effects via disrupting sex hormone synthesis, and verified this hypothesis by *in vitro* and *in vivo* assays. Effects of MC-LR (1, 10, 100, 500, 1000 and 5000 μg/L) on steroidogenesis were assessed in the H295R cells after 48 h. The contents of 17β-estradiol (E2) and testosterone (T) increased in a non-dose-dependent manner, which showed positive correlations with the expression of steroidogenic genes. In the *in vivo* assay, adult male zebrafish were exposed to 0.3, 1, 3, 10 and 30 μg/L MC-LR for 30 d. Similarly, E2 and T contents in the testis were increased, accompanied by extensive up-regulation of steroidogenic genes, especially *cyp19a*. Meanwhile, the percentage of spermatid in the testis declined. In the liver, the *vtg1* gene was significantly up-regulated while both the transcriptional and protein levels of the estrogenic receptor (ER) declined. These results indicate that MC-LR induced non-dose-dependent estrogenic effects at environmental concentrations, which may result from steroidogenesis stimulation via a non-ER-mediated pathway. Our findings support a paradigm shift in the risk assessment of MC-LR from traditional toxicity to estrogenic risk, particularly at low concentrations, and emphasize the potential threat to the male reproductive capacity of wildlife in bloom areas.

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1. Introduction

Cyanobacterial blooms are frequently found throughout the world where water conditions range from eutrophic to hypertrophic. Relevant to this range is the production of cyanotoxins, i.e. microcystins (MCs). MCs are a group of harmful compounds composed of seven peptide-linked amino acids (Rudolph-Böhner et al., 1994; Dawson, 1998). They are very stable, soluble in water, and resistant to high temperatures, hydrolysis or oxidation (van Apeldoorn et al., 2007). Among over 100 variants of MCs, microcystin-LR (MC-LR) attracts the most concern because of its strongest toxicity and broad distribution (Meriluoto and Spoof, 2008; Pearson et al., 2010). The concentrations of MC-LR during cyanobacterial blooms often exceed the advisory level of 1 μg/L set by the WHO, e.g., in Lake Taihu of China, MC-LR concentration was up to 35.8 μg/L in October of bloom period (Wang et al., 2010), posing a tremendous threat to the health of aquatic organisms and humans.

MCs can impair various organs of vertebrates (Li et al., 2005, 2012, 2013; Trinchet et al., 2011), with the liver being the main target (Li et al., 2005). Recent research has documented that MCs can induce reproductive endocrine disorder, with results showing a variation in dose, duration, and life stage of tested animals (Wang et al., 2012; Qiao et al., 2013; Zhao et al., 2015; Hou et al., 2016). Wang et al. (2012) reported MC-LR injection (3.75, 7.5, 15 and 30 μg/kg body weigh) elicited an initial increase and subsequent decline of T levels by damaging the hypothalamus and pituitary gland in male BALB/c mice. In zebrafish, sub-chronic immersion to

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environmental levels of MC-LR induced gonadal damages as well as the disorder of E2 and T in both females and males (Qiao et al., 2013; Zhao et al., 2015; Liu et al., 2016). At present, adopted experimental doses have changed from high concentrations to environmentally relevant levels along with the understanding of MC-LR gradual shifts from a classical hepatotoxin to one of endocrine-disrupting chemicals (EDCs).

Some EDCs are specially termed 'environmental estrogens' because of their ability to mimic the effects of E2. Generally, estrogenic effects are induced via two pathways (Rosselli et al., 2000). Classical environmental estrogens equipped with structural features such as the H-bonding ability by the phenolic ring and precise steric hydrophobic centers could directly compete with E2 for estrogen receptors (ER), thus consequently inducing estrogenic responses (Fang et al., 2001). However, xenobiotics without ER-binding ability can also elicit estrogenic effects by altering steroidogenic gene transcriptions or enzyme activities (Connor et al., 1996; Sanderson and Berg, 1998; Hilscherova et al., 2004). To date, there has been very limited information on the estrogenic or anti-estrogenic potency of MC-LR. By using transgenic human cells, Oziol and Bouaïcha (2010) first found weak estrogenic potency of MC-LR available, which was considered as indirect interaction with ER since the luciferase activity was inhibited by an ER antagonist. In contrast, the up-regulation of vitellogenin (VTG) expression, as a classical biomarker for screening estrogenic substances, was not detected in the whole body of adult male zebrafish after subchronic MC-LR exposure (Qiao et al., 2013). In a 90-d life-cycle exposure experiment, 30 μg/L MC-LR elevated testicular E2 content and stimulated hepatic *vtg1* expression in male zebrafish (Su et al., 2016). This study suggested that decreased expression of the receptor genes might cause the problem of recognition and combination. Thus, an alternative explanation is that the estrogenic activity might result from the stimulation of E2 synthesis. So far, the claim that MCs act as xenoestrogens remains uncertain.

A combined *in vitro* and *in vivo* approach is a useful way to gain a complete understanding of the activities of endocrine disruptors. In *in vitro* assays, the H295R human adrenocortical carcinoma cells has been explored for quantification of interference effects on steroidogenesis gene expressions (Fig. S1; Gene acronyms are defined in Table S1, Supplementary information) (Sanderson et al., 2000; Harvey et al., 2007; Gracia et al., 2008). On the other hand, zebrafish have been developed as a systematic, sensitive, and easily operated vertebrate model for risk assessment of EDCs due to the high similarity of that genome to the human genome (Segner, 2009). Like other vertebrates, reproductive processes of zebrafish are regulated by steroidogenesis of gonadal tissues and by coordinated interactions among hormones along the hypothalamic-pituitary-gonad-liver (HPGL) axis (Nagahama and Yamashita, 2008). Therefore, a disruption at key points on the HPGL axis could be reflected by disordered endocrine and reproductive systems (Sofikitis et al., 2008).

In light of the above, combined *in vitro* and *in vivo* assays were employed to reveal the estrogenic potential and underlying molecular mechanisms of MC-LR in this study. In the *in vitro* assay, the production of E2 and T as well as the expression of genes encoding steroidogenesis enzymes were examined using H295R cells after MC-LR exposure. In the *in vivo* assay, the effects of MC-LR on histopathology, sex hormone contents in the testis and gene expressions in the HPGL axis along with ERα protein levels in the liver were investigated in adult male zebrafish (*Danio rerio*). This study provides insights into the endocrine disrupting risks of MC-LR at environmental concentrations and also addresses possible effects on human health.

2. Materials and methods

2.1. Chemicals

MC-LR (purity ≥95%) was obtained from Express Technology Co. Ltd (Taiwan, China), and the purity was further confirmed using HPLC (LC-10A, Shimadzu, Nakagyo-ku, Kyoto, Japan) (Moreno et al., 2005). For the *in vitro* assay, MC-LR was dissolved in dimethyl sulfoxide (DMSO) to form a 0.01 M stock solution. Forskolin and prochloraz were purchased from Sigma-Aldrich Co. Ltd. (Shanghai, China). For the *in vivo* assay, MC-LR was dissolved in milliQ water to obtain a 0.5 mg/mL stock solution. All other chemicals utilized in the present study were of analytical grade.

2.2. Cell culture and chemical exposure

H295R cells purchased from ATCC (CRL-2128, USA) were maintained in DMEM/F12 medium, which contains Nu-Serum (2.5%), insulin-transferring sodium selenite plus Premix (1%), penicillin (100 U/mL) and streptomycin (100 μg/mL). For chemical exposure, the cells were grown at 37 °C with 5% CO_2 in 6-well plates, and 2 mL of cell suspension with a density of 3×10^5 cells/mL was added to each well. After 24 h, the medium was refreshed, and 1, 10, 100, 500, 1000 and 5000 μg/L MC-LR were added. All groups received 0.1% DMSO, and treatment with only 0.1% DMSO was set as solvent control (SC). Treatments with 5 μM model inducer forskolin (about 2.05 mg/L) and 30 μM model inhibitor prochloraz (about 0.11 mg/L) were set as positive control (PC) and negative control (NC) respectively. After 48 h exposure, cell viability was measured using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc.), and the culture medium from each cell was transferred and stored at −80 °C for hormone analysis. The remaining cells were washed with ice-cold PBS three times. After centrifugation (4000 r/min, 10 min), cells were immediately frozen in liquid nitrogen for gene expression analysis. All treatments were conducted with six replicates.

2.3. Fish maintenance and toxin exposure

Adult male zebrafish (AB-type, 6 months old) were cultured in glass tanks with 15 L filtered tap water under semi-static conditions at 28 ± 1 °C. The light/dark cycle and feeding protocol was performed as described previously (Hou et al., 2016). After a 2-week acclimation, male zebrafish were exposed to 0, 0.3, 1, 3, 10 and 30 μg/L of MC-LR for 30 d. Three replicates (30 fish per tank) were conducted for each treatment group. Every 3 d, one third of the stale water in each tank was renewed with fresh water containing MC-LR or none, and the concentrations were monitored using a commercial ELISA kit (Beacon, USA) (Table S2, Supplementary information).

During the 30 d exposure, 7 zebrafish died in total (1 in the control group, 3, 1 and 2 in the 3, 10 and 30 μg/L groups, respectively. After 30 d, 66 male fish (22 per tank) were selected randomly from each treatment and anesthetized with 0.02% tricaine methanesulfonate (MS-222) solution. Testes from six fish were dissected and fixed for pathological studies. For the rest of the fish, samples (brains, livers and testes) were weighed and immediately frozen in liquid nitrogen for quantitative analysis of hormones, gene expressions and protein. The gonadosomatic index (GSI) was calculated as weight of testis (g)/body weight (g) × 100%. The experiment was approved by the guidelines of Institutional Animal and Care and Use Committees (IACUC) of Huazhong Agricultural University (permission number: SYXK (Hubei) 2015-0084).

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