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Teratogenic effects of azaspiracid-1 identified by microinjection of Japanese medaka (*Oryzias latipes*) embryos

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Abstract

Azaspiracid-1 (AZA-1) is a newly identified phycotoxin that accumulates in commercially important bivalve molluscs harvested in several European countries and causes severe human intoxications. Molluscan shellfish are known vectors for accumulation and subsequent transfer of phycotoxins such as brevetoxin and domoic acid through various trophic levels within food webs. Finfish can also accumulate phycotoxins, both directly from toxic algae or from consumption of contaminated shellfish and smaller intoxicated fish. To evaluate the teratogenic potential of AZA-1 and its relevancy to toxin accumulation in finfish, we have utilized a microinjection technique to mimic the maternal-egg toxin transfer of an AZA-1 reference standard and a shellfish extract containing azaspiracids in an embryonic Japanese medaka (*Oryzias latipes*) fish model. Microinjection of purified AZA-1 caused dose-dependent effects on heart rate, developmental rate, hatching success, and viability in medaka embryos. Within 4 days of exposure to doses $\geq$40 pg AZA-1/egg, substantial retardation in development was observed as reduced somatic growth and yolk absorption, and delayed onset of blood circulation and pigmentation. Embryos treated to $\geq$40 pg AZA-1/egg had slower heart rates (bradycardia) for the 9 days in ovo period, followed by reduced hatching success. Microinjection of a contaminated mussel (*Mytilus edulis*) extract containing AZAs (AZA-1, -2, and -3), okadaic acid, and dinophysistoxin-2 resulted in similar responses from the fish embryos at equivalent doses. These studies demonstrate that AZA-1 is a potent teratogen to finfish. This work will complement future investigations on AZA-1 accumulation in marine food webs and provide a basis for understanding its toxicity at different trophic levels.

Keywords: Phycotoxin; Azaspiracid; Medaka; Development

1. Introduction

In 1995, a novel marine phycotoxin, azaspiracid (AZA), was identified in the Netherlands following cases of shellfish intoxication from mussels cultivated in Killary Harbour, Ireland (McMahon and Silke, 1998). Azaspiracid was later associated with additional shellfish intoxications along the West coast of Europe and has been shown to accumulate in the muscular and visceral tissues of bivalve molluscs (Magdalena et al., 2003; James et al., 2002). Following consumption by humans, AZA induced gastrointestinal symptoms such as nausea, vomiting, and diarrhea. Following intraperitoneal

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(i.p.) injection, mice showed additional neurological symptoms including respiratory difficulty, spasms, and limb paralysis, as well as pathological effects (Ito et al., 2000, 2002).

While little is known about this new phycotoxin, it is thought to be produced by the marine dinoflagellate *Protoperidinium crassipes* (James et al., 2003), though direct evidence is lacking. Accumulation of AZAs in molluscan shellfish suggests the possibility of food web transfer, as is the case with several other marine phycotoxins including brevetoxins, ciguatoxins, domoic acid that have been documented in both shellfish and finfish tissues (Bargu and Silver, 2003; Lehane and Lewis, 2000). As is the case with these toxins, AZAs can remain in tissues for extended periods of time, forcing shellfish closures for as long as 8 months (James et al., 2000; McMahon and Silke, 1998).

Lipid soluble toxins such as the brevetoxins and ciguatoxins are capable of accumulating and amplifying through the food web in concentrations dependent upon factors such as the rate of dietary uptake, absorption efficiency, and depuration (Tester et al., 2000). Typically, older, often larger predators within a given environment carry the highest toxin body burdens. While toxin storage in lipid-rich, visceral tissues such as the liver and gonads may have little or no effect on the adult, mobilization of fat stores during reproduction may provide a route of exposure from the parent to the offspring. This is particularly true in oviparous fishes, which use gonadal (fat) stores to generate the lipid-rich yolk that serves as the primary food source for developing embryos (Niimi, 1983; Smith and Cole, 1973). Lipophilic contaminants, such as DDT and PCBs have been demonstrated to accumulate in fish eggs at concentrations that are proportional to the contaminant body burden levels of the adult (Miller, 1993). Tropical ciguatoxins, which adversely affect finfish development by inducing severe spinal curvatures and death (Colman et al., 2004; Edmunds et al., 1999a), have been shown to undergo biomagnification in food webs yielding the highest toxin loads in top level reef predators. Ciguatoxins have been reported to partition into fish eggs at levels comparable to those found in the liver (Colman et al., 2004; Lehane and Lewis, 2000), even though such toxins are not readily bioavailable to the adult while stored in the gonad. However, as they are transferred to the yolk during oogenesis, the toxins become part of the direct food source to the embryo. The sensitive processes involved in embryogenesis can be disrupted, leading to downstream developmental abnormalities and ultimately to the death of the exposed fish. The maternal transfer of toxins during oogenesis could lead to overall reductions in the number of healthy offspring able to pass into subsequent age classes.

While AZAs have not been identified in fish tissues, *Protoperidinium* spp. are a known prey species for a variety of copepods and ciliates (Jeong, 1999; Jeong et al., 2002), which constitute a fundamental trophic level in marine food webs. It can therefore be inferred that planktivores such as fish may accumulate AZAs in their tissues. In this study, we characterized the effects of the most common azaspiracid congener, AZA-1, as well as extracts from mussels (*Mytilus edulis*) naturally contaminated with AZA-1, -2, -3, okadaic acid (OA), and dinophysistoxin-2 (DTX-2) on embryonic finfish development using microinjection to mimic maternal toxin transfer.

2. Materials and methods

2.1. Medaka

Breeding sets (6 females:4 males) of wild type Medaka (*Oryzias latipes*) were obtained from Carolina Biological Supply (Burlington, NC). Fish were housed in 8-l aquaria in a balanced salt solution (17 mM NaCl, 0.4 mM KCl, 0.2 mM $CaCl_2$, 0.3 mM $MgSO_4$, 0.24 mM $NaHCO_3$) under a 16:8 h light:dark cycle. Water temperature was maintained at 25–28 °C during the light phase and declined approximately 3 °C during the dark phase. These conditions were optimal for day-length and temperature induced reproduction of breeders. Medaka were fed twice daily with Wardley's Spirulina Plus flake food or live *Artemia*. Eggs were collected from the female fish each morning and inspected for fertilization. Healthy fertilized eggs were selected for microinjection.

2.2. Sample preparation

Azaspiracid (AZA-1) was extracted from 2 kg mussels (*Mytilus edulis*) that were collected in 1996 from Killary Harbour, on the west coast of Ireland, and Bantry Bay in 1999 on the southwest coast of Ireland. Toxins were extracted in 2001, as described by Satake et al. (1998) and Ofuji et al. (1999) with slight modifications. Stock AZA-1 (2.4 mg) was determined to be $>93\%$ pure by NMR and showed $<1\%$ impurity of other AZA subtypes/congeners by liquid chromatography–mass spectrometry (LC–MS). Subsequent LC–MS analysis comparing this lot of AZA-1 to a previously prepared lot found no statistically significant differences.

Crude extracts were prepared from both non-toxic and AZA-contaminated mussels (*Mytilus edulis*) originating from Bantry Bay, Ireland in August 2001. Extracts were prepared by tissue homogenization with water, extraction with 2 mL of 80% methanol per gram of homogenate, vortexing and sonication, followed by centrifugation (1000*g* for 10 min at 4 °C), and 0.22 μm filtration of the supernatant. To prepare the samples for injection, extracts were dried down under nitrogen gas and resuspended in acetone. Using extraction methods employed by the Irish Marine Institute, the contaminated mussel extract was previously shown by LC–MS to contain 0.17 μg/g AZA-1, 0.03 μg/g AZA-2, 0.04 μg/g AZA-3 (0.24 μg/g AZA_{total}) in addition to 0.19 μg/g okadaic acid (OA) and 1.15 μg/g dinophysistoxin-2 (DTX-2).