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Note

Distribution of Tetrodotoxin in Pufferfish Collected from Coastal Waters of Sihanouk Ville, Cambodia

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In Cambodia, fatal food poisonings associated with the consumption of pufferfish have occurred for decades, but the causative species or toxins have never been documented. Herein, we investigated the toxicity of three pufferfish species of the genus *Lagocephalus* collected from the coastal waters of Sihanouk Ville, one of the main regions where poisonings have occurred. *L. wheeleri* and *L. spadiceus* were non-toxic, whereas *L. lunaris* was toxic and all of its body tissues exhibited toxicity levels exceeding the safety limit for human consumption (10 mouse units/g). Tetrodotoxin (TTX) was identified as the main toxin in this species; no paralytic shellfish poison(s) were detected. Consequently, we can confirm pufferfish to be a hazardous reservoir of TTX in Sihanouk Ville. It is likely that *L. lunaris* is one of the causative species of past pufferfish poisonings that have occurred in Cambodia.

Key words: pufferfish; genus *Lagocephalus*; *Lagocephalus wheeleri*; *Lagocephalus spadiceus*; *Lagocephalus lunaris*; food poisoning; tetrodotoxin; Cambodia

Introduction

Tetrodotoxin (TTX; $C_{11}H_{17}N_3O_8$, molecular weight = 319) is a lethal marine toxin with no known antidote, and TTX poisoning, which is mainly caused by consumption of certain species of pufferfish, is a serious health issue in many coastal areas of the Asia-Pacific region[1), 2)]. In addition to pufferfish, TTX is also contained in other aquatic animals, such as octopus, gastropod, xanthid crab, and horseshoe crab, which are thought to bioaccumulate TTX through the food chain, starting from bacteria that naturally produce TTX in marine environments[2), 3)].

In Cambodia, poisoning incidents arising from the consumption of pufferfish are common and sometimes result in human fatalities (at least 5 fatal poisoning incidents occurred between 2003 and 2007, involving a total of 57 patients with 9 deaths), but the causative species or toxins have never been documented. In Sihanouk Ville (Fig. 1), one of the main regions where pufferfish poisonings have occurred so far, large quantities of morphologically similar pufferfish of the genus *Lagocephalus* (Fig. 2) are sporadically on sale in local fish-landing markets, even though some of these fish are known to be toxic, and to have caused many human fatalities in Asian-Pacific countries, especially Japan and Taiwan[2), 4)-6)]. Therefore, this study was undertaken in order to clarify the toxicity of Cambodian marine pufferfish species.

Materials and Methods

Pufferfish specimens

All the pufferfish specimens of the genus *Lagocephalus* ($n = 117$) were collected along the inshore fish-landing sites in Sihanouk Ville (Fig. 1) from April–May 2005 (rainy season), December 2005–January 2006 and December 2006–January 2007 (dry season). Different species were identified according to their morphological characteristics, *i.e.*, distribution patterns of small spines in their dorsal bodies (*L. wheeleri*: elliptical shape that is apart from the base of the dorsal fin ray; *L. spadiceus*: tadpole-like shape whose tail reaches the base of the dorsal fin ray; *L. lunaris*: elliptical shape that extends to the base of the dorsal fin ray) (Fig. 2). The collected specimens were immediately frozen, transferred by air to our Laboratory of Food Hygiene in Nagasaki University, Japan, and subsequently stored below −20℃ until required for toxin analyses.

Toxicity assessment

After thawing, the specimens were dissected into different anatomic tissues, and then the toxicity of each tissue was assessed by mouse bioassay, according to the official guidelines of the Japan Food Hygiene Association[7)]. Lethal potency was expressed in mouse units (MU), where 1 MU is defined as the amount of

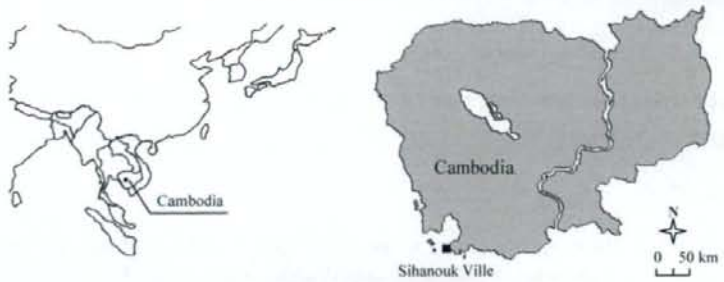

Fig. 1. Maps showing the location where marine pufferfish specimens were collected (■).

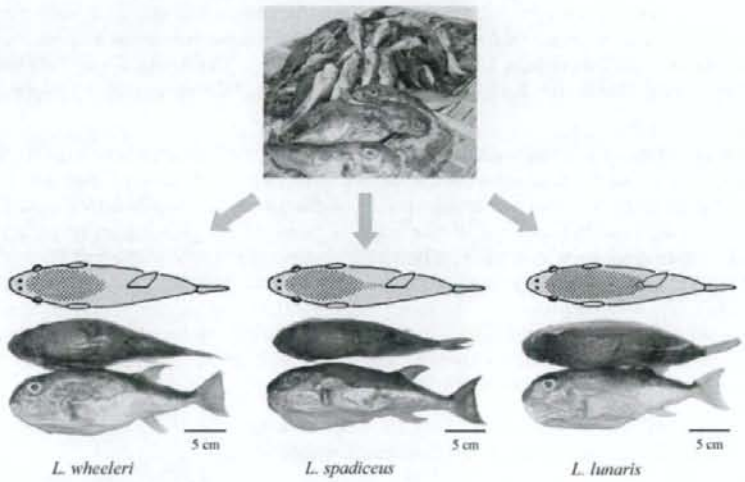

Fig. 2. Pictures of pufferfish of the genus *Lagocephalus* collected from the coastal water of Sihanouk Ville (Fig. 1). The species identification for *L. wheeleri*, *L. spadiceus* and *L. lunaris* are based on the morphological characteristics, *i.e.*, distribution patterns of small spines in their dorsal bodies.

toxin that kills a 20-g male mouse of the ddY strain within 30 min after intraperitoneal injection.

Toxin analyses

After mouse bioassay, a small amount (1–2 mL) of each tissue extract was filtered through a cellulose acetate membrane (0.45 μm; Toyo Roshi Co., Ltd., Japan), and submitted to toxin profile analyses as described below.

For detection of TTX, liquid chromatography/mass spectrometry (LC/MS) was performed on an Alliance separation module (Waters Corporation) equipped with a ZSpray™ MS 2000 detector (Micromass Limited) as reported by Ngy *et al.*[8] Reference of TTX (purity more than 90%; Wako Pure Chemical Industries Ltd., Japan), whose specific toxicity had been calibrated previously by mouse bioassay, was used as an external standard to identify and quantify the TTX contained in each sample.

For the detection of paralytic shellfish poison(s) (PSP), high-performance liquid chromatography with post-column fluorescence derivatization (HPLC-FLD) was conducted on a Hitachi L-7100 HPLC system using the methods described previously[9, 10], with some modifications. Reference samples of gonyautoxins 1–4 (GTX1–4), decarbamoylgonyautoxins 2, 3 (dcGTX2, 3), and neosaxitoxin (neoSTX), which were provided by the Fisheries Agency, Ministry of Agriculture, Forestry and Fisheries of Japan, as well as saxitoxin (STX) and decarbamoylsaxitoxin (dcSTX), prepared as reported previously[11], were used as standards to identify and quantify the individual analogues.

Results

The pufferfish specimens ($n=117$) collected in this study were identified as three different species, *Lagocephalus wheeleri* ($n=20$), *L. spadiceus* ($n=15$) and *L. lunaris* ($n=82$), which are common species in Cambodian and Asian-Pacific coastal waters.

Toxicity assessment by mouse bioassay revealed that *L. wheeleri* and *L. spadiceus* were non-toxic (less than 2 MU/g; data not shown), whereas *L. lunaris* was toxic (Table 1), irrespective of the collection season. No marked individual or seasonal variation in toxicity was observed, except for some specimens whose internal tissue toxicities were noticeably higher than those of the other specimens. Overall, the highest toxicities were generally found mainly in the liver (2–257 MU/g),

Table 1. Anatomic distribution of toxicity in Cambodian specimens of *L. lunaris*

Collection data	Specimen no.	Sex	Body size		Toxicity assessed by mouse bioassay (MU/g)					
			Weight (g)	Length (cm)	Skin	Muscle	Liver	Intestine	Testis	Ovary
Apr–May 2005	1	♂	350	21.8	3	3	9	5	—	—
(rainy season)	2	♂	340	19.0	14	34	48	63	18	—
	3	♂	300	20.3	3	9	9	<2	—	—
	4	♂	130	14.7	24	67	45	34	52	—
	5	♀	420	22.0	<2	<2	<2	<2	—	43
	6	♀	310	19.5	3	<2	5	3	—	62
	7	♀	305	19.5	21	62	46	14	—	222
	8	♀	255	19.3	2	<2	12	3	—	—
	9	♀	205	18.0	3	<2	<2	<2	—	19
	10	♀	187	16.8	7	41	16	28	—	—
	11[a]	♂/♀	56±5	10.8±1.2	3	2	2	2	—	—
	12[a]	♂/♀	54±7	9.7±1.1	3	3	<2	<2	—	—
	13[a]	♂/♀	50±6	11.1±1.0	3	15	8	6	—	—
	14[a]	♂/♀	47±5	10.3±0.9	<2	6	3	7	—	—
	15[a]	♂/♀	41±4	10.6±0.8	20	65	257	123	—	—
	16[a]	♂/♀	39±3	10.2±0.6	3	8	8	4	—	—
	17[a]	♂/♀	36±3	10.0±0.8	21	44	24	37	—	—
Dec 2005–Jan 2006	18	♀	180	16.3	3	2	4	<2	—	140
(dry season)	19	♀	35	10.1	6	33	17	11	—	17
	20[b]	♂/♀	61±6	15.0±1.4	<2	4	4	<2	—	—
	21[b]	♂/♀	51±5	13.3±1.3	<2	3	<2	2	<2	<2
	22[b]	♂/♀	36±5	11.0±0.9	3	7	3	<2	<2	<2
Dec 2006–Jan 2007	23	♂	223	22.0	<2	<2	<2	3	—	—
(dry season)	24	♂	125	17.9	3	5	3	3	<2	—
	25	♂	59	13.7	<2	3	<2	2	—	—
	26	♀	163	20.3	25	29	25	38	—	238
	27	♀	95	15.5	5	8	8	10	—	50

—: Not available nor applicable.

[a] Pooled specimens of 5 individuals due to their small size. Values presented are means ± standard deviation ($n=5$).

[b] Pooled specimens of 10 individuals due to their small size. Values presented are means ± standard deviation ($n=10$).

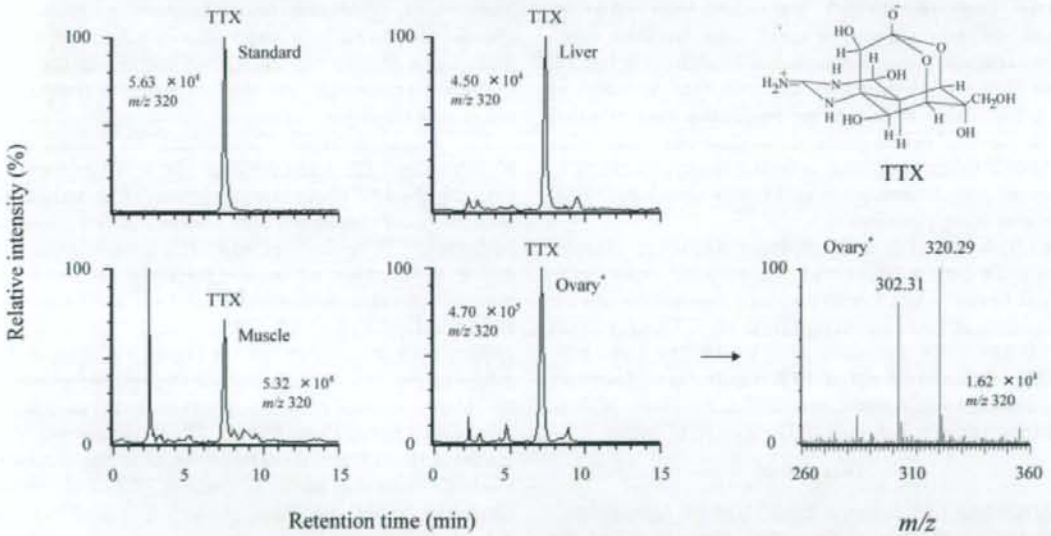

Fig. 3. LC/MS analysis for toxin profile of various toxic tissues extracted from *L. lunaris*. Selected ion (m/z 320) mass chromatograms of standard TTX [$(M+H)^+=320$], and of extracts of muscle, liver and ovary* (and selected mass spectrum). The ion intensity of the base peak (100%, relative intensity) is presented in each mass chromatogram.

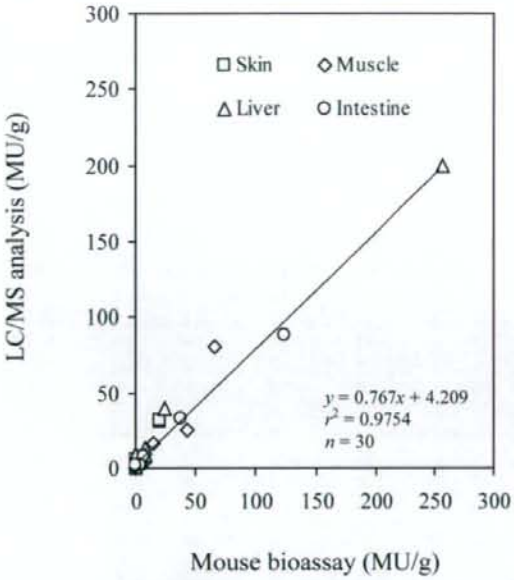

Fig. 4. Comparison of toxicity scores obtained from mouse bioassay and LC/MS analysis.

ovary (17–238 MU/g) and intestine (2–123 MU/g), followed by the muscle (2–67 MU/g), testis (18–52 MU/g) and skin (2–25 MU/g). These results indicate that the Cambodian *L. lunaris* is a hazardous species, as toxicity levels above 10 MU/g are considered as unsafe for human consumption[7].

The toxin profile of various toxic tissues from *L. lunaris* is depicted in Fig. 3. The muscle, liver and ovary tissue extracts showed a clear peak in their mass chromatograms scanned at *m/z* 320, and the retention time (7.34 min) was consistent with that of standard TTX ($[M+H]^+=320$). No PSP analogues were detected by HPLC–FLD in this study (data not shown). Likewise, neither TTX nor PSP was detected in any tissues of *L. wheeleri* and *L. spadiceus* in LC/MS and HPLC–FLD analyses (data not shown).

As depicted in Fig. 4, a significant positive correlation (Pearson's test; $n=30$, $r=0.9876$, $p<0.001$) was established between the toxicity scores assessed by mouse bioassay and those calculated from the TTX peak areas in LC/MS. The regression line, $y=0.767x+4.209$ ($r^2=0.9754$), demonstrates that TTX was the toxic principle in Cambodian *L. lunaris*, accounting for about 80% of the total toxicity detected in the mouse bioassay.

Discussion

According to the Japan Food Hygiene Association[7], pufferfish with a toxicity of less than 10 MU/g are considered as "non-toxic" and those with a toxicity above 10 MU/g are considered as "toxic", *i.e.*, unsafe for human consumption. In this context, the Cambodian *L. wheeleri* and *L. spadiceus*, whose toxicities were less than 2 MU/g, were determined to be non-toxic, irrespective of the collection season. Similarly, the toxicities detected in Japanese, Malaysian, Hong Kong, Bangladeshi and Thai specimens were less than 10 MU/g and these specimens were also considered to be non-toxic,[4,12–15]. On the contrary, the Cambodian *L. lunaris*, whose toxicity often exceeded 10 MU/g, was determined to be toxic. The morphology of *L. lunaris* is very similar to *L. wheeleri* and *L. spadiceus* (Fig. 2), so correct identification is important for consumers. Poisoning incidents due to the mistaken consumption of *L. lunaris* in the belief that it was non-toxic *L. wheeleri* or *L. spadiceus* has already been documented in Japan and Taiwan, involving more than 10 poisoning victims with one death[4–6].

As in many species of marine pufferfish reported previously[2,3], the toxin was contained mainly in the liver and ovary (Table 1), and the toxicity scores, which ranged from less than 2 to 257 MU/g, were somewhat similar to those found in Taiwanese (less than 4 to 1,300 MU/g)[16] and Thai (less than 5 to 2,920 MU/g)[15] specimens, though not Bangladeshi species, whose toxicity scores were reported to be always less than 5 MU/g[14]. Interestingly, in the months (December to February) when low toxicity of less than 5 MU/g was reported for Thai specimens[15], the Cambodian ones were highly toxic. These findings clearly confirm that the levels and tissue distribution of the toxin in pufferfish vary with season and location, even for the same species in the same region[2,3,17,18]. Because the minimal lethal dose of pufferfish (TTX) toxicity in humans is estimated to be 10,000 MU[4], consumption of only about 50 to 200 g of the edible tissues (*i.e.*, liver, ovary, or muscle) of *L. lunaris* (with toxicity above 50 MU/g) can be fatal. Therefore, *L. lunaris* can be desingnated as a hazardous species unsuitable for human consumption, and might have been one of the causative species in the past pufferfish poisonings that have occurred in the coastal regions of Sihanouk Ville.

The main toxin in Cambodian *L. lunaris* was identified as TTX, accounting for about 80% of the total toxicity. No PSP components, such as STXs and GTXs, which were detected in some other marine pufferfish[19–23], were detected in the present study. A minor peak observed in the electrospray ionization mass spectrum at *m/z* 302 of LC/MS (Fig. 3) appeared to be due to anhydroTTX ($[M+H-H_2O]^+=302$), whose specific toxicity is about 1/50 of that of TTX[24], and this might be responsible for a part of the remaining toxicity. Many researchers have reported that Taiwanese[2], Japanese[3], Bangladeshi[14] and Thai[15] specimens contained only TTX and its derivatives, as in the currently studied Cambodian ones. Therefore, TTX could be the causative toxin in past pufferfish poisonings in Sihanouk Ville.

Consequently, we can confirm pufferfish in the coastal water of Sihanouk Ville to be a hazardous reservoir of TTX. Health authorities and the general public must be made aware of the correct identification of toxic and non-toxic pufferfishes, as well as other poten-

tially TTX-contaminated aquatic animals. Although *L. wheeleri* and *L. spadiceus* were tentatively identified as two different species based on their morphological characteristics, further studies on their identification using protein and genome-based techniques[4] are in progress.

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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, $y = 0.9874x + 7.301$ ($r^2 = 0.9295$) 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).

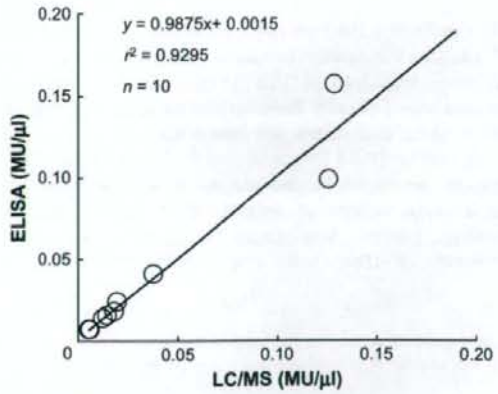

Fig. 1. Comparison of TTX amounts determined by LC/MS and ELISA.

2.5. Immunohistochemical observation

A part of the skin of each fish collected at 120 h after toxin administration was submitted to the immunohistochemical observation under light microscope according to the previously reported method (Tanu et al., 2002; Mahmud et al., 2003a,b) using the anti-TTX antibody.

3. Results

Changes in the toxin content (MU/g or MU/ml) of each pufferfish tissue during the rearing period are shown in Fig. 2. Changes in the toxin content of the liver differed between the PTTX and CTTX groups. In both groups, the toxin content was 12 MU/g at 1 h after administration. In the PTTX group, the toxin content gradually decreased until only a small amount (0.5–1.5 MU/g) remained after 12 h. In the CTTX group, however, the toxin content increased, reaching a maximum (around 21 MU/g) at 8–12 h; thereafter, the toxin content decreased, but 6.4 MU/g remained at the end of the rearing period (168 h). Changes in the skin toxin content also differed between groups. In both groups, the toxin content increased remarkably between 1 and 72 h and then remained at about 15 MU/g. The onset of the increase in the PTTX group occurred earlier than that in the CTTX group (after 12 h). The toxin content of the muscle, which was the site of administration, rapidly decreased and after 8 h was below the detection limit (0.01 MU/g) in both groups. The toxin content of the blood plasma was highest at 1 h (5.9 MU/ml in the PTTX group, 9.8 MU/ml in the CTTX group), and rapidly decreased thereafter.

Changes in the anatomic distribution of TTX, demonstrated by the relative amount of toxin retained in each tissue [% of the administered toxin (50 MU/individual)], are shown in Fig. 3. The total amount of toxin remaining in the whole body at 1–4 h was around 60% of the administered toxin (50 MU/individual) in both groups. The amount first began to decrease at 8–12 h, and then increased to approximately 60%–80% at 24–168 h. Changes in the amount of toxin in the liver tissues differed between the PTTX and CTTX groups. In the PTTX group, the amount of

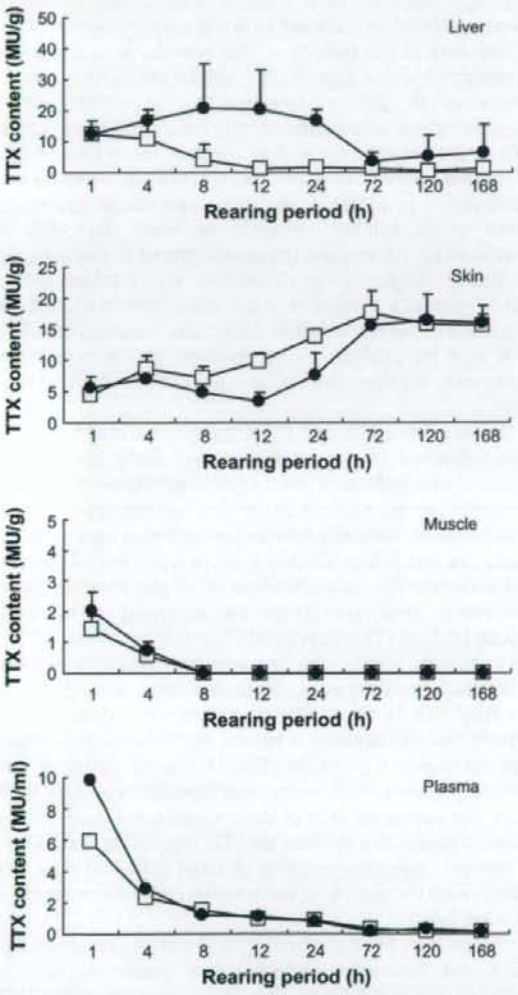

Fig. 2. Changes in the content of TTX (MU/g or MU/ml) retained in each tissue of the *T. rubripes* specimens during the rearing period after toxin administration. —□—: PTTX group; —●—: CTTX group. The toxin content in blood plasma was determined using a combined sample of 5 individuals for each point.

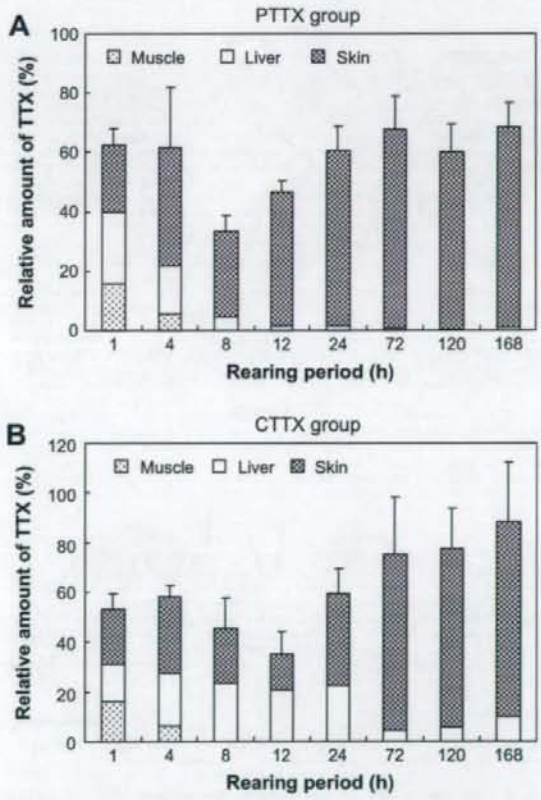

Fig. 3. Changes in the relative amount of TTX [% of the administered amount (50 MU/individual)] retained in each tissue of the *T. rubripes* specimens during the rearing period after toxin administration. A: PTTX group; B: CTTX group.

toxin in the liver rapidly decreased from 1 to 8 h, becoming less than 1.6% of the administered toxin (<3.2% of the remaining toxin) after 12 h. In the CTTX group, the amount of toxin in the liver did not decrease for up to 24 h and accounted for 15%–23% of the administered toxin (28%–58% of the remaining toxin). The amount of toxin in the skin gradually increased during the rearing period in both groups, and accounted for most (PTTX group, 98%; CTTX group, 89%) of the remaining toxin at 168 h.

Light micrographs of representative skin sections at 120 h after toxin administration are shown in Fig. 4. The epidermal layer of the skin was comprised of two distinct cell types, basal cells and succiform cells (Tanu et al., 2002; Mahmud et al., 2003a,b), and no gland or gland-like structure was observed. In both the PTTX and CTTX groups, positive reactions for TTX (brown color) were localized at basal cells along the basement membrane. No positive reaction was observed in the succiform cells, or in the skin sections of negative control, i.e., the fish without toxin administration (data not shown).

4. Discussion

TTX intramuscularly administered to non-toxic cultured *T. rubripes* rapidly transferred to other body tissues, and the toxin content of the liver and skin exceeded that of 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 transferred mainly via the bloodstream. The fact that muscles in toxic wild specimens of *T. rubripes* are not toxic indicates that the muscles of this species either do not retain and accumulate TTX or have a mechanism for eliminating TTX.

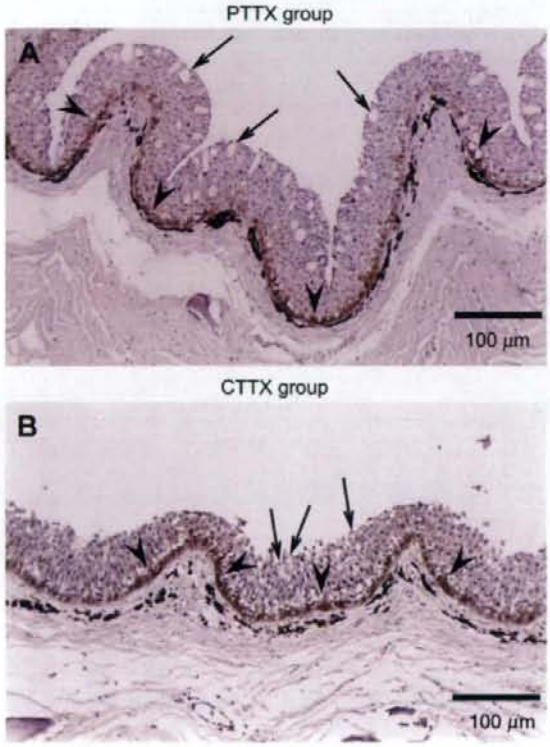

Fig. 4. Light micrographs of representative skin sections (×100) of the test fish collected at 120 h after toxin administration, showing TTX-positive basal cells (arrow heads) and TTX-negative succiform cells (arrows). A: PTTX group; B: CTTX group.

The toxin transfer profiles from 4 to 72 h differed between the PTTX and CTTX groups. In the PTTX group, little TTX was retained in the liver, and most of the toxin transferred to, and accumulated in, the skin after 12 h, whereas in the CTTX group, a considerable amount of toxin was transferred to, and retained in, the liver for up to 24 h. Although most of the toxin transferred to, and accumulated in, the skin thereafter, some toxin remained in the liver even at 168 h. Matsui et al. (1981) reported that when non-toxic cultured specimens of *T. rubripes* are fed diets containing crystalline TTX or crude extract of toxic pufferfish ovary, only the test fish fed with the toxic pufferfish ovary accumulate TTX in their liver. The liver tissue of *T. rubripes* is equipped with a specific TTX uptake mechanism (Nagashima et al., 2003; Matsumoto et al., 2005, 2007), suggesting that some substance(s) coexisting in the crude TTX might enhance the uptake mechanism or change TTX molecules into a form that is more easily processed by this mechanism, resulting in the above mentioned difference. Kono et al. (2008b) recently reported that 40% of purified TTX intramuscularly administered to the cultured pufferfish *Takifugu* (*Fugu*) *niphobles* was transformed to 4,9-anhydroTTX within 4 days after administration. It is unclear, however, whether such transformation between TTX and its analog(s) was involved in the concerned difference. Further studies are needed to clarify these points.

In both the PTTX and CTTX groups, most of the toxin that remained in the body was eventually transferred/accumulated in the skin. Wild adult specimens of *T. rubripes* generally possess no toxin in the skin, but toxicity of several tens MU is occasionally detected in juveniles (unpublished data). The test fish used in the present study were 4 months old and therefore considered to be in the juvenile stage. Therefore, these specimens are assumed to have an immature skin TTX-excreting ability or liver and ovary TTX-accumulating ability. Our previous immunohistochemical investigations (Tanu et al., 2002; Mahmud et al., 2003a,b) revealed that in the pufferfish having toxic skin, such as *T. vermicularis*, *Chelonodon patoca*, *Tetraodon steindachneri*, and *Tetraodon nigroviridis*, TTX is mainly found in the secretory glands or secretory cells (succiform cells) of their skin. The succiform cells of the present test fish, however, showed no positive reaction for TTX, suggesting that TTX, in *T. rubripes* juveniles, remains at basal cells and does not easily reach the succiform cells, which provably excrete TTX in adult fish.

When non-toxic cultured specimens of *Tetraodon turgidus*, the freshwater pufferfish that possesses paralytic shellfish toxin (PST) in the skin, are intramuscularly administered PST, a toxin similar to that used in the present study, the PST rapidly transfers from muscle to other tissues and accumulates mostly in the skin at the end of rearing

period (Ngy et al., 2008). Interestingly, when *T. turgidus* specimens were administered the same dosage of TTX, all died within 3–4 h, and more than half of the TTX administered remained in the muscle in the dead specimens. It is therefore inferred that marine pufferfish that ingest TTX are thus endowed with a mechanism by which they transport TTX specifically and actively, and freshwater pufferfish that ingest PST are endowed with a mechanism that processes PST. TTX/PST-binding proteins have been isolated from the blood plasma of marine pufferfish (Matsui et al., 2000; Yotsu-Yamashita et al., 2001), and may be involved in the transportation mechanism.

In both the PTTX and CTTX groups, a temporary decrease in the total amount of toxin remaining in the whole body was observed at 8–12 h. A similar decrease was observed when *T. turgidus* was administered PST (Ngy et al., 2008). Watabe et al. (1987) reported that when *T. rubripes* was intraperitoneally administered [^{3}H]-TTX, the amount of TTX in the gallbladder was greatly increased 6 days after administration. The temporary decrease may be due to the temporary storage of a large amount of TTX in a particular organ or tissue other than muscle, liver, and skin. This point, along with the properties of the TTX/PST-binding proteins and specific toxin transportation/accumulation mechanisms, remains to be elucidated. Further studies are in progress.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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