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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.

Capsule

MC-LR induced non-dose-dependent estrogenic effects via stimulating steroidogenesis in both *in vitro* and *in vivo* conditions.

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Conflict of Interest

The authors declare that there are no conflicts of interest in the present experiment.

Keywords

microcystins; estrogenic effect; H295R cell; zebrafish; endocrine disruption

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; Trinchet et al., 2011; Li et al., 2012; Li et al., 2013), 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 weight) 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 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 *vgt1* 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 xeno-estrogens 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% CO₂ 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) $\times$ 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).

2.4. Pathological analysis and morphometry of testis

Samples were fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned (5 µm), and stained with haematoxylin and eosin (H&E). Pathological analysis was performed on a light microscope (Nikon H600L, Japan).

The percentages of three major stages of spermatogenesis including spermatogonia (SPG), spermatocytes (SPC) and spermatids (SPD) were quantitatively analyzed. The volume

density was determined using a 441-intersection grid. Ten fields chosen randomly (4410 points) were scored at 400× magnification per fish. The percentage of each cell type was calculated for six individual fish.

2.5. Quantification of hormone, gene expression and protein levels

E2, T levels in the H295R cell culture medium and zebrafish testis samples were analyzed as previously described (Lun et al., 1998; Ma et al., 2012; Hou et al., 2016). The expression of steroidogenic genes in H295R cells and the brain, liver, testis of zebrafish were measured using quantitative real-time PCR according to the previous methods (Livak and Schmittgen, 2001; Hou et al., 2014), and the gene-specific primers are presented in Table S3, Supplementary information. Western blot analysis was conducted to measure ER α protein levels in the liver of male zebrafish after MC-LR exposure. Details of the quantification of hormones, gene expressions and protein can be found in Supplementary information.

2.6. Statistical analyses

Statistical analysis was done using SPSS 20.0. All values were expressed as mean $\pm$ standard error (SE). Normality and variance homogeneity were verified. Data were subjected to one-way analysis of variance (ANOVA) followed by Dunnett's test. To screen which genes (pathways) were responsible for the differences detected in sex hormone levels after MC-LR exposure, spearman correlation analysis was undertaken independently for *in vitro* and *in vivo* results. The strength of association between two variables is considered very strong if the coefficient (r) ranges from 0.95 to 1, strong 0.8 to 0.95, moderate from 0.5 to 0.8, weak from 0.2 to 0.5, and very weak when less than 0.2. Differences were considered statistically significant at $P < 0.05$ (*) or $P < 0.01$ (**) against the control.

3. Results

3.1. In vitro study

3.1.1 H295R steroidogenesis assay—Cell viabilities in MC-LR treatment groups were all above 80% of the SC (Fig.S2, Supplementary information). As shown in Fig.1, E2 and T production in the medium showed a dual change with an increase at low doses and a decrease at high doses. Levels of medium E2 were significantly elevated in the supernatant of H295R cells exposed to 1 and 10 $\mu\text{g/L}$ MC-LR compared to the SC ($P < 0.05$). In contrast, significant decreases in E2 contents were observed in the 500, 1000 and 5000 $\mu\text{g/L}$ groups ($P < 0.05$). T levels increased significantly at 10 $\mu\text{g/L}$ ($P < 0.05$), and then gradually declined to the SC levels. E2 and T levels in the PC and NC groups showed moderate increase and decrease, respectively.

MC-LR induced a non-dose-dependent damage in gene expressions along the sex steroidogenesis pathway in H295R cells (Fig.1C; see Table S4, Supporting Information). After exposed to 1 $\mu\text{g/L}$ MC-LR, the expression of *StAR*, *CYP11A*, *CYP17*, *17 β -HSD1* and *17 β -HSD4* were significantly up-regulated (*CYP17*, $P < 0.05$; *StAR*, *CYP11A*, *17 β -HSD1* and *17 β -HSD4*, $P < 0.01$). In the 10 $\mu\text{g/L}$ group, all the genes except *3 β -HSD2* exhibited significant up-regulations and reached their peaks ($P < 0.01$), among which *StAR* and *17 β -HSD1* dramatically increased more than 5-fold. At the concentration of 100 $\mu\text{g/L}$, *StAR*,

CYP11A and *CYP17* remained significant up-regulated, while the expression of other genes dropped back. With the further increase of MC-LR concentrations (range from 500 to 5000 µg/L), the expression of all genes gradually declined, and significant down-regulations were observed in the expression of *CYP11A*, *CYP19A* and *17β-HSD4* ($P < 0.01$).

3.1.2 Correlation analysis—Gene expressions in H295R cells were positively correlated with concentrations of E2 extracted from the culture medium (Table S5, Supplementary information). *CYP19A* and *3β-HSD2* showed very strong positive correlations ($r = 0.95$; $P < 0.01$) and *CYP17* showed a strong positive correlation ($0.8 < r < 0.95$; $P < 0.01$). *StAR* and *CYP11A* exhibited moderate positive correlations ($0.5 < r < 0.8$; $P < 0.01$) and *HMGR*, *17β-HSD1*, *17β-HSD4* showed weak correlations ($r < 0.5$; $P < 0.01$). In addition, the expression of *StAR*, *CYP11A*, *CYP17*, *CYP19A* and *3β-HSD2* exhibited strong positive correlation with T levels ($0.8 < r < 0.95$; $P < 0.01$), while *HMGR*, *17β-HSD1*, *17β-HSD4* showed moderate positive correlations ($0.5 < r < 0.8$; $P < 0.01$).

3.2. In vivo study

3.2.1 Pathological analysis and GSI—In the control group, the seminiferous tubules in the testis were filled with abundant mature spermatids (SPD). Other stages of development of sperm cell bundles were observed, including spermatogonia (SPG), and spermatocytes (SPC) (Fig. 2A). Testes in the 0.3, 1 and 3 µg/L groups maintained their normal morphology (Fig. 2B, C and D). In the 10 µg/L exposure group, seminiferous tubules were disorderly and cell density was reduced, resulting in widened intercellular spaces (Fig. 2E). The pathological alterations in the 30 µg/L exposure group were similar to those in the 10 µg/L group but more severe, and the degeneration of interstitial cells and seminiferous tubule epithelium including sustentacular or Sertoli cells was observed (Fig. 2F). In addition, no ovotestis was found after MC-LR treatment.

For GSI, only zebrafish in the 30 µg/L MC-LR groups showed a significant decline compared to the control ($P < 0.05$) (Fig. 3A). On the other hand, the percentage of spermatogonia (SPG), spermatocytes (SPC), and spermatids (SPD) showed different trends after MC-LR exposure (Fig. 3B). Percentages of SPC fluctuated without showing any significance in male zebrafish testes after MC-LR exposure. The percentage of SPG gradually increased with the ascent of MC-LR concentration, and peaked at the 30 µg/L MC-LR group ($P < 0.01$). The percentage of SPD showed an opposite, concentration-dependent decrease, and a significant decline was observed after exposed to 30 µg/L MC-LR ($P < 0.01$).

3.2.2. In vivo steroidogenesis assay—Exposure to MC-LR significantly altered sex hormone production in the testis of zebrafish (Fig. 4 A and B). E2 contents significantly increased in the 3 µg/L group ($P < 0.05$), peaked at 10 µg/L ($P < 0.01$) and then remained high levels in the 30 µg/L group ($P < 0.05$). T contents exhibited a similar but modest up-regulation, and a significant increase was observed at 10 µg/L ($P < 0.05$).

The expression of HPGL-axis genes in male zebrafish varied substantially among tissues (Fig. 4C; see Table S6, Supplementary information). In the brain of male zebrafish, exposure to 0.3, 1 and 3 µg/L MC-LR only caused significant down-regulation of *ar* and *erβ* ($P <$

0.01), while 10 and 30 µg/L of MC-LR significantly decreased the expressional levels of all genes except *gnrh2* and *gnrh3r*. In the testis, significant up-regulations were observed in steroidogenesis-related genes including *star*, *cyp11a*, *cyp17*, *cyp19a* and *fshr*. Among which, *cyp19a* in the 1, 3, 10 and 30 µg/L groups maintained more than a 2-fold increase at the transcription level. The other gene expressions peaked at 3 µg/L ($P < 0.01$), then gradually fell back to the control level, or even lower (not significant) after exposure to 30 µg/L MC-LR. In the liver, the expressions of *era* and *erβ* decreased in a dose-dependent manner, which were less than 0.5-fold after exposure to 30 µg/L MC-LR ($P < 0.05$ and $P < 0.01$, respectively). However, *vtg1* expression in the liver exhibited an opposite trend, which dramatically increased to more than 5-fold ($P < 0.01$) at 3 and 30 µg/L.

3.2.3 Correlation analysis—As shown in Table S7, Supplementary information, all gene expression in the brain of male zebrafish showed weak or very weak correlation to E2 contents in the testis ($r < 0.5$). In the testis, the expression of *star*, *cyp11a*, *cyp17*, *cyp19a*, *fshr* and *lhre* exhibited moderate positive correlations ($0.5 < r < 0.8$; $P < 0.05$). In the liver, only *vtg1* expression was moderately correlated with testicular E2 contents ($0.5 < r < 0.8$; $P < 0.01$), and the expression of *era* and *erβ* were weakly correlated with E2. Correlation between HPGL-related gene expressions and T levels presented a similar trend. In the brain, only *gnrh3* showed moderate positive correlation to T ($0.5 < r < 0.8$; $P < 0.05$). The expression of all the genes in the testis were moderately correlated with T contents ($0.5 < r < 0.8$; $P < 0.05$). Again, in the liver *vtg1* was found positively correlated with T contents in the testis ($0.5 < r < 0.8$; $P < 0.01$) while *era* and *erβ* exhibited very weak correlations ($r < 0.2$).

3.2.4 Western blot analysis—In the liver of male zebrafish, the protein expressions of ERα showed significant declines after 30 d exposure to MC-LR at 10 and 30 µg/L ($P < 0.01$) (Fig.5).

4. Discussion

The potential for harmful compounds produced by cyanobacteria to disrupt endocrine function has been a topic of concern in recent years (Sychrová et al., 2012), however, the estrogenicity of MC-LR remains controversial (Oziol and Bouaïcha, 2010; Rogers et al., 2011; Qiao et al., 2013; Su et al., 2016). By combining *in vitro* and *in vivo* assays, the present study demonstrated that MC-LR at environmental concentrations could elicit estrogenic responses via disrupting steroidogenesis.

H295R cells are a valuable tool for rapid screening endocrine disrupting potency and exploring mechanisms of toxicants (Zhang et al., 2005; Kraugerud et al., 2010; Ma et al., 2012). In the present *in vitro* study, E2 and T contents significantly increased at certain low concentrations (1 and 10 µg/L) but gradually declined with the increase of MC-LR concentrations. This result implied strong toxic effects of MC-LR at high concentrations, as suggested by Oziol and Bouaïcha (2010). It was noted that the increase of E2 content at low doses was positively correlated with extensive up-regulation of gene expression along the sex steroidogenesis pathway. In detail the expression of *CYP19A* showed a very strong positive correlation with medium E2 in our study. Induction of E2 via interference with steroidogenic enzymes, such as aromatase, the rate-limiting enzyme converting T to E2, is a

novel mechanism for estrogenicity apart from binding ER (Sanderson et al., 2000; Hilscherova et al., 2004; Spanò et al., 2004). One such example is atrazine, a herbicide which induces estrogenic effects without binding ER (Connor et al., 1996; Sanderson and Berg, 1998). Thus, our *in vitro* results confirmed the estrogenic risk of MC-LR specifically at low concentrations, which motivated us to validate these responses *in vivo* for comparing the differences between the two systems.

Our previous study documented that MC-LR caused endocrine disruption with widened intercellular spaces and delayed sperm maturation in the testis of male zebrafish after a 90 d life-cycle exposure (Su et al., 2016). Here, the current *in vivo* study further confirmed dose-dependent testicular damage in adult males, characterized by spermatogenesis suppression and the degeneration of interstitial and sustentacular cells. These pathological changes, as symptoms of male reproductive impairment, were consistent with extensive down-regulation of brain genes involved in upstream regulation along the HPGL axis. The HPGL axis plays a crucial role in the regulation of steroidogenesis, and is a specific target for EDCs (Grillasca, 2012; Saunders et al., 2014, 2015). Our result suggested that MC-LR could directly affect the synthesis of GnRH, FSH and LH in the hypothalamus and pituitary, posing a threat to the neuroendocrine function of the brain.

Similar to our *in vitro* results, sex hormone levels and steroidogenesis gene expression in zebrafish testis exhibited concurrent remarkable increases in the *in vivo* assay, and the critical estrogen threshold is between 1 and 30 µg/L. According to the correlation analysis, the expression levels of testicular genes exhibited stronger positive correlation with E2 and T contents than those in the brain and liver, indicating that MC-LR induced steroidogenesis disruption primarily by affecting the synthesis and autocrine in the testis. Among these steroidogenesis genes, *cyp19a*, as a special target for EDCs (Sanderson et al., 2002), showed the most significant increase, which could be responsible for elevated E2 in zebrafish testis. Previous *in vitro* and *in vivo* studies on environmental estrogens such as polychlorinated biphenyls (PCBs) and bisphenol AF (BPAF) also found that the increase of *cyp19a* expression coincided with up-regulated E2 levels (Kraugerud et al 2010; Shi et al., 2015). Furthermore, significantly increased hepatic *vtg1* expression, as a symbol of estrogenic effect, was found positively correlated with testicular E2 and T levels after MC-LR exposure. It was also noted that both ER gene expressions (*era* and *erβ*) and protein levels (ERα) in the liver exhibited no significant correlation with the E2 and T content, indicating the existence of ER-independent mechanisms for the estrogenic potency of MC-LR. On the other hand, MC-LR is a cyclic peptide with chemical structures that are not similar to certain natural and synthetic oestrogen-mimicking substances such as nonyl phenol (NP) and bisphenol A (BPA) (Satoh et al., 2002; Ogawa et al., 2006; Liu et al., 2007), and its hydrophilic feature reduces the possibility that it functions as an ER ligand (Fang et al., 2001). Thus, MC-LR is unlikely to cause estrogenic responses via binding ER. Taken together, we speculate that MC-LR is likely to induce E2 increases via stimulating steroidogenesis rather than via ER-mediated pathway. Further research is needed to validate whether this estrogenic risk of MC-LR could result in gender imbalance in eutrophic water bodies subject to *Microcystis* blooms.

5. Conclusions

Our study revealed that MC-LR could exhibit non-dose-dependent estrogenic effects through interfering with steroid hormone synthesis and steroidogenic gene expressions in both *in vitro* and *in vivo* conditions. The critical estrogen threshold of MC-LR at the hormone and gene transcriptional level is between 1 and 30 µg/L, which is considered to be environmentally relevant level. The non-dose-dependent trait was an integrated manifestation of the endocrine disrupting effect and cytotoxicity of MC-LR. These findings support a paradigm shift in the risk assessment of MC-LR, from traditional toxicity to estrogenic risk, emphasizing the potential threat to the reproductive capacity of wildlife and human in bloom area.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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Fig.1.

E2 (A) and T (B) production and gene transcription along sex steroidogenesis pathway (C) in H295R cells after 48 h exposure to MC-LR at the concentrations of 1, 10, 100, 500, 1000 and 5000 μ g/L. Forskolin and prochloraz were used as PC and NC. The colors describe different fold thresholds.

Fig.2.

H&E stained sections of zebrafish testis after exposed to 0, 0.3, 1, 3, 10 and 30 µg/L MC-LR for 30 d. Bars represent 50 µm. (A) Control group; (B) 0.3 µg/L exposure group; (C) 1 µg/L exposure group; (D) 3 µg/L exposure group; (E) 10 µg/L exposure group; (F) 30 µg/L exposure group. SPG, Spermatogonia; SPC, Spermatocyte; SPD, spermatids. Arrows indicate widened interstitial space; stars indicate cellular degeneration.

Fig.3. GSI (A) and percentages of three major stages of spermatogenesis in zebrafish testis (B) after exposed to 0, 0.3, 1, 3, 10 and 30 μg/L MC-LR for 30 d.

Fig.4.

The contents of E2 (A) and T (B) and gene transcriptions along the HPGL axis (C) in male zebrafish after exposed to 0 (Control), 0.3, 1, 3, 10 and 30 µg/L MC-LR for 30 d. The colors describe different fold thresholds.

Fig.5.

Effects of MC-LR on the protein levels of ER α in the liver of male zebrafish exposed to 0 (control), 0.3, 1, 3, 10 and 30 $\mu\text{g/L}$ MC-LR for 30 d. (A) Western blot sample ER α and GAPDH, (B) the ER α /GAPDH fold change resulted from the average of all values in each treatment group.