Environmental Pollution 236 (2018) 12–20

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Effects of MCLR exposure on sex hormone synthesis and reproduction-related genes expression of testis in male *Rana nigromaculata*☆

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ARTICLE INFO

Article history:
Received 22 August 2017
Received in revised form
12 January 2018
Accepted 17 January 2018

Keywords:
Microcystin-LR
Reproductive toxicity
Steroidogenesis
Sex hormone receptors
Rana nigromaculata

ABSTRACT

Microcystin-leucine-arginine (MCLR) is the most popular and toxic variant among microcystins, which can cause severe reproductive toxicity to animals. However, the mechanisms of reproductive toxicity induced by MCLR in amphibians are still not entirely clear. In the current study, toxicity mechanisms of MCLR on the reproductive system of male *Rana nigromaculata* followed by low concentration (0, 0.1, 1, and 10 μg/L) and short-term (0, 7, and 14 days) MCLR exposure were shown. It was observed that MCLR could be bioaccumulated in the testes of male frogs, and the theoretical bioaccumulation factor values were 0.24 and 0.19 exposed to 1 μg/L and 10 μg/L MCLR for 14 days, respectively. MCLR exposure significantly decreased testosterone (T) concentrations and increased estradiol (E2) concentrations exposed to 1 and 10 μg/L MCLR for 14 days. The mRNA levels of *HSD17B3* were downregulated, and *HSD17B1* and *CYP19A1* mRNA expression levels were upregulated, respectively. Only 10 μg/L MCLR group showed significant induction of follicle-stimulating hormone (FSH) levels and cyclic adenosine monophosphate (cAMP) content. Moreover, *AR* and *ESR1* mRNA expression levels were significantly upregulated exposed to 1 and 10 μg/L MCLR for 14 days, respectively. Our results suggested that low-concentration MCLR induced transcription changes of *CYP19A1*, *HSD17B3*, and *HSD17B1* led to endocrine disorders, and caused interference of spermatogenesis by the decrease of T and abnormal gene expressions of AR and ESR1 in the testes of *R. nigromaculata*.

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1. Introduction

Cyanobacteria booms have become serious problems for the aquatic environment and human environmental health due to the production of toxic secondary cyanotoxins (van Apeldoorn et al., 2007). Microcystins (MCs) are the main reported groups of cyanotoxins (Sabatini et al., 2015), and microcystin leucine-arginine (MCLR) is the most popular and toxic variant among more than 100 MC isoforms (Vesterkvist et al., 2012). MCLR causes wide public concern because of its toxicity that poses a threat to people and animals (Zhao et al., 2015). The World Health Organization has recommended a drinking water safety limit of 1 μg/L for MCLR. In recent years, many studies have indicated that a concentration value of approximately 1 μg/L MCLR was still not safe for animals (Jia et al., 2014; Zhang et al., 2006).

A recent study reported that 10.1 nM MCLR presented estrogenic properties in MCF-7 human breast carcinoma cell by MELN bioassay (Oziol and Bouaïcha, 2010). It was also reported that low-concentration MCLR could disrupt the balance of serum hormones including testosterone (T), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) in mammals by damaging the hypothalamic-pituitary-gonadal (HPG) axis (Wang et al., 2012), leading to the decline of sperm quality (Li et al., 2008). Testosterone, estradiol (E2), luteinizing hormone and follicle-

☆ This paper has been recommended for acceptance by Dr. Harmon Sarah Michele.

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https://doi.org/10.1016/j.envpol.2018.01.057
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stimulating hormone are important hormones in the HPG axis, which mediate steroidogenesis, and spermatogenesis in male animals (Ascoli et al., 2002; Ruwanpura et al., 2010). When vertebrates were exposed to environmental contamination, the HPG axis plays a crucial role in maintaining regulation of the serum hormones (Chen et al., 2016). Steroidogenic acute regulatory protein (STAR), cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1), cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1), 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2), 17β-hydroxysteroid dehydrogenase type 3 (HSD17B3), and cytochrome P450 aromatase (CYP19A1) are important steroidogenic enzyme profiles along the HPG axes in testes (Miller and Auchus, 2011). The investigation of the steroidogenic enzyme is important to understand disorder differentiation, reproduction, fertility, and physiological homeostasis, etc (Miller and Auchus, 2011). In addition, estrogen receptor (ER) and 17β-hydroxysteroid dehydrogenase type 1 (HSD17B1) are considered estrogen-related genes that are mainly involved in regulating sperm concentration and sperm motility (Lee et al., 2011). Androgen receptor (AR) is another important factor for initiating and maintaining spermatogenesis and male fertility (Wang et al., 2009). Many studies have investigated whether environmental antiandrogens could cause the disruption of AR (Ding et al., 2017; Zhuang et al., 2016, 2017) and estrogen receptor 1 (ESR1), (Lv et al., 2017). However, little is known about MCLR-induced reproduction toxicity mechanisms in amphibians thus far. The investigation of the effects of MCLR exposure on these gene expressions and hormone levels can clarify the exact mechanisms of MCLR-induced reproduction toxicity, including endocrine disorders and spermatogenesis disruption.

With the development of industry and agriculture, loss and degradation of the habitat of amphibians are the factors threatening the largest number of amphibian species (Ficetola et al., 2015). The number of *R. nigromaculata* species has declined in China and Japan; this species is designated as a near-threatened species by the International Union for the Conservation of Nature and Natural Resources red list (Xie et al., 2007). Endocrine disruption is another potential reason for the decrease in the number of species of amphibians (Hayes et al., 2010; Jia et al., 2014). Due to the high sensitivity to contamination and environmental change-related stress, *R. nigromaculata* are the excellent bio-indicators of environmental contamination and ecosystem health (Hopkins, 2007). Furthermore, the highest total cyanobacteria density was observed in summer accorded with the period of *R. nigromaculata* growth and development (Cong et al., 2006), so that *R. nigromaculata* are vulnerable to MCLR. Our previous studies have found that low-concentration MCLR exposure in vivo could lead to testis cell apoptosis, endocrine disruption, and a decline in sperm quality of *R. nigromaculata* (Zhang et al., 2013; Jia et al., 2014). Therefore, it is suitable using *R. nigromaculata* to investigate the mechanisms of MCLR induced-reproduction toxicity.

In the current study, the goal was to identify the mechanisms of low MCLR exposure on the reproductive toxicity in testes of male *R. nigromaculata*. The bioaccumulation of MCLR in testes was evaluated by using high-performance liquid chromatography-mass spectrometry (HPLC-MS) and the hormones including T, E2, LH, and FSH were measured by enzyme-linked immunosorbent assay (ELISA). Meanwhile, the expression of reproduction-related genes involved sex hormones synthesis and sex hormone receptors were also determined. This is the first work to investigate the exact mechanisms of MCLR-induced reproduction toxicity including endocrine disruption and spermatogenesis disruption in amphibian. This study would be helpful to assess the toxicological risk of MCLR to amphibians.

2. Material and methods

2.1. Chemicals

Standard MCLR (≥95% purity; HPLC) was obtained from Enzo Life Sciences (Enzo Biochem, lnc, USA). T, E2, LH, FSH, and cyclic adenosine monophosphate (cAMP) ELISA assay kits were purchased from Nanjing Jiancheng Bioengineering, Inc. (Nanjing, Jiangsu, China).

2.2. Animals, animal treatment, and hormone analysis

Healthy adult male frogs (*R. nigromaculata*, 2 years old) were obtained from Zhejiang Changxing Creative Ecological Agriculture Development Co., Ltd. (Huzhou, Zhejiang, China). The frogs were kept in aquariums ($60\ cm \times 40\ cm \times 35\ cm$) filled with dechlorinated tap water (temperature at $21 \pm 1\ ^\circ C$; pH at 6.5 ± 0.5; dissolved oxygen content at 7 ± 1 mg/L) at a depth of 3 cm for 7 days prior to all experiments. Subsequently, strong and healthy frogs (average body length of 7.50 ± 0.20 cm; average body weight of 52.06 ± 3.15 g) were selected and randomly divided into seven groups ($n = 20$ per group). Among these groups, three groups were assigned as control groups and were exposed to dechlorinated tap water for 0, 7, and14 days, respectively. Two groups were exposed to 1 μg/L MCLR for 7 and 14 days, and the last two groups were exposed to 0.1 and 10 μg/L of MCLR for 14 days, respectively. The frogs were fed with *Eisenia fetida* twice a day and full-exposure solutions were renewed daily by static displacement method. Natural light/dark cycle conditions were provided. After the end of the exposure time, the frogs were respectively euthanized by pithing, which aimed to destroy brain tissue in the cranial cavity and destroy spinal cord in the vertebral canal. The following frog dissection procedures were supported by People for the Ethical Treatment of Animals. The testes were removed from the frogs with clearing of adhering tissues, quickly stored at $-80\ ^\circ C$ with liquid nitrogen in preparation for cAMP assay and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) assay. Frog blood was collected, separated, and stored at $-20\ ^\circ C$ until the assays for T, E2, LH, and FSH were completed. All procedures on animals followed the guidelines for humane treatment set by the association of laboratory animal sciences.

Testosterone, E2, LH, and FSH were measured using ELISA kits based on the double-antibody sandwich method from Nanjing Jiancheng Bioengineering (Nanjing, Jiangsu, China). 40 μL serum samples, 10 μL hormone antibodies labeled with biotin, and 50 μL of streptavidin-streptavidin-horseradish peroxidase (HRP) were added to test wells in special 96-well plates, which have been precoated with the respective hormone antibodies. Meanwhile, 50 μL of each standard and streptavidin-HRP were added to standard wells. The 96-well plates were incubated at $37\ ^\circ C$ for 60 min and washed five times. Subsequently, each well was added with 50 μL chromogen solutions A and B and then incubated for 10 min at $37\ ^\circ C$ away from light. 50 μL of the stop solution was added to terminate the reaction in each well, and the optical density was measured under 450 nm wave lengths with a microplate reader. Hormone analysis was repeated in triplicate.

2.3. Determination of MCLR concentrations in testes by HPLC-MS

After lyophilized for 72 h, the testes were extracted with 80% methanol (MeOH) and then centrifuged and filtered. The extracted procedures were repeated twice and the supernatants were combined for further treatment. After evaporation, the dried residues were redissolved in 5% MeOH and applied to a preconditioned hydrophilic lipophilic balanced solid-phase cartridge. The cartridge

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