The Red Tide Toxin, Brevetoxin, Induces Embryo Toxicity and Developmental Abnormalities

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Brevetoxins are lipid-soluble, polyether, marine toxins that have an excitatory action on voltage-sensitive sodium channels (1). Brevetoxins are produced by the marine dinoflagellate Gymnodinium breve, and their neurotoxic effects on adult animals have been documented. In this study, we characterized adverse developmental effects of brevetoxin-1 (PbTx-1) using an exposure paradigm that parallels the maternal oocyte transfer of toxin. Medaka fish (Oryzias latipes) embryos were exposed to PbTx-1 via microinjection of toxin reconstituted in a triolein oil droplet. Embryos microinjected with doses of 0.1-8.0 ng/egg (ppm) of brevetoxin-1 exhibited pronounced muscular activity (hyperkinesis) after embryonic day 4. Upon hatching, morphologic abnormalities were commonly found in embryos at the following lowest adverse effect levels: 1.0-3.0 ppm, lateral curvature of the spinal column; 3.1-3.4 ppm, herniation of brain meninges through defects in the skull; and 3.4-4.0 ppm, malpositioned eye. Hatching abnormalities were also commonly observed at brevetoxin doses of 2.0 ppm and higher with head-first, as opposed to the normal tail-first, hatching, and doses > 4.1 ng/egg produced embryos that developed but failed to hatch.

Given the similarity of developmental processes found between higher and lower vertebrates, teratogenic effects of brevetoxins have the potential to occur among different phylogenetic classes. The observation of developmental abnormalities after PbTx-1 exposure identifies a new spectrum of adverse effects that may be expected to occur following exposure to G. breve red tide events.

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day: 22–25°C night, which has been determined optimal for breeding (21). Each 10-gallon aquarium had four mature female and two mature male fish. Fertilization occurs externally, with the eggs attached to the abdomen of the female. Eggs were collected each morning from breeding females and kept in embryo-rearing solution (133 mM NaCl, 2.7 mM KCl, 2.1 mM CaCl2, 0.2 mM NaHCO3), where they were separated and inspected for fertilization. Eggs were placed in 2% agarose in 12.5% Hank’s solution (w/v) for stabilization during injection.

Aluminosilicate micropipettes (O.D. 1 mm; Sutter Instrument Co., Navato, CA) were pulled with a micropipette puller (P-87; Sutter). Individual micropipettes were front loaded with either triolein oil (95% pure; Sigma, St. Louis, MO) or with PbTx-1 (Calbiochem, La Jolla, CA) reconstituted into triolein oil. PbTx-1 was concentrated into triolein oil by layering the PbTx-1 (100 µg/mL in methanol) on top of the triolein and evaporating the MeOH under nitrogen to yield a final concentration of 3 µg/µL. Injections were made into the yolk space with the aid of a gas pico-injector system using an injection pressure of 7.8 psi (PLI-100; Harvard Apparatus, Holliston, MA). Balance pressure was maintained at 0.3 psi and increased to 3.2 psi before insertion through egg chorion to compensate for increased pressure within the egg. Eggs were placed in 2% agarose prepared with 12.5% Hank’s balanced salts before injection. Eggs were injected 6–8 hr after fertilization, and both vehicle-injected controls (the same amount of methanol without PbTx-1 in triolein oil) as well as noninjected controls were performed along with the PbTx-1 injections. We injected 20–30 eggs per treatment group. Injected droplet diameters were measured via micrometer and the injected volumes calculated. We calculated the concentration of PbTx-1 injected as the product of the concentration of the toxin in the oil droplet and the volume of the oil droplet. After injections, the eggs were removed from the stabilizing agarose and washed in 12.5% Hank’s balanced salts for 1 min. Eggs were then placed in 24-well plates containing 1.5 mL rearing solution and maintained in a 16 hr light: 8 hr dark cycle at 26°C. Embryos were observed for development daily for 2 weeks with the aid of a stereomicroscope (MZ12; Leica), and spinal hyperkinetic events were recorded on embryonic day 4 over a 3-min time interval. Digital images were captured with an autoexposure CCD camera (Microlmage Video Systems Co., Boyertown, PA).

Results

PbTx-1 was administered to medaka eggs at 6 hr postfertilization by microinjection (Figure 1). Larval survivability at embryonic day 4 was 91% in noninjected animals and 90% of animals injected with triolein. A dose of PbTx-1 caused a dose-dependent effect on larval survivability, with a half maximal effect at approximately 4.5 ng/egg (Figure 2). Hatching of medaka normally occurred on embryonic days 9–12 and occurred successfully in 79% of noninjected embryos and 75% of embryos injected with triolein. A dose of PbTx-1 caused a dose-dependent inhibition of hatching with a half maximal effect at approximately 3 ng/egg (Figure 2).

PbTx-1 also elicited a hyperkinetic activity, observed on embryonic day 4. This hyperkinesis was manifested as tail and body twitching and was monitored over a 3-min interval. Fewer than five spinal hyperkinetic twitches per 3 min were observed in both the noninjected and triolein-injected vehicle

![Figure 1. Microinjection of medaka egg. (A) Uninjected egg 6 hr after fertilization. The droplets inside the egg are the natural oil droplets that have coalesced. (B) Insertion of a microinjection tip into the egg and the injection of a 2 nL droplet of triolein.](image1)

![Figure 2. Effects of PbTx-1 on larval survivability and hatching. Fertilized eggs were microinjected with eight dose ranges of brevetoxin-1. Larval survivability was determined at embryonic day 4 and hatching at days 9–12. Twenty to 30 eggs were injected with each dose.](image2)

![Figure 3. Effects of PbTx-1 on hyperkinesis. On embryonic day 4, fewer than five spinal hyperkinetic twitches per embryo were monitored in both the noninjected and triolein-injected control groups over a 3-min interval. Abbreviations: NI, noninjected; Veh, vehicle. Bars represent the mean ± SEM for >5 embryos in each dose range. *Mean response is significantly different from the vehicle (p<0.05, Tukey multiple comparison test).](image3)
control groups. A concentration-dependent increase was observed for PbTx-1 in the frequency of events. The lowest observable effect occurred in the range of 0.1–0.9 ng/egg (ppm), and a maximal effect of 50 twitches over the 3-min time interval was observed at the dosing range of 5.0–5.9 ng/egg (Figure 3). Hyperkinesis declined at doses > 6.0 ng.

Increasing concentrations of 0.1–7.9 ng/egg PbTx-1 elicited several morphologic effects on the embryos, some of which affected hatching success. PbTx-1, given at doses in the range of 0.1–3.0 ng/egg induced a lateral curvature of the spinal column in embryos, the severity of which was dependent on the concentration of the dose. Figure 4A shows an embryo hatched from an egg injected with 1.1 ng PbTx-1, and Figure 4B shows an embryo hatched from an egg injected with 1.5 ng PbTx-1. Although this degree of anatomical deformity did not interfere with the hatching success of these embryos, their swimming ability was greatly impaired, leading to their inability to survive longer than 10 days. Concentrations of PbTx-1 between 1.5 and 3.0 ng/egg led to the development of embryos with an even greater degree of spinal curvature. In these cases, the embryos seemed to no longer be able to hatch out in the normal tail-first fashion, but instead hatched out in an abnormal head-first fashion (Figure 5). Although these embryos survived the hatching process, they too were unable to survive due to their greatly impaired swimming ability. Eggs that were injected with > 4.0 ng PbTx-1 showed progressing embryonic development but failed to hatch.

A more severe developmental effect of PbTx-1 on medaka embryos was seen at doses > 3.0 ng/egg. Eggs injected with doses from 3.1–4.0 ng/egg of PbTx-1 produced embryos that suffered from herniation of the brain and meninges, possibly through skeletal defects in the skull (Figure 6). It is unknown whether the cranial herniation was due in part to the abnormal head-first hatching of this embryo. However, eggs injected with lower concentrations did not produce embryos with similar defects, even though the embryos had also hatched out head first. Eggs injected with 3.4–4.0 ng of PbTx-1 or higher produced embryos that exhibited malpositioned eyes, and an apparent lack of a frontal skull (Figure 7). A summary of the effects at the different doses is provided in Table 1.

**Discussion**

The objective of this study was to quantify the adverse developmental effects of PbTx-1 using an exposure paradigm that parallels the maternal oocyte transfer of toxin. We have previously used this paradigm to examine the developmental effects of a related polyether toxin, ciguatoxin. Through the use of microinjection, we were able to determine that the lowest observable adverse effect level of PbTx-1 was 0.8–1.0 ng/egg, which is equal to 0.8–1.0 ppm (wet weight embryo = 1.0 mg). With increasing toxin loads of 1.0–3.0 ng/egg, we saw a direct increase in the incidence of lethal spinal defects and an increase in the occurrence of abnormal hatching. Embryos exposed to 3.1–4.0 ng of PbTx-1 exhibited deleterious morphologic abnormalities such as herniation of the brain and meninges and malpositioning of the eyes. Embryos exposed to > 4.0 ng/egg exhibited embryonic development, but failed to hatch. Although we calculated the reported toxin dosages administered at the time of injection, we did not know the exact amounts absorbed by the embryos. Injected oil droplets may have different solubility properties from natural oil droplets. Microinjection of the toxin directly into the natural oil droplet may increase absorption efficiency. Accordingly, PbTx-1 may have even more potent effects than described in this report.

Red tide effects have been reported to affect larval stages of finfish. Riley et al. (22) found that the density of red drum larvae (Scianops ocellatus) declined precipitously in the Aransas Pass Channel (Port Aransas, TX) after the September 1989 red tide. These investigators also examined the effects of G. breve on the hatching and larval survivability of laboratory spawned red drum. No effects on hatch rate were found at cell concentrations up to 5,600 cells/mL; however, effects on larval development were observed at lower cell concentrations.
however a cell concentration of 23 cells/mL was lethal to larvae. These results indicate that the larvae, but not the eggs, are susceptible to G. breve.

In contrast, an earlier study with the sea urchin (Lytechinus variegatus) reported that lysates of G. breve administered to eggs did induce developmental abnormalities (23). This study found that sperm motility, egg fertilization, and development through the blastula stage were unaffected; however, mortality and developmental (axial) abnormalities occurred in about 50% of the gastrula-stage embryos and 80% of pluteus-stage embryos. The reason for the difference between the study with the red drum eggs and the sea urchin eggs may result from the use of G. breve cells and lysates.

The effect levels we report for brevetoxin are about one-half to one-fifth the in vitro inhibition constant ($K_i$) and median effective dose (ED$_{50}$) for binding and voltage-dependent sodium channel-directed cytotoxicity (24). The effect levels are also 1,000 times lower than those we observe for the related toxin ciguatoxin (CTX). CTX injected at 1–9 ppb into medaka eggs increased spinal hyperkinesis and produced induced spinal defects (19). CTX is reported to be 20 times more effective than brevetoxin as an ichthyotoxin (0.5 nM vs. 10 nM for LD$_{50}$) when administered in the aquarium water (25). The higher relative effect by microinjection may be due to differences in the two routes of administration. An unexpected difference between the developmental toxicity of both ciguatoxins and brevetoxins was that PbTx-1, but not CTX, caused cranial and optic deformities. The reason for this difference has not been elucidated, but it may be due to the biodistribution of microinjected toxin or perhaps to channel subtype specificity.

Red tides in the Gulf of Mexico are dense concentrations of G. breve beginning at 0.1 million cells/L and leading to discoloration at 2.0 million cells/L (13). Blooms containing 0.2 million cells/L kill fish (26). In culture G. breve produces approximately 10–15 pg of brevetoxin/cell (27). Thus, an ichthyotoxic bloom would contain approximately 5 nM brevetoxin. This value is close to the 60-min LD$_{50}$ for Poecilia reticulata for PbTx-1 (4.0 ng/mL) in aquarium water (6). Assuming a maximal tolerable concentration of 5 nM (or approximately 5 ppm) for adult fish, developmental effects such as the ones described in this paper could be expected to occur in animals having a bioaccumulation and oocyte transfer factor of approximately 0.1. Brevetoxin accumulation in fish tissue has not been reported; however, ciguatoxin added to aquarium water has been determined to accumulate 9-fold in fish tissue (24). Radioisotopic distribution studies for brevetoxin have been conducted, but distribution to oocytes cannot be inferred from the analysis of the gonads because these studies were not conducted on sexually mature female fish (28,29). However, oocyte transfer factors of 0.1–1.0 have been reported for organochlorines in salmon and lake trout based on body burden measurements and are generally related to egg lipid content (30).

The observation of developmental abnormalities in fish after PbTx-1 exposure identifies a new spectrum of adverse effects that may be expected to occur after exposure to G. breve red tide events. Adverse effects

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**Table 1.** Summary of adverse effects of brevetoxin-1 by dose.

| Dose | Characteristic adverse effect |
|------|------------------------------|
| Noninjected | Hatched normally between days 9 and 12 |
| Triolein | Hatched normally between days 9 and 12 |
| 0.1–0.9 | Hatched normally between days 9 and 12 |
| 1.0–1.9 | Hatched normally, but delayed to days 10–15; spinal curvature present |
| 2.0–2.9 | Hatched abnormally head first with spinal curvature, delayed to days 10–15 or HD; clumping of erythrocytes in vessels at day 4 |
| 3.0–3.9 | Hatched abnormally head first; brain herniation/malpositioned eyes present; clumping of erythrocytes in vessels |
| 4.0–4.9 | Abnormally hatched dead (herniation); clumping of erythrocytes in vessels; heart continued to pump up to day 18; clumping of erythrocytes in vessels |
| 5.0–5.9 | NH; heart continued to pump up to day 18; clumping of erythrocytes in vessels |
| 6.0–6.9 | NH; often failed to develop to later stages; clumping of erythrocytes in vessels |
| 7.0–7.9 | NH; often failed to develop to later stages; clumping of erythrocytes in vessels |

Abbreviations: HD, hatched dead; NH, did not hatch.
on larval life stages have been long suspected. The persistence of red tides from the late fall until early spring has suggested that the spawning of some marine species may be subject to the adverse effect of red tide toxins. Steidinger et al. (10) also emphasized the need for attention to the effects of red tide outbreaks on migratory species, as many species seek estuaries for breeding and nursery grounds. However, we also raise the potential of lifetime accumulation of sublethal body burdens of brevetoxins. Based on studies with other classes of fat-soluble contaminants, somatic stores of toxin in fish are transferred during oogenesis and lead to larval toxicity (30). This would be expected to have a more pronounced effect on the first brood; however, such effects may not necessarily impact successful recruitment of fish populations in successive years (31).

B. breve red tides have been associated with mortality events of many aquatic animals including finfish, sea turtles, and sea birds during their adult stages (4, 9, 10, 15, 22, 26, 32–34). These animals have been known to bioaccumulate substantial body burdens of fat-soluble contaminants at times, and in certain cases transfer toxicity to offspring during oogenesis. Mammals including manatees, bottlenose dolphins, and humans are affected by red tide toxins and also accumulate substantial body burdens of fat-soluble contaminants such as organochlorines. A well-recognized means of contaminant transfer in mammals is by lactation, with placental transfer also being a potential risk (35). As the developmental processes of both higher and lower vertebrates is similar, the potential exists for developmental toxicity and abnormalities to occur among different phylogenetic classes as a result of cumulative exposure to G. breve red tide events.

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