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Transfer profile of intramuscularly administered tetrodotoxin to non-toxic cultured specimens of the pufferfish *Takifugu rubripes*

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ABSTRACT

Tetrodotoxin (TTX) was intramuscularly administered to non-toxic cultured specimens of the pufferfish *Takifugu rubripes* to investigate TTX transfer/accumulation profiles in the pufferfish body. In two groups of test fish administered either 50 MU/individual of TTX standard (purified TTX; PTTX) or crude extract of toxic pufferfish ovary (crude TTX; CTTX), TTX rapidly transferred from the muscle via the blood to other organs. The toxin transfer profiles differed between groups, however, from 4 to 72 h. In the PTTX group, little TTX was retained in the liver, and most (>96%) of the toxin remaining in the body transferred/accumulated in the skin after 12 h, whereas in the CTTX group, a considerable amount of toxin (15%–23% of the administered toxin or 28%–58% of the remaining toxin) was transferred/retained in the liver for up to 24 h, despite the fact that 89% of the remaining toxin transferred/accumulated in the skin at the end of rearing period (168 h). The total amount of toxin remaining in the entire body at 1–4 h was approximately 60% of the administered toxin in both groups, which decreased at 8–12 h, and then increased again to approximately 60%–80% at 24–168 h. Immunohistochemical observation revealed that the toxin accumulated in the skin was localized at the basal cells of the epidermal layer.

1. Introduction

The pufferfish *Takifugu rubripes*, as well as many marine pufferfish of the family Tetraodontidae, possess a potent neurotoxin, tetrodotoxin (TTX). In wild adult *T. rubripes*, the liver and ovary usually have strong toxicity, whereas the muscle, skin, and testes are non-toxic and are safe for human consumption (Noguchi and Arakawa, 2008). TTX is originally produced by marine bacteria, and distributed over a wide variety of animals other than pufferfish, including gobies, blue-ringed octopuses, carnivorous gastropods, starfish, toxic crabs, horseshoe crabs, flat worms, and ribbon worms (Miyazawa and Noguchi, 2001). The facts that pufferfish become non-toxic when fed non-toxic diets in an environment in which the invasion of TTX-bearing organisms has been eliminated (Matsui et al., 1982; Saito et al., 1984; Noguchi et al., 2006), and that such non-toxic pufferfish become toxic when orally administered TTX (Matsui et al., 1981; Yamamori et al., 2004; Honda et al., 2005; Kono et al., 2008a), indicate that TTX is exogenous in pufferfish and is derived from the food chain that starts from bacteria (Noguchi and Arakawa, 2008). The transfer, accumulation, and elimination mechanisms of TTX taken up into the pufferfish body via food organisms remain unclear. In our studies to clarify this point, we investigated the short-term transfer and accumulation profiles of TTX intramuscularly administered to non-toxic cultured specimens of *T. rubripes*. In oral administration

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experiments, long-term toxin accumulation is observed, but these experiments are not suitable for tracing short-term inter-tissue toxin transfer, because it is difficult to accurately administer a single large dose of toxin (Honda et al., 2005). To overcome this problem in the present study, we administered the TTX intramuscularly. Matsui et al. (1981) reported that when non-toxic cultured specimens of *T. rubripes* are fed diets containing crystalline TTX or crude toxic pufferfish ovary extract, only the test fish fed the crude extract of toxic pufferfish ovary accumulated TTX in their liver. Based on this information, we administered two types of toxins, 'purified TTX' and 'crude TTX', to evaluate whether the transfer profiles differed after entering the pufferfish body.

2. Materials and methods

2.1. Pufferfish specimens

Non-toxic cultured specimens of *T. rubripes* (approximately 4 months old; body weight, 13.2 ± 3.4 g; body length, 7.1 ± 0.6 cm; $n = 80$) (Noguchi et al., 2006) were purchased from a culture farm in Toishi, Nagasaki Prefecture, Japan. The specimens were acclimatized in aerated tanks for several days before administration of the toxin.

2.2. Preparation of toxin solutions

Toxic ovaries of the pufferfish *Takifugu vermicularis* were extracted with 1% acetic acid in 80% methanol, and the extract was defatted with dichloromethane and evaporated to make a condensed toxin solution (designated crude TTX). The toxicity of the crude TTX was evaluated using a mouse bioassay according to the official guidelines of the Japan Food Hygiene Association (2005). Lethal potency was expressed in mouse units (MU), where 1 MU was defined as the amount of toxin required to kill a 20-g male ddY strain mouse within 30 min after intraperitoneal administration. Liquid chromatography/mass spectrometry (LC/MS) analysis (Nakashima et al., 2004) revealed that the crude TTX was composed mainly of TTX and its analogs, such as 4-*epi*TTX and 4,9-anhydroTTX; TTX alone accounted for more than 90% of the total toxicity (data not shown).

Both TTX standards, purchased from Wako (purity > 90%; designated purified TTX) and crude TTX, were dissolved or diluted individually with a physiologic saline solution containing 1.35% NaCl, 0.06% KCl, 0.025% $CaCl_2$, 0.035% $MgCl_2$, and 0.02% $NaHCO_3$ at a concentration of 500 MU/ml and used in the following toxin administration experiments.

2.3. Toxin administration experiments

The acclimatized pufferfish specimens were divided into two groups of 40 individuals; one group was administered purified TTX (PTTX group) and the other was administered crude TTX (CTTX group). The groups were then maintained separately in two aerated 90-l tanks. Each fish was intramuscularly administered 0.1 ml (50 MU) of either purified or crude TTX solution and immediately returned to the tank (total handling time <30 s/individual to minimize stress to the fish). Then, 5 fish from each group were randomly collected at 1, 4, 8, 12, 24, 72, 120, and 168 h after toxin administration and toxin quantification was performed as described below.

2.4. Toxin quantification

Using a syringe precoated with sodium heparin, blood was withdrawn from the portal vein of each fish and centrifuged at 4200 g for 10 min. As TTX is partially binding to the TTX/PSP-binding protein in pufferfish blood plasma (Matsui et al., 2000; Yotsu-Yamashita et al., 2001), the supernatant (blood plasma) obtained was added with acetic acid at a final concentration of 0.1% to cut the binding, ultrafiltered through an Ultrafree-MC 5000 NMWL (Millipore Corp., Bedford, MA), and then submitted to enzyme-linked immunosorbent assay (ELISA) for TTX. After blood collection, all specimens were dissected into different anatomic tissues (liver, skin, and muscle), which were extracted with 0.1% acetic acid (Japan Food Hygiene Association, 2005). Each tissue extract was filtered through a USY-1 membrane (0.45 μm; Toyo Roshi Co., Ltd., Japan) and submitted to ELISA.

ELISA was performed according to the previously reported method (Ngy et al., 2008) using a monoclonal anti-TTX antibody developed by Kawatsu et al. (1997). The amount of TTX (in ng) determined by ELISA was converted to MU based on the specific toxicity of TTX (220 ng/MU). In a preliminary experiment using crude liver extracts ($n = 10$) of the pufferfish *Takifugu poecilonotus*, a significant and positive correlation (Pearson's test; $r = 0.9641$, $p < 0.01$) was observed between the TTX amounts determined by ELISA and those calculated from the TTX peak areas in LC/MS (Nakashima et al., 2004) (Fig. 1). The regression line, $y = 0.9874\,x + 7.301$ ($r^2 = 0.9295$) indicated that TTX was selectively quantified by ELISA in the presence of some TTX analogs including 4-*epi*TTX, 4,9-anhydroTTX, and deoxyTTXs (Yotsu-Yamashita, 2001) that were detectable in the extracts by LC/MS (data not shown).

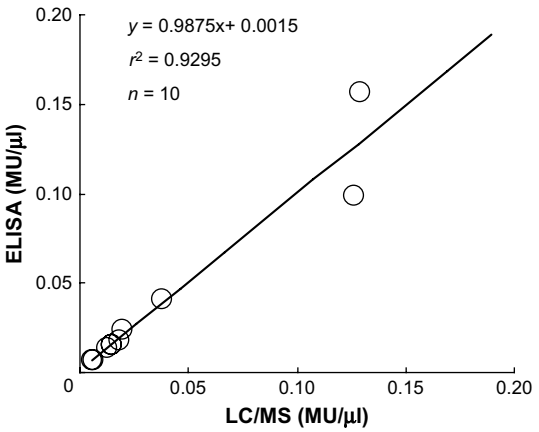

Fig. 1. Comparison of TTX amounts determined by LC/MS and ELISA.