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Transfer profile of intramuscularly administered tetrodotoxin to artificial hybrid specimens of pufferfish, *Takifugu rubripes* and *Takifugu niphobles*

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Abstract

Tetrodotoxin (TTX) was intramuscularly administered to artificially hybridized specimens of the pufferfish *Takifugu rubripes* and *T. niphobles* to investigate toxin accumulation in hybrids, and TTX transfer/accumulation profiles in the pufferfish body. In the test fish administered 146 MU TTX in physiologic saline, TTX rapidly transferred from the muscle via the blood to other organs. Toxin transfer to the ovary rapidly increased to 53.5 MU/g tissue at the end of the 72-h test period. The TTX content in the liver and skin was, at most, around 4 to 6 MU/g tissue, and in the testis it was less than 0.01 MU/g tissue. On the other hand, based on the total amount of toxin per individual (% of the administered toxin), the skin and the liver contained higher amounts (20%-54% and 2%-24%, respectively), but the amount in the liver rapidly decreased after 8 to 12 h, and fell below the level in the ovary after 48 h. These findings suggest that part of the TTX is first taken up in the liver and then transferred/accumulated in the skin in male specimens and in the ovary in female specimens.

*Keywords:* Tetrodotoxin; hybrid; pufferfish; *Takifugu rubripes*; *Takifugu niphobles*; Intramuscular administration
1. Introduction

Marine pufferfish of the genus *Takifugu* possess a potent neurotoxin, tetrodotoxin (TTX). Although TTX is exogenous in pufferfish and is derived from the food chain (Noguchi and Arakawa, 2008), the transfer, accumulation, and elimination mechanisms of TTX taken up into the pufferfish body via food organisms remain unclear. TTX administered intramuscularly to non-toxic cultured specimens of *Takifugu rubripes* is first transferred to the liver and then to the skin via the blood (Ikeda et al., 2009). Kono et al. (2008b) conducted a similar toxin administration experiment using cultured *T. niphobles* specimens. Unlike general non-toxic fish, the liver tissue of *T. rubripes* has a specific TTX-uptake mechanism (Nagashima et al., 2003; Matsumoto et al., 2005, 2007). TTX introduced into the pufferfish body is rapidly taken up into the liver via the blood (Matsumoto et al., 2008a, 2008b).

In wild pufferfish, the liver and ovary usually have strong toxicity, whereas the muscle and testis are weakly toxic or non-toxic (Noguchi and Arakawa, 2008), indicating sexual differences in pufferfish toxicity, and that maturation may affect toxin kinetics in the pufferfish body. We recently investigated seasonal changes in tissue toxicity, as well as the amount and forms of TTX in the blood plasma using wild specimens of the pufferfish *T. poecilonotus*, and demonstrated that maturation greatly affects the inter-tissue transfer and/or accumulation of TTX via the blood stream in nature (Ikeda et al., 2010). In the above-mentioned toxin administration experiments or the pharmacokinetic studies, however, juveniles or non-mature young fish were used, and the influence of aging or maturation was not considered.

Among *Takifugu* pufferfish, natural hybrids occasionally appear. Although some morphologic or genetic studies of these hybrids have been conducted (Masuda et al., 1991; Yokogawa and Urayama, 2000), little information is available on their toxicity. In our studies, to clarify the toxicity, toxin accumulation ability, and the inherited characteristics of hybrid pufferfish, as well as the transfer/accumulation/elimination
mechanisms of TTX in the pufferfish body, we administered TTX intramuscularly to artificially hybridized *T. rubripes* and *T. niphobles* offspring, and investigated the toxin transfer/accumulation profile of the hybrid fish. In a previous toxin-administration experiment (Ikeda et al., 2009), we used young and small *T. rubripes* specimens, which are relatively easy to rear and handle, and are most suitable for this type of experiment. The maturation of *T. rubripes*, however, is very slow, and the sexual differences in the toxin transfer/accumulation profile could not be clarified with young specimens. In the present study, we attempted to elucidate this point using the hybrid specimens produced by crossbreeding *T. rubripes* with *T. niphobles*, which matures earlier than *T. rubripes*.

2. Materials and Methods

2.1. Pufferfish specimens

A female specimen of ‘torafugu’ *T. rubripes* (3 years old; body weight, 2010 g) that had been cultured in Nagasaki Prefectural Institute of Fisheries, and a wild male specimen of ‘kusafugu’ *T. niphobles* (unknown age; body weight, 58 g) collected from Omura Bay, Nagasaki Prefecture, were used as the parent fish. After long-day treatment (14L10D), the female fish was intramuscularly administered a luteinizing hormone-releasing hormone analog (400 µg/kg body weight), and ovulated eggs were artificially fertilized by the dry method (Takushima et al., 2003) with sperm obtained from the male fish. Larvae that hatched from the fertilized eggs were reared in the institute for approximately 10 months with rotifers, brine shrimp, or an artificial diet depending on the growth stage. Thirty-one specimens (body weight, 71.5±15.1 g; body length, 12.7±0.6 cm) of the artificial hybrid pufferfish were obtained (designated ‘torakusa’, whose morphologic and genetic characterization is currently in progress) and transported to the laboratory at Nagasaki University, and acclimatized in aerated tanks for several days. TTX levels were quantified in 4 of the
31 specimens as described below for the non-administration (NA) group, and the remaining 27 specimens were subjected to the toxin administration experiment.

2.2. Preparation of toxin solution

The toxicity of TTX standard purchased from Wako (purity >90%) was calibrated 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.

The TTX standard was dissolved in a physiologic saline solution containing 1.35% NaCl, 0.06% KCl, 0.025% CaCl₂, 0.035% MgCl₂, and 0.02% NaHCO₃ at a concentration of 1460 MU/ml and used in the following toxin administration experiments.

2.3. Toxin administration experiment

Each fish was intramuscularly administered 0.1 ml (146 MU) of TTX solution [a dose (approximately 40 MU/20 g body weight) equal to about 1/10 of the minimum lethal dose of TTX to T. rubripes (Noguchi and Arakawa, 2008)], and immediately returned to the tank (total handing time < 30 s to minimize stress to the fish). Subsequently, 3 to 4 specimens were randomly collected at 1, 4, 8, 12, 24, 48, and 72 h after toxin administration, and toxin quantification was performed as described below.

2.4. Toxin quantification

Using a syringe precoated with sodium heparin, all of the blood of each specimen was withdrawn from the portal vein and centrifuged at 4200g for 10 min. The supernatant (blood plasma) obtained was subjected to enzyme-linked
immunosorbent assay (ELISA) for TTX (Kawatsu et al., 1997; Nyg et al., 2008). After blood collection, all specimens were dissected into different anatomic tissues (muscle, skin, liver, and gonads), and extracted with 0.1% acetic acid (Japan Food Hygiene Association, 2005). Each extract of the muscle, liver, and gonads was filtered through a USY-1 membrane (0.45 μm; Toyo Roshi Co., Ltd., Japan) and subjected to liquid chromatography/mass spectrometry (LC/MS) analysis (Nakashima et al. 2004), while that of the skin was subjected to ELISA. The amount of TTX (in ng) determined by LC/MS or ELISA was converted to MU based on the specific toxicity of TTX (220 ng/MU). In a preliminary experiment, a significant and positive correlation (Pearson’s test: $r = 0.9641$, $p < 0.01$) was observed between the TTX amounts determined by ELISA and LC/MS, with the regression line of $y = 0.9874x + 7.301$ ($r^2 = 0.9295$), as described previously (Ikeda et al., 2009).

2.5. Assessment of gonadosomatic index (GSI)

The GSI (%) of each fish was calculated from its gonad weight (GW) and body weight (BW) using the following equation: $\text{GSI} = \frac{\text{GW}}{\text{BW}} \times 100$.

3. Results

The GSI of the female ‘torakusa’ specimens used in the present study was $0.40 \pm 0.06\%$ and that of the male specimens was $5.96 \pm 2.41\%$. TTX was not detected in any tissues of the four specimens of the NA group.

Changes in the toxin content (MU/g or MU/ml) of each tissue, except the gonads, of the TTX-administered ‘torakusa’ specimens during the rearing period are shown in Fig. 1 (refer to Fig. 3 for the number of specimens tested at each rearing period). In both females and males, little toxin remained in the muscle, which was the site of toxin administration, even 1 h after toxin administration, and the toxin content of the muscle became lower than the detection limit (0.01 MU/g) after 4 h. In contrast,
the toxin content of both skin and liver reached around 4 MU/g at 1 h, and further increased to a maximum at 4 to 8 h. The toxin content of the liver significantly decreased thereafter. The regression lines of females and males during 12 to 72 h were expressed as $y = -0.0581x + 4.3393 \ (n = 8, \ r^2 = 0.7534, \ p < 0.05)$ and $y = -0.0500x + 3.8610 \ (n = 7, \ r^2 = 0.8668, \ p < 0.05)$, respectively, with no significant difference between the two lines. On the other hand, the toxin content of the skin decreased once, but remained at the same level in females, and had a significant increasing trend ($y = 0.0807x + 1.1054, \ n = 7, \ r^2 = 0.7341, \ p < 0.05$) again in males after 12 h.

The toxin content of the blood plasma was highest at 1 h, and rapidly decreased thereafter [female: $y = -3.271ln(x) + 13.631 \ (n = 13, \ r^2 = 0.876, \ p < 0.05)$; male: $y = -2.874ln(x) + 12.443 \ (n = 14, \ r^2 = 0.915, \ p < 0.05)$; no significant difference between the two regression lines].

Changes in the toxin content of the gonads are shown in Figure 2. The toxin content of the ovary was strikingly higher compared with the other tissues, and continued to rapidly increase ($y = 12.716ln(x) - 2.0188, \ n = 13, \ r^2 = 0.9148, \ p < 0.05$) throughout the rearing period to reach 53.5 MU/g at the end of the period (72 h). In contrast, little toxin transferred to the testis, whose toxin content was lower than the detection limit after 4 h.

Changes in the anatomic distribution of TTX, demonstrated by the relative amount of toxin retained in each tissue [% of the administered toxin (146 MU/individual)], are shown in Figure 3. In both females and males, the total amount of toxin remaining in the whole body was 60% to 80% of the administered toxin up to 8 h, and decreased a little to 45% to 65% thereafter. The amount of toxin in the skin, followed by the liver, was generally high (20%-54% and 2%-24%, respectively), but the amount in the liver rapidly decreased after 8 to 12 h. During this period, the toxin transfer profile was different between females and males: in females, the amount of toxin in the ovary gradually increased, exceeding that in the liver after 48
h, and then reached 9.8% at 72 h, whereas in males the toxin did not transfer to the testis, but instead the toxin amount in the skin increased gradually.

4. Discussion

The GSI of the male ‘torakusa’ specimens used in the present study was much higher than that of the female specimens. In wild *T. niphobles* and *T. poecilonotus*, the GSI of both females and males is generally less than 2% prior to maturation, but rapidly rises when maturation begins and reaches as high as 10-20% (Yu and Yu, 2002; Ikeda et al., 2010), indicating that maturation had begun to occur, at least in the male specimens. It takes more than 2 years for cultured *T. rubripes* to mature (unpublished data), whereas *T. niphobles* usually mature within 1 year (Honma et al., 1980). Therefore, in terms of maturation, the hybrid specimens (10 months old) seem to be closer to *T. niphobles*. The GSI of females normally begins to increase about 1 month later than that of males (Yu and Yu, 2002; Ikeda et al., 2010), suggesting that the present female specimens were in a very early stage of maturation, just before the GSI began to increase. Although histologic observation of the ovary is necessary to clarify this point, histology was not performed because the ovaries were still very small and the whole ovary was used for toxin quantification.

TTX was not detected in the four specimens of the NA group. TTX is exogenous in pufferfish, and cultured *T. rubripes* or *T. niphobles* do not have detectable levels of TTX (Noguchi and Arakawa, 2008). It was confirmed that ‘torakusa’ also become non-toxic when they are reared with non-toxic food under conditions in which toxic food organisms are completely excluded.

Intramuscularly administered TTX in the ‘torakusa’ specimens was rapidly transferred to other body tissues, and the toxin content of the skin, liver, and ovary exceeded that of the muscle within as little as 1 h after administration. At 1 h after intramuscular administration, a high concentration of TTX was present in the blood
plasma, indicating that TTX was transferred mainly via the bloodstream. The fact that muscles in toxic wild specimens of *T. rubripes* are not toxic, and those of *T. niphobles*, whose toxicity is usually higher than *T. rubripes*, are also non-toxic or weakly toxic (Noguchi and Arakawa, 2008) suggests that the muscles of their hybrid have little ability to retain and accumulate TTX.

In both females and males, the toxin content of the skin and liver reached a maximum at 4 to 8 h after TTX administration. Although the toxin content of the liver gradually decreased thereafter, the toxin content of the skin remained at the same level in females, and tended to increase in males after 12 h. This suggested that the toxin transferred to the skin is retained there for a long period, whereas the toxin that transferred to the liver is then transferred to other organs within a relatively short period. We observed a similar phenomenon when a TTX standard (purified TTX) was intramuscularly administered to *T. rubripes* (Ikeda et al., 2009). When the crude extract of toxic pufferfish ovary (crude TTX) was administered, however, the toxin was retained in the liver at a higher concentration for a longer period of time than the purified TTX. Moreover, in previous oral TTX-administration experiments (Honda et al., 2005; Kono et al., 2008a), pufferfish retained TTX in the liver for a long period, even after stopping the feeding of a TTX-containing diet, indicating that the form and/or uptake route of TTX affects its transfer/accumulation to the liver, though the mechanism remains to be elucidated.

On the other hand, the toxin continued to transfer to the ovary throughout the rearing period, and accumulated there at an extraordinarily higher concentration than in the skin and liver. The amount of toxin transferred to the gonads could not be examined because the tissues were undeveloped in the experiment with *T. rubripes*. Therefore, the present study is the first study to demonstrate that the intramuscularly administered toxin transfers rapidly and in large quantities to the ovary. We recently investigated seasonal changes in tissue toxicity using wild
specimens of the pufferfish *T. poecilonotus*, and determined that the TTX absorbed from toxic food organisms into the female pufferfish body is actively transported and accumulated in the ovary during the maturation period, as liver toxicity was high prior to maturation, and that of the ovary was high during the maturation period (Ikeda et al., 2010). The female ‘torakusa’ specimens used in the present study seem to be in the very early stage of maturation, as expected, in which such a toxin transportation mechanism would have begun to function. To further clarify the effect of maturation on the transfer and accumulation of TTX, comparative studies of completely non-matured and/or fully matured ‘torakusa’ specimens are needed.

In the males, most of the toxin remaining in the body, including the toxin that was first in the liver, was eventually transferred/accumulated in the skin; a toxin transfer profile that is essentially very similar to that of the immature *T. rubripes* specimens (Ikeda et al., 2009). Wild adult specimens of *T. rubripes* generally possess no toxin in the skin, but toxicity of several tens of mouse units is occasionally detected in juveniles (unpublished data). Most of the toxin was also transferred/accumulated in the skin during the toxin administration experiment. Therefore, the species to which the toxin accumulating ability of the ‘torakusa’ is closer is unclear; *T. rubripes* or *T. niphobles*, which have highly toxic skin. The testis, like muscle, seems to have little ability to retain or accumulate toxin, as little toxin transferred to the testis despite the fact that the GSI of males was much higher than that of the females. In contrast, the amount of toxin in the ovary of females gradually increased, and exceeded that of the liver after 48 h. Although statistically not significant, the sum of the toxin amount in the skin and ovary in female specimens seemed to correspond to the change to the toxin amount in the skin in the male specimens. In addition, the toxin content of the skin remained at the same level in females, but tended to increase in males after 12 h, suggesting that a part of the TTX that should be, after first being taken up into the liver, transferred/accumulated
into the skin in male specimens is transferred to the ovary in female specimens.

TTX-binding proteins are found in the blood plasma of toxic marine pufferfish (Matsui et al., 2000; Yotsu-Yamashita et al., 2001) and may be involved in toxin transportation. Detailed inter-tissue transfer mechanisms of TTX, especially those involved in the specific and powerful uptake of the ovary during the maturation period, however, remain to be elucidated. Further studies are in progress.

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Conflicts of interest

The authors declare that there are no conflicts of interest.
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Fig. 1. Changes in the TTX (MU/g or MU/ml) content retained in each tissue, except for gonads, of the ‘torakusa’ specimens during the rearing period after toxin administration. The TTX content was determined for each individual, and the symbols indicate the mean value (refer to Fig. 3 for specimen numbers for each rearing period).
Fig. 2. Changes in the content (MU/g) of TTX in the gonads of ‘torakusa’ specimens during the rearing period after toxin administration. The TTX content was determined for each individual, and the symbols indicate the mean value (refer to Fig. 3 for specimen numbers for each rearing period).
Fig. 3. Changes in the relative amount of TTX [% of administered amount (146 MU/individual)] retained in each tissue of the ‘torakusa’ specimens during the rearing period after toxin administration. The values over each column indicate the number of tested specimen for each rearing period.