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In vivo effects of *Aphanizomenon flos-aquae* DC-1 aphantoxins on gas exchange and ion equilibrium in the zebrafish gill

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ABSTRACT

Aphantoxins, neurotoxins or paralytic shellfish poisons (PSPs) generated by *Aphanizomenon flos-aquae*, are a threat to environmental safety and human health in eutrophic waters worldwide. The molecular mechanisms of neurotoxin function have been studied; however, the effects of these neurotoxins on oxidative stress, ion transport, gas exchange, and branchial ultrastructure in fish gills are not fully understood. Aphantoxins extracted from *A. flos-aquae* DC-1 were detected by high-performance liquid chromatography. The major ingredients were gonyautoxins 1 and 5 and neosaxitoxin, which comprised 34.04%, 21.28%, and 12.77% of the total, respectively. Zebrafish (*Danio rerio*) were administered *A. flos-aquae* DC-1 aphantoxins at 5.3 or 7.61 μg saxitoxin equivalents (eq)/kg (low and high doses, respectively) by intraperitoneal injection. The activities of Na^+-K^+-ATPase (NKA), carbonic anhydrase (CA), and lactate dehydrogenase (LDH), ultrastructural alterations in chloride and epithelial cells, and reactive oxygen species (ROS) and total antioxidative capacity (T-AOC) were investigated in the gills during the first 24 h after exposure. Aphantoxins significantly increased the level of ROS and decreased the T-AOC in zebrafish gills from 3 to 12 h post-exposure, suggesting an induction of oxidative stress and inhibition of antioxidant capacity. Reduced activities of NKA and CA demonstrated abnormal ion transport and gas exchange in the gills of aphantoxin-treated fish. Toxin administration also resulted in increased LDH activity and ultrastructural alterations in chloride and epithelial cells, suggesting a disruption of function and structure in zebrafish gills. The observed abnormalities in zebrafish gills occurred in a time- and dose-dependent manner. These findings demonstrate that aphantoxins or PSPs may inhibit ion transport and gas exchange, increase LDH activity, and result in ultrastructural damage to the gills through elevations in oxidative stress and reduced antioxidant capacity. These effects of aphantoxins in the gills of zebrafish suggest an induction of respiratory toxicity. The parameters investigated in this study may be also considered as biomarkers for studying aphantoxin/PSP exposure and cyanobacterial blooms in nature.

1. Introduction

Blooms of toxicogenic cyanobacteria predominated by *Aphanizomenon flos-aquae* (*A. flos-aquae*), which synthesize cyanobacterial neurotoxins or paralytic shellfish poisons (PSPs), have been encountered in many bodies of freshwater around the world and have become a global problem (Ballot et al., 2010; Gkelis and Zaoutsos, 2014). The primary threats of cyanobacterial blooms are the direct negative impacts on the aqueous environment and ecosystem, in addition to the secretion of toxic secondary metabolites (neurotoxins) into the water (Gkelis and Zaoutsos, 2014). Blooms predominated the *A. flos-aquae* DC-1 strain have occurred frequently in recent decades in Dianchi Lake, which supplies freshwater for agriculture, industry, recreation, and tourism to a population of more than five million in nearby Kunming city, Yunnan Province of China (Liu et al., 2006; Zhang et al., 2013a). These blooms have triggered serious disturbances in environmental safety and human health for those living near the lake due to the direct harmful impacts of the blooms and the secretion of

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cyanobacterial neurotoxins or paralytic shellfish poisons (PSPs) into the water (Liu et al., 2006; Zhang et al., 2013b).

Paralytic shellfish poisons are potent alkaloid neurotoxins that are found primarily in saltwater where they are secreted by dinoflagellates, but these toxins are also found in freshwater where they are generated by freshwater cyanobacteria and bacteria (Prol et al., 2009; Ballot et al., 2010; Hackett et al., 2013). The primary problem with the presence of PSPs in the aquatic environment is that they bioaccumulate through the food chain to high levels in aquatic organisms in which they may have no detrimental effect; however, PSPs may lead to neurotoxicity when humans or other animals consume these PSP-contaminated aquatic animals (Ferrão-Filho and Kozlowsky-Suzuki, 2011). In addition, many aphantoxins or PSPs may not be completely eliminated through normal food preparation techniques such as cooking owing to their resistance to heat, acids, and bases (Gastro et al., 2004). Efficient methods for the elimination and treatment of aphantoxin or PSP toxicity as a result of the consumption of contaminated foodstuffs are also lacking, and intravenous fluids and artificial respiration remain the only medical treatments available (Wiese et al., 2010).

Fish are the most important top predators in aquatic food chains and are reliable indicators of aquatic ecosystem health owing to their trophic status (Oliva et al., 2013). Previous studies have shown that PSPs may enter the fish body and accumulate in the tissues via the consumption of PSP-contaminated food (Jiang et al., 2007). Dietary aphantoxins or PSPs were reported to accumulate in fish muscle and have a number of toxic effects, including increased rates of delayed hatching, malformation, and mortality in fish embryos, disrupted function in the brain and liver, and impaired swimming ability (Samson et al., 2008; Bakke et al., 2010; Zhang et al., 2013a,b,c, 2014, 2015).

Fish gills are important organs with many functions including respiratory gas exchange, excretion of nitrogenous wastes, and the maintenance of ionic, osmotic, and acid–base balance (Shimomura et al., 2012). The gills are also a primary site for the absorption of pollutants and toxicants into the body. Therefore, alterations in branchial physiological function may serve as crucial biomarkers of exposure to environmental challenges (Brunelli et al., 2011). Alterations in gill ultrastructure, reactive oxygen species (ROS), total antioxidative capacity (T-AOC), and the activities of carbonic anhydrase (CA), Na^+-K^+-ATPase (NKA), and lactate dehydrogenase (LDH) are sensitive and valuable parameters that can be used to assess the impacts of exposure to many anthropogenic or natural pollutants (Pandey et al., 2008; Shimomura et al., 2012; Topal et al., 2014). Previous studies have associated abnormalities in these parameters with exposure to pesticides, metals, and other toxicants (Suvetha et al., 2010; Atli and Canli, 2013; Kaur and Kaur, 2015). However, data are lacking regarding the effects of cyanobacterial neurotoxins or PSPs on the aforementioned parameters and respiratory function in the gills of fish.

In this study, two concentrations of toxins were selected; 5.3 and 7.61 μg saxitoxin equivalents (STXeq)/kg dry weight (dw) to reflect those in natural waters. These doses were derived from the sampled, isolated, cultured, extracted, and purified aphantoxins of *A. flos-aquae* DC-1 from Dianchi Lake. This strain has been confirmed to secret PSPs, with an extracted toxin concentration of 6.51 μg STXeq/g dw. Aphanotxins or PSPs are often secreted and reach high concentrations during blooms dominated by *Aphanizomenon* in freshwater. For example, in Crestuma-Lever reservoir, Portugal, the concentration of aphantoxins was recorded at 4.7 μg STX eq/g dw of *Aphanizomenon* cellular extracts (Ferreira et al., 2001), slightly lower than selected in the present study. In lakes in Denmark, the average concentration was found to be 34.1 (range: 5.9–224.1) μg STX eq/g dw; among these, two lakes contained values as high as 182.5 and 224.1 mg STX eq/g dw, with STX constituting more than 95% of the total toxin content, considerably higher than that selected in the present study (Kaas and Henriksen, 2000). In Dianchi Lake, China, the content of aphantoxins produced by *A. flos-aquae* DC-1 was 3.2 mg STX eq/g dw (Liu et al., 2006), lower than the doses selected in the present study. However, during 2007 and 2008, the concentration of aphantoxins reached 13.12 mg STX eq/g dw (unpublished data, Su et al., 2008), higher than the present study. Therefore, the selected doses of aphantoxins in the study are considered to reflect the normal range of aphantoxin in natural lakes subjected to the occurrence of algal blooms dominated by *Aphanizomenon*.

The main purpose of the present study was to investigate alterations in gill ultrastructure, LDH, ROS, T-AOC, CA, and NKA to recognize respiratory toxicity related to ion permeation and gas exchange in the gills of zebrafish during the first 24 h following the administration of a sublethal dose of aphantoxins or PSPs isolated from *A. flos-aquae* DC-1. The findings of this study will further the understanding of respiratory function in fish gills following exposure to cyanobacterial neurotoxins or PSPs.

2. Materials and methods

2.1. Chemicals

The PSP reference standards were purchased from the Canadian National Research Council (Halifax, NS, Canada) and consisted of saxitoxins (dcSTX, STX, neoSTX) and gonyautoxins (GTX1-5, dcGTX2, 3). All other reagents, unless declared otherwise, were of the highest grade available from other commercial suppliers.

2.2. Toxin preparation

The *A. flos-aquae* DC-1 strain was isolated from water samples collected during blooms in Lake Dianchi and were cultured in sterile BG11 medium for approximately 35 d (25 ± 2 °C, 16 h:8 h light:dark cycle, light intensity 40 μmol photon m^{-2} s^{-1}). Following culture, the cells were harvested by centrifugation (6000*g*, 10 °C, 10 min) and stored at −20 °C for analysis.

The toxins were extracted using a 0.01 M acetic acid solution (1:9, w:v; 4 °C) and purified via a series of precipitation, filtration, and concentration with a rotary evaporator (R-210; Buchi, Flawil, Switzerland), followed by passing through seppak C18 cartridges (Waters, Milford, MA, USA).

The toxins were examined using an LC20A HPLC system (Shimadzu, Kyoto, Japan) with fluorescence monitoring (LC20A, RF-10AXL, Shimadzu). Data were analyzed using Shimadzu Class-CR10 software (Shimadzu).

The STX and GTX toxins extracted from the *A. flos-aquae* DC-1 strain were examined by comparison to the chromatograms of the reference standards. The concentration of each toxin was determined using factor response (peak area/toxin concentration) obtained by injecting reference standards (Diener et al., 2006). The overall toxicity of the extracts was calculated as STX equivalents on the basis of the amount of toxin and its relative toxicity compared with STX (Asp et al., 2004; Usup et al., 2004). Purified toxins were frozen at −20 °C.

2.3. Selection of toxin dosage

The methods used for the selection of the toxin dosage followed previously published methods (Lu and Tomchik, 2002) using a total of approximately 200 zebrafish (*D. rerio*). Two toxin doses were finally determined: 5.3 and 7.61 μg STX eq/kg body weight, which were referred to as the low- and high-doses, respectively. The lower dose was chosen owing to evident behavioral changes as a result of toxicant exposure without death, whereas the higher dosage was selected because of severe behavioral alterations in all fish with low