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Dualistic immunomodulation of sub-chronic microcystin-LR exposure on the innate-immune defense system in male zebrafish

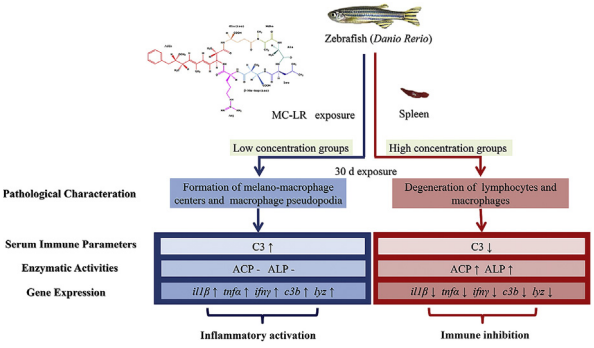
Wang Lin [a], Jie Hou [a], Honghui Guo [a], Yuming Qiu [a], Li Li [a, b, c, d, *], Dapeng Li [a, b, c, d], Rong Tang [a, b, c, d]

[a] College of Fisheries, Huazhong Agricultural University, Wuhan 430070, PR China
[b] Key Laboratory of Freshwater Animal Breeding, Ministry of Agriculture, Wuhan 430070, PR China
[c] Hubei Provincial Engineering Laboratory for Pond Aquaculture, Wuhan 430070, PR China
[d] Freshwater Aquaculture Collaborative Innovation Center of Hubei Province, Wuhan 430070, PR China

HIGHLIGHTS

- Macrophages might be the main action site of MC-LR in the spleen.
- MC-LR has concentration-dependent dualistic influences on fish innate immune system.
- Inflammatory activation in low exposure concentration but immune inhibition at high concentration.
- Interference of environmental concentrations of MC-LR in the immune system should not be neglected.

GRAPHICAL ABSTRACT



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ABSTRACT

Microcystins (MCs), produced by toxic cyanobacterial blooms that appeared world wildly in eutrophication waters, have often caused fish illness and even massive death cases. Among at least 90 structural variants, microcystin-LR (MC-LR) is the most common and toxic variant. In order to better understand innate immune responses in fish disrupted by environmental concentrations of MC-LR, male zebrafish (*Danio rerio*) were exposed to 0, 0.3, 1, 3, 10 and 30 μg/L MC-LR for 30 d, and the changes in splenic pathology and immunological gene expression as well as serum immune parameters were studied. In the low concentration groups (0.3, 1 and 3 μg/L), zebrafish displayed splenic inflammatory changes including the formation of melano-macrophage centers and the increase of macrophage pseudopodia, remarkable elevation of serum C3 levels, and significantly upregulated expression of innate immune-related genes (*c3b*, *lyz*, *il1β*, *tnfα* and *ifnγ*). In contrast, high concentrations of MC-LR (10 and 30 μg/L) resulted in the degeneration of splenic lymphocytes and macrophages, and down-regulation of immune-related genes as well as significant decreases in the level of serum C3. Furthermore, significant increases in the activity of serum ACP and ALP suggested that high concentrations of MC-LR increased permeability of macrophage plasma membrane or cellular necrosis, and subsequently decreased innate immune function. Our findings illustrated that sub-chronic exposure of MC-LR has dualistic influences on fish innate immune system with inflammatory activation at low exposure concentrations but turned to immune inhibition with the increases of exposure concentration.

* Corresponding author.
E-mail address: foreverlili78@mail.hzau.edu.cn (L. Li).

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1. Introduction

Cynobacterial blooms caused by water eutrophication have become a worldwide environmental problem (Paerl et al., 2001). Produced by cyanobacteria like *Microcystis*, *planktothrix* and *Anabaena*, microcystins (MCs) are the most common toxins found in natural water, which can exert various toxic effects on aquatic organisms including behavioral changes, histological damage, circulatory disturbance and even mass mortality (Zimba et al., 2001; Baganz et al., 2004; Li et al., 2009a, 2013). More than 90 analogues of MCs have been identified (Ufelmann et al., 2012), among which microcystin-LR (MC-LR) is the most toxic, common and also extensively studied variant (Gupta et al., 2003). The maximal safety concentration of MC-LR in drinking water was set at 1 μg/L by the World Health Organization (WHO). However, the concentrations of dissolved MCs in eutrophic lakes normally range from 0.1 to 10 μg/L, and reach to 35.8 μg/L or higher following the collapse of a large, highly toxic blooms (Lahti et al., 1997; Wang et al., 2010). Moreover, MCs can accumulate and transfer in food chain, posing potential health risks to aquatic organisms and even human beings (Van Liere and Mur, 1980; Chen et al., 2009; Zhang et al., 2009).

It is well known that the liver is the most important target organ of MCs, whereas the kidney, gastrointestinal tract, cardiovascular system and reproductive system as well as nervous system are also vulnerable to MCs exposure (Ding et al., 2006; Qiu et al., 2009; Feurstein et al., 2009; Li et al., 2013; Hou et al., 2014). Several studies have found that MCs are accumulated in the mammalian spleen and cause immunotoxicity (Shen et al., 2003; Li et al., 2012). Until now, there is only limited information on the immune toxicity of MC-LR in fish and the details of its mechanism are still lacking (Wright et al., 2004; Rymuszka et al., 2007; Sierosławska et al., 2007; Wei et al., 2009; Trinchet et al., 2011). Rymuszka et al. (2007) revealed that the proliferation of lymphocytes isolated from the spleen of rainbow trout (*Oncorhynchus mykiss*) was inhibited after application of MC-LR at a concentration of 40 mg/ml, but significantly increased at a concentration of 1 mg/ml in comparison to the control group. Also, MC-LR caused time- and dose-dependent cell viability decrease when *in vitro* phagocytic cells were exposed at the concentration of 1, 5, 10, 20 μg MC-LR/mL (Sierosławska et al., 2007). On the contrary, Wright et al. (2004) found no changes in the number and proliferation in isolated lymphocytes from pronephros in Murray cod (*Maccullochella peelii peelii*) after the administration with low concentrations of MC-LR at 0.05 and 0.5 μg/ml. In an *in vivo* experiment, the significant down-regulation in transcriptional levels of splenic immune-related genes revealed inhibition effects of MC-LR on immune function in grass carp intraperitoneally injected with 50 μg/kg MC-LR body weight (Wei et al., 2009). Li et al. (2009b) documented remarkable induction of MC-LR on transcription of several genes essential for early lymphoid development (Rag1, Rag2, Ikaros, GATA1, Lck, TCRα) and heat shock proteins (HSP90, HSP70, HSP60, HSP27) in zebrafish larvae after 168 h exposure to 800 μg/L MC-LR. In a chronic contamination of the medaka (*Oryzias latipes*) with a low concentration of MC-LR (5 μg/L), a cellular immune response was suggested by the inflammatory cells (macrophages) found in the spleen parenchyma (Trinchet et al., 2011). However, previous studies basically focused on responses of different types of immune cells *in vitro* or changes of immunological indices *in vivo* after a short term exposure to high concentrations of MCs. It is imperative to systematically assess the immunotoxic potential of MCs at environmentally relevant concentrations and long-term exposure in fish since MCs producing waterblooms can be present for a majority of the year.

The fish, because of its earliest evolution, mostly depends on non-specific (innate) immunity and its specific (acquired) immune system is less well developed (Fletcher and Secombes, 1999; Magnadóttir, 2006). As the largest immune organ, the spleen plays a vital role to maintain normal immune function in fish (Manning and Turner, 1994). In light of the above, the aims of this study are (1) to evaluate splenic pathological characterization in zebrafish after 30 d of sub-chronic exposure to MC-LR at the concentrations of 0.3, 1, 3, 10 and 30 μg/L respectively, (2) to determine responses of serum immune parameters and transcription levels of splenic innate immune-related genes and (3) to discuss the possible molecular mechanism of MCs immunotoxicity from the perspective of the innate immune defense system.

2. Materials and methods

2.1. Chemicals

The cyanobacterial toxin MC-LR was obtained from Express (Express Technology Co. Ltd, TaiWan), with a purity greater than 95% confirmed by the high performance liquid chromatography (HPLC, LC-10A, Shimadzu, Nakagyo-ku, Kyoto, Japan) following the method by Li et al. (2005). The toxin used in the experiment was suspended in milliQ water to acquire a stock solution at the concentration of 0.5 mg/ml. All of the other chemicals utilized in the experiment were of analytical grade.

2.2. Zebrafish maintenance and treatment protocol

Adult healthy male zebrafish (AB strain, 4 month old) were obtained from Institute of Hydrobiology, Chinese Academy of Sciences. Zebrafish were acclimated in charcoal-filtered aerated water for 14 days prior to the experiment. Water temperature was maintained at 28 ± 0.5 °C and the photoperiod was adjusted to a 14:10 h (Light: Dark) cycle. During the acclimation, fish were fed daily with freshly hatched *Artemia* nauplii and commercial flake food (Tetra, Germany) at a rate of 5.0% of body weight.

Six exposure concentrations of MC-LR were set at 0, 0.3, 1, 3, 10 and 30 μg/L with consideration of the environmental relevance and other reference reports (Baganz et al., 2004; Wang et al., 2010; Hou et al., 2016). Each concentration had three replicates. Each replicate consisted of 30 male adult zebrafish and 15 L of filtered tap water dechlorination with or without MC-LR under semi-static conditions. The experiment lasted for 30 d and the other conditions were as same as described above for the acclimation period. In order to keep the MC-LR concentration stable and comparatively close to the target doses, one third of stale water from each tank was replaced every 3 d with fresh water containing the corresponding MC-LR concentration, or no MC-LR. During the whole experimental period, MC-LR concentrations within the experimental waters were detected every 6 d using an MC-LR ELISA kit (Beacon Analytical System, Inc., Saco, ME, USA) with a minimum detection limit of 0.1 μg/L (data not shown). The measured real concentration in each MC-LR treatment group deviated by less than 20% from the nominal concentration, which conformed with the proposed of the OECD guideline 204 for fish prolonged toxicity test (OECD, 1984).

After 30 d of exposure, 60 male zebrafish were selected completely randomly from each treatment group and anesthetized with a 0.02% tricaine methanesulfonate (MS-222) solution. Blood samples were first taken from the caudal vein using capillary pipet to separate the serum. And then spleens were excised and weighed to determine the spleen index as [weight of spleen (g)/body weight (g) × 100%]. In each treatment group, serum samples from 10 fish were pooled as one replicate for the analysis of immune parameters analysis, and 3 fish's spleens were pooled as one replicate and stored at −80 °C for the analysis of gene expression. For the pathological study, 6 fish's spleens were fixed in 10% neutral buffered