Evaluation of the tetrodotoxin uptake ability of pufferfish *Takifugu rubripes* tissues according to age using an *in vitro* tissue slice incubation method

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Highlights

> Tetrodotoxin (TTX) uptake ability of *Takifugu rubripes* tissues was examined

> TTX uptake ability was similar in the skin, intestine, and liver

> TTX uptake in the skin was ~2-fold higher in young fish than in adult fish

> The TTX uptake pathway in each tissue was evaluated using immunohistochemistry

ABSTRACT

The tetrodotoxin (TTX) uptake ability of pufferfish *Takifugu rubripes* tissues and its growth-associated changes were investigated using an *in vitro* tissue slice incubation method. Tissue slices prepared from the liver, skin, and intestine of a non-toxic cultured adult *T. rubripes* (20 months old) and incubated with incubation buffer containing 25 μg/mL TTX for 1-48 h showed a time-dependent increase in the TTX content in all tissues. The TTX contents of the skin and intestine slices were comparable to or slightly higher than that of the liver slices, with a similar transition pattern, suggesting similar TTX uptake ability among the skin, intestine, and liver. The TTX uptake ability of the liver and intestine did not differ significantly between young (8 months old) and adult (20 months old) fish, but the skin slices of young fish took up approximately twice as much TTX as that of adult fish, suggesting that the TTX uptake ability of the skin is involved in the growth-dependent changes in the toxin distribution inside the body in *T. rubripes*. To estimate the TTX uptake pathway in each tissue, an immunohistochemical technique was used to observe temporal changes in the intra-tissue microdistribution of TTX during incubation. The findings suggested that TTX is transferred and accumulates from pancreatic exocrine cells to hepatic parenchymal cells in the liver, from connective tissues to basal cells in the skin, and from villi epithelial cells via the lamina propria to the muscle layer in the intestine.

Keywords: Tetrodotoxin, Pufferfish, *Takifugu rubripes*, Tissue slice, Immunohistochemistry

1. Introduction

Marine pufferfish of the family Tetraodontidae generally possess a potent neurotoxin, tetrodotoxin (TTX). TTX is lethal to humans and causes muscle paralysis by specifically blocking voltage-gated sodium channels (Geffeney and Ruben, 2006; Narahashi, 2001). Several studies have revealed that (1) the toxicity of pufferfish exhibits remarkable individual and regional variation (Miyazawa and Noguchi, 2001); (2) TTX is distributed over a wide variety of marine organisms in addition to pufferfish, including certain species of gobies, octopuses, gastropods, starfish, crabs, flatworms, and ribbon worms (Noguchi and Arakawa, 2008); (3) TTX originates in marine bacteria (Magarlamov et al., 2017); (4) pufferfish such as *Takifugu rubripes* and *Takifugu alboplumbeus* (formerly known as *Takifugu niphobles*) become non-toxic when artificially reared with non-toxic diets after hatching (Matsui et al., 1982; Noguchi et al., 2006); and (5) such non-toxic pufferfish become toxic when orally administered TTX (Honda et al., 2005; Yamamori et al., 2004). These findings indicate that the toxification of pufferfish is exogenous and derived from a food chain that begins with marine bacteria (Noguchi and Arakawa, 2008).

The distribution of TTX inside the pufferfish body varies depending on the species (Noguchi and Arakawa, 2008), and is also affected by the maturation of individuals even in the same species. In the natural environment, *Takifugu flavipterus* (formerly known as *Takifugu poecilonotus*), *T. alboplumbeus*, and *Takifugu pardalis* typically have high concentrations of TTX in the liver and skin, but during maturation females accumulate TTX mainly in the ovary and skin, and males accumulate TTX mainly in the skin and liver, with the total TTX amount being higher in females (Gao et al., 2018; Ikeda et al., 2010; Itoi et al., 2016). Wang et al. (2011) reported that TTX administered intramuscularly to hybrid specimens produced by crossbreeding *T. rubripes* with *T. alboplumbeus*, which matures earlier than *T. rubripes*, is first taken up in the liver and then transferred to and accumulates in the ovary in females and the skin in males.

The distribution of TTX in the pufferfish body also changes with the growth of the individuals. In wild adult *T. rubripes*, the liver and ovary are generally strongly toxic, and the skin, muscle, and testis are non-toxic (Noguchi and Arakawa, 2008), but in wild young fish, the skin is the main toxin-accumulating tissue (Ikeda, 2009; Tatsuno, 2012). In TTX administration experiments using non-toxic cultured young *T. rubripes*, much of the TTX is transferred to the skin (Honda et al., 2005; Ikeda et al., 2009). Tatsuno et al. (2013a) conducted an *in vivo* oral gavage TTX administration experiment in *T. rubripes* of different ages, and found that the administered TTX was mainly transferred to the skin in young fish (6 months old), whereas most of it was transferred to and accumulated in the liver in adult fish (15 months old). They speculated that because the liver is undeveloped and has low TTX-accumulating ability in young fish, the TTX mainly accumulates in the skin for elimination, but as the

liver develops, TTX accumulates and is stored in the liver.

Nagashima et al. (2003) and Matsumoto et al. (2005, 2007), using an *in vitro* tissue slice incubation method, demonstrated that liver tissues of marine *Takifugu* pufferfish, unlike those of general marine fish, take up a considerable amount of TTX. Kiriake et al. (2016) used this same method to test the hypothesis of Tatsuno et al. (2013a). They prepared liver tissue slices from young (4 months old) and adult (18 months old) *T. rubripes* to compare the TTX uptake ability, but found no significant differences between the two. They concluded that rather than the TTX uptake ability, it is the ability to retain or metabolize TTX that changes with the development of the liver. They did not consider the TTX uptake ability of the intestine, however, which serves as the first barrier when TTX in the food is absorbed into the pufferfish body, or of the skin, which is a main transfer destination of TTX absorbed into the body.

In the present study, to clarify the mechanisms involved in the unique kinetics of TTX in the pufferfish body and growth-associated changes, we first investigated whether the *in vitro* tissue slice incubation method is applicable for evaluating the TTX uptake ability of not only the liver but also the skin and intestine, and then compared the TTX uptake ability of these tissues between young (8 months old) and adult (20 months old) *T. rubripes*. Moreover, to estimate the TTX uptake pathway in each tissue, an immunohistochemical technique (Tanu et al., 2002) was used to observe temporal changes in the intra-tissue microdistribution of TTX during incubation.

2. Materials and methods

2.1. Pufferfish

Non-toxic cultured young (8 months old; body length, 13.5 ± 0.4 cm; body weight, 80.6 ± 5.1 g; n=4) and adult (20 months old; body length, 28.4 ± 0.8 cm; body weight, 743 ± 61 g; n=3) *T. rubripes* were used for the tissue slice incubation experiments described below.

2.2. TTX preparation

TTX extracted from the ovaries and livers of *T. pardalis*, and purified by solvent partitioning, activated charcoal treatment, and Bio-Gel P-2 (Bio-Rad Laboratories, Hercules, CA, USA) column chromatography according to a previously reported method (Arakawa et al., 1994) was used for the tissue slice incubation experiments. Crystalline TTX (Nacalai Tesque, Inc., Kyoto, Japan) was used as a standard for the TTX quantification analysis described below.

120 *2.3. Tissue slice incubation experiments*

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122 To confirm whether the *in vitro* tissue slice incubation method is applicable for evaluating TTX
123 uptake ability of not only the liver but also the skin and intestine, these tissues were collected from one
124 of the adult fish, and an incubation experiment was conducted according to the method of Matsumoto
125 et al. (2007). Briefly, 12 tissue slices (8 mm in diameter, ~1 mm in thickness) were prepared from the
126 liver, dorsal skin, and intestine, which was first sliced longitudinally to form a sheet. Each slice was
127 incubated with a 1.5 ml of incubation buffer (160 mM NaCl, 4.8 mM KCl, 23.8 mM $NaHCO_3$, 0.96 mM
128 KH_2PO_4, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 12.5 mM HEPES, and 5.0 mM D-glucose; adjusted to pH 7.4
129 with NaOH solution) containing 25 μg/mL TTX in a 15-mL plastic tube aerated with O_2 and CO_2 at a
130 9:1 ratio at 20°C for a maximum of 48 h. During the incubation, 3 slices of each tissue were collected
131 at 1, 8, 24, and 48 h, washed with neutral phosphate buffer (0.15 M NaCl and 0.01 M Na_2HPO_4; adjusted
132 to pH 7.0 with 0.15 M NaCl and 0.01 M NaH_2PO_4), and weighed. Then, 1 ml of 0.1% acetic acid was
133 added to each slice, and the slices were ultrasonicated and heated in a boiling water bath for 10 min.
134 After centrifugation at 830*g* for 15 min, the supernatant was passed through an HLC-DISK membrane
135 filter (0.45 mm, Kanto Chemical Co., Inc., Tokyo, Japan), and then applied to liquid chromatography-
136 tandem mass spectrometry (LC-MS/MS) analysis as described below. In a preliminary experiment, all
137 the tissues were confirmed to remain viable for over 48 h using an alarmarBlue™ Cell Viability Reagent
138 (ThermoFisher Scientific, Tokyo, Japan) assay (Nagashima et al. 2003).
139 To investigate whether the TTX uptake ability of each tissue differed according to the age of the fish,
140 three tissue slices were similarly prepared from the liver, skin, and intestine of the three young fish and
141 the remaining two adult fish, and an incubation experiment was conducted. As the previous experiment
142 revealed that TTX uptake advanced sufficiently even at 8 h of incubation, the incubation time was set
143 at 8 h, and after combining the data of 8-h incubation in the previous experiment, the TTX amount taken
144 up into each tissue was compared between the young and adult fish.
145 To observe the microdistribution of TTX taken up into each tissue, six tissue slices were similarly
146 prepared from the liver, skin, and intestine of the remaining one young fish, respectively, and incubated
147 for a maximum of 8 h. During the incubation, 2 slices of each tissue were collected at 0.5, 2, and 8 h,
148 and submitted to immunohistochemistry as described below.

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150 *2.4. TTX quantification*

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152 TTX was quantified by LC-MS/MS analysis according to the previously reported method (Gao et al.,
153 2018), in which chromatography was carried out using an Alliance 2690 Separations Module (Waters,

Milford, MA, USA) with a Mightysil RP-18 GP column (2.0 x 250 mm, Kanto Chemical Co., Inc., Tokyo, Japan) and mobile phase comprising 30 mM heptafluorobutyric acid in 1 mM ammonium acetate buffer (pH 5.0) at a flow rate of 0.2 ml/min. The eluate was introduced into a Quattro microTM API detector (Waters) in which the TTX was ionized by positive-mode electrospray ionization with a desolvation temperature of 350°C, source block temperature of 120°C, and cone voltage of 50 V, and monitored at *m*/*z* 162 (for quantitative) and 302 (for qualitative) as product ions (collision voltage 38 V) with *m*/*z* 320 as a precursor ion through a MassLynxTM NT operating system (Waters).

2.5. Immunohistochemical observation

Tissue sections (6-µm thick) were prepared from the incubated tissue slices by conventional histologic procedures, and immunostained according to the previously reported method (Gao et al., 2018; Tanu et al., 2002). Briefly, the sections were successively treated with 10% H_2O_2 in water and 25% goat serum in 0.01 M phosphate-buffered saline (Iatron Lab. Inc., South Bend, IN, USA), and then incubated with a monoclonal anti-TTX antibody (Kawatsu et al., 1997), followed by a polymer, EnVision+ (Dako North America Inc., Carpinteria, CA, USA) for 60 min. For a negative control, mouse IgG (Vector Laboratories Inc., Burlingame, CA, USA) was used instead of the anti-TTX antibody. After treating the sections with 0.017% 3,3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries Ltd., Tokyo, Japan) substrate solution in 0.01 M phosphate-buffered saline, they were counter-stained with Mayer's hematoxylin (Merck, Darmstadt, Germany), and observed under an optical microscope (BZ-X700, Keyence Corp., Osaka, Japan). TTX-positive signals were indicated by a brown color.

2.6. Statistical analysis

Statistical analysis was performed by combining the data from the 8-h incubation in the first incubation experiment and the data from the second incubation experiment. Namely, for each tissue, Student's *t*-test was performed between young fish and adult fish using the mean TTX content of each individual (mean TTX content of the 3 slices).

3. Results

Changes in the TTX content of liver, skin, and intestine slices of adult *T. rubripes* during the incubation are shown in Fig. 1. The TTX content temporally increased in all tissues. The TTX content

of the liver was 4.7 ± 1.7, 9.3 ± 2.5, 10.7 ± 0.5, and 13.6 ± 1.2 μg/g at 1, 8, 24, and 48 h of incubation, respectively, and was highest among the three tissues at 1 h, but the content of the skin and intestine exceeded that of the liver at 8 h and thereafter. The TTX content of the intestine was highest at 8 and 24 h (12.1 ± 1.7 and 17.3 ± 1.3 μg/g, respectively), and that of the skin was highest at 48 h (18.8 ± 1.4 μg/g).

The TTX content of the tissue slices of the young and adult *T. rubripes* after 8 h of incubation is shown in Fig. 2. In the young fish and adult fish, the TTX content was 12.6 ± 1.2 and 11.0 ± 1.6 μg/g in the liver, 26.9 ± 2.7 and 11.6 ± 1.7 μg/g in the skin, and 15.5 ± 3.0 μg/g and 12.7 ± 4.8 μg/g in the intestine, respectively. The TTX content in the liver and intestine did not differ significantly between the young fish and adult fish, while it was significantly higher in the skin of the young fish compared with the adult fish ($p<0.05$).

Changes in the microdistribution of TTX in the liver, skin, and intestine tissue slices during the incubation are shown in Figs. 3-5. In the liver, weak TTX-positive signals were observed at the pancreatic exocrine cells at 0.5 h of incubation, and the signal became stronger in the pancreatic exocrine cells and spread to surrounding hepatic parenchymal cells at 2 h. At 8 h, the whole section was stained brown. In the skin, weak positive signals were observed at the connective tissue on the muscle side (data not shown), but the epidermis and dermis layer were not stained at 0.5 h. Although there was no obvious change between 0.5 h and 2 h, strong TTX-positive signals were confirmed at basal cells between the epidermis and dermis at 8 h. In the intestine, weak positive signals were observed at the epithelial cells and lamina propria of the intestinal villi at 0.5 h, then the signals became stronger at 2 h and extended to the muscular layer at 8 h.

4. Discussion

In the present study, the *in vitro* tissue slice incubation method developed by Nagashima et al. (2003) for liver tissue was applied to the skin and intestine tissues as well, and demonstrated for the first time that the TTX uptake ability is similar between the skin, intestine, and liver of *T. rubripes*, and is higher in the skin of young fish than in the skin of adult fish. In addition, temporal changes in the microdistribution of TTX in each tissue slice were successfully visualized using immunohistochemistry.

Nagashima et al. (2003) reported that when the liver slices of several general fish species were incubated in the same TTX concentration as used in the present study (25 μg/mL), ~3-4 μg/g TTX was detected at 0.5 h, but the amount changed little thereafter. In contrast, in the liver slices of *T. rubripes*, the TTX content increased over time, and reached up to ~12 μg/g at 24 h and ~15 μg/g at 48 h. After that, the TTX content did not decrease even when incubated in incubation buffer containing no TTX.

These findings led them to conclude that the liver tissue of *T. rubripes* is endowed with high TTX uptake ability. In the present study, TTX was taken up into the liver slices at nearly the same level at the same incubation times (~11 μg/g at 24 h, ~14 μg/g at 48 h), confirming the high reproducibility of this experimental system. The TTX content in the skin and intestine slices was comparable to or slightly higher than that in the liver slices, with a similar transition pattern between the three tissue types. The tissue structures and properties differ between the liver and skin/intestine, and it is unlikely that such a liver-like TTX uptake profile was caused by mere physical diffusion of TTX. Therefore, we concluded that the tissue slice incubation method can be applied for evaluating the TTX uptake ability of the skin and intestine, and that the TTX uptake ability of the skin and intestine of *T. rubripes* is similar to that of the liver. In future studies, the TTX uptake ability of the skin and intestine should be evaluated in non-toxic pufferfish and in general fish as well.

Wild adult *T. rubripes* accumulate high levels of TTX in the liver and ovary, but the skin, muscle, and testis are generally non-toxic (Noguchi and Arakawa, 2008). According to studies by Ikeda (2009) and Tatsuno (2012), however, the TTX amount in the skin accounts for more than 90% of the total TTX amount in wild young *T. rubripes* (small-sized fish with a body weight of 20.9 ± 3.9 g). Medium-sized fish (body weight 261 ± 66 g) have a lower TTX ratio in the skin than small-sized fish, and the TTX amount in the liver accounts for 15%-86%. Therefore, it is presumed that the skin is rather the main toxin accumulation tissue in young *T. rubripes*, but the liver becomes the main toxin repository as the fish grows. Similarly, in a rearing experiment in which cultured young (under 1 year old) and adult (under 2 years old) *T. rubripes* were fed a TTX-containing diet for 60 days, the TTX accumulation rate in the skin was higher in the young fish than in the adult fish (Honda et al., 2005). When TTX was administered intramuscularly to cultured young *T. rubripes*, most of the toxin was transported to the skin where it accumulated (Ikeda et al., 2009). On the basis of their *in vivo* TTX administration experiment using *T. rubripes* of different ages, Tatsuno et al. (2013a) assumed that the growth-dependent changes in the toxin distribution between the skin and liver were due the undeveloped liver in young fish, making TTX less likely to accumulate in the liver than in adult fish, and rather to transfer to the skin. Furthermore, Kiriake et al. (2016) performed an *in vitro* tissue slice incubation experiment and found no difference in the TTX uptake ability of the liver between young and adult fish, and presumed age-dependent differences in the ability to retain or metabolize TTX after uptake. In the present study, like in Kiriake et al. (2016), the TTX uptake ability in the liver did not differ significantly between young and adult fish. The TTX uptake ability of the intestine also differed little between young and adult fish. In contrast, the skin of the young fish took up about twice as much TTX as the skin of the adult fish. This finding strongly suggests that the TTX uptake ability of the skin is involved in the growth-dependent changes in the toxin distribution inside the body in *T. rubripes*, although the ability of the

256 liver to retain and metabolize TTX requires further investigation.

257 From the temporal change in the microdistribution of TTX in each tissue slice, the TTX uptake
258 pathway in each tissue can be estimated to some extent. Tatsuno et al. (2017) reported that in an *in vivo*
259 TTX administration experiment using *T. rubripes*, TTX-positive signals were obtained in the whole
260 hepatic parenchymal cells and pancreatic exocrine cells in the liver only when administered at a high
261 dose (300 μg/fish). They concluded that TTX overflowing from the hepatic cytoplasm was transferred
262 to the pancreatic exocrine cells. When temporal changes are considered, however, the reverse would be
263 true; TTX is first taken up into pancreatic exocrine cells, and then spreads to hepatic parenchymal cells.
264 In the skin, TTX seems to be first taken up into the connective tissues, and is then transferred to and
265 accumulates in the basal cells. Many pufferfish have secretory glands or secretory cells (sacciform cells)
266 in the skin (Itoi et al., 2012; Kodama et al., 1986; Mahmud et al., 2003; Tanu et al., 2002) and release
267 TTX from the skin in response to external stimuli (Kodama et al., 1985; Saito et al., 1985), but in *T.*
268 *rubripes*, no glandular structure is observed in the skin, and TTX-positive signals are found only in the
269 basal cells (Ikeda et al., 2009; Okita et al., 2013). Therefore, it is highly likely that the basal cell
270 properties are involved in the difference in the TTX uptake ability of the skin between young and adult
271 fish, consistent with the findings of the present study. In the intestine, TTX was assumed to be taken up
272 from the epithelial cells of the villi into the lamina propria, and gradually transferred to the muscle layer.
273 It is unclear, however, how such uptake of TTX by the intestine slices is involved in intestinal TTX
274 absorption *in vivo*. This point requires further clarification to apply the TTX uptake ability of the
275 intestine slices as an index of TTX absorption ability or TTX selectivity in the intestine

276 The findings of the present study indicate that the TTX uptake ability is similar among the skin,
277 intestine, and liver of *T. rubripes*, and is higher in the skin of young fish than in the skin of adult fish.
278 The molecular mechanisms involved in the age-dependent difference in the skin accumulation of TTX,
279 however, remain to be elucidated. A toxin-binding protein (puffer fish saxitoxin and tetrodotoxin binding
280 protein; PSTBP) was separated from the blood plasma of *T. pardalis* (Yotsu-Yamashita et al., 2001), and
281 genes homologous to PSTBP were found in *T. rubripes* and other *Takifugu* pufferfish (Hashiguchi et al.,
282 2015; Tatsuno et al., 2013b). These toxin-binding proteins could be involved in toxin transportation to
283 the skin and toxin absorption at the intestine (Yotsu-Yamashita et al., 2013). On the basis of an *in vitro*
284 experiment using liver tissue slices, Matsumoto et al. (2007) hypothesized that carrier-mediated
285 transport is responsible for the specific uptake of TTX in the pufferfish liver. Because the toxin transfer
286 profile to the skin and liver is different when TTX is administered to *T. rubripes* at different
287 concentrations, Tatsuno et al. (2017) speculated that the molecular mechanisms involved in the
288 transfer/accumulation of TTX differ between the skin and liver tissues. Very recently, Gao et al. (2019)
289 conducted *in vivo* toxin administration experiments using artificially reared specimens of the marine

species *T. pardalis* and the freshwater pufferfish *Pao suvattii*. Their findings indicated that *T. pardalis*, which naturally harbors TTX, selectively accumulates TTX, and *P. suvattii*, which naturally harbors paralytic shellfish toxin (PST), selectively accumulates PST. The stage at which the absorption, transportation, and accumulation of such toxin selectivity is exerted and the mechanism of the toxin selectivity, however, require further investigation. The *ex vivo* toxin administration method using cultured tissue slices, which was applied in this study, will be a powerful tool for addressing these questions and 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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Figure captions

Fig. 1. Changes in TTX content in the liver, skin, and intestine slices of adult *T. rubripes* during incubation for 48 h. Data are shown as means (symbols) and SD (error bars). Twelve tissue slices (8 mm in diameter, ~1 mm in thickness) were prepared from the liver, skin, and intestine of a non-toxic cultured adult *T. rubripes* (20 months old), and each slice was incubated with a 1.5 ml of incubation buffer containing 25 μg/mL TTX at 20°C for a maximum of 48 h. During the incubation, 3 slices of each tissue were collected at 1, 8, 24, and 48 h, and the TTX content was quantified by LC-MS/MS analysis.

Fig. 2. TTX content in the liver, skin, and intestine slices of young and adult *T. rubripes* after 8 h of incubation. Data are shown as means (columns) and SD (error bars). Asterisk indicates significant difference (*t*-test, $p<0.05$). Three tissue slices were prepared from the liver, skin, and intestine of three young (8 months old) and two adult (20 months old) *T. rubripes*, and an incubation experiment was conducted. As the previous experiment (Fig. 1) revealed that TTX uptake advanced sufficiently even at 8 h of incubation, the incubation time was set at 8 h, and after combining the data of 8-h incubation in the previous experiment, the TTX amount taken up into each tissue was compared between the young and adult fish.

Fig. 3. Changes in the microdistribution of TTX in liver slices of young *T. rubripes* during incubation for 8 h. A positive immunoreaction was visualized as a brown color. NC indicates negative control. Letters h and p indicate hepatic parenchymal cells and pancreatic exocrine cells, respectively. Scale bars indicate 50 μm.

Fig. 4. Changes in the microdistribution of TTX in skin slices of young *T. rubripes* during incubation for 8 h. A positive immunoreaction was visualized as a brown color. NC indicates negative control. Letters b, d, and e_d indicate basal cells, dermis layer, and epidermis, respectively. Scale bars indicate 50 μm.

Fig. 5. Changes in the microdistribution of TTX in intestine slices of young *T. rubripes* during incubation for 8 h. A positive immunoreaction was visualized as a brown color. NC indicates negative control. Letters e_t, l, and m indicate epithelial cells of villi, lamina propria, and muscular layer, respectively. Scale bars indicate 50 μm.

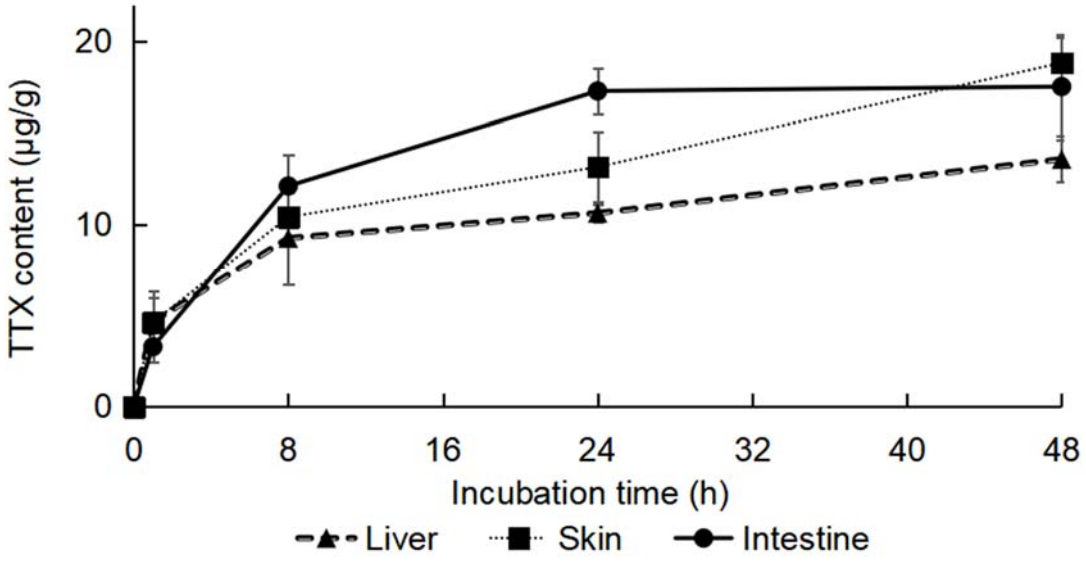
TTX content (μg/g)
20
10
0
0
8
16
24
32
40
48
Incubation time (h)
Liver
Skin
Intestine

Fig. 1

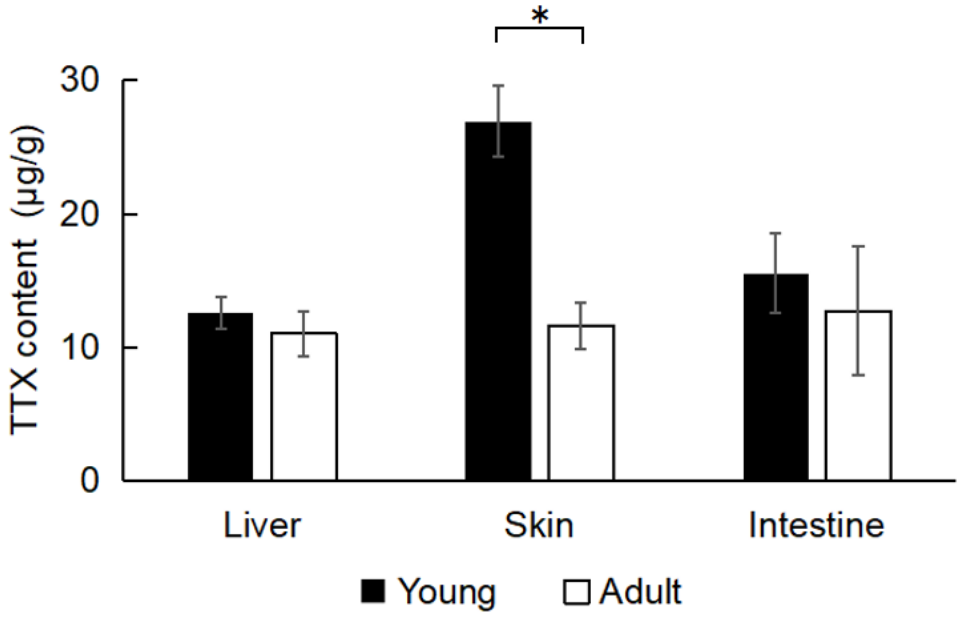
*
TTX content (μg/g)
30
20
10
0
Liver
Skin
Intestine
Young
Adult

Fig. 2

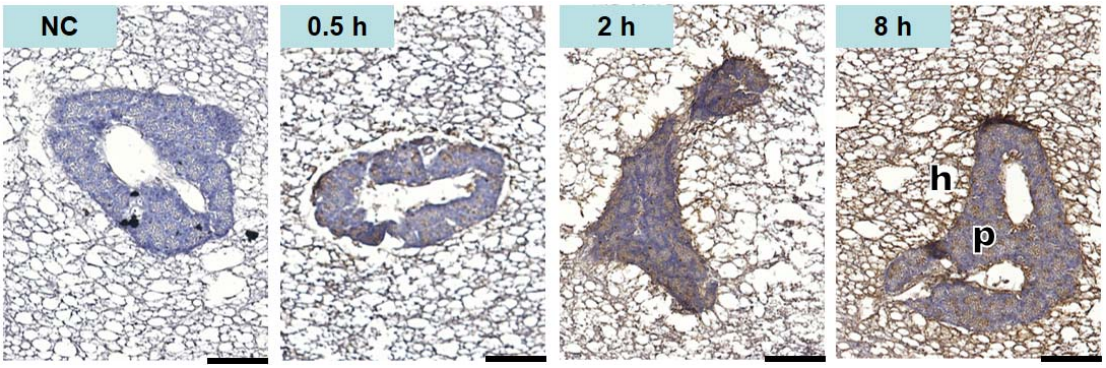

Fig. 3

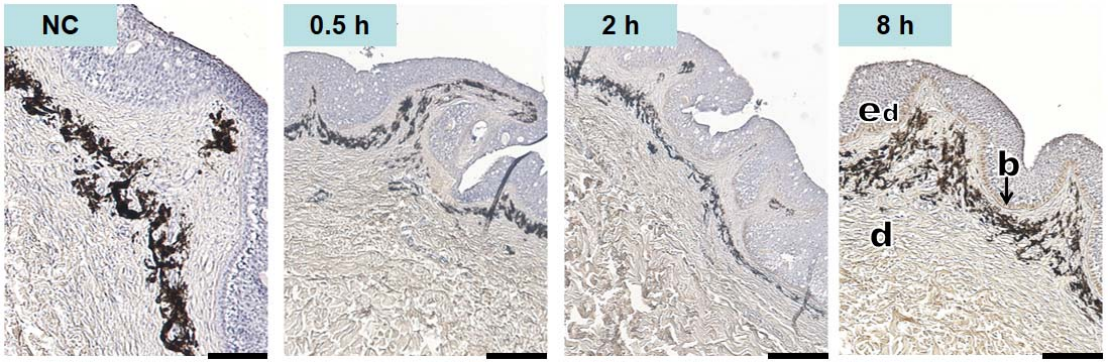

Fig. 4

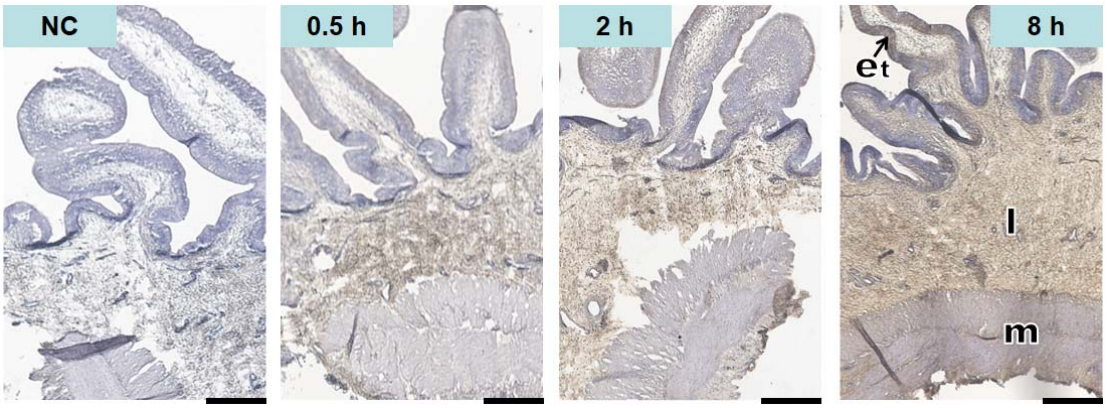

Fig. 5