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INTRAVENOUS LIPID EMULSION TREATMENT REDUCES SYMPTOMS OF BREVETOXICOSIS IN TURTLES (*TRACHEMYS SCRIPTA*)

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Abstract: Harmful algal blooms (HABs) occur when excess nutrients allow dinoflagellates to reproduce in large numbers. Marine animals are affected by blooms when algal toxins are ingested or inhaled. In the Gulf of Mexico, near annual blooms of *Karenia brevis* release a suite of compounds (brevetoxins) that cause sea turtle morbidity and mortality. The primary treatment at rehabilitation facilities for brevetoxin-exposed sea turtles is supportive care, and it has been difficult to design alternative treatment strategies without an understanding of the effects of brevetoxins in turtles *in vivo*. Previous studies using the freshwater turtle as a model species showed that brevetoxin-3 impacts the nervous and muscular systems, and is detoxified and eliminated primarily through the liver, bile, and feces. In this study, freshwater turtles (*Trachemys scripta*) were exposed to brevetoxin (PbTx-3) intratracheally at doses causing clear systemic effects, and treatment strategies aimed at reducing the postexposure neurological and muscular deficits were tested. Brevetoxin-exposed *T. scripta* displayed the same behaviors as animals admitted to rehabilitation centers for toxin exposure, ranging from muscle twitching and incoordination to paralysis and unresponsiveness. Two treatment regimes were tested: cholestyramine, a bile acid sequestrant; and an intravenous lipid emulsion treatment (Intralipid®) that provides an expanded circulating lipid volume. Cholestyramine was administered orally 1 hr and 6 hr post PbTx-3 exposure, but this regime failed to increase toxin clearance. Animals treated with Intralipid (100 mg/kg) 30 min after PbTx-3 exposure had greatly reduced symptoms of brevetoxicosis within the first 2 hr compared with animals that did not receive the treatment, and appeared fully recovered within 24 hr compared with toxin-exposed control animals that did not receive Intralipid. The results strongly suggest that Intralipid treatment for lipophilic toxins such as PbTx-3 has the potential to reduce morbidity and mortality in HAB-exposed sea turtles.

Key words: Brevetoxin, cholestyramine, intravenous lipid emulsion, *Trachemys scripta*, red tide, sea turtle.

INTRODUCTION

Harmful algal blooms (HABs, red tides), which occur when populations of single-celled algae (dinoflagellates) rapidly increase due to an overabundance of nutrients, have been increasing in frequency and distribution in recent years worldwide.[15] The dinoflagellates release neurotoxins that are responsible for a variety of ailments, including neurotoxic shellfish poisoning and respiratory symptoms from ocean aerosols.[1,12,16,17] In the Gulf of Mexico, *Karenia brevis* releases a suite of toxins known as brevetoxins (PbTx).[13] *K. brevis* blooms occur nearly annually, and last anywhere from a few weeks to longer than a year.

Marine animals are also negatively impacted by HABs, with morbidity and significant mortalities reported in fish, seabirds, dolphins, manatees, and sea turtles.[5,9,13,19,23,30,31] Brevetoxin readily crosses membranes and affects excitatory tissues including the nervous, cardiac, and muscular systems.[22] Symptoms of brevetoxicosis include muscle twitching, ataxia, paralysis, and coma.[2] Not only are these animals affected by the toxins directly, but impacts on behavior that reduce foraging ability, as well as negative effects on the immune system, may make animals more susceptible to other diseases. In a previous study investigating the immune system of loggerhead sea turtles (*Caretta caretta*) exposed to brevetoxins, a number of genes involved in oxidative stress were upregulated, indicating disruption to normal-functioning cells and cellular signaling pathways.[31] Stranded animals thought to be suffering from red tide exposure are taken to rehabilitation facilities for treatment, which generally consists of supportive care: placing the animals in a toxin-free environment, and maintaining their heads above water to prevent drowning while the toxins clear.[21] In loggerhead sea turtles experiencing edema, dehydration using furosemide has also proven to be effective.[21] The severity of the response to brevetoxin exposure can vary by individual, likely due to

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differences in immune response, diet, body mass, species, and how much of the toxin they have absorbed.[9] A study that investigated brevetoxin concentrations in the plasma of sea turtles admitted for rehabilitation showed the toxins cleared from their systems over 5–80 days; however, there was no correlation between brevetoxin concentrations and whether or not the animal survived.[9] The ability to design treatment protocols for sea turtles has been limited because little is known of the toxin distribution, effects, or clearance, and this can be very challenging to address experimentally due to their listing as threatened and endangered species. Toxin distribution studies have only been performed on necropsied animals, while clearance studies are limited to blood samples.[9] In one study using dehydration therapy as a method for treating PbTx-exposed loggerhead sea turtles, 71% of the animals that were treated showed a decrease in neurological symptoms, and therefore survived and were able to be released.[21] Two other treatments attempted for neurotoxin exposures are activated charcoal and mannitol, which have shown limited success in decreasing clinical symptoms.[21]

Because of the limitations on working directly with sea turtles, the freshwater turtle *Trachemys scripta* was developed as a model for brevetoxin exposure in sea turtles.[6,7] The clinical signs observed in the laboratory after oral and intratracheal exposures were similar to those reported in the literature for mammals exposed to high doses of brevetoxin and for sea turtles admitted to rehabilitation centers, including muscle twitching and uncoordinated movements, swimming in circles, head bobbing, rigidity of the limbs and partial to complete paralysis.[9,29] In these laboratory studies, neurological symptoms post brevetoxin-3 (PbTx-3) appeared within 2–5 min after inhalation exposures and approximately 30 min after oral exposures. The evident clinical symptoms last for greater than 6 hr post toxin exposure and disappear within 24 hr; over 24–48 hr post exposure the animals still show reduced activity and lethargy.[6] These results mirror data from enzyme-linked immunosorbent assay (ELISA) analysis which confirmed that PbTx-3 distributes to all organ systems and for these relatively acute exposures, is rapidly cleared out of most systems 24–48 hr post exposure while concentrations increase in the bile and feces over 24–48 hr, indicating the primary route of excretion.[6]

Using behavioral data, organ system impact, clearance rates, and histopathology analyses post PbTx-3 exposure; two different potential treatment strategies were devised and tested. Because the bile was an important route of excretion, and plays an important role in the absorption of fat-soluble substances such as lipophilic PbTx-3, cholestyramine was utilized in an attempt to increase clearance rates of PbTx-3. Cholestyramine is an anion exchange resin that strongly binds to bile salts to prevent bile reabsorption.[25] It was hypothesized that cholestyramine would become bound to the bile and allow for a more rapid PbTx-3 clearance; several case studies in domestic animals report successful use of the compound in cases of toxin exposure, and it is possible that the continued high plasma levels of toxin in sea turtles admitted for brevetoxicosis could be due to continued recirculation after reabsorption from the gut.[9,26,28]

The second potential treatment relied on the fact that brevetoxin is a lipid-soluble molecule that easily passes through the cell membranes. Intravenous lipid emulsion (ILE) therapy using Intralipid® (Fresenius Kabi AB, 75320 Uppsala, Sweden), a lipid compound consisting primarily of soybean oil and egg yolk protein, is thought to draw toxins out of the fatty membranes of cells by creating a high volume of lipids in the extracellular compartment.[27] This approach has been reported in several veterinary case studies as an antidote for animals that have ingested lipophilic toxins.[9,10,14] A dog that ingested bromethalin, a neurotoxic rodenticide, was successfully treated with ILE therapy; toxin levels in the serum were reduced by 75% post ILE treatment.[18] Intravenous lipid emulsion also has been used successfully in cats exposed to permethrins and with two juvenile green sea turtles exposed to domoic acid, another neurotoxin produced by algae (Manire, pers. obs.).[9] Because ILE therapy has been used in a number of successful cases, it was hypothesized that it would reduce the neurological symptoms associated with brevetoxicosis in turtles.

MATERIALS AND METHODS

Experimental animals

A total of 46 *T. scripta* were used for these experiments (Table 1); all work was approved by the Florida Atlantic University Institutional Animal Care and Use Committee. Animals were acclimated to the laboratory for 2 wk prior to conducting any experiments. Mixed-sex *T. scripta*, approximately 12–15-cm straight carapace length and weighing 0.2–0.4 kg were obtained from a commercial supplier (Niles Biological Inc, Sacramento, CA 95829, USA) and maintained in fresh-

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Table 1. Number of animals (*Trachemys scripta*) per control and treatment groups.

Experimental group	*N*
Controls: no PbTx-3, no treatment	5
Intralipid (ILE[a]) controls 100 mg/kg, no PbTx-3	3
PbTx-3 1-hr IT controls, no ILE	3
PbTx-3 24-hr IT controls, no ILE	3
PbTx-3 + ILE 30 min post PbTx-3 IT 1-hr harvest; ILE: 50 mg/kg	3
PbTx-3 + ILE 30 min post PbTx-3 IT 1-hr harvest; ILE: 100 mg/kg	4
PbTx-3 + ILE 30 min post PbTx-3 IT 24-hr harvest; ILE: 50 mg/kg	2
PbTx-3 + ILE 30 min post PbTx-3 IT 24-hr harvest; ILE: 100 mg/kg	3
ILE 30 min before PbTx-3 + PbTx-3 IT 1-hr harvest; ILE: 100 mg/kg	4
ILE 30 min before PbTx-3 + PbTx-3 IT 24-hr harvest; ILE: 100 mg/kg	4
Cholestyramine control 50 mg/kg (no PbTx-3)	3
PbTx-3 + cholestyramine 50 mg/kg	4
PbTx-3 + cholestyramine 20 mg/kg	5

[a] ILE, intravenous lipid emulsion; IT, intratracheal instillation.

water tanks at room temperature, 22°C (±3°C), 50% (±4%) relative humidity on a 12-hr day–night cycle. Aquaria were cleaned according to standard husbandry methods and the animals fed commercial aquatic food three times weekly to satiety. Turtles were given individual identification numbers and randomly assigned to treatment groups.

Brevetoxin

Brevetoxin-3 was purchased from LKT Laboratories (St. Paul, MN 55130, USA), dissolved in ethanol and mixed with 0.9% NaCl to a final concentration of 0.05 μg/μl. Turtles were restrained by hand and administered 10.53 μg PbTx-3/kg body mass by intratracheal instillation (IT) approximately 1.5 cm into the trachea at the base of the tongue as previously described to mimic inhalation.[6] Animals received one dose of PbTx-3 either prior to or after the treatment drug. One control group was exposed to PbTx-3 without additional treatment (exposure controls), while a second group of animals received sham doses of physiological saline solution mixed with ethanol to a final concentration to 0.1% EtOH and treatment drugs (treatment controls).[7]

Cholestyramine

Cholestyramine powder for oral suspension was purchased from PAR Pharmaceutical Companies, Inc (Chestnut Ridge, NY 10977, USA). Cholestyramine powder (4 g resin per 9 g powder) was mixed with deionized water to make a final dosage of either 20 mg/kg or 50 mg/kg. Turtles were administered one intratracheal dose of PbTx-3 (10.53 μg/kg) prior to receiving oral cholestyramine via an esophageal tube at both 2 hr and 6 hr post PbTx-3 exposure. Animal tissues and fluids were collected 24 hr post toxin exposure and tissues and fluids were analyzed via ELISA. Treatment control animals were administered two doses of 50 mg/kg of cholestyramine 4 hr apart and sacrificed 24 hr post cholestyramine.

Intralipid emulsion

Intralipid (Baxter Healthcare Corp., Deerfield, IL 60015, USA) is a 20% fat emulsion consisting of 20% soybean oil, 1.2% phospholipids (from powdered egg yolk), 2.25% glycerin, USP, water for injection q.s., with pH 8 (6–8.9) adjusted with NaOH. Prior to or after a single intratracheal exposure to PbTx-3, turtles were administered ILE via the subcarapacial vein, injected slowly to prevent lipid embolism. Intralipid (50 or 100 mg/kg) was administered to animals 30 min before or 30 min after toxin exposure. Animals were monitored every 5 min for the first hour, every 30 min for up to 8 hr, and at 24 hr to compare brevetoxin symptoms post ILE treatment versus untreated animals (exposure controls: PbTx-3 without ILE). Animals were sacrificed either 1 hr or 24 hr post toxin exposure and tissues and fluids were collected to determine PbTx-3 tissue distribution, PbTx-3 concentrations in each tissue/fluid and rates of clearance. Control animals received one dose of ILE (100 mg/kg) and tissues were analyzed via ELISA 24 hr post toxin exposure.

Tissue collection

All turtles were euthanized by decapitation and the following tissues and fluids were collected for ELISA: kidney, urine, liver, intestines, bile, brain, heart, spleen, lung, trachea, and plasma. Sedation was not used prior to decapitation as the effect of additional neuroactive compounds on the toxin-exposed turtles is unknown. Whole blood was collected by exsanguination, feces were removed from the large intestine, and other fluids were collected directly from the organ with a syringe post mortem. Tissues were flash frozen in liquid nitrogen and stored at −80°C. Plasma was extracted from whole blood by centrifugation (10 min at

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1,398 *g*) and the plasma and other fluid samples were frozen at −80°C. Tissue and fluid samples were shipped overnight to the Florida Fish and Wildlife Research Institute (St. Petersburg, FL 33701, USA) on dry ice and remained frozen at −80°C until processing.

ELISA

A competitive ELISA was used to detect brevetoxins in turtle tissues and biological fluids with extractions performed as previously described.[9] Brevetoxins and brevetoxin-like compounds were quantified in all sample extracts using a competitive ELISA (Marbionc, Wilmington NC 28403, USA) with modifications as previously described.[9,24] Toxin concentrations were calculated using a PbTx-3 standard curve, and results are reported in PbTx-3 equivalents. The limits of detection as described were approximately 1–2 ng/ml in plasma or urine, 10 ng/ml in bile, and 10 ng/g for feces or tissue.

Behavioral analysis

Turtles grouped in the 1-hr postexposure experiments (exposure controls) were monitored for clinical signs of brevetoxicosis and video recorded immediately and at 5 min, and every 10 min thereafter for 1 hr post toxin exposure. Animals that were part of the 24-hr exposure group were monitored and video recorded at 5 min, then every 10 min for the first hour, and every hour after that up to 8 hr post PbTx-3 and then again at 24 hr.

Animals that were administered PbTx-3 followed by cholestyramine were monitored and video recorded immediately after each PbTx-3 treatment and every hour for the first 8 hr post toxin administration and then at 24 hr.

Animals that were treated with ILE (before and after PbTx-3) were monitored and clinical signs were video recorded 5 min post PbTx-3, and every 10 min for the 1-hr exposures. Animals grouped in the 24-hr post-PbTx-3 exposures were video recorded for clinical signs every 30 min after 1 hr and up to 8 hr post PbTx-3. Turtles were again recorded at 24 hr.

Statistical analysis

Tissue and fluid concentration data were analyzed using one-way analysis of variance (ANOVA, $\alpha = 0.50$) followed by Holm-Sidak Test (Holm-Sidak) pairwise comparison test using Sigma Plot 11.0 (Systat Software, Inc, San Jose, CA 15131, USA). Treatments were compared with control PbTx-3 exposures and to each other within each individual tissue or fluid. Samples in which brevetoxin was not measurable were given a value of half the level of detection (2.5 ng per g tissue or ml bile or 0.5 ng per ml fluid) to calculate mean.

RESULTS

PbTx-3-exposure controls

Turtles that were administered one dose of intratracheal PbTx-3 exhibited muscular and neurological symptoms consistent with brevetoxicosis including head bobbing, muscle twitching, ataxia, circling, and paralysis (Table 2). The onset of symptoms was within 2–5 min post PbTx-3 exposure and lasted over 6 hr in untreated animals, showing a decline in symptoms over a 24-hr period as previously described.[6] Tissues and fluids showed a distribution to most organ systems within the first 1 hr post exposure to PbTx-3, with toxin accumulating higher in the intestines, bile, feces, and urine 24 hr post PbTx-3 exposure compared with 1 hr post PbTx-3 exposure (Fig. 1). PbTx-3 rapidly clears from most tissues over 24 hr post toxin exposure.

PbTx-3 + cholestyramine treatment

In the cholestyramine-treated animals post PbTx-3 exposure, there was no reduction in clinical signs for either the 20 mg/kg or 50 mg/kg doses as observed over 24 hr when compared with PbTx-3-exposure controls. Cholestyramine-treatment controls (no PbTx-3) exhibited normal behavior. The levels of PbTx-3 varied widely in the tissue and fluids of PbTx-3 control animals, consequently no statistically significant ($P < 0.05$) changes were observed in PbTx-3 concentrations in the bile post cholestyramine treatment for either dose of cholestyramine (20 mg/kg or 50 mg/kg) compared with the PbTx-3 controls (Fig. 2). While there was an overall decrease in PbTx in the detoxification and excretion systems (kidney, liver, and feces), these differences were not significant ($P < 0.05$) between either cholestyramine treatment groups compared with the PbTx-3 controls. The brain, heart, and fat also showed a decrease in PbTx-3 concentration after both dosing treatments with cholestyramine, but again these changes were not statistically significant ($P < 0.05$). Treatment control exposures (cholestyramine, no PbTx) did not have any PbTx-3 in tissues or fluids that were above the level of detection (data not shown).

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Table 2. Behavioral outcomes due to PbTx-3, with pre– and post–intravenous lipid emulsion (ILE) treatment. PbTx-3 was administered to *T. scripta* one time by intratracheal instillation and ILE was given 30 min before or after PbTx-3 (50 mg/kg or 100 mg/kg) and monitored for behavioral changes. Behavioral symptoms for PbTx-3 + ILE groups (where ILE was administered after PbTx-3 exposure) in some animals were video recorded periodically for the first 6 hr post PbTx-3, though all animals were observed and symptoms noted for up to 8 hr. Animal behavior was recorded again at 24 hr post treatment. In ILE + PbTx-3 groups (where ILE was administered prior to PbTx-3 administration) significant behavioral changes were noted in the first 1–2 hr post ILE exposure and symptoms were greatly reduced by 6 hr.

Experimental group	Time point post PbTx-3	ILE dose (mg/kg)	Behavior	*N*
PbTx-3-exposure control	1 hr	—	Significant muscle twitching, circling, head bobbing, paralysis, ataxia	3
PbTx-3-exposure control	6 hr	—	Significant muscle twitching, circling, head bobbing, paralysis, ataxia	2
PbTx-3-exposure control	24 hr	—	Some paralysis and ataxia, lethargy	3
Sham control + ILE	1 hr	100	Normal behavior	3
PbTx-3 + ILE	1 hr	50	Reduced ambulation, moderate twitching, slight head bobbing	3
PbTx-3 + ILE	1 hr	100	Reduced ambulation, mild twitching, slight head bobbing	4
ILE + PbTx-3	1 hr	100	Mild to no twitching	4
PbTx-3 + ILE	6 hr	50	Normal ambulation, slight lethargy	2
PbTx-3 + ILE	6 hr	100	Normal ambulation, no lethargy	4
PbTx-3 + ILE	24 hr	50	Normal ambulation, no lethargy	2
PbTx-3 + ILE	24 hr	100	Normal ambulation, no lethargy	4
ILE + PbTx-3	24 hr	100	Normal ambulation, no lethargy	4

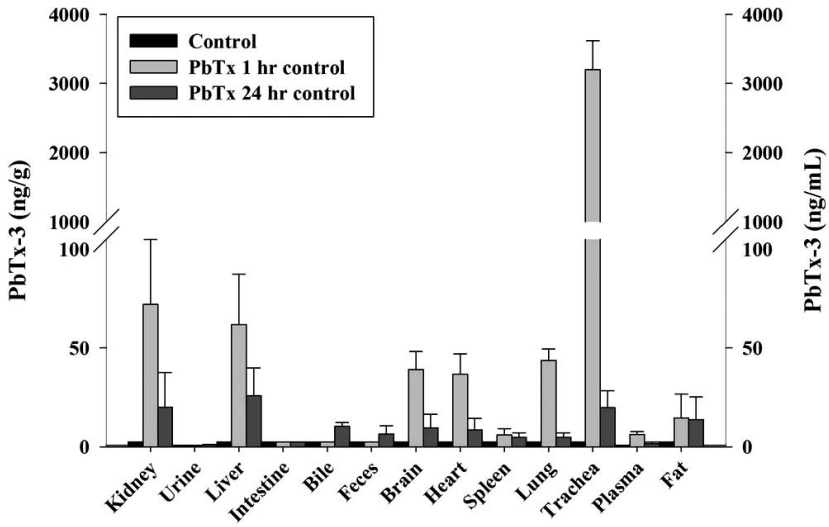

Figure 1. Distribution of brevetoxin (ng PbTx-3 eq. per g or ml) in the tissues and fluids of *Trachemys scripta* collected for sham controls, 1-hr post-PbTx-3 controls, and 24-hr post-PbTx-3 controls. PbTx-3 was administered one time via intratracheal instillation (10.53 μg/kg). Error bars represent standard error of the mean. There was no significant difference ($P < 0.05$) between the 1-hr and 24-hr exposure groups in any of the individual tissues or fluids due to the high variability of the data. Samples in which PbTx-3 was not detectable, including all sham controls, were given a value of half the level of detection (2.5 ng/g of tissue or ml bile or 0.5 ng/ml fluid) to calculate the mean.

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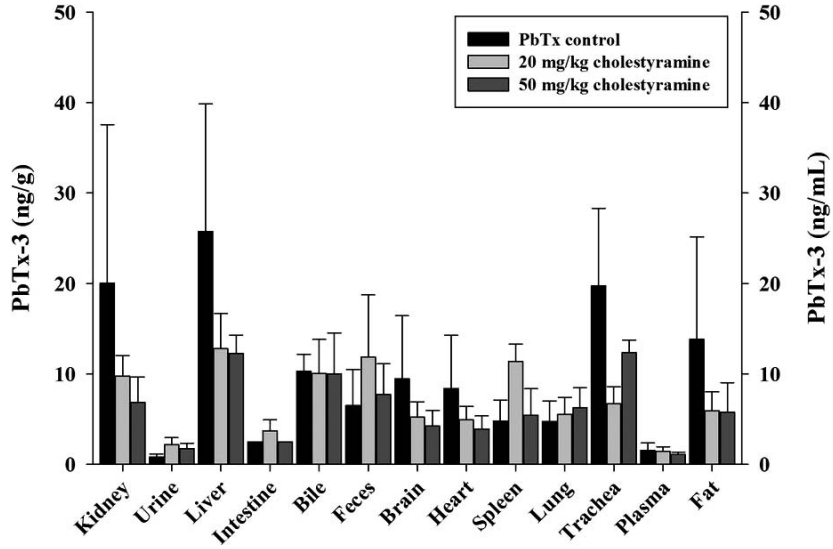

Figure 2. Distribution of brevetoxin (ng PbTx-3 eq. per g or ml) in the tissues and fluids of *Trachemys scripta* collected 24 hr post PbTx-3 (exposure controls) and PbTx-3 + cholestyramine (20 mg/kg or 50 mg/kg) treatments. PbTx-3 was administered one time via intratracheal instillation (10.53 μg/kg) and cholestyramine was given 2 hr and 6 hr post PbTx-3. Error bars represent standard error of the mean. There was no significant difference ($P < 0.05$) between the PbTx-3 and cholestyramine treatment groups in any of the individual tissues or fluids. Samples in which brevetoxin was not detectable were given a value of half the level of detection (2.5 ng/g of tissue or ml bile or 0.5 ng/ml fluid) to calculate the mean.

PbTx-3 + ILE treatment

Animals that received the ILE treatment (100 mg/kg) 30 min after PbTx-3 exposure had greatly reduced symptoms of brevetoxicosis within the first 1-2 hr compared with animals that did not receive the treatment (PbTx-3-exposure controls); in some individuals improvement was noticed within the first 10 min post ILE treatment (Table 2). By 6 hr post toxin exposure, untreated animals still displayed the same clinical signs as they did upon initial toxin administration (twitching, limb rigidity, ataxia), whereas ILE-treated animals were no longer showing those deficits and were moving around their tanks actively and in a coordinated manner. Intralipid was also tested at a dosage of 50 mg/kg, and while toxin impacts were reduced in comparison with untreated PbTx-3 exposure controls, the animals responded better to the 100 mg/kg dose, with an earlier and greater reduction in signs of brevetoxicosis.

ILE + PbTx-3 treatment

Animals that received the ILE treatment 30 min prior to PbTx-3 showed little to no symptoms of PbTx-3 exposure. Animals initially had slight changes in motor responses but were moving around and appeared clinically normal within the first hour.

Tissue distribution of PbTx-3

Aside from the reduced clinical signs associated with the ILE treatments, the tissue distribution of PbTx-3 in ILE-treated animals versus PbTx-3-only animals was also investigated. As in control PbTx-3-exposure animals, tissues and fluid analysis showed there was distribution to most organ systems within the first 1 hr post exposure to PbTx-3 (Fig. 3), with movement into the detoxification and excretory systems over 24 hr (Fig. 4). While not significantly different ($P < 0.05$) due to the high variability of tissue toxin levels in control PbTx-3 exposures, there is clearly an increase in PbTx-3 in the bile and feces 24 hr post ILE treatment compared with nontreatment PbTx-3-exposed animals (Fig. 4), suggesting that the toxin is clearing at a quicker rate post ILE. PbTx-3 concentrations in the kidney, liver, brain, and heart decreased more at the higher ILE dose of 100 mg/kg compared with the lower dose of 50 mg/kg 1 hr post toxin exposure (Fig. 5); 24 hr after toxin exposure, concentrations were higher in the detoxification and excretion organs with the 100 mg/kg ILE treatment versus the 50 mg/kg dose of ILE (Fig. 6). Together these results

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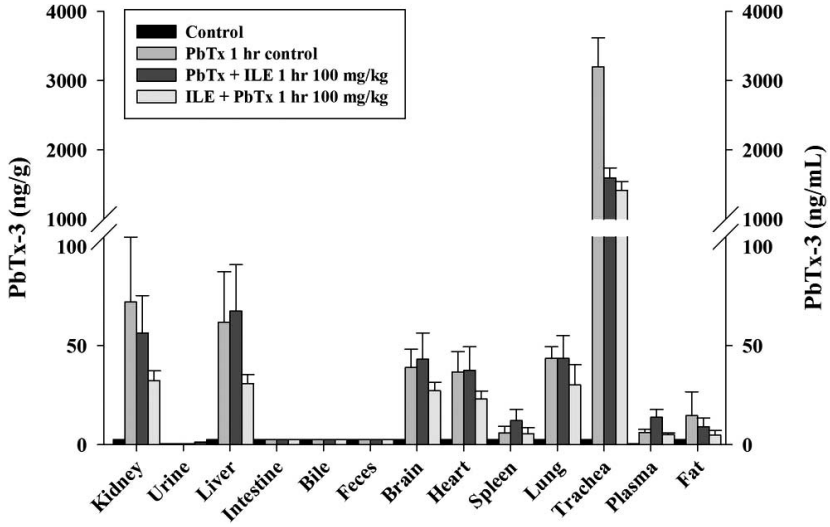

Figure 3. Distribution of brevetoxin (ng PbTx-3 eq. per g or ml) in the tissues and fluids of *Trachemys scripta* collected for intravenous lipid emulsion (ILE, 100 mg/kg) sham controls, 1-hr post-PbTx-3-exposure controls, 1-hr post-PbTx-3 + ILE (100 mg/kg, dosed following exposure), and for ILE (100 mg/kg) + PbTx-3 (dosed prior to exposure). PbTx-3 was administered one time via intratracheal instillation (10.53 μg/kg). Error bars represent standard error of the mean. There was no significant difference ($P < 0.05$) between the PbTx-3-exposure controls and the ILE-treatment groups in any of the individual tissues or fluids. Samples in which brevetoxin was not detectable was given a value of half the level of detection (2.5 ng/g of tissue or ml bile or 0.5 ng/ml fluid) to calculate the mean. ILE control exposures were all below the level of detection.

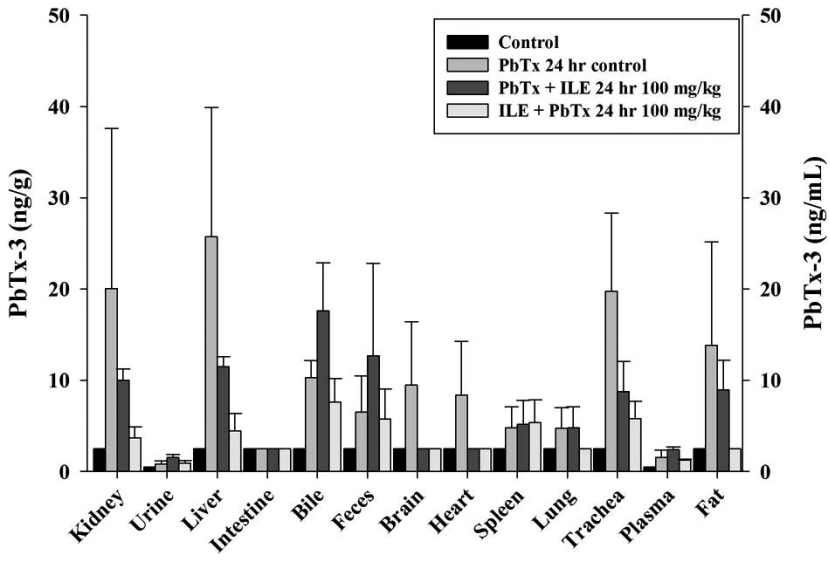

Figure 4. Distribution of brevetoxin (ng PbTx-3 eq. per g or ml) in the tissues and fluids of *Trachemys scripta* collected for intravenous lipid emulsion (ILE) sham controls, 24-hr post-PbTx-3 controls, and for 24-hr post-PbTx-3 + ILE (dosed following exposure) and ILE + PbTx-3 (dosed prior to exposure) where ILE was given at a 100 mg/kg dose. PbTx-3 was administered one time via intratracheal instillation (10.53 μg/kg). Error bars represent standard error of the mean. There was no significant difference ($P < 0.05$) between the PbTx-3-exposure controls and the ILE-treatment groups in any of the individual tissues or fluids. Samples in which brevetoxin was not detectable was given a value of half the level of detection (2.5 ng/g of tissue or ml bile or 0.5 ng/ml fluid) to calculate the mean. ILE control exposures were all below the level of detection.

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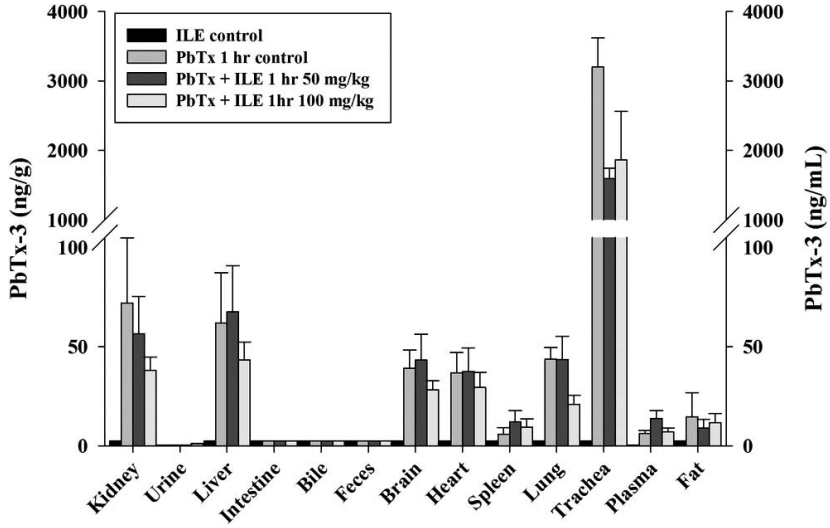

Figure 5. Distribution of brevetoxin (ng PbTx-3 eq. per g or ml) in the tissues and fluids of *Trachemys scripta* collected for intravenous lipid emulsion (ILE) sham controls, 1-hr post-PbTx-3 controls and 1-hr post-PbTx-3 + ILE (50 mg/kg and 100 mg/kg). PbTx-3 was administered one time via intratracheal instillation (10.53 μg/kg). Error bars represent standard error of the mean. There was no significant difference ($P < 0.05$) between the PbTx-3-exposure controls and the ILE-treatment groups in any of the individual tissues or fluids. Samples in which brevetoxin was not detectable was given a value of half the level of detection (2.5 ng/g of tissue or ml bile or 0.5 ng/ml fluid) to calculate the mean. ILE control exposures were all below the level of detection.

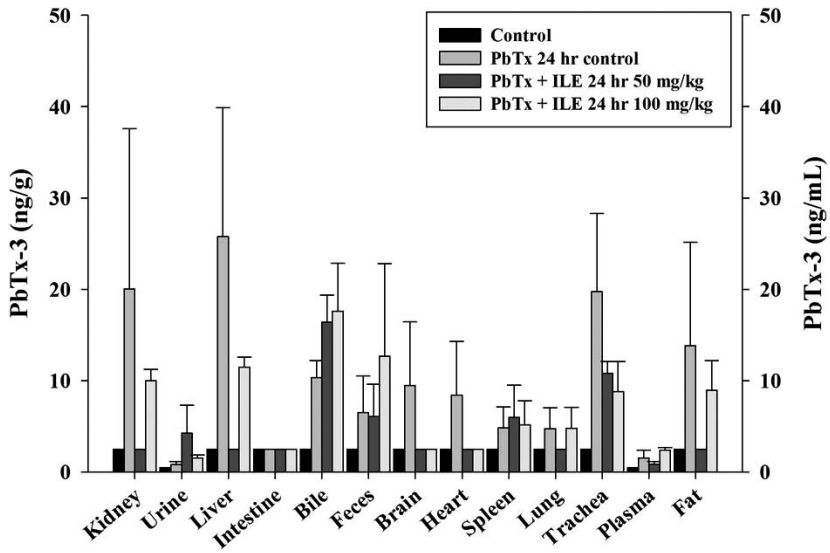

Figure 6. Distribution of brevetoxin (ng PbTx-3 eq. per g or ml) in the tissues and fluids of *Trachemys scripta* collected for intravenous lipid emulsion (ILE) sham controls, 24-hr post-PbTx-3 controls and 24-hr post-PbTx-3 + ILE (50 mg/kg and 100 mg/kg). PbTx-3 was administered one time via intratracheal instillation (10.53 μg/kg). Error bars represent standard error of the mean. While toxin levels were clearly lower with either ILE treatment 24 hr post-PbTx-3 exposure, the differences were not significant ($P < 0.05$) in any of the individual tissues or fluids due to the high variability of the PbTx-3 control values 24 hr post exposure. Samples in which brevetoxin was not detectable were given a value of half the level of detection (2.5 ng/g of tissue or ml bile or 0.5 ng/ml fluid) to calculate the mean. ILE control exposures were all below the level of detection.

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suggest that the higher ILE dose of 100 mg/kg is more effective in clearing out PbTx-3 from tissues than the 50 mg/kg dose of ILE. In animals in which ILE was administered prior to the toxin, toxin concentrations were lower overall in all tissues and fluids tested (Figs. 3, 4), although these differences were not statistically significant ($P < 0.05$).

DISCUSSION

Brevetoxicosis is a serious health hazard for animals that inhabit areas where HABs occur. Blooms can last for weeks to months, and animals may be affected even after a bloom dissipates due to vectors still carrying the toxin. In one mass mortality event of Florida manatees (*Trichechus manatus latirostrus*) and bottlenose dolphins (*Tursiops truncatus*) that occurred months after a red tide bloom disappeared, postmortem studies determined deaths were likely related to toxin still present at high levels in fish and on seagrasses.[11,13] This potential for biomagnification and bioaccumulation also likely affects sea turtles; PbTx-3 concentrations have been reported in the tissues of necropsied animals greater than 100 ng/g and in the feces of up to 61,078 mg/g.[9] Sea turtles may be highly susceptible to brevetoxicosis because many species prey on filter-feeding invertebrates that can accumulate brevetoxin, while green sea turtles (*Chelonia mydas*) graze on seagrasses that also may accumulate an algae coat.[4,13] Sea turtles may also inhale toxins aerosolized near the sea surface; both loggerhead and green turtles have large tidal volumes.[20] Mass mortalities of hundreds of sea turtles thus have been linked to HABs, with increased strandings resulting in more admissions to rehabilitation facilities.[9]

Animals taken to rehabilitation facilities after exposure to brevetoxicosis exhibit behaviors identical to those observed in these experiments, including jerky body movements, ataxia, and unresponsiveness.[9] Mammals experimentally exposed to high doses of PbTx-2 (up to 100 μg/kg) show similar symptoms, along with irregular heart rates and other cardiac effects, ventilation changes, and decreased body temperatures.[29] It is not surprising that the behaviors resulting from brevetoxin exposure are similar in reptiles and mammals, because it has been shown that the mode of action is similar; PbTx-3 binds to and opens voltage-gated sodium channels and thus depolarizes electrically active tissues.[3,7] In the laboratory, behavioral deficits after PbTx-3 exposure are severe but decrease rapidly over 24 hr as the toxin is cleared by the detoxification and excretion systems.[6]

However, treatments for sea turtles that are suspected of suffering brevetoxicosis have had mixed results, with some success utilizing dehydration therapy in cases of edema, but otherwise consisting primarily of supportive care.[9,21] The degree of exposure for wild animals is largely unknown, while clearance rates can only be determined from blood samples and vary widely. In some sea turtles that died in rehabilitation, brevetoxin levels in the tissues and fluids were lower than in animals that survived, with clearance taking from 5 to 80 days.[9] While the highest brevetoxin concentrations in necropsied sea turtles were found in the feces, stomach, and liver, histopathology revealed no definitive pathological lesions, as also was noted in laboratory-exposed *T. scripta*.[6,9] The lack of such lesions emphasizes the difficulty in analyzing the distribution and effects of brevetoxin in sea turtles. Thus, a series of studies were performed to determine initial organ system impacts and rates of clearance post PbTx-3 exposure both orally and intratracheally using the *T. scripta* as a model in order to devise better treatment strategies.[6,7]

In the current study one intratracheal dose of brevetoxin was administered; animals began experiencing severe signs of brevetoxicosis within 2–5 min that lasted for greater than 6 hr post exposure; symptoms decreased between 6 and 24 hr as the toxin cleared. Inhalation exposure was selected for these experiments as the impacts are evident much more rapidly than in animals exposed by toxin ingestion (and occur at a lower dose), which made the effects of potential treatments more immediately obvious.[6] The previous studies showed that PbTx-3 distributes to all organ systems, after both oral and intratracheal exposure. PbTx-3 concentrations in the bile and feces, post oral and IT exposure, increased over 48 hr, while other tissues showed a rapid decline in toxin concentrations.[6] Due to this demonstrated increase of PbTx-3 in the bile over time, cholestyramine was first tested as a potential treatment, on the hypothesis that it would bind PbTx-3 in the bile and increase the rates of clearance compared with control animals. However, the results from this study did not show any significant changes in bile PbTx-3 levels over a 24-hr period. While the kidney, liver, heart, lung, and brain did show a decrease in PbTx-3 over 24 hr, these differences were not significant ($P < 0.05$) compared with the PbTx-3 control groups. In this study, though, the turtles were only given one

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dose of PbTx-3 prior to cholestyramine treatments and the levels in the tissues were not as elevated following a single dose (Fig. 1) as they were following multiple doses; in the previous studies turtles received 12 doses over a 4-wk period.[6] Repeated dosing over a 4-wk period resulting in higher final toxin levels in the tissues and fluids may provide a better test of PbTx-3 clearance rates over time post cholestyramine. In addition, mammalian protocol was followed with either 20 mg/kg or 50 mg/kg doses given at the same time points (1 hr and 6 hr) post toxin exposure, but as the PbTx-3 dose employed in the turtle studies was considerably higher than is used in mammalian laboratory studies, it is also possible that the cholestyramine doses were not high enough to see clear effects on clearance.[6,26] Additional doses of cholestyramine, or higher dosages, may prove more effective and should be tested in future studies. Based on tissue levels in necropsied animals, sea turtles in the wild are exposed to even higher toxin levels than under these controlled laboratory exposures, thus cholestyramine could still be a possible treatment for animals entering rehabilitation facilities suffering from brevetoxin exposure.[9]

Turtles exposed to PbTx-3 experimentally in this study showed obvious behavioral deficits symptomatic of brevetoxicosis for at least 10–12 hr post exposure, but generally recover within 24 hr except for persistent lethargy. As the toxin has been shown to distribute to all tissues including such critical organs as the brain, liver, kidney, and heart, ILE was tested as a potential treatment strategy to remove toxins from the tissue. ILE has been used successfully in veterinary practices to treat cases of toxin ingestion in domestic animals. For example, in cats exposed to permethrins, which are neurotoxins that also act on sodium channels and cause ataxia, tremors, and seizures, ILE treatment reduced clinical symptoms and restored normal vital signs within a 24-hr period.[8] Unlike cholestyramine, posttoxin administration of ILE provided rapid amelioration of neurological and muscular deficits and increased toxin elimination compared with animals that did not receive ILE, while in experiments where ILE was administered prior to the toxin, virtually no signs of brevetoxicosis presented. Signs of brevetoxin exposure declined dramatically over a 6-hr period; by 24 hr post PbTx-3 + ILE, the turtles were moving normally within their aquaria. The rapid recovery of ILE-treated animals was reflected in the tissues and fluids post ILE treatment as well, which, though not significant ($P < 0.05$) due to the variability of the data, showed an increase in PbTx-3 in the bile and feces over a 24-hr period of time compared with non-ILE-treated animals (Fig. 4). The intravenous lipid emulsion may thus be helping the toxin clear at a quicker rate by drawing it out of the tissues for excretion. The reduction of behavioral deficits and increased rates of clearance were visible in animals treated with both 50 and 100 mg/kg ILE, but the improvements were more dramatic at the higher dose and clearance appeared to be greater as well (Fig. 5).

While not practicable from a rehabilitation standpoint, the efficacy of ILE as a protective agent against PbTx-3 was also tested by administering the treatment 30 min prior to the toxin. The administration of Intralipid before brevetoxin resulted in overall lower concentrations in all tissues and fluids tested (Figs. 3, 4) compared with control animals or those receiving ILE after toxin exposure, suggesting that ILE treatment prior to the toxin prevents it from collecting in the tissues at as high a concentration compared with animals given ILE post PbTx-3. Behavioral symptoms were likewise nearly absent in animals in which ILE was administered prior to the toxin, reinforcing its potential as an aid to detoxification in rehabilitation facilities. Thus, while the cholestyramine treatment was unsuccessful in increasing clearance in these experiments, ILE for brevetoxicosis in *T. scripta* was highly successful in reducing toxin symptoms and thus has the potential to positively impact survival in turtles; we anticipate that this treatment will be implemented at rehabilitation centers worldwide for toxin-exposed sea turtles.

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