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ACUTE TOXICITY AND BIOACCUMULATION OF TRIBUTYLTIN IN TISSUES OF *Urolophus jamaicensis* (YELLOW STINGRAY)

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Tributyltin oxide (TBTO) is the main constituent of tin-based antifouling marine paint used on the hulls of ships to prevent the growth of fouling organisms. TBTO was shown to be hazardous to nontarget organisms. The stingray, Urolophus jamaicensis*, may represent the ideal study organism for the adverse effects of TBTO to elasmobranches. This study investigated the toxicity and accumulation of tin in the gill tissue of the stingray* U. jamaicensis *after acute exposure to TBTO. This work demonstrates the alterations in the morphological architecture of the gill using electron and light microscopy, the induction of stress proteins, and peroxidative damage in response to tributyltin (TBT) exposure. A captured population of* U. jamaicencis *was housed in isolated, static tank systems. After a minimum 30-d acclimation period, the animals were exposed to one of 5 experimental doses of TBTO (4 μg/L, 2 μg/L, 1 μg/L, 0.5 μg/L, or 0.05 μg/L). A sixth group served as a control population. At 3h following treatment, animals were sacrificed and gill tissue was extracted, processed, and stored for analysis. Results indicate that* U. jamaicensis *is hypersensitive to TBT exposure. The elasmobranch gill showed a distorted, swollen epithelium with exfoliation following acute exposure to as little as 0.05 μg/L TBTO for 3 h. Graphite furnace atomic absorption spectroscopy (GFAAS) results indicate that tissues of treated animals contained a significantly increased tin concentration as compared to controls. Western blot analysis demonstrated the induction of the stress proteins Hsp 70 and HO1. 4-Hydroxynonenol (4HNE) adduct formation determined by Western blot analysis provides further evidence that observed membrane degradation is a result of lipid peroxidation.*

Heavy metals deposited in the environment pose a serious threat to global ecosystems. Organometallic compounds are an increased risk due to their potential reactivity, bioconcentration, and bioaccumulation. Tributyltin (TBT) compounds are used by industry in a variety of applications for their biocidal properties. These involve uses in agriculture, water treatment, textiles, and breweries (Fent & Muller, 1991). Tributyltin compounds are also involved in the manufacture of marine antifouling paint, as a stabilizing resin in PVC pipe, disinfectants, and wood preservatives, and in food storage containers. As a constituent of marine paint, tributyltin oxide (TBTO) is used to inhibit the growth of fouling organisms that would otherwise readily attach to the hulls of ships, piers, and buoys. Tin-based antifouling paints are formulated to leach

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TBTO at a constant rate as the surface paint undergoes hydrolysis by seawater (Bennett & Zelder, 1966).

Mounting evidence of the threat of TBTO toxicity in the marine environment led to the Anti-fouling Paint Control Act of 1988, limiting the use of TBT antifouling paints to commercial ships greater than 25 m (82 f) in length. Reports of significantly elevated concentrations of TBT in both water column and global sediments indicate that even in the presence of safer alternatives, TBT is still in use. Although reports of TBT contamination have steadily decreased in many areas due to regulations, there are many ports and harbors that have moderate to high levels of TBT in sediments as well as in the water column. Examples of observed TBT levels in global marine water samples include: 200 ng/L, Mediteranean Sea, Corsica (Michel et al., 2001); 242 ng/L, Mondavi estuary, India (Bhosle et al., 2004); 610 ng/L, Yam O, Japan (Cheung et al., 2003); 3.20 μg/L, Singapore (Basheer et al., 2002); and 14.7 μg/L, Bahrain (Alzieu, 1998). Global sediment levels are much higher; some examples include: 670 ng/g, São Paulo, Brazil (Godoi et al., 2003); 560 ng/g, Yam O, Japan (Cheung et al., 2003); 5.0 μg/g, Barcelona, Spain (Diez et al., 2002); and 340 μg/g, Great Barrier Reef, Australia (Haynes & Loong, 2002).

Under normal salinity and pH conditions in the marine environment, TBT exists primarily in a hydro-chelated form (Alzieu, 1998). The bioavailability of TBT to marine organisms is highest in this form. Studies have shown that TBT sequestered in top sediment layers may diffuse to the water column. Stang and Seligman (1987) reported diffusion of TBT from sediment to water column in Pearl Harbor, Hawaii. These observations were supported by a recent study by Burton et al. (2005), who report pore water concentrations of released TBT from the 0–6 cm of sediment with a total TBT burden of 220 to 8750 μg/kg to range from 0.05 to 2.35 μg/L. Once introduced into the environment, TBT may persist in area sediment from 5 to over 20 yr (Clarkson, 1991). Even after the proposed worldwide ban of TBT by the World Maritime Organization in 2003, the effects of TBT use will last for decades.

TBTO has low water solubility and binds strongly to suspended material, eventually precipitating to bottom sediment. The high lipid solubility of TBT allows for rapid membrane permeability and affects the intracellular environment, inducing cytotoxicity (Gadd, 2000). Organotin has a high specificity for the hippocampus, and was found to elevate reactive oxygen species (ROS) in the hippocampus of treated rats (LeBel et al., 1990). Organotin-induced immunotoxicity was attributed to the elevation of ROS, resulting in apoptosis in the thymus (Raffray et al., 1993).

TBT exposure was shown to be involved in endocrine disruption, immune suppression, and infertility in a variety of species (Blaber, 1970; Evans et al., 2000; Mensink et al., 2002; McAllister & Kime, 2003; Vos et al., 1990). The deleterious effects seen in invertebrates, including mussels, clams, oysters, and lobsters, include shell thickening, growth abnormalities, infertility, and death with exposure to as little as 0.08 μg/L of dissolved TBT (Huggett et al., 1992).

Heavy metals catalyze the cleavage of hydrogen peroxide via the Fenton reaction, producing the hydroxyl radical and labile metal complexes. High-valence metal oxidants are the most reactive in biological systems (Koppenol, 1994). The production of hydroxyl radicals ($HO^{\bullet}$) by metal ions leads to the disruption of lipid membranes (Roméo & Gnassia-Barelli, 1997). A series of hydrogen abstraction reactions results in the generation of lipid peroxyl ($LOO^{\bullet}$) and lipid alkoxyl ($LO^{\bullet}$) radicals, producing membrane degradation. These reactions are thought to be the driving force in lipid peroxidation. In addition to lipid radical formation, reactive aldehydes including 4-hydroxynonenal (4HNE) are a unique by-product of lipid peroxidation.

The toxicity of TBTO in marine fish is variable. LC_{50} values were shown to range from 1.5 to 36 μg/L (IPCS, 1990). Marine teleosts such as *Cyprinodon variegatus* (sheepshead minnow) showed 40% necrosis in gill tissue after 28-d exposure to 4 μg/L TBTO (Bryan & Gibbs, 1991). A study in *Platichthys flesus* (flounder) revealed that a concentration of 17.3 μg/L produced mortality after 7 to 12 d (Grinwis et al., 1997).

Recent studies comparing the accumulation of metals in tissues of marine teleosts and elasmobranchs indicate susceptibility of the elasmobranch gill to metals. It was shown that the elasmobranch gill accumulates water-borne copper 40 to 50-fold, as compared to a 3-fold increase in the sculpin (Grosell et al., 2003). Similar results were demonstrated in the exposure of the elasmobranch *Squalus acanthias* to silver when compared to marine teleosts (De Boeck et al., 2001). Elasmobranchs possess significantly lower antioxidant enzyme activities and a reduced capacity to neutralize hydroxyl radicals (Gorbi et al., 2004).

The elasmobranch *Urolophus jamaicensis* (yellow stingray) inhabits the Caribbean Sea where commercial fishing and cruise ship activity, a primary source of TBT, is high. Stingrays inhabit the sediment in relatively shallow bays and harbors, where TBT levels are known to be elevated. Apex predators such as sharks and rays have a slow reproduction rate and are sensitive to environmental changes. Elasmobranchs play an important role in the health and stability of the marine ecosystem. The pressures of overfishing, habitat disruption, and competition for food sources have caused a global decline in some species of elasmobranchs. Chemical pollutants such as TBTO are an added threat that may be more devastating to these species, and over time may be irreversible. *Urolophus jamaicensis* may therefore represent an ideal indicator organism for the adverse effects of TBT to marine vertebrates.

The focus of this study was to demonstrate the effects of tin in the gill tissue of *U. jamaicensis* following acute exposure to TBTO. Morphological alterations in the architecture of gill tissue using electron and light microscopy were studied, as well as biochemical markers for cytotoxicity. Two biomarkers studied were the stress protein heme oxygenase (HO-1), which was shown to be induced following metal exposure, and Hsp70, a stress protein used to evaluate cellular response (Gilberti & Trombetta, 2000). In addition, alterations in oxidative defense were studied. The generation of 4HNE adduct formation,

unique to lipid peroxidation, was evaluated due to the relationship of altered oxidative defense and metal-induced toxic injury in fish (Stripp & Trombetta, 1994).

MATERIALS AND METHODS

Animals and Treatment

Individuals of a captured population of *U. jamaicencis* were housed in the Animal Care Center of St. John's University in isolated static tank systems, with individual filtration and 12-h photoperiod. Water quality parameters including pH, salinity, dissolved oxygen, and temperature were monitored daily. After a minimum 30-d acclimation period, the animals were exposed by water-column pulse exposure to 1 of 5 experimental doses of TBTO (4 μg/L, 2 μg/L, 1 μg/L, 0.5 μg/L, or 0.05 μg/L). TBTO was introduced directly to the water column of treatment tanks, each containing one organism. A sixth group was comprised of individual organisms housed in nontreatment tanks as a control population. Three hours following treatment, animals were sacrificed by decapitation, and gill tissue was removed, processed, and appropriately stored for analysis.

Light Microscopy

Tissue collected from animals for light microscopy was immediately fixed in 2% phosphate buffered (pH 7.4) glutaraldehyde for 3 h at 4°C, washed in buffer, and postfixed in 1% phosphate-buffered (pH 7.4) osmium tetroxide for 1 h at 4°C. Tissue was washed in buffer and dehydrated in a series of water/acetone mixtures to 100% acetone. The tissue was then infiltrated in LX112-Araldite, embedded in BEEM capsules, and placed in a 60°C oven for 4 d. Using a Dupont Sorvall Porter-Blum MT-1 ultramicrotome, tissue blocks were sectioned and the sections were collected and mounted on slides. The methylene blue/azure B-stained sections were viewed on an Olympus BH2 microscope and images were captured digitally using a Spot insight camera.

Transmission Electron Microscopy

Tissue was processed as previously described for light microscopy, and thin sections were collected on 400 mesh copper grids. Tissue sections were stained with uranyl acetate followed by lead citrate, and observed using a Jeol JEM-1200EX transmission electron microscope (TEM) at 80 kV.

Scanning Electron Microscopy

Tissue collected from animals was immediately fixed in 2% phosphate-buffered (pH 7.4) glutaraldehyde for 3 h at 4°C, washed in buffer, and postfixed in 1% phosphate buffered (pH 7.4) osmium tetroxide for 1 h at 4°C. Tissue was washed in buffer and dehydrated in a series of water/acetone mixtures to 100% acetone. Tissue was critical-point dried using CO_2 as the transition

fluid. Specimens were mounted on steel stubs, attached with silver paint, and coated with 40 nm platinum. The tissue was observed using a goniometer stage on a Hitachi S-530 scanning electron microscope (SEM) at 25 kV coupled with Evex analytical software. Images were captured digitally.

Graphite Furnace Atomic Absorption Spectrophotometry

Tissues collected for GFAAS were immediately frozen in liquid nitrogen and stored at −80°C for analysis. Prior to analysis, tissues were lypholyzed for 48 h, weighed, and digested in 50% HCl (12 *N*), and slowly evaporated on a warming plate for 72 h. The sample was then resuspended in 2.0 ml of 6% HCl and .015 mg Pd + 0.01 mg $Mg(NO_3)_2$ (matrix modifier) for analysis. Tissues were analyzed for tin using a Perkin Elmer HGA-400 graphite furnace coupled with a Perkin Elmer 2380 atomic absorption spectrophotometer, at a wavelength of 286.3 nm, slit 0.7 nm, and an injection volume of 10 µl.

Analysis of Stress Proteins and Lipid Peroxidation

Western blot analysis was performed on gill tissue samples to demonstrate the induction of two stress proteins, heme oxygenase (HO-1) and Hsp70, using a method described by Gilberti and Trombetta (2000). Increase of 4-hydroxynonenol (4HNE) as a marker for lipid peroxidation was analyzed by Western blotting using a method described by Hardej and Trombetta (2002). Total protein concentrations were determined by the Bradford protein assay, and a total of 20 µg protein of each sample was loaded in all wells for analysis. All primary and secondary antibody concentrations were dilutions of 1:2000. Horseradish peroxidase-labeled secondary antibodies included in a chemiluminescent Western blotting kit from Amersham Biosciences allowed visualization of signal using an Alpha Innotech chemiluminescent imager.

Chemicals and Antibodies

All reagents and standards for tin analysis were purchased from Sigma-Aldrich, including a protease inhibitor cocktail used in the homogenizing buffer. Stress protein antibodies and standards were purchased from Stressgen, and the antibody for 4-hydroxynonenal was purchased from Alpha Diagnostic International.

Statistical Analysis

One-way analysis of variance (ANOVA) and two-tailed *t*-tests were performed to determine statistical significance among and between groups. All statistical analysis was performed using GraphPad Prism version 4.00 (GraphPad Software, Inc.). The criterion for significance was set at $p < 0.05$.

RESULTS

Results indicate that *U. jamaicensis* is hypersensitive to TBTO exposure. At all doses there were observable changes in behavior, swimming patterns, and

respiration rates. In the treatment groups of 1 μg/L TBTO and greater, labored respiration, convulsions, impaired swimming, and disorientation were observed within 15 min of exposure. It is important to note that in preliminary trials, animals exposed to 1 μg/L TBTO and greater died within 4 to 5 h following treatment. The 0.5-μg/L TBTO exposure resulted in death within 6 h, and the 0.05-μg/L TBTO exposure proved fatal 8 h following treatment.

The elasmobranch gill showed a distorted, swollen epithelium with exfoliation following acute exposure to as little as 0.05 μg/L TBTO for 3 h, as compared to control animals (Figures 1, 2, and 3). As seen in the micrographs of the highest and lowest treatment concentrations, the outer epithelium as well as internal membrane integrity is compromised. Photomicrographs demonstrate exfoliation of outer epithelial tissues, accompanied by loss of internal membrane integrity in gill tissues following 3-h exposure to as little as 0.05 μg/L TBTO. In doses greater than 1 μg/L the loss of outer epithelium is so extensive that basement membranes become exposed to the external environment. The internal cellular membranes are also compromised. The highly complex internal compartmentalization of the tissue within the lamellae is lost. This highly organized internal environment is achieved through the presence of an intricate network of membranes. The degradation of these membranes by lipid peroxidation following TBT exposure induces a rapid loss of function of the gills. The mitochondria-rich cells (MRC) of the gill tissue are responsible for gas exchange and osmotic balance. There is a loss of structure in the cristae and overall mitochondrial swelling within the cells of treatment animals. This is consistent with oxidative stress, resulting in the loss of ATP production leading to cell death.

To quantify the concentration of tin in the gill tissues following exposure, tissues were analyzed by graphite furnace atomic absorption spectrophotometry (GFAAS) (Figure 4). In addition, the accumulation of tin was measured in brain and blood samples following exposure to 4 μg/l TBTO for 3 h (Figures 5

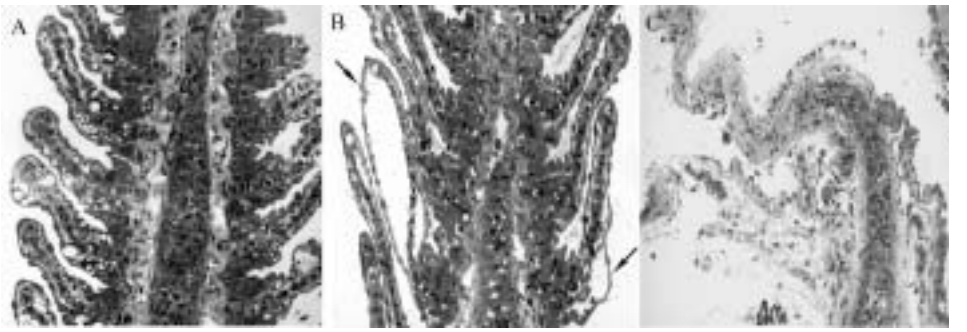

FIGURE 1. Light micrographs of control and treated stingray gill, magnification 20×. (A) Control, (B) 3-h treatment with .05 μg/L TBT, and (C) 3-h treatment with 4 μg/L TBT. Initial separation of outer epithelium is evident even in the lowest acute dose as shown by arrows in (B). In higher doses there is a complete exfoliation and loss of outer epithelium, and an overall loss of structure of lamellae as seen in (C).

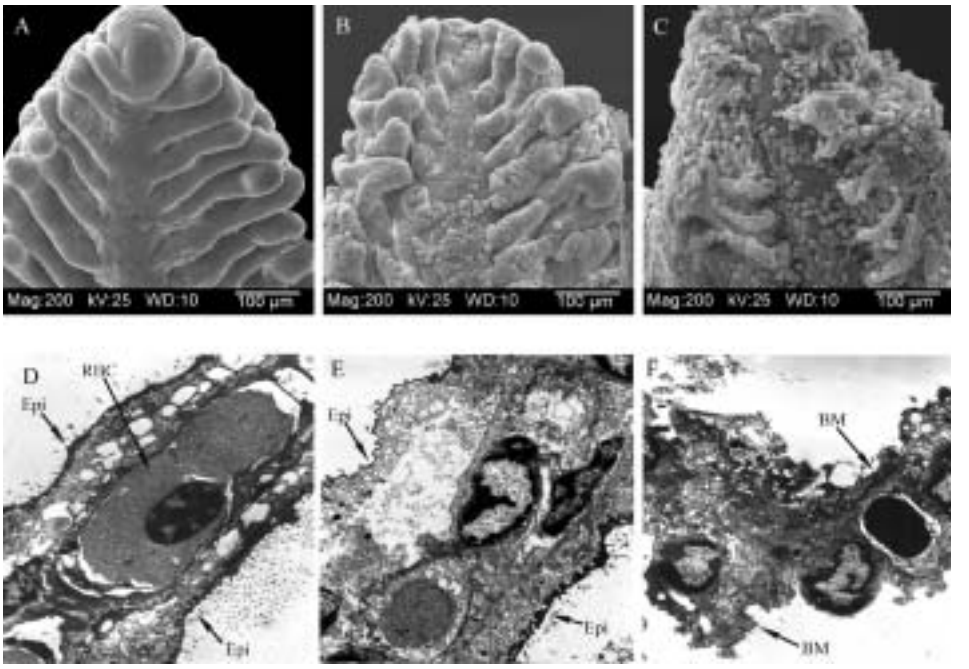

FIGURE 2. Photomicrographs of the gills of control and treated stingrays. (A, D) Controls; (B, E) 3-h treatment with 0.05 µg/L TBT; (C, F) 3-h treatment with 4 µg/L TBT. (A, B, C) Scanning electron micrographs, magnification 200×. (D, E, F) Transmission electron micrographs, magnification 7500×. There is significant exfoliation and swelling of the epithelium, and a loss of both internal and outer membrane integrity in all treatment groups. As seen in (C) and (F), exfoliation is to the extent that basement membranes are exposed. Epi, epidermis; RBC, red blood cell; BM, basement membrane.

and 6). Significant increases in tin concentration following exposure were observed in the tissues of all treatments groups when compared to control tissues.

Western blot analysis was performed to determine the induction of the stress proteins heme oxygenase (HO–1), and Hsp70 (Figure 7). HO-1 was absent in control tissues, and induced in all treatment groups. Hsp70 was present in both control and treatment groups, with an increase in expression in treated animals. Western blot was also used to determine 4-hydroxynonenal (4HNE) adduct formation (Figure 8). There was an increase in the formation of 4HNE adducts in all treatment groups.

DISCUSSION

The initial events leading to the progression of disease following exposure to environmental toxins play an important role in risk assessment. Cellular damage as a result of peroxidative stress is a critical early event that may lead

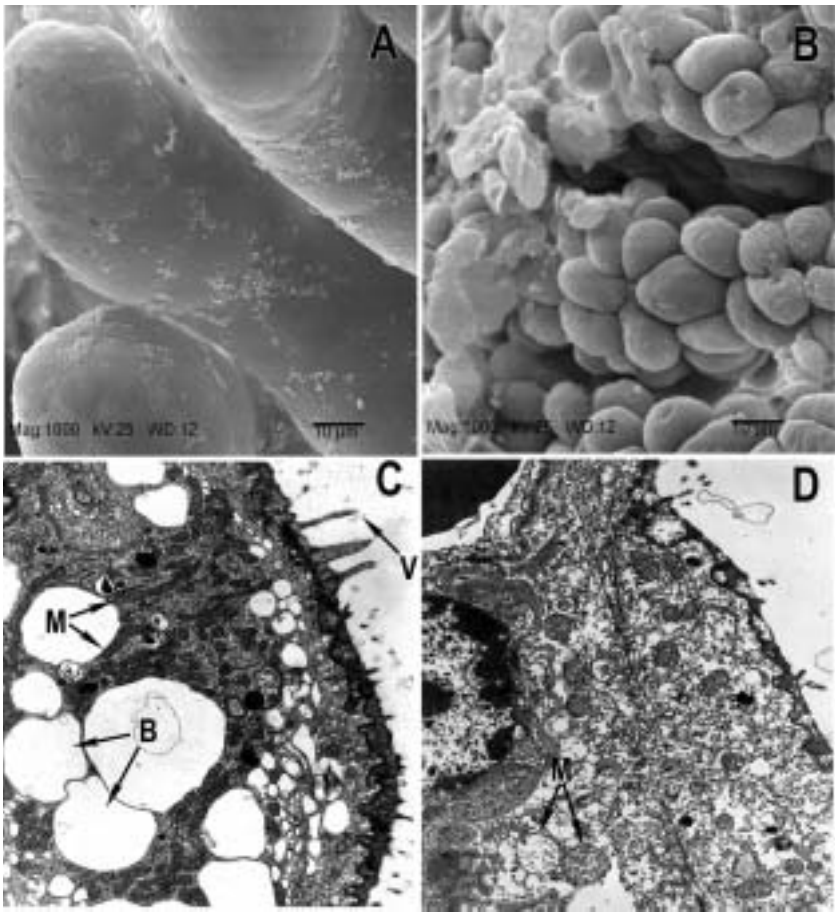

FIGURE 3. Scanning and transmission electron micrographs of the gills of control (panels A and C) and 3-h 4-μg/L TBT treated (panels B and D) stingrays. (A, B) Scanning electron micrographs, magnification 1000×. (C, D) transmission electron micrographs, magnification 7500×. As seen in (A) and (B), there is a marked change in the morphology of epithelial cells between controls and treated animals. The normally smooth, flat epithelium is swollen, and cells are rounded. In comparing (C) and (D), mitochondria (M) are swollen, and disrupted cristae are present in treated animals. The blood sinuses (B) as well as internal membrane compartments are compromised, and outer microvilli (V) are lost.

to pathology. The morphological changes observed in the gill tissues of *U. jamaicensis* support the hypothesis that acute exposure to tributyltin oxide results in lipid peroxidation. The exfoliation of outer epithelium, the disruption of internal membranes, and the swelling of mitochondria are consistent with peroxidative injury. To verify increased lipid peroxidation, Western blot analysis confirmed the production of the reactive aldehyde 4HNE. 4HNE adduct formation increased in treatment animals as compared to controls.

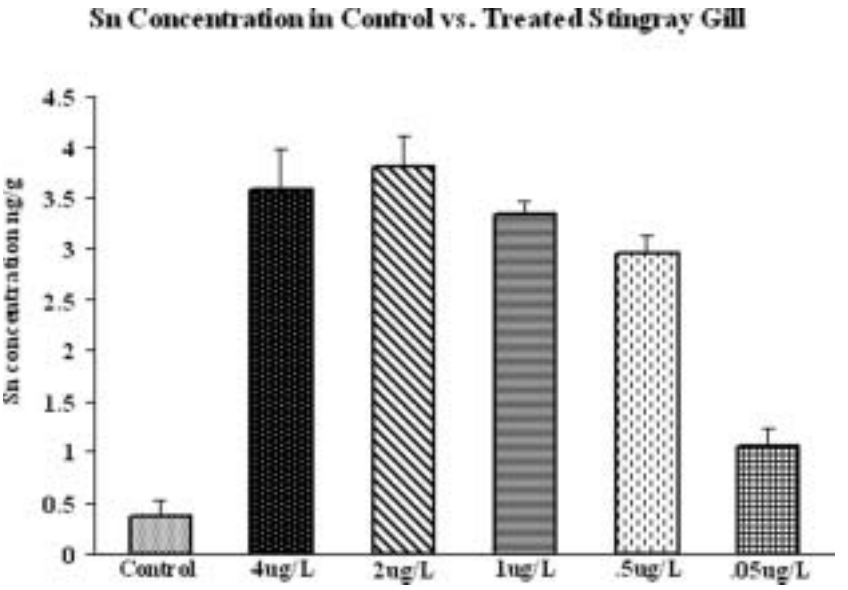

FIGURE 4. Tin (Sn) concentration (ng/g dry weight) in control and treated stingray gill tissues. There is a significant increase of Sn in the gills of all treatment groups following 3-h exposure to TBT ($n = 4$, $P < .05$).

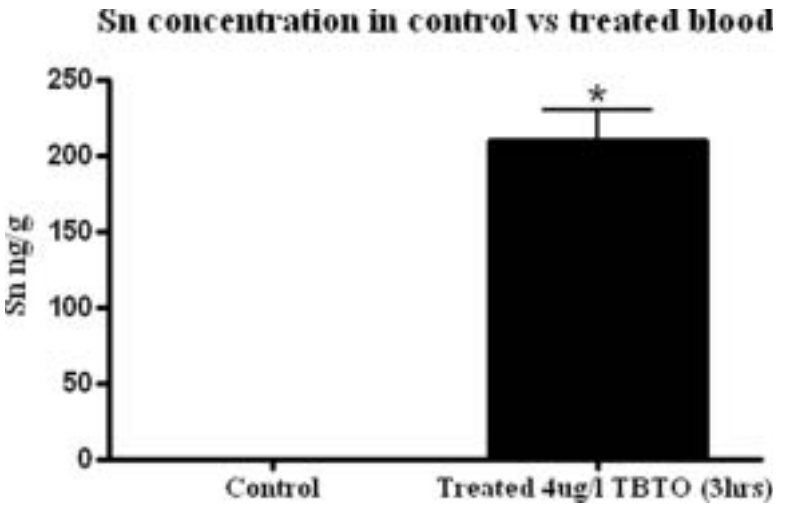

FIGURE 5. Tin (Sn) concentration (ng/g dry weight) in control and treated stingray blood. There is a significant increase in Sn concentration in the blood of treated animals compared to controls ($n = 4$). Asterisk indicates significant difference from control ($P < .05$).

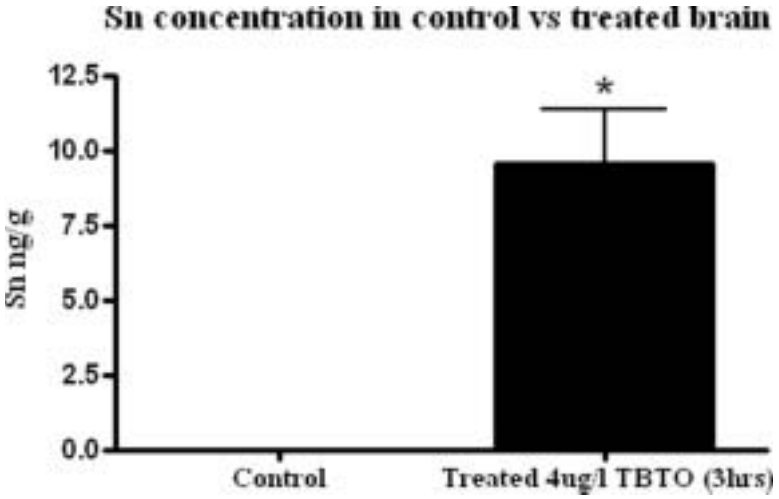

FIGURE 6. Tin (Sn) concentration (ng/g dry weight) in control and treated stingray brain tissue. There is a significant increase in Sn concentration in the brain of treated animals compared to controls ($n = 4$). Asterisk indicates significant difference from control ($P < .05$).

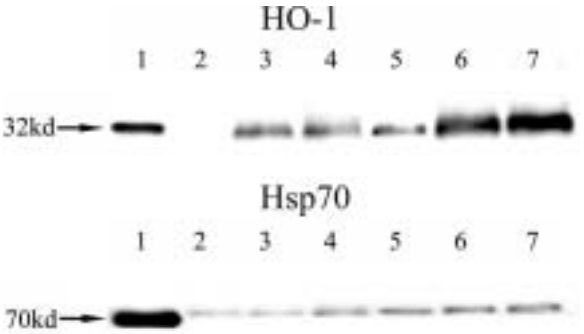

FIGURE 7. Western blot analysis of stress protein induction in the gills of control and treated stingrays. All sample protein concentrations equilibrated to 20 μg. Top: Heme oxygenase (HO-1); bottom: Hsp70. Lane 1, standard; lane 2, control; lane 3, 4 μg/L TBT treatment (3 h); lane 4, 2 μg/L TBT treatment (3 h); lane 5, 1 μg/L TBT treatment (3 h); lane 6, 0.5 μg/L TBT treatment (3 h); lane 7, 0.05 μg/L TBT treatment (3 h).

Graphite furnace atomic absorption spectroscopy (GFAAS) results demonstrate the rapid uptake of TBTO from the tank environment. The high levels of tin detectable in gill tissues of treated animals compared to controls confirm that the resulting tissue degradation is attributable to TBTO exposure. In addition to the accumulation of tin in gill tissues, there is a significant increase in tin concentration in both brain and blood following exposure. To further verify the uptake of TBTO, seawater and sediment samples were taken from each

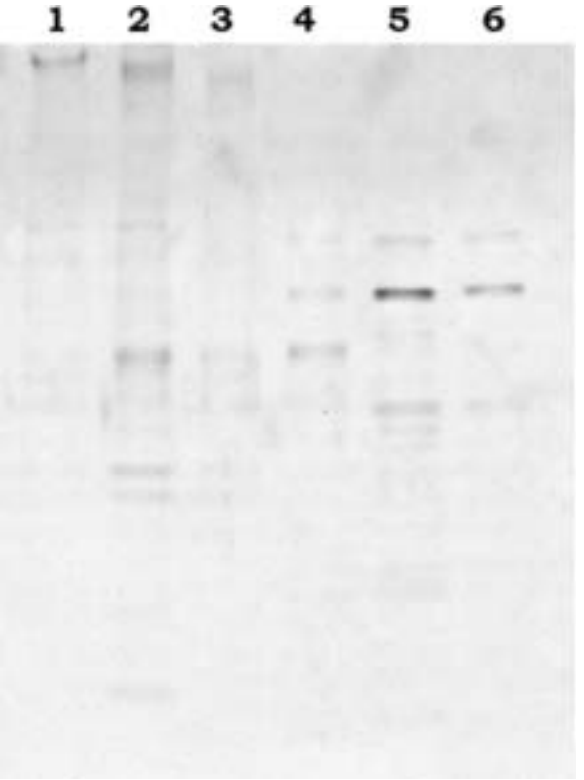

FIGURE 8. 4-Hydroxynonenal (4HNE) adduct formation detected by Western blot analysis. Lane 1, control; lane 2, 4 µg/L TBT treatment (3 h); lane 3, 2 µg/L TBT treatment (3 h); lane 4, 1 µg/L TBT treatment (3 h); lane 5, 0.5 µg/L TBT treatment (3 h); lane 6, 0.05 µg/L TBT treatment (3 h).

treatment tank following sacrifice and analyzed by GFAAS. There were no detectable levels of tin in any treatment tanks following exposure. This indicates rapid and almost complete uptake of TBTO from the ambient seawater.

The hypersensitivity of *U. jamaicensis* to low concentrations of TBTO is consistent with recent findings demonstrating a reduced cellular defense mechanism in elasmobranch species as compared to marine teleosts (Grosell et al., 2003; De Boeck et al., 2001; Gorbi et al., 2004). The evaluation and determination of the specific enzyme deficiencies that result in this increased susceptibility of elasmobranches to environmental toxins will result in a greater understanding of the antioxidant defense systems that have evolved in many marine and terrestrial organisms.

To identify common biomarkers following exposure to environmental toxins, and their correlation to the development of pathology, changes in the levels of the stress proteins heme oxygenase (HO-1) and Hsp70 were evaluated. These results are consistent with the hypothesis that an increase in stress

proteins would be observed following exposure to TBTO. It is important to note that although there seems to be less expression of these proteins at higher doses, this is probably due to the extreme membrane degradation. Released proteases degrade many of the existing proteins to such an extent that they can no longer bind to the specific antibodies that were used for detection. It is clear, however, that there is induction of HO-1 in all tissues from treatment groups as compared to controls, as well as an increase in the expression of Hsp70 in all treatment groups.

In determining the risk factors to nontarget marine and terrestrial organisms, including humans, it is necessary to determine the effects of TBT exposure in relation to species-specific defense mechanisms. This work demonstrates that organisms that have a decreased ability to defend against oxidative stress will be at greater risk from environmental toxins such as TBTO.

Further studies will focus on identifying additional early detection biomarkers for toxicity induced by TBTO exposure. In addition, the identification and characterization of the antioxidant response element (ARE) will be pursued to determine differences between elasmobranch and teleost species. Finally, the study of low-level chronic exposure of *U. jamaicensis* to TBTO will provide further insight into the mechanisms and pathology that accompany exposure.

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