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Early developmental toxicity of saxitoxin on medaka (*Oryzias melastigma*) embryos

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ABSTRACT

Saxitoxin (STX) is the most potent paralytic shellfish poisoning toxin in crustaceans and molluscs, and is known to cause intoxication to humans and marine animals due to its neurotoxicity. However, the extent of its early developmental toxicity to marine species remains unknown. In this study, we examined the early developmental toxicity of STX using marine medaka (*Oryzias melastigma*) embryos as model. The medaka embryos were exposed to STX for four days, from the early blastula stage onwards, and this exposure period covered the main developmental stage of the central nervous system and somites. After exposure, the treated medaka eleutheroembryos at 15 day post fertilization exhibited abnormal growth with longer body length and relatively smaller yolk sac size. High cell proliferation, neuron development, and metabolism were confirmed using whole-mount immunostaining and two-dimensional electrophoresis. In summary, STX disturbed the normal growth of medaka embryos probably by affecting the metabolic rate in the exposed medaka embryos.

1. Introduction

Paralytic shellfish poisoning (PSP) toxins, produced by harmful dinoflagellates, are a series of neurotoxins that accumulate mainly in shellfish and cause human intoxication through the intake of toxic mollusks. Vectors have been found involving clams, mussels, oysters, gastropods, cockles, fish, scallops, whelks, lobsters, copepods, crabs and so on, with a wide geographical distribution around the world (Kumar-Roiné et al., 2011). They are the commonest and the most lethal bio-toxins among the marine algal toxins, and pose serious public health threat in the affected area (Wong et al., 2011). Apart from humans, it affects marine fish and mammals as well, through either direct exposure or dietary uptake (Castonguay et al., 1997; Reyero et al., 1999; White, 1981). Among the PSP toxins, saxitoxin (STX) is the most potent. It is difficult to degrade and highly stable even after heating (Falconer and Humpage, 2005). The specific properties of STX make it a public health hazard. Pregnant women and young children are more vulnerable to STX. Various symptoms after direct exposure to STX, such as fever, eye irritation, abdominal pain, and skin rashes, have been found in infants and children (Rapala et al., 2005). When brain cells are destroyed and the endocrine is disrupted during the developmental stage

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in infants and children, the resulting dysfunction can be permanent and irreversible (Landrigan et al., 1999). Thus, it is important to study the early developmental toxicity of STX. Recent studies show that STX exposure results in edema and body curvature in zebrafish embryos, and sensorimotor impairments and paralysis in herring larvae (Lefebvre et al., 2004, 2005). STX is known to exert its neural toxicity by blocking the voltage-gated sodium channel in excitable cells, due to its high binding affinity to the soluble sites of sodium channels in the nervous system and muscles. Its neural toxicity may partly explain its toxic effects on the developing fish embryos and larvae, however, the knowledge on its developmental effects and relevant mechanisms is still limited.

Marine medaka (*Oryzias melastigma*), a small teleost fish, is a popular model fish widely used for the study of human diseases and marine toxins. Their embryos are easy to culture and have a relatively short generation time, which make them an appropriate marine model for rapid toxicity screening. Medaka embryos are optically clear, and their developmental stages are thoroughly characterized, and thus, are convenient for the observation of organogenesis and the developmental process (Shi and Faustman, 1989). Based on their morphological and pathological changes, medaka embryos have been successfully used to study the teratogenic effects of azaspiracid, one novel marine phycotoxin, and the developmental toxicity of okadaic acid, a diarrhetic shellfish poisoning toxin (Colman et al., 2005; Escoffier et al., 2007). Usually combined with pathological observations in model animals, the two-dimensional electrophoresis (2-DE) approach is widely used in toxicological studies to present a whole protein profile and elucidate molecular responses to toxicants. It has proved to be effective in identifying protein biomarkers with high resolution, statistical confidence and good compatibility with mass spectrometry. For example, in the study of the toxicity of freshwater microcystin-LR to zebrafish, 2-DE helped display diverse proteins responding to the toxin, and revealed the reactive oxygen species pathway might be the main toxic pathway (Wang et al., 2010). Therefore, to elucidate the molecular mechanism of STX on fish embryo development, we adopted 2-DE technique in this study.

Currently, there are limited studies on the developmental toxicity of STX on children and marine species. In order to reveal the developmental toxicity of STX, medaka fish embryos are adopted as the developmental model, and investigated using various techniques. This study will contribute to the knowledge on STX intoxication during developmental stages. Furthermore, the unique protein patterns at different developmental stages provide potential biomarkers for the rapid screening of marine toxins.

2. Materials and methods

2.1. Chemicals

Purified STX powder (≥95% in purity) was obtained from Prof. H. N. Chou (Institute of Fisheries Science, National Taiwan University, Taipei, Taiwan), and dissolved in Milli-Q water as the stock solution. An STX dihydrochloride standard (65 ± 3 μM) was purchased from the National Research Council Canada (CRM-STX-e, NRC Institute for Marine Biosciences, Canada) and stored at −20 °C. The concentration of STX in the stock solution was quantified using high performance liquid chromatography (Waters) with a fluorescence detector (Waters 2475).

2.2. Medaka fish maintenance and four-day consecutive static exposure

Adult medaka fish were maintained in a large aquarium (40 cm × 60 cm × 40 cm) filled with continuously aerated water at 26 °C with a 14 h:10 h light–dark cycle. They were fed with commercial dry feed twice per day. The medaka embryos were directly collected from the females. The embryos were examined using a dissection microscope, and normal fertilized medaka eggs at Stage 10, early blastula stage, 0 day post fertilization (dpf) were cleaned and transferred to a sterile 96-well cell culture plate for subsequent exposure experiments. Each well, which contained 10 embryos, received either 840 or 1260 μg/L of STX, or none. The dose adopted was that suggested to induce abnormalities in zebrafish larvae (Lefebvre et al., 2004). Fifteen replicates were set up for each concentration. The control and exposure media were renewed every two days, and exposure lasted for four days. Following the period of exposure, the control and treated embryos were transferred to a clean 6-well cell culture plate, and the embryos were collected at different developmental time points: 4, 8, 12 and 16 dpf (newly hatched larvae, which are also named eleutheroembryos) for the subsequent proteomic study.

2.3. Body length measurement and morphological observations

The measurement of body length was performed according to Cheng et al. (2007). The body length of the medaka embryos exposed to STX and control medaka embryos were measured at the 15th day of development. Ten embryos were randomly selected from each condition and anaesthetized with 0.016% (w/v) tricaine (Sigma). They were mounted in lateral view in a culture medium on clean slides, and then photographed with an Olympus disk scanning unit (Olympus, Tokyo, Japan). The pictures were then quantitatively analyzed with the public domain NIH Image program (developed by the US National Institutes of Health and publicly available on the Internet at http://rsb.info.nih.gov/nih-image/). Using this software, the overall body length of the embryos was manually quantified by point to point measurement. Meanwhile, early life parameters, such as the melanophores, dorsal fins and yolk sacs, were examined and photographed.

2.4. Whole-mount immunostaining

Neurogenesis and cellular proliferation at 4 dpf in the embryos exposed to 840 μg/L of STX were examined using whole-mount immunostaining, and performed according to Chen et al. (2011). Primary antibodies used included a proliferating cell nuclear antigen (PCNA) (DAKO, 1:1000) and Zn-12 (DSHB, 1:100). AlexaFluor 488-conjugated goat