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Toxic effects of pure anatoxin-a on biomarkers of rainbow trout, *Oncorhynchus mykiss*

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ABSTRACT

Anatoxin-a is a neurotoxin produced by various bloom-forming cyanobacteria. Although it shows widespread occurrence and is highly toxic to rodents, its mechanisms of action and biotransformation, and effects in fish species are still poorly understood. The main aim of this study was, thus, to investigate sub-lethal effects of anatoxin-a on selected biochemical markers in rainbow trout fry in order to get information about the mechanisms of toxicity and biotransformation of this toxin in fish. Trout fry were administered sub-lethal doses of anatoxin-a (0.08–0.31 μg g^{-1}) intraperitoneally. Livers and muscle tissue were collected 72 h later for quantification of key enzyme activities as biochemical markers. Enzymes assessed in muscle tissues were related to cholinergic transmission (acetylcholinesterase [AChE]), energy metabolism (lactate dehydrogenase [LDH] and $NADP^+$-dependent isocitrate dehydrogenase [IDH]). Enzymes assessed in the liver were involved in biotransformation (ethoxyresorufin-*O*-deethylase [EROD] and glutathione *S*-transferases [GST]). The results indicated a significant increasing trend for AChE activity with the dose of anatoxin-a, possibly representing an attempt to cope with overstimulation of muscle activity by the toxin, which competes with acetylcholine for nicotinic receptors binding. Anatoxin-a was also found to significantly induce the activities of liver EROD and GST, indicating the involvement of phase I and II biotransformation in its detoxification. Likewise, lactate dehydrogenase activity recorded in fry muscle increased significantly with the dose of anatoxin-a, suggesting an induction of the anaerobic pathway of energy production to deal with toxic stress induced by the exposure. Altogether, the results suggest that under continued exposure in the wild fish may experience motor difficulties, possibly becoming vulnerable to predators, and be at increased metabolic demand to cope with energetic requirements imposed by anatoxin-a biotransformation mechanisms.

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1. Introduction

Cyanobacteria are ubiquitous prokaryotes that can proliferate at high densities (cyanobacterial blooms) in aquatic environments and particularly in freshwater systems. The main concern about these microorganisms is their ability to produce secondary metabolites that are

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toxic – cyanotoxins – and may cause death in humans and in aquatic and terrestrial organisms (Azevedo et al., 2002). Cyanotoxins have been classified according to their main target in mammals as neurotoxins, hepatotoxins, dermotoxins and cytotoxins. Anatoxin-a is a neurotoxin produced by various bloom-forming cyanobacteria, including *Anabaena*, *Oscillatoria* and *Aphanizomenon* (Osswald et al., 2007 and references herein). Despite their widespread occurrence and high toxicity (10 min LD50 of 250 μg kg^{-1}, following intraperitoneal injection) (Rogers et al., 2005), little attention from researchers has been paid to anatoxin-a, probably because it has seldom been implicated in toxicosis and appears to be less frequent than hepatotoxins (Codd et al., 2005). Nevertheless, some studies have described high incidences of anatoxin-a in different parts of the globe, some of them causing animal deaths (Puschner et al., 2008). Considering the current climate change scenario, with the expected increases in water temperature and higher risk of formation of toxic blooms, more knowledge on the effects of anatoxin-a particularly in aquatic organisms is urgently needed. In this context our team has been studying the effects of anatoxin-a in aquatic animals, including fish that are a crucial link in aquatic food webs (Osswald et al., 2007, 2008, 2009, 2011).

Anatoxin-a is an alkaloid and a potent nicotinic agonist (Thomas et al., 1993) that acts by blocking the cholinergic neurotransmission. In the last decades its mode of action has been described and characterized, mainly in rodents (Campos et al., 2010; Fawell et al., 1999; Spivak et al., 1980). Anatoxin-a mimes the neurotransmitter acetylcholine (ACh) present in cholinergic synapses of the peripheral and central nervous systems of vertebrates. In the absence of anatoxin-a, when a nervous impulse reaches the synapses ACh is released into the synaptic cleft and binds to the receptors on the effector organs. After transmission of the electrical impulse, ACh is hydrolyzed by acetylcholinesterase (AChE) allowing for signal termination and consequent re-establishment of the postsynaptic resting potential. Since anatoxin-a is not degraded by esterases and it competes with ACh for the same cholinergic receptors, when anatoxin-a is present the effector organ remains in a state of activation. In case of exposure to lethal doses the animal ends up dying by respiratory arrest (Carmichael et al., 1979). Because fish are vertebrates possessing nicotinic receptors and ACh, they are expected to show a reaction to anatoxin-a similar to that of rodents. However, despite its toxicity and interference with cholinergic transmission, the mode of action, biotransformation and effects of anatoxin-a and/or its metabolites are still poorly described, both in rodents and fish species.

The aim of this work was, therefore, to investigate whether sub-lethal doses of anatoxin-a could induce alterations in relevant biochemical markers in rainbow trout (*Oncorhynchus mykiss*), an animal model widely used in aquatic toxicology. Changes in the activity of some key enzymes were used as biomarkers. The selected enzymes were AChE, lactate dehydrogenase (LDH) and $NADP^{+}$- dependent isocitrate dehydrogenase (IDH) in muscle tissue, and ethoxyresorufin-*O*-deethylase (EROD) and glutathione *S*-transferases (GST) in the liver. AChE is the cholinesterase present in rainbow trout brain and white muscle (Sturm et al., 2007), being involved in the cholinergic neurotransmission as described above. LDH and IDH are involved in cellular respiration, respectively in the glycolysis and the citric acid cycle, and their activities may be used as indicators of the metabolic state of organisms under chemical or natural stress (Castro et al., 2004; Polakof et al., 2006; Almeida et al., 2010). EROD activity is a widely used indicator of xenobiotics uptake in fish, providing evidence of receptor-mediated induction of cytochrome P450-dependant monooxygenases, namely the CYP1A subfamily responsible for the metabolization of various xenobiotics in liver phase I biotransformation (Van der Oost et al., 2003; Smith and Wilson, 2010). GST play an important role in liver phase II biotransformation, catalyzing the conjugation of glutathione with a wide variety of xenobiotics, rendering them more soluble and facilitating their excretion (Van der Oost et al., 2003). It is expected that this set of enzymes could provide novel information on the toxic effects and biotransformation pathways of anatoxin-a in fish.

2. Materials and methods

2.1. Holding of fish

One month old *O. mykiss* of the same parenthood were kindly provided by an aquaculture facility (Castro & Gabero at River Coura) in the north of Portugal. They were maintained under controlled conditions in the bioterium (BOGA-CIIMAR) for two months, in a tank with recirculation of aerated and dechlorinated tap water at 14 °C ± 1 °C. During this period fish were fed ad libitum with standard commercial pellets (A. Coelho & Castro, Portugal). No deaths or disease symptoms were recorded during this period of acclimatization. Therefore, fish were concluded to be healthy and proper for experimentation. Two weeks before starting the experiments, 3 months old fry (average total length and fresh weight of 4.8 cm and 1 g, respectively) were randomly separated in groups according to the treatments described below (sections 2.3.1–2.3.3). Each group was placed in their respective floating cage, inserted in the same tank under the same conditions as in the acclimatization period. Feeding was halted 24 h before the experiments.

2.2. Injection solutions

Phosphate buffer saline (PBS) (pH 7.4) was prepared using p.a. chemicals from Sigma–Aldrich®. After adding the chemicals and confirming the pH, this solution was sterilized by autoclave and kept at 4 °C for 24 h before use in the experiments.

A stock solution of anatoxin-a fumarate (Tocris Bioscience, U.K.) was prepared by dilution in PBS to a concentration of 0.5 μg μL^{-1} of pure anatoxin-a. Final anatoxin-a solutions were prepared within 12 h prior to injection, by diluting the sterile stock solution in PBS to the desired test concentration.

2.3. Bioassays

2.3.1. Range-finding bioassay

Based on the results of previous studies (Osswald et al., 2011), four doses (0.005, 0.05, 0.5 and 5 μg g^{-1} of fish fresh

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