Effects of paralytic shellfish toxins on the middle intestine of Oncorhynchus mykiss: Glutathione metabolism, oxidative status, lysosomal function and ATP-binding cassette class C (ABCC) proteins activity

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ABCC
Saxitoxin
Multixenobiotic resistance
Lysozyme
Toxicokinetics
Rainbow trout

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A B S T R A C T

We studied the absorption, cytotoxicity and oxidative stress markers of Paralytic Shellfish Toxins (PST) from three extracts from Alexandrium catenella and A. ostenfeldii, in middle Oncorhynchus mykiss intestine in vivo and ex vivo preparations. We measured glutathione (GSH) content, glutathione S-transferase (GST), glutathione reductase (GR) and catalase (CAT) enzymatic activity, and lipid peroxidation in isolated epithelium exposed to 0.13 and 1.3 μM PST. ROS production and lysosomal membrane stability (as neutral red retention time 50%, NRRT50) were analyzed in isolated enterocytes exposed to PST alone or plus 3 μM of the ABCC transport inhibitor MK571. In addition, the concentration-dependent effects of PST on NRRT50 were assayed in a concentration range from 0 to 1.3 μM PST. We studied the effects of three different PST extracts on the transport rate of the ABCC substrate DNP-SG by isolated epithelium. The extract with highest inhibition capacity was selected for studying polarized DNP-SG transport in everted and non-everted intestinal segments. We registered lower GSH content and GST activity, and higher GR activity, with no significant changes in CAT activity, lipid peroxidation or ROS level. PST exposure decreased NRRT50 in a concentration-depend manner (IC50 = 0.0045 μM), but PST effects were not augmented by addition of MK571. All the three PST extracts inhibited ABCC transport activity, but this inhibition was effective only when the toxins were applied to the apical side of the intestine and DNP-SG transport was measured at the basolateral side. Our results indicate that PST are absorbed by the enterocytes from the intestine lumen. Inside the enterocytes, these toxins decrease GSH content and inhibit the basolateral ABCC transporters affecting the normal functions of the cell. Furthermore, PST produce a strong cytotoxic effect to the enterocytes by damaging the lysosomal membrane, even at low, non-neurotoxic concentrations.

1. Introduction

Paralytic shellfish toxins (PST) are neurotoxic compounds that inhibit voltage-gated sodium channels interrupting the action potential in nerve and muscle cells (Catterall, 1980, for a review). PST comprise several structural variants, such as saxitoxin (STX), sulfated gonyautoxins (GTX) and N-sulfocarbamoyl toxins (B- and C-toxins), of which STX is the most neurotoxic one (Thottumkara et al., 2014). These compounds are produced by marine dinoflagellates and freshwater cyanobacteria (Lagos, 2003, for a review; Kellmann et al., 2013), and can reach high levels in blooms or surface scums, which can particularly affect the use of water for tap water production, aquaculture and recreational activities (Testai et al., 2016a, for a review). PST may be accumulated in aquatic organisms and incorporated into the trophic chain (Ferrao-Filho and Kozlowsky-Suzuki, 2011; Lopes et al., 2014; Madigan et al., 2018; Suárez-Isla, 2016). In fish, besides neuromotor effects (Bakke and Horsberg, 2007; Bakke et al., 2010; Lefebvre et al., 2004), PST can produce oxidative stress (Clemente et al., 2016; da Silva et al., 2011) and lethal effects (Montoya et al., 1997; White, 1980).

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The highest amounts of these toxins are mostly found in gut content and liver (Deeds et al., 2008) although accumulation of PST in muscle has been reported to occur in two freshwater fish species used for human consumption, the Nile tilapia (Oreochromis niloticus) (Galvão et al., 2009) and Geophagus brasiliensis (Calado et al., 2020). Therefore, it is important to know the capacity of fish used in aquaculture for biotransformation and excretion of PST, in order to avoid possible risks of human poisoning (Etheridge, 2010). For example, the Atlantic salmon (Salmo salar) and the Atlantic cod (Gadus morhua) can excrete PST from their organs (Bakke and Horsberg, 2010), but the biotransformation and excretion mechanisms involved are not clear yet.

The biotransformation of PST has been reported to occur in bacteria, marine invertebrates and mammals (Garcia et al., 2010, 2004), through chemical reactions that include glucuronidation, oxidation, epimerization and reduction (Bricelj and Shumway, 1998, for a review; Lukowski et al., 2019). Fish like white seabream (Diplodus sargus) are able to biotransform the N-sulfocarbamoyl PST analogues, incorporated through the diet, into B1 and decarbamoyl saxitoxin (dcSTX) (Costa et al., 2011). This kind of biotransformation was reported to occur in in vivo preparations of O. mykiss intestine and liver (Bieczynski et al., 2014; Painefilú et al., 2019). The aim of this work was to evaluate the absorption and intracellular effects of PST in intestinal epithelial cells of O. mykiss middle intestine by measuring lysosomal membrane stability, ROS production, GSH content, GR and GST activity, and lipid peroxidation. Furthermore, we studied the interaction between PST and the MXR system through ABCC transport experiments in ex-vivo and in vitro preparations.

2. Materials and methods

2.1. Chemicals

1-chloro-2,4-dinitrobenzene (CDNB), MK571 sodium salt hydrate, glutathione (GSH), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), Dithiothreitol, Bovine serum albumin, Nicotinamide adenine dinucleotide phosphate (NADPH), Neutral red, Trypan blue, 2,7-dichlorofluorescein diacetate (DCFDA) and butylhydroxytoluene were purchased from Sigma (USA). Tris (hydroxymethyl) aminomethane (Tris) was purchased from Serva (Germany). 5-Sulfosalicylic acid was purchased from Mallinckrodt Chemicals (Ireland). Folin Ciocalteu reagent was purchased from Biopack (Argentina). Ethylenediaminetetraacetic acid (EDTA) and acetic acid were purchased from Cicarelli (Argentina). Glutathione di-sulfide (GSSG) was purchased from Santa Cruz Biotechnology (USA). Tris (hydroxymethyl) aminomethane (Tris) was purchased from Biopack (Argentina). Ethylenediaminetetraacetic acid (EDTA) and acetic acid were purchased from Cicauco (Argentina). Glutathione di-sulfide (GSSG) was purchased from Santa Cruz Biotechnology (USA). Trichloroacetic acid and Thiobarbituric acid were purchased from Merck (Germany). Dimethyl sulfoxide (DMSO) was purchased from Anerda (Argentina). Sodium citrate (Na3C4H5O7) was purchased from Stanton (Argentina). Lysing matrix D was purchased from MP Biomedicals (USA).

2.2. Paralytic shellfish toxin extracts

PST were extracted from cultures of Alexandrium catenella (PST 1) and Alexandrium ostenfeldii (PST2 and PST3) (Table 1). The cell pellet

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ABCC</td>
<td>ATP-binding cassette class C protein</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CDNB</td>
<td>1-chloro-2,4-dinitrobenzene</td>
</tr>
<tr>
<td>DCFDA</td>
<td>2,7-dichlorofluorescein diacetate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNP-SG</td>
<td>(dinitrophenyl-S-glutathione)</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5′-dithiobis (2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>GR</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione disulfide</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NRRT50</td>
<td>Neutral Red Retention Time 50%</td>
</tr>
<tr>
<td>PST</td>
<td>Paralytic Shellfish Toxins</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl) aminomethane</td>
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Table 1

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<tr>
<th></th>
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<th>A. ostenfeldii</th>
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<td>92.64</td>
<td>79.77</td>
</tr>
<tr>
<td>PST2</td>
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<td>B1</td>
<td>35.13</td>
<td>92.64</td>
<td>79.77</td>
</tr>
<tr>
<td>NEO</td>
<td>35.13</td>
<td>92.64</td>
<td>79.77</td>
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<tr>
<td>STX</td>
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was lysed with 500 μL of 0.03 N acetic acid and 0.9 g lysing matrix D in a sample disruption instrument (FastPrep®, Thermo Savant, Illkirch, France) and homogenized by reciprocal shaking at 6.5 ms\(^{-1}\) for 45 s. After this, samples were centrifuged at 16,100 \(\times\) g at 4 °C, for 15 min. The supernatant was filtered (0.45 μm pore size) and centrifuged at 800 \(\times\) g for 30 s. The toxins contained in the extract were determined by HPLC/FLD with post-column derivatization (Column: Phenomenex Luna C18, 5 μm, 250 \(\times\) 4.6 mm) a detailed in Van de Waal et al. (2015).

### 2.3. Fish and experimental models

*O. mykiss* individuals (280 ± 40 g) were maintained in tanks with circulating freshwater (\(T^\circ = 17 ± 3.6\) °C, pH 7.13 ± 0.7, DO = 9.84 ± 0.24 mg L\(^{-1}\)) from Chimehuin river, in the Centro de Ecología Aplicada del Neuquén (CEAN, Argentina). Fish were fasted for 24 h before sacrificed by a blow to the head and decapitation. The middle intestine was removed, rinsed in Cortland solution and used for obtaining intestinal segments, isolated epithelium or isolated enterocytes. For all these preparations, the intestinal of each individual was used to obtain the control and the treated preparations. Six to nine individuals were used for each experiment. These experimental protocols were approved by the Bioethics Committee, Faculty of Biochemical and Pharmaceutical Sciences, National University of Rosario, Argentina (6060/116).

The use of different experimental models allowed the measurement of a broad range of variables with more realistic to more detailed approaches. The intestinal segments allow to maintain the epithelium polarity to differentiate apical from basolateral transport. We used the isolated epithelium in ex vivo assays to avoid any interference from the sub-epithelial muscle layers and to obtain sensitive responses. Finally, the isolated enterocytes allowed the determination of reactive oxygen species (ROS) levels and lysosomal membrane stability.

#### 2.3.1. Intestinal segments

According to Bieczynski et al. (2014), to prepare paired everted and non-everted preparations, the middle intestine was separated and cut into two transversal segments (one for control and the other for the treatment), weighed, ligated at both ends and filled with Cortland solution with a Teflon cannula attached to a Hamilton syringe. For the everted preparations, the segment was everted by sliding a crochet needle inside the intestine tube.

#### 2.3.2. Isolated epithelium

The middle intestine was opened longitudinally and cut into strips. From each strip, the epithelial layer was separated from the muscular layer with a 170 μm thick PVC sheet. The isolated epithelium was incubated with PST according to the variable to be measured. After each incubation, the epithelium was put in ice-cold homogenization buffer (Tris 40 mM; KCl 150 mM; 0.5 mM EDTA), pH 8.6, and homogenized with a Cole-Parmer LabGen 7 homogenizer at 35,000 rpm. Homogenates were centrifuged at 9700 \(\times\) g for 15 min at 4 °C and supernatants were used for determinations.

#### 2.3.3. Isolated enterocytes

Enterocytes were isolated according to the protocol described by Kwong and Niyogi (2012), with slight modifications. The middle intestine was cut into strips and incubated for 5 min in 5 mL of citrate buffer (pH 7.4) and 20 min in 5 mL of isolation buffer (154 mM NaCl, 10 mM NaO\(_2\)HPO\(_4\), 2 mM EDTA, 2 mM dithiothreitol, 5.5 mM glucose, pH 7.4). After this, enterocytes were removed from the muscular layer using a 170 μm needle inside the intestine tube.

2.4. Intracellular effects

2.4.1. Protein content in isolated epithelium

The results of the experiments conducted with isolated epithelium were referred to total soluble protein, which was the standardization variable with lowest variability in preliminary assays. Total protein content was measured in the supernatant of epithelium homogenates by the method of Lowry et al. (1951), using bovine serum albumin as standard and were expressed as mg protein mL\(^{-1}\).

2.4.2. Glutathione and associated enzymes

We measured GSH content, GST activity, and glutathione reductase (GR) activity in isolated epithelium incubated for 1 h in glass vials with 3 mL of Cortland solution alone or plus 0.13 μM (50 μg L\(^{-1}\)) or 1.3 μM (500 μg L\(^{-1}\)) PST1. These concentrations were defined according to cellular PST contents and cell numbers reported for several dinoflagellate blooms (Cembella et al., 2002; Sephton et al., 2007; Testai et al., 2016b; for a review).

GSH content was measured according to Ellman’s method (1959), modified by Venturino et al. (2001). Supernatants (section 2.3.2) were deproteinized by treatment with 5% sulfosalicylic acid, final concentration and centrifuged at 6700 \(\times\) g for 10 min. These supernatants were incubated for 5 min with 2 mM DTNB, final concentration, in 250 mM phosphate buffer, pH 8, and absorbance was read at 412 nm. The results were expressed in nmol GSH mg protein\(^{-1}\).

GST activity was measured according to Habig et al. (1974). The absorbance was measured through 180 s in 100 mM phosphate buffer, pH 6.5, with 100 mM GSH and 100 mM CDNB as substrates. Activity was expressed in nmol s\(^{-1}\) mg protein\(^{-1}\).

GR activity was measured according to Schaede and Bassham (1977), with modifications. Absorbance was read at 340 nm for 300 s in 143 mM phosphate buffer plus 6.3 mM EDTA, pH 7.5, with 0.13 mM NADPH and 10 mM GSSG as substrates. The activity was expressed in nmol s\(^{-1}\) mg protein\(^{-1}\).

2.4.3. Oxidative stress biomarkers

We measured catalase (CAT) activity and lipid peroxidation in supernatants of homogenates from isolated epithelium (section 2.3.2) incubated for 1 h in glass vials with 3 mL of Cortland solution alone or plus 0.13 μM or 1.3 μM of PST1 extract and ROS production in isolated enterocytes (section 2.3.3) incubated for 1 h in glass vials with 1 mL of Cortland solution alone, plus 0.13 μM PST1 extract, or plus 0.13 μM PST1 extract with 3 μM MK571.

CAT activity was measured according to Aebi (1984), with modifications. Absorbance was read at 240 nm for 30 s in 50 mmol L\(^{-1}\) phosphate buffer, pH 7 with 0.3 M H\(_2\)O\(_2\) as substrate. Activity was expressed in nmol s\(^{-1}\) mg protein\(^{-1}\).

Lipid peroxidation was measured according to Fraga et al. (1988) by the thiobarbituric acid reactive substances (TBARS) method. Absorbance was read at 532 nm in a reaction mixture with 26 mM thiobarbituric acid, 2% HCl, 15% trichloroacetic acid and 100 mM butylhydroxytoluene. The results were expressed as μmol mg protein\(^{-1}\).

ROS production was measured according to Amado et al. (2009) and Moss and Bassem (2006), with modifications. A suspension of isolated enterocytes (10\(^5\) viable cells mL\(^{-1}\)) was added to the reaction buffer (30 mM Hepes, 200 mM KCl, 1 mM MgCl\(_2\), pH 7.2), with 40 μM DCFDA as substrate. Fluorescence was recorded in a Qubit fluorometer (Invitrogen, USA) at 485/530 nm for 60 min. Fluorescence units measured along time were adjusted to a second order polynomial function and the area under the curve (AUC) was calculated and standardized to 5 \(\times\) 10\(^4\) viable cells mL\(^{-1}\) (Amado et al., 2009). Data are shown as percentage of the control.
2.4.4. Lysosomal membrane stability

Isolated enterocytes (10^6 viable cells mL\(^{-1}\), final concentration) were incubated for 1 h in glass vials with 0.5 mL of Cortland solution and PST1 at seven increasing concentrations from 0 to 1.3 µM. Then, the lysosomal membrane stability was evaluated using the Neutral Red Retention Time assay (NRRT50), according to Mamaca et al. (2005) and Dayeh et al. (2003), with modifications. After incubation with PST, the isolated enterocytes were incubated with 66 µM (final concentration) neutral red dye for 5 min. Each sample was observed under a light microscope and the number of red stained cells was counted every 10 min, until reaching 50% of the total cells. The results were expressed as neutral red retention time 50% (NRRT50, min). Data were adjusted to a non-linear regression analysis of NRRT50 (as percentage of the control) vs. log PST concentration.

2.5. ABCC activity

The experiments of this section are based on that CDNB is a membrane permeant compound, which diffuses into the cell, where it is conjugated with GSH in a reaction catalyzed by GST. The conjugation product, dinitrophenyl-S-glutathione (DNP-SG) is exported by ABCC proteins from the cell to the external bath, where it is detected spectrophotometrically (Fig. 1).

2.5.1. DNP-SG transport in isolated epithelium

We studied the effects of three PST extracts (PST1, PST2 and PST3) on DNP-SG transport rate in isolated intestinal epithelium. We incubated isolated epithelium strips in glass vials with 3 mL of Cortland solution plus 200 µM of CDNB. All PST extracts were applied at final concentrations of 0.13 µM for PST1 and 0.11 µM, for PST2 and PST3. These concentrations are equivalent to 50 µg L\(^{-1}\) for all extracts. Transport rate results were expressed as nmol DNP-SG mg protein\(^{-1}\) min\(^{-1}\).

2.5.2. DNP-SG transport in intestinal segments

The effects of PST on the apical (luminal) and basolateral DNP-SG transport were studied in paired everted and non-everted intestinal segments, respectively. In both types of preparation, the PST were applied either at the apical or at the basolateral side, in order to study the permeability of both membranes to PST. In all cases, CDNB was added in the bathing solution (Fig. 1). PST2 was selected for this experiment because it was the most effective in the previous experiment with isolated epithelium.

DNP-SG absorbance was measured at 340 nm every 10 min for 1 h and the DNP-SG transport rate was calculated as the slope of a cumulative absorbance vs. time plot, and using an extinction coefficient of 9.6 mM cm\(^{-1}\). The transport rate was normalized to wet tissue mass and the result for each individual was calculated as percentage of the corresponding control. The specificity of the assay for DNP-SG transport was previously confirmed in similar preparations by HPLC analysis (Bieczyński et al., 2014).

2.5.3. Effects of ABCC transport inhibition on PST cytotoxicity

In order to investigate whether PST are exported by ABCC transporters, we studied NRRT50, as a cell toxicity marker, in enterocytes treated with PST and the ABCC transport inhibitor MK571. We incubated a suspension of isolated enterocytes (10^6 viable cells mL\(^{-1}\), final

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**Fig. 1.** Ex-vivo exposure design used to study polarized DNP-SG transport in *Oncorhynchus mykiss* middle intestine. Different preparations allowed the study of different possible routes of PST uptake and exportation by the cell a) Everted segments. b) Non-everted segments. In all the preparations DNP-SG concentration was measured in the bathing solution.
concentration) for 1 h with 0.0033 μM PST1, with or without 0.1 μM MK571, dissolved in DMSO. This MK571 concentration was selected from a preliminary study of the concentration-dependent effects of this inhibitor on NRRT50 in isolated enterocytes (Fig. 6a) and on DNP-SG transport assays (Fig. 6b). In NRRT50 assays, we included a solvent control (DMSO). In both, NRRT50 and DNP-SG transport assays, the DMSO concentration was lower than 0.002%.

2.6. Statistics

Bartlett’s test was used to check homogeneity of variance and Kolmogorov-Smirnov test was used for checking normality. Differences among treatments were tested by paired Student’s t-test, or repeated measures one-way ANOVA. We used Dunnett’s pos hoc comparisons when the treatments were compared against the control, and Tukey’s pos hoc comparisons for comparing among treatments. P < 0.05 was considered as statistically significant (Zar, 1999). The arcsine square root transformation was applied before analyzing percentage data.

3. Results

3.1. Intracellular effects

3.1.1. Glutathione and GSH-associated enzymes

The intracellular glutathione metabolism was affected by 1 h of ex vivo exposure to PST1 in the intestinal epithelium. The GSH content was diminished by 16.33% after exposure to 0.13 μM PST1 and by 16.24%, with 1.3 μM PST1 (repeated measures, one-way ANOVA and Dunnett’s pos hoc comparisons, F1,9, 1.5 = 1.50, p < 0.05 and p < 0.01, respectively, n = 9) (Fig. 2a). GST activity decreased 19.3% after exposure to 0.13 μM PST1 (repeated measures, one-way ANOVA and Dunnett’s pos hoc comparisons, F1,9, 1.6 = 4.639, p < 0.05, n = 7) but was not affected by 1.3 μM PST1 (Fig. 2b). GR activity increased 14.27% with 0.13 μM PST1 and 15.64% with 1.3 μM PST1 but the effect was statistically significant only at the highest PST1 concentration (repeated measures, one-way ANOVA and Dunnett’s pos hoc comparisons, F1,7, 1.6 = 6.483, p < 0.05, n = 8) (Fig. 2c).

3.1.2. Oxidative stress biomarkers

There were no significant effects of PST1 on the oxidative status of the middle intestine epithelium. Neither CAT activity nor lipid peroxidation in isolated epithelium were affected by PST1 (Fig. 3). ROS production in isolated enterocytes was not significantly augmented by 0.13 μM PST1, neither by PST1 plus 3 μM MK571 (Fig. 3).

3.1.3. Lysosomal membrane stability

Lysosomal membrane stability was evaluated in order to find a sensitive marker for intracellular effects of PST. We registered a concentration-dependent effect of PST1, which fitted to a non-linear regression in isolated epithelium were affected by PST1 (Fig. 3). ROS production in isolated enterocytes was not significantly augmented by 0.13 μM PST1, neither by PST1 plus 3 μM MK571 (Fig. 3).

3.2. ABCC activity

3.2.1. DNP-SG transport

All the tested PST extracts inhibited DNP-SG transport in isolated intestinal epithelium with respect to the control preparations (30.08, 50.66 and 32.26% for PST1, PST2 and PST3, respectively) (repeated measures, one-way ANOVA and Dunnett’s pos hoc comparisons, F2,2, 13.1 = 16.82, p < 0.05, p < 0.01 and p < 0.05, respectively, n = 7) (Fig. 5a).

According to these results, PST2 was selected for performing the assays with everted intestine segments. PST2 inhibited the basolateral DNP-SG transport by 17.62%, but only when it was applied at the apical side (paired Student’s t-test, t3 = 3.99, p < 0.01, n = 8) (Fig. 5b). There was no effect of PST2 on apical DNP-SG transport.

3.2.2. Effects of ABCC transport inhibition on PST cytotoxicity

In a preliminary experiment with, isolated enterocytes, the exposure to 0.1–3 μM of the ABCC transport inhibitor MK571, decreased NRRT50 in a concentration-dependent fashion (Fig. 6a). In addition, 0.1 μM MK571 proved to be effective for inhibiting DNP-SG transport (one-way ANOVA and Dunnett’s post hoc comparisons, F2, 21 = 4.74, p < 0.05, n = 6–9) (Fig. 6b). Consequently, we chose the lowest MK571 concentration (0.1 μM) for the following assays, in order to avoid the effect of MK571 on NRRT50 as much as possible.

In the isolated enterocytes exposed to 0.1 μM MK571, NRRT50 was
reduced by 18.17%, with respect to the control, but this effect was not statistically significant. In turn, in the isolated enterocytes exposed to 0.0033 μM PST1, NRRT50 was 51.40% lower than in control enterocytes (Tukey’s post hoc comparisons, $F_{3, 15} = 27.99, p < 0.0001, n = 6$). The co-exposure to 0.0033 μM PST1 plus 0.1 μM MK571 reduced NRRT50 by 65.85%, with respect to the control (Tukey’s post hoc comparisons, $F_{3, 15} = 27.99, p < 0.0001, n = 6$). There were not significant differences between the effects of PST1 alone and PST1 plus 0.1 μM MK571 (Fig. 6c).

4. Discussion

This study provides evidence on the absorption of PST by the middle intestine epithelium of *O. mykiss* obtained in ex vivo and in vitro experiments. The obtained results show, for the first time, that these toxins are
taken up by the cells of fish intestine epithelium, affect glutathione metabolism and produce cytotoxic effects even after a short exposure (1 h) to PST concentrations below the recommended values for drinking water. We also show, for the first time, that PST interfere with the MXR defense system by inhibiting ABCC-mediated transport.

Several works report that in mammals, including humans, PST are absorbed in the intestine through paracellular transport (Andrinolo et al., 2002a, 2002b; Torres et al., 2007) and that their elimination takes place mostly in the kidney by glomerular filtration. However, reports on the presence of PST in bile (García et al., 2004, 2010; Gessner et al., 1997) and the report of STX being transported through ABCB1 (Pgp) in a mouse hippocampal cell line (Ramos et al., 2018) suggest that PST can be transported transcellularly by active transporters such as ABC proteins.

In contrast, PST intestinal absorption in fish has received little attention. Gao et al. (2019) reported that the seawater pufferfish Takifugu pardalis fed with tetrodotoxin (TTX) and STX rapidly accumulates TTX in specific tissues while most STX remains in the intestine. The opposite occurs with the freshwater pufferfish Pao suvattii, which accumulates STX in skin, ovary and intestine but keeps little or no TTX in the body (Gao et al., 2019). These studies do not analyze the mechanisms by which a specific toxin can cross the intestinal wall, while the other cannot, but suggest the selectivity of fish intestine for different toxins. However, as far as we know, there are no reports on the transcellular uptake of PST in vertebrates’ intestine, neither on intracellular effects of PST in this organ.

GSH depletion is generally regarded as indicative of pro-oxidant conditions since GSH is oxidized to GSSG in the presence of ROS (Deponte, 2013; Meister and Anderson, 1983; Wu et al., 2004). We have found that 0.13 μM PST1 causes a mild but significant decrease of intracellular GSH content, but the effect is not increased at a higher PST1 concentration (1.3 μM PST1). Accordingly, the increase in GR activity at the highest PST1 concentration could explain the lack of concentration dependence in GSH content through augmented recycling of GSSG to GSH. The fact that the increase in GR activity seems to stabilize the GSH content but does not reverse the depletion suggests that GSH is being consumed or oxidized in some biotransformation reaction. In this sense, the conversion between PST analogues, such as GTXs into SXTs, with the formation of thiol-conjugated intermediates, has been demonstrated by Sato et al. (2000) and Sakamoto et al. (2000) for bacteria and in laboratory reactions. In bivalves, N-sulfocarbamoyl toxins (C1 and C2) are reduced to carbamoyl toxins (GTX1-GTX4, NEO and STX) (Cembella et al., 1994; Asakawa et al., 1987). In fish, this kind of biotransformation has been suggested in the Atlantic salmon (Salmo salar) and in the white seabream (Diplodus sargus), respectively Gubbins et al. (2000) and Costa et al. (2011). Considering that PST1 contains 39% GTX1/4 and 35% of C1/2, the decrease in GSH content observed in our experiment could reflect the occurrence of biotransformation reactions that involve GSH conjugation and/or oxidation in O. mykiss enterocytes. Another alternative explanation for the observed decrease in GSH content of enterocytes could be the exportation of GSH from the cells as a co-substrate for the transport of toxins through ABCC proteins.

The participation of GST in the detoxification of PST is still unclear. In fish, GST activity has been reported to increase after exposure to PST (Calado et al., 2020; Clemente et al., 2010; Gubbins et al., 2000), or to decrease (Silva et al., 2011; Barbosa et al., 2019), while in mice and in...
cladoceran crustaceans, PST have no effect on GST activity (Ferreão-Filho et al., 2017; Hong et al., 2003). An increase in GST activity can be related with increased antioxidant and/or biotransformation activity (Calado et al., 2020; Stephensen et al., 2002; Painefilú et al., 2019). In the present work, we observe a decrease in GST activity with 0.13 μM PST1, which could be related to GSH depletion; however, this effect is not evident at higher PST1 concentration (1.3 μM). At this concentration, GR activity is significantly increased and could provide GSH by reducing GSSG to maintain GST activity, at least in the short term (Li et al., 2011; Vieira et al., 2009). However, we could not measure GSSG content to further support this idea.

All the PST extracts studied in this work inhibited the DNP-SG transport. According to their composition (see Table 1), PST2 and PST3 should be 6-fold less neurotoxic than PST1. On the other hand, PST2 resulted the most potent transport inhibitor (50% at 0.11 μM). In the experiments with polarized preparations, PST2 was able to inhibit basolateral but not apical DNP-SG transport, which differs from previous reports by our group, in which microcystin-LR and arsinite have inhibited DNP-SG transport at both sides of O. mykiss intestinal epithelium (Bieczynski et al., 2014; Painefilú et al., 2019). In addition, PST2 inhibited basolateral transport only when it was applied to the apical side. This suggests that at least one of the toxins contained in PST2 can cross the apical membrane of the enterocytes and inhibit DNP-SG transport through basolateral ABC proteins either as competitive (substrate) or noncompetitive inhibitor. This also suggests that PST do not enter the enterocytes from the blood.

Garcia et al. (2010, 2009) have proposed that PST can be detoxified by oxidation followed by glucuronidation, which is considered an important process for detoxification of xenobiotics in rainbow trout (Clarke et al., 1991, for a review; Lahiti et al., 2011). Besides, glucuronide conjugates have been described as substrates for basolateral ABC transporters, such as ABC3 and ABC4 (Bai et al., 2004; Dereeley et al., 2006, for a review; Zamek-Gliszczynski et al., 2006, for a review). Unlike the apical ABC2, human basolateral ABCs, especially ABC3 have more affinity for glucuronide conjugates than for GSH conjugates such as DNP-SG (Zamek-Gliszczynski et al., 2006). Thus, the possible transport of glucuronide-conjugated PST through ABC3 and/or ABC4 in O. mykiss deserves further analysis.

In order to gather more evidence about the possible transport of PST or their metabolites through basolateral ABC proteins, we have studied the effects of PST1 alone or combined with the ABC inhibitor MK571. PST1 significantly reduces NRRT50, but blocking ABCs with MK571 does not increase this effect, as it would be expected, if PST were transported through ABCs (Bieczynski et al., 2014, 2016). Thus, PST components would more likely inhibit basolateral ABC-mediated transport by a non-competitive mechanism.

The strong concentration-dependent effect of PST1 on lysosomal neutral red retention (IC50 = 0.0045 μM (1.34 μg STXeq L−1)) shows that these toxins are absorbed into O. mykiss enterocytes and are cytotoxic at concentrations that are lower than the guideline values for drinking water (3 μg STXeq L−1 for children, and 20 μg STXeq L−1 for adults, WHO, 2019). Besides the considerations for human health, this finding suggests that PST producing blooms could affect the digestive system of fish, particularly in aquaculture facilities, at non-neurotoxic concentrations (FAO, 2016; Testai et al., 2016b). The observed cytotoxic effects could be explained by toxin binding to voltage-gated Na+ and/or Ca2+ channels in the lysosomal membrane, which are critical for lysosomal functions (Xu and Ren, 2016). This also suggests NRRT50 in O. mykiss enterocytes as a sensitive end-point for PST toxicology.

5. Conclusions

In O. mykiss, PST present in the intestinal lumen are absorbed into the epithelial cells and produce cytotoxic effects, even at low concentrations. This means that the digestive physiology of fish can be altered when are exposed to blooms or surface scums of PST producing microorganisms, e.g. in aquaculture facilities. These alterations can produce adverse consequences in fish health and growth, in the absence of visible neurotoxic effects. In addition, the fact that PST inhibit the basolateral ABCC transporters suggests that these toxins can enhance the toxicity of other toxins by delaying their transport out of the intestinal cells. Particularly, microcystin-LR can be produced together with PST by freshwater cyanobacteria and are exported from fish intestinal cells through ABCC proteins.

We can conclude that PST are not eliminated from O. mykiss intestinal cells by ABCCC transporters but affect their function. Thus, the participation of other xenobiotic transporters, which are expressed in the intestine and in other organs of fish, such as ABCB1 and ATP-binding cassette class G protein 2 (ABCG2), together with biotransformation enzymes, should be investigated in order to understand PST detoxification and transport in fish.

Ethics approval

The experimental protocols were approved by the Bioethics Committee, Faculty of Biochemical and Pharmaceutical Sciences, National University of Rosario, Argentina (6060/116).

Credit author statement


Declaration of competing interest

The authors declare that they have no conflict of interest.

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