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Absence of neurotoxic effects in leopard sharks, *Triakis semifasciata*, following domoic acid exposure

P. Schaffer [a], C. Reeves [b], D.R. Casper [c], C.R. Davis [d,*]

[a] *College of Veterinary Medicine, University of Tennessee, 2407 River Drive, Knoxville, TN 37996, USA*
[b] *Department of Ecology & Evolutionary Biology, University of California, 1156 High Street, Santa Cruz, CA 95064, USA*
[c] *Long Marine Laboratory, University of California, Santa Cruz. 100 Shaffer Road, Santa Cruz, CA 95060, USA*
[d] *Department of Comparative Medicine, Stanford University School of Medicine, RAF1, Quad7 Building 330, Stanford, CA 94305-5410, USA*

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Abstract

Domoic acid (DA), a potent neurotoxin produced by select species of algae and diatoms, kills neurons bearing kainic acid-type glutamate receptors. Studies have shown that DA bioaccumulates in invertebrates and fish that consume the diatoms. In every vertebrate species tested or observed in the wild, dietary or systemic DA causes neuronal damage or clinical signs of neurotoxicity. Sharks, like marine birds and mammals, are exposed to DA through their diet; however, no research has demonstrated the effect of DA on shark behavior or physiology. In this study, juvenile leopard sharks (*Triakis semifasciata*) were given DA by intracoelomic injection at doses of 0, 1, 3, 9, and 27 mg/kg and observed for 7 days. The sharks failed to demonstrate behavioral or histological changes in response to the toxin. We identified putative brain glutamate receptors by probing western blots with an antibody specific for kainic acid-type glutamate receptors and demonstrated receptor localization in the cerebellum with immunohistochemistry. Blood levels of DA in three sharks dosed at 9 mg/kg fell rapidly within 1.5 h of injection. We show that leopard sharks possess the molecular target for DA but are resistant to doses of DA known to be toxic to other vertebrates.

Keywords: Domoic acid; *Pseudo-nitzschia*; *Triakis semifasciata*; Leopard shark; Glutamate receptor; Kainic acid; Neurotoxin

1. Introduction

Domoic acid (DA) is a naturally occurring neurotoxin produced by the red algae *Chondria armata* and three species of diatoms in the genus *Pseudo-nitzschia*, abundant in algal blooms worldwide (Takemoto and Daigo, 1958; Clark et al., 1999). DA concentrates in various filter feeders such as fat innkeeper worms (*Urechis caupo*), blue mussels (*Mytilus edulis*), razor clams (*Siliqua patula*), and northern anchovies (*Engraulis mordax*) (Goldberg, 2003; Grimmelt et al., 1990; Wekell et al., 1994; Lefebvre et al., 1999). Vertebrates, including humans, that eat fish and shellfish, make up the commonly recognized cases of DA toxicity. Massive mortalities of pelicans (*Pelecanus occidentalis*) and sea lions (*Zalophus californianus*) in Mexico and California were linked to DA in planktivorous fish, and debilitation in southern sea otters (*Enhydra lutris nereis*) has been linked to DA ingestion (Sierra Beltran et al., 1997; Scholin et al., 2000; Lefebvre et al., 1999; Kreuder et al., 2005). DA acts as an analogue of the neurotransmitter glutamate and toxicity is thought to be mediated through high-affinity binding to kainic acid-type glutamate receptors in the brain (Ganakas et al., 1994; Kwak et al., 1992) resulting in neuronal excitation and uncontrolled intracellular calcium increases (Nijjar and Nijjar, 2000). Seizures and

* Corresponding author. Tel.: +1 650 723 3308; fax: +1 650 725 0940.
E-mail address: crdavis@stanford.edu (C.R. Davis).

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neuronal necrosis are the hallmarks of DA toxicity in rats, mice, and non-human primates (Dakshinamurti et al., 1991; Strain and Tasker, 1991; Sutherland et al., 1990; Scallet et al., 1993) similar to what is seen in humans exposed to DA through shellfish consumption (Teitelbaum et al., 1990).

Interestingly, reports of DA toxicity in large piscivorous teleost fish and sharks are lacking, although these animals possess the molecular targets of DA, brain kainic acid-type glutamate receptors (London et al., 1980). Lack of observed deaths could be due to the fact that these animals do not frequent the ocean's surface to breathe and they may live in deep off-shore waters. Intoxicated teleost fish and sharks may also be promptly eaten if they become incapacitated. However, it is interesting that leopard shark mass mortalities have not been reported despite the fact that these sharks inhabit shallow inshore and estuarine waters and feed on the fat innkeeper worm, *U. caupo*, a filter feeder that can concentrate DA to 750 mg/l during *Pseudo-nitzschia* blooms (Goldberg, 2003).

In this study, we examined leopard sharks for behavioral changes and morphological brain changes after dosing with intracoelomic DA. We confirmed the presence of kainic acid-type glutamate receptors and demonstrated their distribution in leopard shark brains.

2. Materials and methods

2.1. Animals

All animals were housed and handled according to Stanford University and University of California Administrative Panel on Laboratory Animal Care guidelines. Juvenile leopard sharks were collected by trawl net from the San Francisco Bay and transported in seawater to the California Department of Fish and Game Facility, Long Marine Laboratories, UC Santa Cruz. Sharks were housed in two covered, 8550 l tanks supplied with air stones and filtered seawater from the Monterey Bay replenished at 20 l/min. Sharks were acclimated until they demonstrated regular feeding and swimming behavior before further handling. All sharks were anesthetized by immersion in aqueous tricaine methanesulfonate (MS222) (Finquel™), colored T-tags were inserted through a scalpel incision lateral to the dorsal fin and each shark was weighed (avg = 82 ± 20 g). Tagging allowed identification of individuals that were housed together in a large community tank.

2.2. Domoic acid

Domoic acid was purchased from BioVectra, Canada, dissolved in sterile water and stored in stock solutions at 4 °C.

2.3. Exposure

Four sharks were administered 1, 3 (approximate ED50 for anchovies), 9, and 27 mg/kg of DA, respectively, by intracoelomic (i.c.) injection. Injection volume was 0.25 ml. Four sham control sharks were given 0.25 ml sterile water i.c. The dosing schedule of 1, 3 and 9 mg/kg was initially designed as a pilot study to confirm an appropriate dose range for a more comprehensive exposure with multiple animals per dosage group; however, when the shark injected with 9 mg/kg DA showed no ill effects, an additional animal was given 27 mg/kg. When this animal also showed no ill effects, we determined that we would not be able to find a feasible toxic dose, considering the extreme expense of purified DA used in the injections.

2.4. Behavioral observation

Sharks were observed three times daily for swimming activity and ability. Close attention was paid during feeding, which occurred every other day, when the animals were most physically active and thus likely to show signs of neurotoxicity. Digital videos were taken of feeding and swimming behavior.

2.5. Histology and Immunohistochemistry

Seven days after injections sharks were euthanized in an aqueous solution of MS222. Brain, gill, heart, liver, spleen, pancreas, kidney, rectal gland, stomach, duodenum, spiral valve, colon, muscle, and skin were collected from each shark and fixed in 10% neutral buffered formalin. Tissues were routinely processed into paraffin, sectioned at 4 μm and stained with hematoxylin and eosin.

Standard biotin–streptavidin immunohistochemistry was performed on selected brain sections using MAB379 (Chemicon International, Temecula, CA), a monoclonal antibody recognizing kainic acid-type glutamate receptors (GluR 5, 6, 7). Rat brain served as the positive control. Primary antibody raised against desmin was used as a negative control.

2.6. Brain protein extraction and western blots

Two untreated juvenile leopard sharks were euthanized in an aqueous solution of MS222 and brains were removed and frozen at −80 °C until processed. Proteins were extracted from the two leopard shark brains and one rat brain (positive control) for western blotting as previously described (Tolwani et al., 2002). Extracted proteins were denatured and separated in 7.5% SDS-PAGE and were transferred for 40 min to a nitrocellulose membrane (Invitrogen). After blocking the membrane for 1 h (5% milk in PBS) the membrane was immunoblotted for 1 h at room temperature with a 1:1000 dilution of MAB379. The membrane was incubated with biotinylated goat-anti-mouse

IgG for 1 h at room temperature, incubated for 30 min with streptavidin and developed with 3,3′-diaminobenzidine.

2.7. Uptake study

To confirm that DA was absorbed from the injection site, four juvenile leopard sharks were injected with 9 mg/kg DA and euthanized for terminal blood collections at 30 min ($n=2$) and 90 min ($n=2$) after injection. One unexposed control shark was also sampled. Terminal bleeds were necessary to collect appropriate volumes for analysis; the sharks were too small to take serial non-terminal blood collections. Samples were centrifuged immediately, the serum separated and shipped on dry ice to the California Animal Health and Food Safety Laboratory (CAHFS), Davis, California for quantification of DA by liquid chromatography–mass spectrophotometry (LC–MS/MS).

3. Results

3.1. Behavior

All exposed and control sharks maintained normal swimming and feeding behavior for the 7-day duration of the study.

3.2. Histology, immunohistochemistry, western blots

All organs from exposed and control animals appeared within normal limits grossly and histologically. Immunohistochemistry of brain using MAB379 was most strongly positive in the cytoplasm of cerebellar Purkinje cells and in the neuropil of the molecular cell layer (Fig. 1). Cell cytoplasm and processes in the cerebellar granular cell layer and the cytoplasm of individual astrocytes in cerebellar white matter were moderately positive. Small numbers of scattered telencephalic and diencephalic neurons and select brainstem and diencephalic nuclei comprised of large neurons demonstrated mild to moderate cytoplasmic staining. The neuropil of the telencephalon and diencephalon and the white matter of the cerebellum demonstrated no staining. The choroid plexus was strongly positive, but this tissue frequently yields nonspecific positive results on a variety of immunohistochemical reactions (personal observation). The apical tips of the ependymal cells in the cerebellum were strongly positive while ependymal cells lining more rostral ventricles were negative.

In the western blots of shark brain, three specific protein bands of approximately 115, 97, and 69 kDa reacted with the monoclonal antibody that recognizes specific kainic acid-type glutamate receptors (GluR 5, 6, 7). These bands corresponded to bands of approximately 123, 100, and 74 kDa in the rat brain that was used as a positive control. The intermediate-sized bands were faint in both species (Fig. 2).

3.3. DA uptake

Serum levels of DA in four sharks after i.c. injection are summarized in Table 1. These results confirm that DA was absorbed from the coelomic cavity and that the injection protocol delivered DA into the bloodstream. Circulating DA levels dropped to trace or undetectable levels by 90 min after injection. The method detection limit was 5 μg/l.

4. Discussion

Juvenile leopard sharks demonstrated no discernable ill effects after receiving DA at dosages exceeding those known to be toxic in other fish and mammalian species. A single i.c. or i.p. injection of 4 mg/kg DA was neurotoxic to northern anchovies (*E. mordax*), mice (*Mus musculus*) and

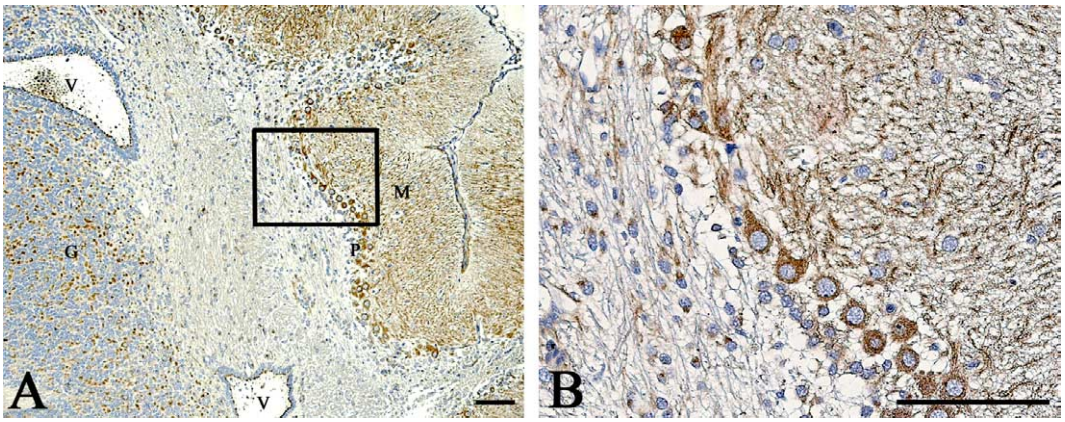

Fig. 1. Leopard shark cerebellum. A. Immunohistochemical stain with MAB379 recognizing kainic acid-type glutamate receptors GluR 5, 6, 7. Strong specific staining is evident in Purkinje cells, granular cells and fibers in the molecular layer. Purkinje cells (P), granular cell layer (G), molecular layer (M), and fourth ventricle (V). Light microscopy. Bar = 100 μm. B. Magnified insert. Bar = 100 μm.

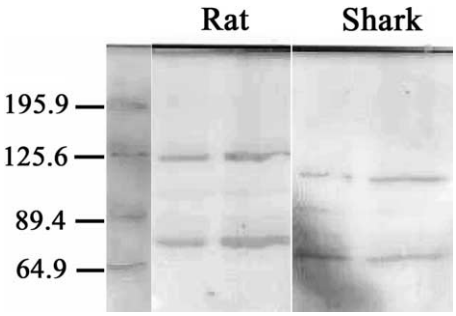

Fig. 2. Western blot of rat (positive control) and leopard shark brain protein extract probed with MAB379, recognizing kainic acid-type glutamate receptors GluR 5–7. Three specific bands are seen in both rat and shark samples. The intermediate-sized band is faint in both species. Molecular weight standards are in the left lane.

Cynomolgus monkeys (*Macaca fascicularis*) (Lefebvre et al., 2001; Strain and Tasker, 1991; Tryphonas et al., 1990). Ours is the first report of a vertebrate that is apparently resistant to DA toxicity and raises several interesting questions.

First, are the appropriate molecular targets for DA present in leopard sharks? Our western blot and immunohistochemical results using a monoclonal antibody recognizing an epitope common to glutamate receptors 5–7 confirm that the specific kainic acid (KA)-type glutamate receptor targets for DA toxicity are present in the leopard shark brain. The molecular weights of the three specific bands detected in the leopard shark correlate well with those in rat control tissue. Ex vivo studies demonstrating high affinity of DA for leopard shark KA-type glutamate receptors would further support the role of these receptors as DA targets. Our observed distribution pattern of receptors in the shark brain is consistent with London et al. (1980) who reported the highest level of KA binding in the cerebellum with less in forebrain and least in the medulla of spiny dogfish (*Heterodontus fransisci*) and smooth dogfish (*Mustelus californicus*), both estuarine sharks. Although the hippocampus of higher vertebrates is a site of KA-type glutamate receptors, the hippocampus is not developed in sharks, so specific analysis of the hippocampus is not relevant in these species.

Table 1
Serum domoic acid (DA) levels in sharks dosed with 9 mg/kg i.c. and sampled at 30 and 90 min after injection

DA (mg/kg)	Minutes	Plasma conc. (μg/l)
9	30	38
9	30	9
9	90	Trace
9	90	ND
0	30	ND

The control shark received sterile water and was sampled 30 min after injection. Detection limit = 5 μg/l. ND, not detected.

Secondly, were the dosages and route of administration appropriate and do excretion rates differ significantly in sharks? The range of DA doses administered to our sharks exceeded the ED_{50} (exposure dose affecting 50% of the population) for mice (4 mg/kg by i.p. injection) (Strain and Tasker, 1991) and anchovies (3 mg/kg by i.c. injection) (Lefebvre et al., 2001). By testing serum samples at two time points after i.c. injection, we confirmed that DA was readily absorbed from the coelomic cavity and that circulating DA levels fell rapidly. Similar data in rodents after i.p. injection is not readily available. In rats given DA orally, almost the entire dose was excreted in the feces, consistent with poor absorption from the gastrointestinal tract (Iverson et al., 1990), but when given i.v., blood levels of DA dropped in a linear fashion and the entire DA dose was recovered in the urine as parent compound 160 min after injection (Suzuki and Hierlihy, 1993), implicating glomerular filtration as the primary clearance mechanism. In sharks, it is likely that the gills play a significant role in excretion of DA; however, no published studies have confirmed this. If glomerular filtration were the primary route of excretion in leopard sharks, the sharks may be expected to excrete DA more slowly than mammals due to low systemic blood pressure and consequent low glomerular filtration rate (GFR). Single nephron filtration rates are lower in sharks than in rats (Dantzler, 1989) and GFR is lower in sharks than in mice when corrected for body weight (Wells et al., 2002; Qi et al., 2004). Total numbers of glomeruli are also lower in sharks when compared to rats (Nash, 1931; Vimtrup, 1928). The clearance in our leopard sharks seems rapid in light of these comparisons. Sharks have a renal portal system, which may drain the caudal coelomic cavity, but the renal portal veins do not contribute to the afferent arterioles of the glomeruli; rather, they drain into the renal sinusoidal capillaries (Hamlett, 1999). Thus, rapid clearance via glomerular filtration before entering the systemic circulation is not expected with an i.c. injection of DA. Although renal secretion is not thought to play a significant role in DA excretion in mammals, it is possible that some degree of tubular secretion contributed to clearance in the leopard sharks.

Thirdly, does the shark blood–brain barrier (BBB) exclude DA entry? The vertebrate BBB consists of endothelial cells and glial foot processes (Bundgaard and Cserr, 1981). In mammals, the permeability barrier and the role of active transport reside in the endothelial cells (Reese and Karnovsky, 1967). In the shark BBB, the endothelium is fenestrated and the glial cells provide the permeability barrier and active transport functions (Hashimoto, 1972; Bundgaard and Cserr, 1981). The mammalian BBB is poorly permeable to DA, which circulates in a charged state, and no evidence for active transport has been identified (Preston and Hynie, 1991). Comparative studies indicate that the shark BBB may not be as well developed nor as exclusive of polar compounds as the mammalian BBB (Cserr et al., 1972). These conclusions suggest that

exclusion of DA from the shark brain is unlikely. However, in the present study we did not conclusively demonstrate that DA entered the shark brain and interacted with the appropriate receptors. The quantification of DA in brain tissue is technically difficult, although radioactive tracer studies could be useful to confirm entry of DA into the brain.

An intriguing hypothesis is that leopard shark resistance to DA toxicity hinges on the presence of high levels of an endogenous (physiological) ligand, presumably a neurotransmitter, for the KA-type glutamate receptors. London et al. (1980) investigated the distribution of KA receptors among a wide variety of invertebrate and vertebrate species. They found that KA receptors had a wide phylogenetic distribution (from hydras to man) indicating that the receptors play an important role in neuronal function from an evolutionary perspective. This finding also suggests the presence of an endogenous substance resembling KA, since KA is an exogenous substance produced by the seaweed *Digenea simplex*. London et al. (1980) hypothesized that KA binds a specific subpopulation of receptors distinct from those through which glutamate mediates its primary effects. In invertebrates, KA activates a specific set of receptors that potentiate the effect of glutamate (Shinozaki and Shibuya, 1974; Walker, 1976). While total specific binding for KA in human brain ranged from 29 to 193 fmol/mg protein, binding in sharks, *H. francisci* and *M. californicus*, ranged from 455 to 77,200 fmol/mg (London et al., 1980). The only animal with higher specific KA binding was the amphibian *Xenopus laevis*. Subsequently, Migani (1990) reported the existence of an endogenous ligand for the KA receptor in the goldfish, which has 100-fold higher KA brain receptor densities than rats (Migani et al., 1985). This goldfish kainate binding inhibitor (KBI) displaced KA from its binding sites on neurons, but did not impede binding of glutamate.

We suspect that an endogenous ligand for KA receptors is present in the shark brain and that, in light of the extremely high receptor levels in the sharks tested by London et al. (1980), the endogenous ligand may be so prevalent as to provide very strong competitive inhibition to DA binding, thus averting toxic neurological effects. This hypothesis could explain how leopard sharks can routinely feed on prey, like the fat innkeeper worm, that concentrate DA to very high levels. Heterogeneity in KA binding sites among different species and the possibility of structural evolutionary changes have also been suggested (Migani, 1990) and could account for species differences in sensitivity to DA. Sequencing of the shark glutamate receptor gene(s) may elucidate unique coding regions for glutamate and domoic acid binding domains. Finally, determination of the LD_{50} for KA in sharks would offer insight into the magnitude of the resistance to DA toxicity and, because KA is much less expensive than DA, allow the experiment to be performed in larger adult sharks.

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