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Alterations in the intestine of Patagonian silverside (*Odontesthes hatcheri)* exposed to microcystin-LR: Changes in the glycosylation pattern of the intestinal wall and inhibition of multidrug resistance proteins efflux activity

Flavia Bieczynski[a,*], Walter D.C. Torres[b], Julio C. Painefilu[a], Juan M. Castro[a], Virginia A. Bianchi[a], Jimena L. Frontera[d], Dante A. Paz[d], Carolina González[e], Alejandro Martín[e], Silvina S.M. Villanueva[c], Carlos M. Luquet[a]

[a] *Laboratorio de Ecotoxicología Acuática, INIBIOMA – (CONICET-UNCo), CEAN- Ruta 61 km 3, Paraje San Cabao, 8371, Junín de los Andes, Neuquén, Argentina*
[b] *CEAN- Ruta 61 km 3, Paraje San Cabao, 8371, Junín de los Andes, Neuquén, Argentina*
[c] *Instituto de Fisiología Experimental, IFISE-CONICET, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 570, 2000, Rosario, Santa Fe, Argentina*
[d] *Laboratorio de Biología del Desarrollo, IFIBYNE-CONICET, Universidad Nacional de Buenos Aires, Pabellón II, Ciudad Universitaria, 1428, Ciudad Autónoma de Buenos Aires, Argentina*
[e] *Dirección Técnica y de Desarrollo Tecnológico, Agua y Saneamientos Argentinos, Av. Figueroa Alcorta 6081, 1425, Ciudad de Buenos Aires, Argentina*

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ABSTRACT

Accumulation and toxicity of cyanobacterial toxins, particularly microcystin-LR (MCLR) have been extensively studied in fish and aquatic invertebrates. However, MCLR excretion mechanisms, which could reduce this toxin's effects, have received little attention. The Patagonian silverside, *Odontesthes hatcheri*, is an omnivorous-planktivorous edible fish, which has been shown to digest cyanobacterial cells absorbing MCLR and eliminating the toxin within 48 h without suffering significant toxic effects. We studied the effects of MCLR on glycoconjugate composition and the possible role of multidrug resistance associated proteins (Abcc) in MCLR export from the cells in *O. hatcheri* intestine. We treated *O. hatcheri* with 5 μg MCLR g^{-1} body mass administered with the food. Twenty four hours later, the intestines of treated and control fish were processed for lectin-histochemistry using concanavalin A (ConA), *Triticum vulgaris* agglutinin (WGA), and *Dolichos biflorus* agglutinin (DBA). MCLR affected the distribution of glycoconjugates by augmenting the proportion of ConA-positive at the expense of WGA-positive cells. We studied MCLR effects on the transport of the Abcc-like substrates 2,4-dinitrophenyl-*S*-glutathione (DNP-SG) and calcein in ex vivo intestine preparations (everted and no-everted sacs and strips). In treated preparations, CDNB together with MCLR (113 μg MCLR g^{-1} intestine, equivalent to 1.14 μmol L^{-1} when applied in the bath) or the Abcc inhibitor, MK571 was applied for one hour, during which DNP-SG was measured in the bath every 10 min in order to calculate mass-specific DNP-SG transport rate. MCLR significantly inhibited DNP-SG transport ($p < 0.05$), especially in middle intestine (47 and 24%, for luminal and serosal transport, respectively). In middle intestine strips, MCLR and MK571inhibited DNP-SG transport in a concentration dependent fashion (IC_{50} 3.3 and 0.6 μmol L^{-1}, respectively). In middle intestine strips incubated with calcein-AM (0.25 μmol L^{-1}), calcein efflux was inhibited by MCLR (2.3 μmol L^{-1}) and MK571 (3 μmol L^{-1}) by 38 and 27%, respectively ($p < 0.05$). Finally, middle intestine segments were incubated with different concentrations of MCLR applied alone or together with 3 μM MK571. After one hour, protein phosphatase 1 (PP1) activity, the main target of MCLR, was measured. 2.5 μM MCLR did not produce any significant effect, while the same amount plus MK571 inhibited PP1 activity ($p < 0.05$). This effect was similar to that

Abbreviations: ABC, ATP-binding cassette; Abcb1, p-glycoprotein; Abcc, multidrug resistance-associated proteins; Bcrp, breast cancer resistance protein; BSA, bovine seroalbumine; calcein-AM, calcein acetoxymethyl ester; CDNB, 1-chloro-2,4-dinitrobenzene; ConA-FITC, fluorescein labeled *Canavalia ensiformis* agglutinin; DBA, biotinylated-*Dolichos biflorus* agglutinin; DNP-SG, 2,4-dinitrophenyl-S-glutathione; DTT, dithiothreitol; GSH, reduced glutathione; GST, glutathione-S transferase; GS-X pumps, ATP-dependent glutathione S-conjugate exporters; MC, microcystin; MCLR, microcystin-LR; MXR, multixenobiotic resistance; Oatps, organic anion transporting polypeptides; *p*NPP, P-nitrophenyl phosphate disodium salt; PP, protein phosphatases; SPE, solid phase extraction; WGA, biotinylated-*Triticum vulgaris* agglutinin.

* Corresponding author at: LIBIQUIMA (CONICET), Departamento de Química, Facultad de Ingeniería, Universidad Nacional del Comahue, Buenos Aires 1400, (8300) Neuquén, Neuquén, Argentina.
E-mail addresses: 675.bieczynskif@comahue-conicet.gob.ar, flabieczynski@gmail.com (F. Bieczynski).

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of 5 μM MCLR. Our results suggest that in *O. hatcheri* enterocytes MCLR is conjugated with GSH via GST and then exported to the intestinal lumen through Abcc-like transporters. This mechanism would protect the cell from MCLR toxicity, limiting toxin transport into the blood, which is probably mediated by basolateral Abccs. From an ecotoxicological point of view, elimination of MCLR through this mechanism would reduce the amount of toxin available for trophic transference.

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1. Introduction

The gastrointestinal system mediates the absorption, distribution, metabolization and excretion of a wide array of endogenous and exogenous compounds, including toxicants (Barltrop and Brueton, 1990; Klaassen and Aleksunes, 2010; Luckenbach et al., 2014). The luminal side of the intestinal wall is covered by a mucous layer secreted by goblet cells, which is mainly composed of glycoproteins and by the glycocalyx, formed by glycosylated membrane proteins (mucins) attached to the cell (Linden et al., 2008). These glycoproteins vary in composition, depending on the region of the intestine, dietary habits, age, sex, and species (Díaz et al., 2003). Among other functions, mucins protect the epithelium against proteolysis, microorganisms and constitute the first barrier for the uptake of xenobiotics (Díaz et al., 2003, 2008; Loretz, 1995). The second barrier is composed by membrane proteins, which are involved in the uptake and elimination of physiological substrates as well as many drugs and toxicants. Among these proteins, there is a group of transporters which belongs to the ABC superfamily (ATP-binding cassette) that acts in tandem with biotransformation enzymes, such as glutathione S-transferases (GST) and cytochrome P450 enzymes (CYP) (Bard, 2000; Luckenbach et al., 2014). The P-glycoprotein 1 (Pgp; ABCB1), the multidrug resistance-associated proteins 1, 2 and 3 (MRP1-3; ABCC1-3) and the breast cancer resistance protein (BCRP; ABCG2) have been described as toxicologically relevant transporters (Chan et al., 2004; Klaassen and Aleksunes, 2010; Leslie et al., 2005; Luckenbach et al., 2014; Takano et al., 2006). According to the zebrafish nomenclature, in the present work capital letters denote ABC superfamily in general or specific mammalian ABC proteins while lowercase letters denote non-mammalian proteins. ABC proteins have been first described as conferring multidrug resistance (MDR) in mammalian tumor cells (Gottesman and Pastan, 1993). Nevertheless, these proteins are present in normal tissues of a wide range of species, including fish and aquatic invertebrates. The term "multixenobiotic resistance" (abbreviated here as MXR, although in the literature this abbreviation has also been used with other meanings) is applied to the role of ABC proteins in the protection of aquatic animals against environmental toxicants (Kurelec, 1992). Abcb1 is the most studied transporter in relation to xenobiotic defense, although recent studies show that Abcc proteins also play an important role in MXR (Ferreira et al., 2014; Luckenbach et al., 2014; for a review).

In mammals, ABCB1 is generally localized in apical membranes of polarized cells and transports a broad range of moderately hydrophobic compounds, preferentially small sized and cationic ones, including non-metabolized toxic compounds and phase I biotransformation products (Klaassen and Aleksunes, 2010; Takano et al., 2006). In the intestine, as well as in kidney and liver, ABCC2 is localized to the apical membrane while other ABCCs, such as ABCC1 and 3, have basolateral location. ABCC1-3 export conjugated and unconjugated anionic compounds (phase II products), such as 2,4-dinitrophenyl-*S*-glutathione (DNP-SG) and bilirubin glucuronides (Fardel et al., 2005; Klaassen and Aleksunes, 2010). ABCG2 is mainly located at the apical membrane of enterocytes and transports some compounds which are also ABCB1 or ABCC1 substrates (Chan et al., 2004).

Toxic metabolites produced by cyanobacteria (cyanotoxins) represent a threat to life when produced in high concentrations during cyanobacterial blooms. Microcystins (MC) are hepatotoxins usually associated with environmental risk cases and with animal and human intoxication reports (Dietrich and Hoeger, 2005; Dörr et al., 2010; Pavagadhi and Balasubramanian, 2013; Sivonen and Jones, 1999; Wiegand and Pflugmacher, 2005). The characteristic toxic effects caused by microcystin-LR (MCLR), one of the most frequent and toxic MC variants (Codd et al., 2005), are mediated by inhibition of protein phosphatases (PP) 1 and 2A which could affect many cellular functions regulated by protein phosphorylation/dephosphorylation, such as cytoskeletal function and maintenance of hepatocyte ultrastructure (Eriksson et al., 1990; Honkanen et al., 1990; MacKintosh et al., 1995; Runnegar et al., 1995; Williams et al., 1997; among others). Additionally, oxidative stress has also been reported as mediating MCLR toxicity (Amado and Monserrat, 2010). Moreover, the combination of these effects could lead to cytotoxicity and to the activation of tumor promoting cascades (Campos and Vasconcelos, 2010; Carmichael, 1992; Falconer and Yeung, 1992; Hooser et al., 1989; Kuiper-Goodman et al., 1999).

Deleterious effects of MCLR have been studied in many fish species (Atencio et al., 2008; Ferrão-Filho and Kozlowsky-Suzuki, 2011; Fischer and Dietrich, 2000; Malbrouck and Kestemont, 2006; Pavagadhi and Balasubramanian, 2013; Sahin et al., 1996; Soares et al., 2004; Williams et al., 1997; Xie et al., 2004). Surprisingly, in spite of being the main site of MCLR uptake in fish, the intestine has received little attention with respect to MCLR effects and detoxification (Bieczynski et al., 2013, 2014; Bury et al., 1998; Tencalla and Dietrich, 1997; Xie et al., 2004).

In general, fish are less sensitive to MC toxicity than mammals but their sensitivity varies over a wide range, depending on, e.g., the species and the exposure route (reviewed by Malbrouck and Kestemont, 2006). For example, 1.7 μg MCLR g^{-1} body mass (bm), applied by oral gavage, caused hepatic damage and mortality in the common carp, *Cyprinus carpio* but did not cause any toxic effect on the rainbow trout *Oncorhynchus mykiss,* while a higher dose, 6.6 μg MCLR g^{-1} bm, caused severe toxic effects and mortality in both species. On the other hand, intraperitoneal injection of 0.55 μg MCLR g^{-1} bm was lethal for both species (Fischer and Dietrich, 2000). Xie et al. (2004) have reported that the phytoplanktivorous silver carp, *Hypophthalmichthys molitrix*, which actively feeds on toxic cyanobacteria, has reduced intestinal absorption of MCLR. Altogether, the results cited above suggest that resistance to MCs is, at least in part, determined by digestion – absorption – detoxification processes in the digestive tract. Several laboratory and field studies have shown that different fish species are able to reduce MC absorption and/or metabolize and eliminate the toxin accumulated in the tissues (Bieczynski et al., 2013, 2014; Sahin et al., 1996; Soares et al., 2004; Williams et al., 1997; Xie et al., 2004). The comprehension of MCs detoxification mechanisms in different species could help to explain interspecific differences in susceptibility to these toxins. Nevertheless, the mechanisms involved in MC transport

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