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Subacute immunotoxicity of the marine phycotoxin yessotoxin in rats

Sara F. Ferreiro [a], Natalia Vilariño [a, **], Cristina Carrera [a, c], M. Carmen Louzao [a], Germán Santamarina [b, c], Antonio G. Cantalapiedra [b, c], J. Manuel Cifuentes [d], Andrés C. Vieira [a], Luis M. Botana [a, *]

[a] Departamento de Farmacología, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002 Lugo, Spain
[b] Departamento de Ciencias Clínicas Veterinarias, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002 Lugo, Spain
[c] Hospital Veterinario Universitario Rof Codina, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002 Lugo, Spain
[d] Departamento de Anatomía y Producción Animal, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002 Lugo, Spain

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ABSTRACT

Yessotoxin (YTX) is a marine phycotoxin produced by dinoflagellates and accumulated in filter feeding shellfish. YTX content in shellfish is regulated by many food safety authorities to protect human health, although currently no human intoxication episodes have been unequivocally related to YTX presence in food. The immune system has been proposed as one of the target organs of YTX due to alterations of lymphoid tissues and cellular and humoral components. The aim of the present study was to explore subacute immunotoxicity of YTX in rats by evaluating the haematological response, inflammatory cytokine biomarkers and the presence of YTX-induced structural alterations in the spleen and thymus. The results showed that repeated administrations of YTX caused a decrease of lymphocyte percentage and an increase of neutrophil counts, a reduction in interleukine-6 (IL-6) plasmatic levels and histopathological splenic alterations in rats after four intraperitoneal injections of YTX at doses of 50 or 70 μg/kg that were administered every 4 days along a period of 15 days. Therefore, for the first time, subacute YTX-immunotoxicity is reported in rats, suggesting that repeated exposures to low amounts of YTX might also suppose a threat to human health, especially in immuno-compromised populations.

1. Introduction

Yessotoxin (YTX) is a marine phycotoxin that was first isolated from the scallop *Patinopecten yessoensis* in 1987 (Murata et al., 1987). YTX is produced by the phytoplanktonic dinoflagellates *Protoceratium reticulatum, Lingulodinium polyedrum* and *Gonyaulux spinifera* (Paz et al., 2004; Rhodes et al., 2006; Satake et al., 1997) and accumulated in filter feeding mollusks. Presently, no human intoxications have been undoubtedly related to YTXs; however several food safety authorities have limited their levels in shellfish destined to human consumption in order to protect public health (European Comission, 2004; European Comission, 2013; Satake et al., 1997). *In vivo* toxicological data identified the heart as one of the main target organs of this toxin owed to ultrastructural alterations in mouse cardiomyocytes after acute and short-term exposures to YTX (Aune et al., 2002, 2008; Ogino et al., 1997; Terao et al., 1990; Tubaro et al., 2008, 2004, 2003). Moreover, two cardiotoxicity studies in rats showed no heart dysfunction after a single YTX administration (Ferreiro et al., 2015), but marked bradycardia and hypotension associated to ultrastructural cardiac damage after repeated YTX exposures (Ferreiro et al., 2016a). In addition to the heart, the immune system has also been proposed as a YTX target due to histopathological alterations and apparent changes of cell populations in the thymus after acute exposure of mice to lethal and sublethal doses (Franchini et al., 2004).

In vitro toxicological data have demonstrated that YTX cause

Abbreviations: CRM, certified reference material; DAB+, diaminobenzidine; DMSO, dimethyl sulfoxide; DPX, di-n-butyl phthalate in xylene; EMA, European Medicines Agency; HCT, haematocrit; H&E, haematoxylin and eosin; IL-6, interleukin-6; KC-GRO, keratinocyte chemoattractant/human growth-regulated oncogene; LIX, C-X-C chemokine lipopolysaccharide-(CXCL5/LPS)-induced chemokine; LM, light microscopy; PKC, protein kinase C; RBC, red blood cells; PBS, phosphate buffer solution; TNF-α, tumor necrosis factor-α; WBC, white blood cells; YTX, yessotoxin.

* Corresponding author. Departamento de Farmacología, Facultad de Veterinaria, Campus Universitario, 27002 Lugo, Spain.
** Corresponding author. Departamento de Farmacología, Facultad de Veterinaria, Campus Universitario, 27002 Lugo, Spain.
E-mail addresses: natalia.vilarino@usc.es (N. Vilariño), luis.botana@usc.es (L.M. Botana).

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cytotoxicity in many cell lines, inducing cell death through different pathways (Fernandez-Araujo et al., 2015; Korsnes, 2012; Korsnes and Espenes, 2011; Lopez et al., 2012; Rubiolo et al., 2014), as well as alterations of several aspects of cell function and cell signaling such as intracellular calcium movements, cyclic nucleotide levels, disruption of cytoskeleton components as F-actin and E-cadherin and triggering of ribotoxic stress (Alfonso et al., 2003; Callegari and Rossini, 2008; de la Rosa et al., 2001; Korsnes et al., 2007, 2014; Ronzitti et al., 2004; Ronzitti and Rossini, 2008; Rubiolo et al., 2014). Moreover, YTX has been reported to inhibit protein endocytosis and phagocytic activity, to induce the production of inflammatory cytokines or to have immunomodulatory effects trough protein kinase C (PKC) activation (Fernandez-Araujo et al., 2015; Lopez et al., 2011; Orsi et al., 2010).

European medicines Agency (EMA) guidelines to evaluate immunotoxicity recommend that the initial standard toxicity study should include a haematological analysis to quantify leukocytes, a clinical chemistry analysis to estimate globulin levels and a histopathology evaluation of lymphoid tissues as thymus and spleen (EMA, 2006). Furthermore, cytokines, chemokines and growth factors, collectively known as cytokines, have been used as biomarkers to evaluate inflammation or the immune response. Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) are considered primary inflammatory cytokines and they are commonly detectable in blood samples (Tarrant, 2010). Chemokines are also implicated in the control of inflammatory responses through the recruitment of a specific leukocyte population to inflammation sites during organ damage, among other functions. CXCL5/LPS-induced chemokine (LIX) and the keratinocyte chemoattractant/human growth-regulated oncogene (KC-GRO) are chemokines that have been associated to chemotaxis of neutrophils after heart injury (Lukacs et al., 1999).

Overall, *in vivo* and *in vitro* toxicological data suggest that YTX exerts toxic effects on the immune system. The aim of this study was to explore subacute immunotoxicity of YTX in rats by evaluating the haematological response, several plasmatic inflammatory cytokine biomarkers and histological structure of YTX-induced structural alterations in the spleen and/or thymus after YTX repeated administrations.

2. Material and methods

2.1. Reagents

YTX certified reference material (CRM) was supplied by Laboratorio CIFGA S.A. (Lugo, Spain). Sodium chloride solution 0.9% was from Grifols Engineering, S.A. (Barcelona, Spain). Dimethyl sulfoxide (DMSO) was from Sigma-Aldrich Co. LLC. (St. Louis, MO). Milliplex® Map KITs were from Millipore® (Billerica, MA). Anti-ACTIVE® Caspase-3 polyclonal antibody (pAb) was purchased from Promega Biotech Ibérica, SL. (Madrid, Spain). Dako REAL™ Detection System, Peroxidase/DAB+, Rabbit/Mouse was from Dako Diagnostics, S.A. (Barcelona, Spain). All chemicals were reagent grade quality.

2.2. Animals and in vivo experimental design

Female Sprague Dawley rats and housekeeping conditions were as in Ferreiro et al. (2014). The experiment duration was 15 days. YTX and/or vehicle (DMSO) were administered intraperitoneally (i.p.) on days 1, 5, 9, and 13. Doses of 50, 70 and 100 μg/kg YTX were injected to 8, 7 and 1 rats respectively. Each treated rat received the same dose in all injections. For i.p. injection the solvent of the YTX stock solution (methanol) was evaporated and the toxin was reconstituted with DMSO. Saline solution (Grifols Engineering, Barcelona, Spain) was added subsequently to provide a final concentration of 10% DMSO (each rat received 50 μL of DMSO per 200 g of body weight) and 20, 40 or 61 μg/mL YTX (for the 50, 70 and 100 μg/kg doses respectively, final injection volume was 500 μL/200 g in all rats). Eight carrier control rats were injected with 10% DMSO in saline solution. On day 15, they were anesthetized with isoflurane (FI_{ISO} 1.5–2%) and, after a cardiovascular function evaluation (Ferreiro et al., 2016a), one catheter was placed in the jugular vein for blood sample collection. Two blood samples of 400 and 500 μL were collected in EDTA tubes for the detection of biomarkers and haematological analysis respectively. All animals were euthanized by exsanguination at the end of the experiment, except for those that died during the treatment period or those euthanized prematurely to prevent suffering. All animal procedures were approved by the Institutional Animal Care Committee of the Universidad de Santiago de Compostela.

2.3. Haematological analysis

A blood sample collected at the end of every experiment was immediately analyzed in the IDEXX ProCyte Dx® Haematology Analyser for the following parameters: haematocrit (HCT), red blood cells (RBC), reticulocytes and white blood cells (WBC) (neutrophils, lymphocytes, monocytes, eosinophils and basophils). Because this analyzer system was not validated for rats, WBC were also counted in stained blood smears under a microscope (100× magnification) to verify the haematology analyzer results. In each blood smear, 20 microscope fields were randomly chosen and two smears were examined per rat. Lymphocyte and neutrophil percentages were confirmed by optical microscopy. In addition, haematocrit values were similar to those obtained in glass microhaematocrit capillary tubes.

2.4. Inflammatory biomarkers

IL-6, TNF-α, LIX and KC-GRO were measured in one plasma sample collected at the end of each experiment using a commercial assay based on the Luminex XMap® technology. Blood samples were centrifuged immediately after collection to separate the plasma fraction and stored at −80 °C until their analysis. An immunology/immune response rat cytokine/chemokine Milliplex® Map KIT was used for quantification of IL-6, TNF-α, LIX and KC-GRO in plasma (100 μL) following the instructions provided by the manufacturer. All samples were assayed in duplicate.

2.5. Spleen and thymus histology

Samples of spleen and thymus were collected immediately after euthanasia from rats that received repeated administrations of 50 (n = 5), 70 (n = 5) and 100 (n = 1) μg/kg YTX or vehicle alone (n = 1). Macroscopic organ examination was done during the extraction process and the samples were then prepared for light microscopy (LM).

For LM, spleen and thymus samples were fixed by immersion in Bouin's solution for 24 h at 4 °C. Tissue samples were embedded in paraffin according to standard laboratory procedures. Paraffin-embedded sections were cut 3-μm thick, mounted on silanized slides, and dried overnight at 37 °C. Then, they were stained with haematoxylin and eosin (H&E) and examined under the light microscope for routine histochemical and morphological analyses. Several fields were observed from at least 3 non-contiguous sections of all samples, except for controls for which the observations were doubled.

Immunohistochemical detection of activated caspase-3 was performed on deparaffinized tissue sections using a specific