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Localization of microcystin-LR in medaka fish tissues after cyanotoxin gavage

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ABSTRACT

Microcystins (MCs) are toxic monocyclic heptapeptides produced by many cyanobacteria. Over 70 MCs have been successfully isolated and identified, of which MC-LR is the most commonly occurring toxin. Microcystins, especially MC-LR, cause toxic effects in mammals, birds and fish and are a recognized potent cause of environmental stress and pose a potential health hazard in aquatic ecosystems when heavy blooms of cyanobacteria appear. They also constitute a public health threat to people via drinking water and food chains. The concentrations of MC-LR can be very low, even in fish displaying severely disrupted tissues, which makes it essential to devise selective and sensitive histochemical methods for identifying and localizing MC-LR in target organs, such as liver and intestine. The aim of the study reported here was to analyze the presence of MC-LR in contaminated fish tissues using immunohistochemical methods. The present experiment involving subacute exposure confirmed our initial hypothesis that subacute and acute exposure to microcystin contamination can exacerbate physiological stress, induce sustained pathological damage, and affect the immune response in exposed medaka fish.

1. Introduction

Microcystins (MCs) are toxic monocyclic heptapeptides produced by many members of the cyanobacteria including species belonging to the genera *Microcystis*, *Anabaena* and *Planktothrix*. The MCs share a common moiety composed of seven amino acids, and are named according to their variable L-amino acids, so, for example, microcystin-LR (MW: 995) contains leucine (L) and arginine (R). Over 70 MCs have been successfully isolated and identified, of which MC-LR is the most commonly occurring toxin (Fastner et al., 2002). To date, most of the work on microcystins has been conducted with this variant because it is both the most abundant worldwide and the most toxic, with a median lethal dose (LD 50) of 50 μg kg^{-1} bodyweight (mice, IP; Gupta et al., 2003). In several studies, MC-LR has been shown to be a potent inhibitor of protein phosphatases 1 and 2A (Honkanen et al., 1990; Runnegar et al., 1993) and to demonstrate tumor-promoting activity in the rat liver (Ohta et al., 1992). Microcystins, and especially MC-LR, cause adverse effects in mammals, birds and fish, and are recognized as being potent environmental stress inducers and a potential health hazard in aquatic ecosystems when heavy blooms of cyanobacteria producing this toxin appear. They also represent a threat to human public health via drinking water and food chains (Stone and Bress, 2007).

The concentrations of MC-LR found in even severely disrupted fish tissues can be very low, and so it is necessary to devise a selective and sensitive histochemical method for identifying and localizing MC-LR in target organs, such as the liver and intestine. As a complement to the current analytical methods for detecting MC-LR in more or less

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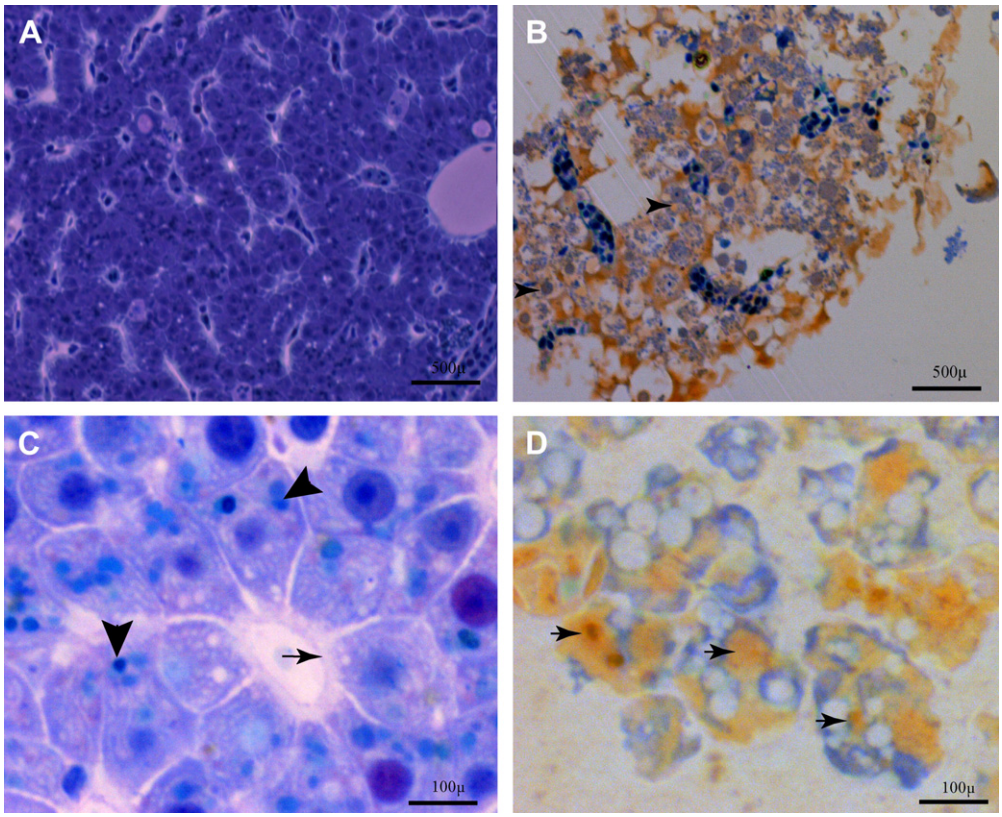

Fig. 1. Semi-thin section of adult medaka liver, Alcyan Blue staining and anti-microcystin labeling (MC10E7) with hydroxyperoxidase. (A) Control (oral gavage with water only). (B) Treated (2 h after oral gavage with microcystin-LR). Hepatocytes are detached and disorganized (arrowhead); the brown color corresponds to positive hydroxyperoxidase anti-microcystin labeled areas. (C) Hepatocytes of a control fish at high magnification. Polysaccharide granules appear dense blue (arrowheads); note the Diss space (small arrow). (D) Hepatocytes of treated medaka. Polysaccharide storage has almost disappeared, and the cells have been more or less completely destroyed, with positive hydroxyperoxidase anti-microcystin labeled areas (arrows).

purified extracts, such as protein phosphatase inhibition assay (PP2A) and liquid chromatography–tandem mass spectrometry (LC/MS/MS), this immunohistochemical examination reveals the precise subcellular localization of MC-LR in contaminated medaka fish.

2. Materials and methods

2.1. Biological material

Adult fishes of the medaka inbred Cab strain were used in all experiments. The fish were raised in 20-L glass aquaria filled with a continuously aerated mixture of tap water and reverse osmosis filtered water (1/3–2/3, respectively) which were changed once a week. The fish were maintained at 25 °C, with a 12 h:12 h light–dark cycle (standard regime).

2.2. Microcystin-LR and fish intoxication

Microcystin-LR purchased from Alexis Corporation (Switzerland) was dissolved in water at 1 mg/mL. After tricaine anesthesia (100 mg/L tricaine, Sigma), 5 μg MC-LR dissolved in 5 μL water per gram bodyweight was administered directly into the fish's stomachs using blunt-tip gavage syringes (Hamilton). Preliminary observations showed that rapid metabolic changes could be detected by a proteomic approach, and that detectable histological modifications appeared subsequently. Four batches of six treated and six control fish were gavaged, and histological studies were carried out. The fish were killed in ice-cold water before liver, spleen and intestine samples were collected.

2.3. Immunohistochemical analysis

For the immunolocalization analysis, all tissues from adult medaka fish were dissected and fixed in a mixture of glutaraldehyde (0.1%) and formaldehyde (2%) in Sörensen buffer 0.1 M, pH 7.4. Two anti-microcystin antibodies were used for immunolocalization: a general monoclonal antibody to microcystins (Adda specific, AD4G2), which recognizes all microcystins, and a monoclonal antibody to microcystin-LR (MC10E7), which recognizes all 4-Arg microcystins. We used a 1:200 dilution for both antibodies. The hybridized antibodies were visualized after the immunoperoxidase reaction using a DakoCytomation EnVision Plus System, Peroxidase-HRP kit. Controls included a serial section of the same slide (stained positive for MC-LR) and not treated with the primary antibody but only with the secondary antibody and the HRP reaction system. They indicated that there is no peroxidase reaction due to the endogenous peroxidase (see Supplementary figure).