Ecotoxicology and Environmental Safety 161 (2018) 729–734

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Effect of a toxic *Microcystis aeruginosa* lysate on the mRNA expression of proto-oncogenes and tumor suppressor genes in zebrafish

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ARTICLE INFO

Keywords:
Cyanobacteria
Gene expression
Carcinogenesis
Microcystin

ABSTRACT

Cyanobacterial blooms of *Microcystis aeruginosa* represent a significant risk to the environment and have become a worldwide concern. *M. aeruginosa* can produce the hepatotoxins microcystins (MCs) with potential for tumor promotion. The present study evaluated the time-dependent effects in the transcription of tumor-related genes in the zebrafish, *Danio rerio*, exposed to dilutions of a *M. aeruginosa* lysate containing 3.5 and 54.6 $\mu g\,L^{-1}$ MCs. We used a cultured *M. aeruginosa* strain, RST 9501, which contains mainly the variant [D-Leu^1] MC-LR and originated from the Patos Lagoon Estuary (RS, Brazil). The exposure caused short-term repression of tumor suppressor genes and long-term repression of proto-oncogenes. These responses were more evident for *p53* that was repressed with exposure for 6, 24 and 96 h, and *fosab* and *myca* that were consistently repressed with exposure for 384 h, when fish were exposed to both *M. aeruginosa* lysate dilutions, compared to controls ($p < 0.05$). The suppressor genes, *baxa* and *gadd45α*, and the proto-oncogene, *junba*, were suppressed mainly at 96 h, where both dilutions of the lysate caused repression compared to controls ($p < 0.05$). The *p53* gene was the only gene to be induced; this occurred in fish exposed to lysate containing 3.5 $\mu g\,L^{-1}$ for 384 h. This is the first study to show that *M. aeruginosa* containing an environmentally relevant concentration of [D-Leu^1] MC-LR could cause time-dependent repression of proto-oncogenes and tumor suppressor genes in fish. The results suggest that short-term repression of tumor suppressor genes could participate in the mechanism of tumor promotion caused by *M. aeruginosa* in fish.

1. Introduction

Over the past few decades, the frequency of occurrence and the global distribution of cyanobacteria blooms in water bodies has become a worldwide problem (Yan et al., 2012). This concern is mainly due to the capacity of several species of cyanobacteria to produce a variety of cyanotoxins (Cui et al., 2011). Microcystins (MCs) are commonly found in the aquatic environment and are produced by species of several genera of cyanobacteria, such *Microcystis sp.* (Amado and Monserrat, 2010). MC-LR is a very common hepatotoxin and its toxicity in diverse biological systems has been investigated (Abdel-Rahman et al., 1993; Qiao et al., 2013).

Studies have demonstrated the appearance of hepatic lesions in fish exposed to MC-LR in the laboratory (Fischer and Dietrich, 2000; Mezhoud et al., 2008). One of the most studied toxic mechanisms for MC-LR is inhibition of the phosphatases PP1 and PP2A, which can lead to protein hyperphosphorylation and cytoskeletal alterations (Campos and Vasconcelos, 2010; Mezhoud et al., 2008). On the other hand, MC-LR could also induces oxidative stress in mammal cells by alteration in intracellular reduced glutathione (GSH), reactive oxygen species (ROS) production and lipid peroxidation (Bouaïcha and Maatouk, 2004).

Tumor promoter activity caused by MCs has been attributed to PP2A inhibition, involving regulation of mitogen activated protein kinases (MAPKs) (Gehringer, 2004; Wang et al., 2013). MAPKs, once activated, regulate the expression of proto-oncogenes. For example, the proto-oncogenes *fosab* and *junba* are well-known targets of the MAPK pathway (Delaney et al., 2008; Zegura et al., 2011) and initiate transcription of genes involved in growth, differentiation and cellular proliferation (Gehringer, 2004; Li et al., 2009; Wang et al., 2013; Zegura et al., 2008). The transcriptional induction of proto-oncogenes has been linked to the promotion of tumor activity in different organs in rats, such as kidney, testis, brain and liver (De Felipe and Hunt, 1994; Li et al., 2009; Wang et al., 2013).

Some studies have provided evidence that MC-LR induces the expression of c-*Jun*, c-*Fos* and another proto-oncogene, called *myca*, in primary cultures of hepatocytes from rats and zebrafish (Li et al., 2009;

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https://doi.org/10.1016/j.ecoenv.2018.06.007
Received 8 March 2018; Received in revised form 23 May 2018; Accepted 3 June 2018
Available online 26 June 2018
0147-6513/

Sueoka et al., 1997; Wei et al., 2008). The protein c-Jun is a positive regulator of proliferation and induces other regulators of cell cycle progression (Szremska et al., 2003; Wei et al., 2008) and c-Fos has oncogenic activity with frequent overexpression in tumor cells (Verde et al., 2007). According to Fan et al. (2014), the altered expression of *myc* proto-oncogene contributes to tumor development. This gene is activated in 20% of all human cancers and has been found to be active in tumors of other species (Dang, 2012; Dang et al., 1999). On the other hand, the organisms possess a variety of defense mechanisms against cellular stressors and tumor suppressor genes are one mechanism responsible for preventing severe damage to the cell. These genes often encode proteins that function as negative regulators of cell proliferation (Smart et al., 2008) and are necessary for maintaining cell integrity and cellular content. The well-known tumor suppressor gene, *p53*, is the gene most often mutated in cancer (Storer and Zon, 2010) and is conserved in structure and function, being very similar in mammals and zebrafish.

The *p53* gene is a central factor in cellular stress responses, governing the adaptive and protective responses following several types of damage, such as DNA damage, hypoxia, nucleotide imbalance and oxidative stress (Levine, 1997). Several reports have argued that oxidative stress is a toxicological consequence of the exposure to MCs in different organisms (Amado and Monserrat, 2010; Yan et al., 2012) and plays a critical role in apoptosis (Fu et al., 2005) and genotoxic potential (Bouaïcha et al., 2005). After oxidative stress status caused by MC-LR, *p53* can be activated and induces the expression of genes related to cell cycle arrest, apoptosis and DNA repair (Fu et al., 2005; Zegura et al., 2008). Similarly to what have been observed for animal cells, MC-LR induced mRNA expression of *p53* in fish too, suggesting its involvement in the promotion of cell cycle arrest and apoptosis (Brzuzan et al., 2012, 2009).

Among the various p53 target genes, *gadd45α* is one that operates in cell cycle control and DNA repair processes. The *gadd45α* gene removes a variety of DNA lesions or interrupts the cell cycle, preventing the replication of damaged DNA (Smart et al., 2008; Svircev et al., 2010; Zegura et al., 2008). If DNA damage is too severe, *p53* can induce apoptosis through the regulation of genes that stimulate apoptotic pathways (Smart et al., 2008), such as *baxa* (Wang et al., 2013). Exposure to MC-LR is reported to cause a persistent increase of its transcriptional and protein levels in hepatocytes and testicular cells and it is responsible for cell death with cytochrome c release and expression of caspases (Fu et al., 2005; Wang et al., 2013).

Previous evidences indicated for biphasic effects of the MC-LR in fish liver, characterized by a severe injury at the beginning of the exposure and long term reconstruction of the liver parenchyma with inflammatory responses (Wozny et al., 2016). Thus, it would be interesting to see if biphasic effects were also observed for tumor-related genes in fish exposed to a toxic a *M. aeruginosa*. The alteration of the expression of proto-oncogenes and tumor suppressor genes has been studied with the aim to improve the understanding of toxicity of MC-LR and its carcinogenic potential (Li et al., 2009; Zegura et al., 2011). The present study evaluated the transcriptional response of the proto-oncogenes, *fosab*, *junba* and *myca*, and tumor suppressor genes, *baxa*, *gadd45α* and *p53*, in zebrafish (*Danio rerio*) after exposure to *Microcystis aeruginosa* lysate containing 3.5 and 54.6 $\mu g\,L^{-1}$ [D-Leu^1] MC-LR for 6, 24, 96 and 384 h.

2. Material and methods

2.1. Microcystis aeruginosa lysate

The cyanobacteria lysate used was obtained from a culture of *M. aeruginosa* originally isolated from the Patos Lagoon Estuary, Rio Grande, RS, Brazil. *M. aeruginosa* cells of RST 9501 strain producing cyanotoxin were cultivated at the Laboratory of Cyanobacteria and Phycotoxins, Oceanographic Institute of Federal University of Rio Grande (IO-FURG). Characterization of the MCs produced was previously reported by Matthiensen et al. (2000). The most abundant MC variant in that strain was a [D-Leu^1] MC-LR (90%), which presents a potency of phosphatase inhibition similar to the common [D-Ala^1] MC-LR (MC-LR) (Matthiensen et al., 2000).

M. aeruginosa was cultured in 5 L of BG-11 medium in FANEM 347 growth chambers at 20 ± 2 °C and in a 12:12 h light and dark cycle. The culture was concentrated 100 times by 11,000 × g centrifugation to yield an aqueous pellet of *M. aeruginosa* lysate with a volume of 50 mL. The lysate was prepared by freezing and thawing the concentrated solution three times. The concentrated lysate was stored at −20 °C.

2.2. Laboratory animal care and experimental design

Adult zebrafish (*Danio rerio*) were obtained commercially, transported to the wet lab at the Institute of Biological Sciences (ICB), Federal University of Rio Grande (FURG), and maintained in tanks containing dechlorinated and aerated water at 28 ± 2 °C, pH 7.0 ± 1 and a 12:12 h light and dark photoperiod. Fish were acclimated for two weeks before the experiments and fed with commercial Tetracolor™ Tropical Granules (Tetra) twice daily.

Working solutions (2 L volume) were prepared using 0.025% and 0.0025% dilutions of the *M. aeruginosa* lysate in dechlorinated water and shaking for 30 min in order to obtain nominal concentrations of 5 and 50 $\mu g\,L^{-1}$. Those estimated nominal concentrations were calculated taking into consideration a previous analysis of the MCs concentration that was made in the lysate using a specific immunoassay QuantiPlate™ Kit for Microcystins (EnviroLogix, Portland, ME, USA) according to the manufacturer's instructions, and a microplate spectrophotometer at 405 nm absorbance (Biotek Lx 800). Dechlorinated water alone was used in the working solution for the control group. The 2 L volumes of working solutions were each divided into 200 mL glass beakers, making ten flasks per experimental group. One fish was immediately added to each beaker and maintained with constant aeration at 28 °C and a 12:12 h light and dark photoperiod. This procedure was repeated four times for each treatment and control group to enable exposure durations of 6, 24, 96 and 384 h. Water in each beaker was renewed every 24 h.

Individual water samples (10 mL) were collected from the three experimental groups after the 6-h exposure experiment and stored at −20 °C. The MC-LR concentration in the water was analyzed in duplicate using a specific immunoassay QuantiPlate™ Kit for Microcystins (EnviroLogix, Portland, ME, USA). The MCs concentration measured in the water of the control group was below the detection limit for the method ($< 0.147\,\mu g\,L^{-1}$ microcystin) and the MCs concentration measured in the treatment groups were 3.5 and 54.6 $\mu g\,L^{-1}$ in the groups exposed to 0.0025% and 0.025% of concentrated *M. aeruginosa* lysate, respectively (nominal concentrations of 5 and 50 $\mu g\,L^{-1}$ MCs, respectively). Based on that measurement, we chose to indicate the real concentrations of exposure solutions of 3.5 and 54.6 $\mu g\,L^{-1}$ MCs, instead of the nominal concentrations, throughout the manuscript.

After the end of each lysate exposure time (6, 24, 96 and 384 h), the fish was anaesthetized using 100 $mg\,L^{-1}$ of MS-222 tricaine, immersed in the water for 5 min and euthanized by a section of the spinal cord. The liver was dissected from fish and stored in 500 μL of TRIzol at −80 °C for subsequent procedures. All the procedures used were approved by the Ethic Committee of Animal Use of FURG (CEUA N° P029/2015, 23116.002456/2015–05 protocol).

2.3. Gene expression evaluation by RT-qPCR

The liver samples from *D. rerio* were homogenized in TRIzol reagent (Invitrogen) and total RNA extraction was conducted according to the manufacturer's instructions. RNA quality and quantity were evaluated using a spectrophotometer (Nanodrop ND1000; NanoDrop