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Intraperitoneal exposure of whitefish to microcystin-LR induces rapid liver injury followed by regeneration and resilience to subsequent exposures

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ABSTRACT

To date, there has been no systematic approach comprehensively describing the sequence of pathological changes in fish during prolonged exposure to microcystin-LR (MC-LR). Towards this aim, juvenile whitefish individuals received an intraperitoneal injection with pure MC-LR, and the injection was repeated every week to maintain continuous exposure for 28 days. During the exposure period, growth and condition of the fish were assessed based on biometric measurements. Additionally, selected biochemical markers were analysed in the fishes' blood, and their livers were carefully examined for morphological, ultrastructural, and molecular changes. The higher dose of MC-LR (100 $\mu g \cdot kg^{-1}$) caused severe liver injury at the beginning of the exposure period, whereas the lower dose (10 $\mu g \cdot kg^{-1}$) caused less, probably reversible injury, and its effects began to be observed later in the exposure period. These marked changes were accompanied by substantial MC-LR uptake by the liver. However, starting on the 7th day of exposure, cell debris began to be removed by phagocytes, then by 14th day, proliferation of liver cells had markedly increased, which led to reconstruction of the liver parenchyma at the end of the treatment. Surprisingly, despite weekly-repeated intraperitoneal injections, MC-LR did not accumulate over time of exposure which suggests its limited uptake in the later phase of exposure. In support, mRNA expression of the membrane transport protein oatp1d was decreased at the same time as the regenerative processes were observed. Our study shows that closing of active membrane transport may serve as one defence mechanism against further MC-LR intoxication.

1. Introduction

Cyanobacterial blooms, which are characterized by excessive proliferation and dominance of harmful species of cyanobacteria, are an expanding environmental problem in aquatic ecosystems (Pitois et al., 2000). Harmful species of blooming cyanobacteria (e.g. *Microcystis*, *Anabaena*, *Planktothrix*, *Chroococcus*) can produce cyanotoxins, with microcystins (MCs) being one of the most commonly occurring toxins; MCs have received worldwide concern in recent decades (van Apeldoorn et al., 2007; Hudnell, 2010). Microcystin-LR (MC-LR) is the most toxic MC variant; it poses health threat to aquatic biota, wild life, and humans, due to swimming in the contaminated water, ingesting the toxic algae, or consuming food (clams) harvested from the polluted sites (Malbrouck and Kestemont, 2006; van Apeldoorn et al., 2007; Miller et al., 2010). Some reports also suggest carcinogenic risk to people through drinking water from the reservoirs polluted with MCs (Nishiwaki-Matsushima et al., 1992; Ueno et al., 1996; Zhou et al., 2002; van Apeldoorn et al., 2007). Considering the increasing environmental problem with cyanobacterial blooms, there is an increasing need for better understanding of how MCs may influence public health and wild life condition.

Fish are particularly vulnerable to MCs exposures. Despite differences in sensitivity to lethal doses of MC-LR, its effects in fish appear to be similar in many respects to those observed in mammals, as they mostly concern the liver as the primary target organ. Studies on acute exposures of fish to MC-LR typically report increased liver weight and pronounced histopathological changes, including disruption of liver parenchyma structure, hepatocellular swelling, loss of cell membrane integrity, necrosis, and inflammatory response (Malbrouck and Kestemont, 2006). On the other hand, more recently reported subcellular effects of MC-LR hepatotoxicity are more consistent with apoptosis, as they include cell shrinkage, membrane blebbing, swelling of the endoplasmic reticulum and mitochondria, chromatin condensation, or

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formation of pyknotic nuclei (Svirčev et al., 2015). Although the hepatotoxic properties of MC-LR have been described in a number of fish species, knowledge concerning mechanisms underlying these histopathological and ultrastructural changes is fragmentary. Most of the studies have been focused on single, short-term period of exposure to acute or sub-acute doses of MC-LR, while only few experiments have explored the chronic effects of MC-LR on fish. Moreover, little attention has been given to regenerative processes that occur after MC-LR-induced liver injury (Fournie and Courtney, 2002; Nince Ferreira et al., 2010; Van Wettere et al., 2013). To date, there has been no systematic approach comprehensively demonstrating the sequence of histopathological changes in the liver during long-term, continuous exposure to MC-LR.

MC-LR is mostly known from its hepatotoxic action for it is rapidly absorbed and accumulated predominantly in the liver (Svirčev et al., 2010, 2015). Due to its spatially large, hydrophilic structure, MC-LR is incapable to passively penetrate through the cell membrane, and thus require active transport via multispecific organic anion transporting polypeptides (OATPs) that are mostly expressed in hepatocytes (Fischer et al., 2005; Komatsu et al., 2007; Steiner et al., 2014). Once preferentially transported into the cell, MC-LR primarily acts as inhibitor of protein serine/threonine phosphatases (PP1 and PP2A), which leads to hyperphosphorylation of numerous proteins that are structurally and functionally involved in different cellular compartments and signalling pathways (Svirčev et al., 2010, 2015; Zhou et al., 2015). Classic mechanism of MC-LR hepatotoxicity considers hyperphosphorylation of cytoskeletal proteins which results in alterations and reorganization of the cytoskeleton, loss of the cell shape and the intercellular junctions, and ultimately disorganization of the liver structure (van Apeldoorn et al., 2007; Svirčev et al., 2015; Zhou et al., 2015). Moreover, oxidative stress has also been shown to play an important role in MC-LR-induced endoplasmic reticulum stress and mitochondrial impairment, mainly through production of reactive oxygen species (ROS) and lipid peroxidation (Zhao et al., 2011; Shi et al., 2015; Faltermann et al., 2016).

In contrast to these destructive processes, MC-LR has been shown to trigger cell specific programs, such as cell cycle arrest or apoptosis both in vivo and in vitro (Komatsu et al., 2007; Li et al., 2009; Takumi et al., 2010). For example, under specific conditions, chronic exposure to low doses of MC-LR may lead to activation of signalling pathway that promotes survival and growth, which result in excessive cell proliferation (Takumi et al., 2010). Although MC-LR is a matter of unceasing research interest, the exact mechanisms that regulate the balance between the destructive, protective, or repair processes during the MC-LR intoxication are not understood. Recent concerns are focused mainly on identification of particular hallmarks of these aberrant processes leading to MC-LR-induced liver injury and disease (Paskerová et al., 2012; Adamovsky et al., 2015; Brzuzan et al., 2016).

In 2013, we began a research project to explore the molecular mechanisms underlying MC-LR hepatotoxicity in fish (Brzuzan et al., 2015; Florczyk et al., 2016; Brzuzan et al., 2016). In the present paper, associated with this project, the aim was to describe in detail the course of pathological changes in the liver of juvenile whitefish during long-term, continuous exposure to pure MC-LR. To address this issue, juvenile whitefish individuals (mass ~ 100 g) were repeatedly injected with MC-LR at a subacute (100 $\mu g \cdot kg^{-1}$ body mass) or chronic dose (10 $\mu g \cdot kg^{-1}$ body mass). Fish injected with physiological saline or only pricked with needle served as control groups. After 1/3 day, and then 1, 2, 7, 14 and 28 days of the exposure, the growth and condition of both the control and MC-LR-challenged fish was assessed based on biometric measures (total length, total mass, CF, and HSI). Additionally, to investigate the health-related effects of MC-LR exposure, selected biochemical markers were analysed in the fishes' blood and their livers were carefully examined for morphological and ultrastructural changes using light and electron microscopy, respectively. To better characterize the MC-LR-induced pathological changes in the liver, we performed quantitative analysis of the liver cells morphology. Furthermore, immunodetection was used to track the MC-LR content in the liver and to explore its possible co-occurrence with the hepatotoxic effects. Finally, to gain insight into the molecular background of MC-LR action, we profiled mRNA expression of genes involved in membrane transport (*oatp1d*), cell proliferation (*pcna*), and apoptosis (*cas3b*). To support these findings, the livers were additionally investigated for hallmarks of apoptosis (DNA laddering) and cell proliferation (immunolocalization of pcna protein).

This study provides a comprehensive description of biochemical, morphological, ultrastructural, and molecular changes in fish during prolonged exposure to MC-LR. It identifies and interprets plausible mechanisms underlying MC-LR hepatotoxicity, bringing us closer to better understanding of biological processes driving liver damage and regeneration.

2. Material and methods

2.1. Fish maintenance, exposure, and collection of samples

The procedures related to the fish maintenance and exposure were conducted in late autumn 2013 at the Department of Salmonid Research in Rutki (Inland Fisheries Institute in Olsztyn; Poland). All fish were housed and handled in compliance with widely accepted guidelines of laboratory animal care. The experiment was approved by the Local Ethical Commission (resolution No. 100/2011 of 23rd November 2011). The fish were acclimated for two weeks in 800 L flow tanks supplied with well (underground) water at the flow of 600 $L \cdot h^{-1}$. Throughout the experiment, all the fish were fed with reduced feeding procedure dependent on the water temperature, caloric content of the feed, and predicted fish mass (according to From and Rasmussen, 1984). Water temperature in the tanks ranged between 8 and 9 °C and oxygen level was above 90% of saturation. The fish were deprived of food 2 days prior to exposure (intraperitoneal injection) or collection of samples.

The doses of MC-LR (10 and 100 $\mu g \cdot kg^{-1}$ of body mass) and set of the treatment periods (1/3, 1, 2, 7, 14, and 28 days) were based on the previous studies on effects of pure microcystins or biomass of blue algae on fish metabolism (Malbrouck and Kestemont, 2006; Ernst et al., 2007), and our experience in studying molecular and physiological responses of whitefish to this toxin (Brzuzan et al., 2009, 2010, 2012). MC-LR (purity ≥95%; HPLC) was obtained from Enzo Life Sciences (Enzo Biochem, Inc.; USA) and dissolved in saline solution (0.8% NaCl) as a solvent vehicle. The prepared solutions contained 1 μg (the lower dose) or 10 μg (the higher dose) of MC-LR per 200 μL of volume set for each intraperitoneal injection.

In the beginning of the experiment (0 days), the juvenile whitefish individuals (of both sexes) were sorted by their mass (~100 g; age 1+) and divided into 4 experimental groups that were then kept in separate tanks: i) control (n = 24), ii) sham-control (n = 24), iii) treated with a lower dose of MC-LR (10 $\mu g \cdot kg^{-1}$ body mass; n = 48), and iv) treated with a higher dose of MC-LR (100 $\mu g \cdot kg^{-1}$ body mass; n = 48). Prior to exposure, the fish were anesthetized by immersion in etomidate solution, and received an intraperitoneal injection of the respective MC-LR solution (10 or 100 $\mu g \cdot kg^{-1}$ body mass, depending on the treatment group) or pure saline solution (control). Fish from the sham-control group were only pricked with a needle but they did not receive any injection. In order to maintain continuous exposure, the injections with MC-LR were repeated every 7 days of the experiment (i.e. on the 7th, 14th, and 21st day).

After each exposure period (1/3, 1, 2, 7, 14, and 28 days), randomly selected individuals (n = 4 from control or sham-control group, and n = 8 from each MC-LR-treated group) were anesthetized, weighed (accuracy ±10 mg) and measured (±0.5 cm). Blood samples were taken from the caudal vein of the anesthetized fish and were immediately processed for plasma isolation (for details, see *Biochemical measurements in blood plasma*). Then, the fish were euthanized, and their body cavity was opened for visual inspection. The liver with gall