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En Lugo, de diciembre de 2023

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ABREVIATURAS

5-HT: serotonina
ACh: acetilcolina
ADJ: uniones adherentes, *adherent junctions*
AO: ácido okadaico
ASP: intoxicación amnésica por consumo de marisco, *Azaspiracid Poisoning*
ARfD: dosis de referencia aguda, *Acute Reference Dose*
AZA: azaspirácido
AZP: intoxicación por azaspirácidos, *Amnesic Shellfish Poisoning*
CFP: Intoxicación de ciguatera por consumo de pescado, *Ciguatera Fish Poisoning*
CI: iminas cíclicas
CPH: ciproheptadina
CTX: ciguatoxina
DA: ácido domoico
DL50: dosis letal 50
DSP: intoxicación diarreica por consumo de marisco, *Diarrethic Shellfish Poisoning*
DTX: dinofisistoxina
ELISA: ensayo inmunoadsorbente ligado a enzimas
ESI: ionización por electrospray
Glu: glutamato
HAB: floraciones algales nocivas, *Harmful Algal Blooms*
LOAEL: nivel más bajo al que se observan efectos adversos, *Lowest-Observed-Adverse-Effect-Level*
MBA: bioensayo en ratón, *mouse bioassay*
MRM: monitoreo de reacciones múltiples
Na_V: canales de sodio dependientes de voltaje
NOAEL: nivel al que no se observan efectos adversos, *No-Observed-Adverse-Effect-Level*
NPY: neuropéptido Y
NSP: intoxicación neurotóxica por consumo de marisco, *Neurotoxic Shellfish Poisoning*
PBS: tampón fosfato salino
PbTx: brevetoxin
PLTX: palitoxina
pNpp: p-nitrofenil fosfato disódico
PP: protein fosfatasa de serina/treonina
PSP: intoxicación paralítica por consumo de marisco, *Paralytic Shellfish Poisoning*
PTX: pectenotoxinas
PYY: péptido YY
STX: saxitoxina
TDI: ingesta tolerable diaria, *Tolerable Dose Intake*
TEF: fde equivalencia tóxica, *Toxic Equivalency Factors*
TJ: uniones estrechas, *tight junctions*
TMB: 3, 3', 5, 5'-tetrametilbencidina
TTX: tetrodotoxina
UHPLC-MS/MS: ultra-alta resolución acoplado a un espectrómetro de masas en tándem.
YTX: yesotoxinas

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SUMMARY

Marine natural products are a rich source of diverse chemical structures with potential application in medicine. Some microalgae can synthetize marine phycotoxins which can cause human poisoning and faunal mortality events. Alteration of some environmental conditions can trigger that these microorganisms can grow exponentially leading to harmful algal blooms. These events are associated with an increase of toxin concentration in water, which can enter the food net by accumulation in filter-feeding organisms such as bivalve molluscs. Consumption of contaminated seafood can cause shellfish poisoning. The symptomatology varies according to the group of toxins responsible. In Galicia, toxins of okadaic acid groups are of relevance since they are recurrent throughout the year and causes the closure of shellfish harvesting areas. They produce Diarrhetic Shellfish Poisoning which is characterised by gastrointestinal symptoms like diarrhoea, vomiting, nauseas and abdominal cramps that lasts for 2-3 days. These toxins are regulated in the European Union and a maximum limit of 160 μg okadaic acid equivalent/kg shellfish meat is permitted. Okadaic acid is the reference compound of the group which is also constituted by the analogues dinophysistoxins 1 and 2. Differences in toxicity among the three toxins have been reported *in vitro* and *in vivo* assays. To compare the toxicity the Toxicity Equivalency Factors (TEFs) are used. They are defined as the toxicity ratio of the analogue and the reference compound of the same group. The European Food Safety Authority established TEFs of 1 for okadaic acid and dinophysistoxin 1 and of 0.6 for dinophysistoxin 2. Based on the lack of toxin distribution after poisoning, in the present thesis, we approached the toxicokinetic of okadaic acid and dinophysistoxins 1 and 2 after oral administration to mice. We detected elevated concentration of the three toxins in the stomach after 24 h, with decreasing concentrations in small and large intestines and liver. Still, dinophysistoxin 2 was below the amount detected of okadaic acid and dinophysistoxin 1. Likewise, dinophysistoxin 2 was the mayor analogue detected in mice faeces, suggesting slower absorption and fast elimination. These phycotoxins inhibit serine/threonine protein phosphatases (PPs) like PP1 and PP2A, which does not fully explain the clinical signs and other targets could be involved. Okadaic acid has been related to neurotoxicity in cells. Therefore, in this thesis we have addressed whether signalling routes in the intestinal wall could be involved in the pathophysiology of diarrhoea in mice. To do so, mice were pretreated with neuropeptide Y, peptide YY(3-36) or cyproheptadine and later treated with okadaic acid. As a result, we obtained a limited effect of neuropeptide Y and peptide YY(3-36) in okadaic acid induced diarrhoea. Conversely, administration of cyproheptadine (antagonist of serotonin receptors 1 and 2) before okadaic acid treatment resulted in a delay or suppression of diarrhoea. This entails the involvement of serotoninergic pathways in the okadaic acid induced diarrhoea. Moreover, we have farther characterised that this toxin causes secretory diarrhoea, which entails increased chloride and sodium concentration in the intestinal lumen. Besides, cytomorphological alterations caused by okadaic acid were prevented by cyproheptadine pretreatment. Likewise, intercellular junction in the intestinal epithelia were barely disrupted by okadaic acid.

Brevetoxins are marine lipophilic phycotoxins non-regulated in Europe. They are responsible for Neurotoxic Shellfish Poisoning, which comprise gastrointestinal and neurologic alterations in human. Dinoflagellates of the genera *Karenia* spp. can synthetise these toxins and its presence have been reported in European coasts. In countries where it is regulated such as in

the US, the mouse bioassay is still a method of detection for these toxins. This consists on the intraperitoneal administration of contaminated shellfish extract and timing the percent of animals that die within 15.5 h. Due to the lack of toxicology studies, in this thesis, we have performed a neurotoxicity assay comparing intraperitoneal and oral administration of PbTx3. Animals developed neuromuscular alterations, such as ataxia, minutes after toxin administration. However, a tendency to recover happen within 24 h. Functional tests also showed that muscular weakness and reduced activity at 6 h, that was improved at 24 h. We could obtain a No-Observable-Adverse-Effect-Level (NOAEL) of 10 μg/kg PbTx3 and Lowest-Observable-Adverse-Effect-Level (LOAEL) of 100 μg/kg PbTx3. These results are of special relevance to provide an adequate legal framework for toxins regulations.

1 INTRODUCCIÓN

El mar es una fuente de compuestos químicos con una amplia diversidad de estructuras. En ocasiones, tienen aplicaciones beneficiosas para la sociedad, como su uso como medicamentos, suplementos alimentarios y en cosmética (Ngo et al. 2012; de Jesus Raposo et al. 2013). No obstante, esta riqueza también implica la presencia de compuestos bioactivos con un impacto negativo tanto en el ecosistema (Bossart et al. 1998), como en la salud de la población y a nivel económico (Ferreira et al. 2014). Entre ellos destacan las biotoxinas marinas causantes de intoxicación por consumo de moluscos. Algunos microorganismos que forman parte del fitoplancton como son dinoflagelados y diatomeas pueden sintetizar estas toxinas. Asimismo, cambios en las condiciones medioambientales como son temperatura, salinidad y disponibilidad de nutrientes pueden desencadenar el crecimiento exponencial de las microalgas (Lee et al. 2016; Ruiz-Villarreal et al. 2016). Estos eventos pueden teñir el agua según los pigmentos predominantes de la especie o especies y se les conoce comúnmente como mareas rojas, aunque también se les denomina floraciones algales. En ocasiones, la especie causante de las floraciones puede producir biotoxinas, en este caso se les denomina floraciones algales nocivas (*Harmful Algal Blooms*, HABs) (Berdalet et al. 2015; Lassus et al. 2015). Las biotoxinas pueden tanto entrar a la cadena trófica mediante organismos filtradores u organismos que se alimentan del fitoplancton, como formar aerosoles causando intoxicaciones por vía aérea o por contacto con la piel (Berdalet et al. 2015).

1.1 INTRODUCCIÓN A LAS FICOTOXINAS MARINAS

La exposición a estas toxinas se debe principalmente al consumo de productos marinos contaminados con las mismas. En función de las síntomas y toxina causante se pueden identificar distintos tipos de intoxicaciones (Tabla 1). Se distinguen la intoxicación diarreica por consumo de marisco (*Diarrhetix Shellfish Poisoning*, DSP), la intoxicación con azaspirácidos por consumo de marisco (*Azaspiracid Shellfish Poisoning*, AZP), la intoxicación neurotóxica por consumo de marisco (*Neurotoxic Shellfish Poisoning*, NSP), la intoxicación amnésica por consumo de marisco (*Amnesic Shellfish Poisoning*, ASP), la intoxicación paralítica por consumo de marisco (*Paralytic Shellfish Poisoning*, PSP), la intoxiación de ciguatera por consumo de pescado (*Ciguatera Fish Poisoning*, CFP), la intoxicación por palitoxinas y la intoxicación por tetrodotoxina (FAO/WHO 2004; Nicolas et al. 2017; Louzao et al. 2017). Según su estructura química las ficotoxinas marinas pueden clasificarse en: ácido okadaico (AO), azaspirácidos (AZAs), saxitoxinas (STX), tetrodotoxinas (TTXs), brevetoxinas (PbTxs), palitoxinas (PLTXs), pectenotoxinas (PTXs), yesotoxinas (YTXs), imicas cíclicas (CIs) y ciguatoxinas (CTXs) (Tabla 1) ((EFSA) 2009b; FAO/WHO 2016; Farabegoli et al. 2018). Asimismo, se pueden diferenciar según sus propiedades físico-químicas en hidrofílicas, lipofílicas y anfifílicas (Tabla 1) (Farabegoli et al. 2018).

Tabla 1. Clasificación de las biotoxinas marinas (FAO/WHO 2004; Lassus et al. 2015).

Intoxicación	Grupo de toxinas	Solubilidad	Organismo productor

DSP	**Ácido okadaico**	Lipofílicas	*Dinophysis* spp., *Prorocentrum* spp.
	Pectenotoxinas	Lipofílicas	*Dinophysis* spp.
	Yesotoxinas	Lipofílicas	*Protoceratium reticulatum, Lingulodinium polyedrum, Gonyaulax spinifera*
	Iminas cíclicas	Lipofílicas	*Karenia selliformis, Alexandrium* spp., *Vulcanodinium rugosum, Prorocentrum* spp.
AZP	**Azaspirácidos**	Lipofílicas	*Azidinium poporum, Azadinium* spp., *Amphidoma* spp.
NSP	**Brevetoxinas**	Lipofílicas	*Karenia* spp., *Chattonella* spp.
CFP	**Ciguatoxinas**	Lipofílicas	*Gambierdiscus* spp., *Fukuyoa* spp.
ASP	**Ácido domoico**	Hidrofílicas	*Pseudo-nitzschia* spp., *Nitzschia* spp., *Amphora* spp.
PSP	**Saxitoxina**	Hidrofílicas	*Alexandrium* spp., *Gymnodinium* spp., *Pyrodinium bahamense, Trichodesmium Erythraeum*
	Tetrodotoxinas	Hidrofílicas	
	Palitoxinas	Anfifílicas	*Ostreopsis* spp.

La intoxicación diarreica (DSP) se caracteriza por una sintomatología que afecta principalmente al aparato digestivo (Yasumoto et al. 1984). El AO y las dinofisistoxinas (DTXs) son las toxinas responsables de esta intoxicación. Se ha demostrado que los análogos del AO inhiben las protein fosfatasas de serina/treonina (PP) (Figura 1) (Takai et al. 1992a), sin embargo, esto no causa los síntomas diarreicos (Munday 2013). Las PTXs también son toxinas lipofílicas que causan desorganización del citoesqueleto mediante su unión a la actina (Allingham et al. 2007). . Cabe destacar que, si bien las PTXs causan toxicidad en ratones tras su administración intraperitoneal, no causa toxicidad a dosis altas por administración oral (Miles et al. 2004) y no hay datos de intoxicación en humanos (FAO/WHO 2016). En el grupo de las toxinas lipofílicas también se incluyen las YTXs cuyo mecanismo de acción se desconoce (FAO/WHO 2016). Los datos de toxicidad e intoxicación en humanos van en línea con el caso de las PTXs: no se han reportado casos de intoxicación en humanos y se cuestiona la toxicidad oral en ratones, pese a que sí es tóxica en el bioensayo en ratón ((EFSA) 2008). Las iminas cíclicas se componen de una amplia variedad de análogos que actúan sobre los canales nicotínicos de acetilcolina (Molgo et al. 2017). Además, se han detectado en moluscos en costas europeas (FAO/WHO 2016).

La intoxicación por azaspirácidos (AZP) comprende mayormente síntomas gastrointestinales (James et al. 2004). Lo causa el consumo de moluscos contaminados con AZAs, aunque se desconoce el mecanismo de acción (Figura 1).

La intoxicación neurotóxica (NSP) se produce por intoxicación con las toxinas lipofílicas PbTxs (Watkins et al. 2008). Las PbTxs se unen a los canales de sodio dependientes de voltaje causando despolarización en neuronas y células musculares, lo que desencadena síntomas neurológicos (Figura 1) ((EFSA) 2010b). Parestesia, vértigo, mareos, diarrea, vómitos, debilidad muscular y ataxia son algunos de los síntomas que pueden causar (Morris et al. 1991). Por otro lado, las ciguatoxinas comparten mecanismo de acción con las PbTxs, se unen también al sitio 5 de los canales de sodio dependientes de voltaje (Hartshorne and Catterall 1981). La intoxicación por ciguatoxinas (CFP) se define por síntomas gastrointestinales, cardiovasculares y neurológicos, siendo característica la inversión de la sensación de la temperatura (Figura 1) ((EFSA) 2010a).

La intoxicación amnésica (ASP) se caracteriza por la pérdida de memoria consecuencia de la neurotoxicidad causada por el ácido domoico (Figura 1). Esta toxina hidrosoluble activa los receptores AMPA/kainato, lo que desencadena la entrada de calcio y la liberación de glutamato

que activa los receptores NMDA (Vale 2014). Además de síntomas gastrointestinales, también produce dolor de cabeza y pérdida de memoria (FAO/WHO 2016).

La intoxicación paralizante (PSP) comprende síntomas tan leves como sensación de hormigueo alrededor de los labios, hasta llegar a causar la muerte ((EFSA) 2009a). La saxitoxina, compuesto de referencia de este grupo, es una molécula hidrosoluble de bajo peso molecular que se une al sitio 1 de los canales de sodio dependientes de voltaje impidiendo físicamente la entrada de sodio (Hartshorne and Catterall 1984; (EFSA) 2009a). En consecuencia, se bloquea la propagación del impulso nervioso y la contracción muscular. La tetrodotoxina produce una sintomatología muy similar, ya que comparte el mecanismo de acción (FAO/WHO 2016).

Finalmente, la intoxicación por palitoxina (PLTX) implica síntomas cardiovasculares, neurológicos, cardiovasculares, musculares, respiratorios y gastrointestinales (Pelin et al. 2016). La PLTX se une a la bomba de Na^+/K^+ estabilizándola en la conformación abierta. Esto favorece el flujo de cationes a favor de gradiente, por lo que la unión de la palitoxina la convierte en un canal no selectivo (Rossini and Bigiani 2011). La bomba de Na^+/K^+ tiene una distribución ubicua y es esencial en el mantenimiento del balance iónico entre el interior y exterior celular. Por consiguiente, la alteración de su funcionamiento puede llegar a causar la muerte.

El trabajo de la presente tesis doctoral se centra en el estudio de las toxinas diarreicas y en las PbTxs, por consiguiente, en las secciones posteriores se explica en detalle la estructura, mecanismo de acción y toxicidad de estos dos grupos de toxinas.

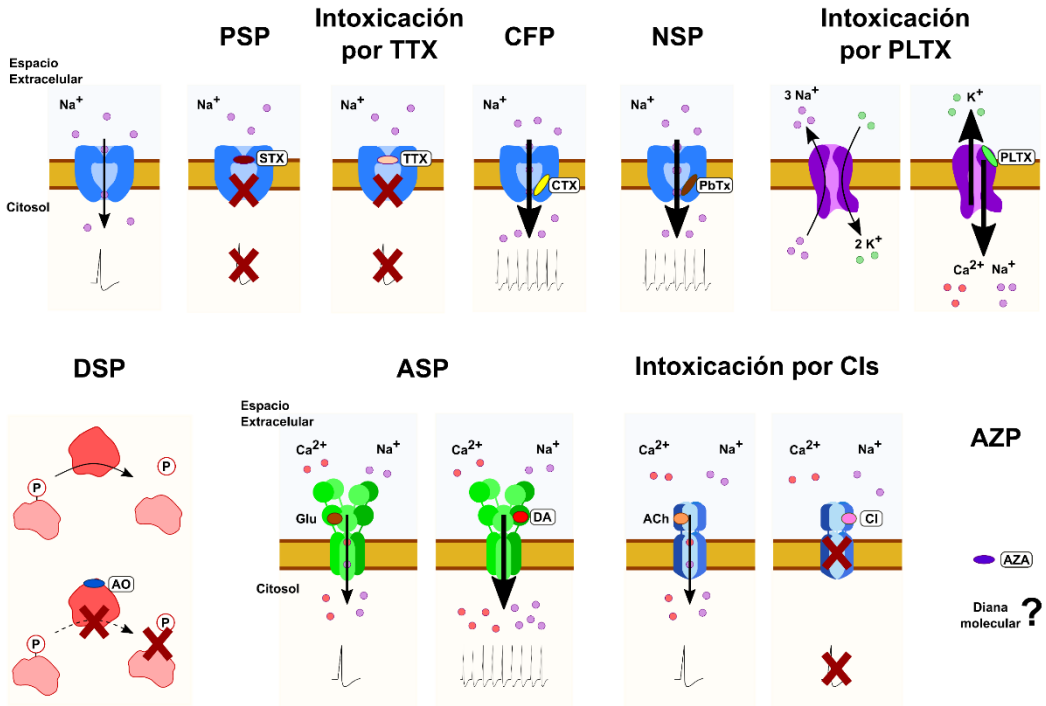


Figura 1. Representación de los mecanismos de acción de las biotoxinas marinas. Las toxinas paralizantes (como la saxitoxina, STX) y la tetrodotoxina (TTX) bloquean la entrada de sodio al interior celular inhibiendo la transmisión nerviosa y contracción muscular. Las ciguatoxinas (CTX) y las brevetoxinas (PbTx) también actúan sobre estos canales, facilitan la entrada de sodio y provocan la intoxicación por Ciguatera y la intoxicación Neurotóxica respectivamente. La palitoxina (PLTX) se une a la ATPasa de Na^+/K^+ alterando su conformación de modo que actúa como un canal catiónico no selectivo. Las toxinas del grupo del ácido okadaico inhiben la acción de las protein fosfatasas de Ser/Thr lo que lleva a un estado de hiperfosforilación en las células. El

ácido domoico (DA) es agonista de los receptores ionotróficos de glutamato (Glu), lo que desencadena hiperexcitabilidad neuronal y la intoxicación Amnésica. Las iminas cíclicas (CIs) son antagonistas competitivos de los receptores nicotínicos de acetilcolina, por lo que bloquean la contracción muscular inducida por la liberación de acetilcolina (ACh). Se desconce la diana molecular de los azaspirácidos (AZAs). Modificado de Louzao et al. (2022).

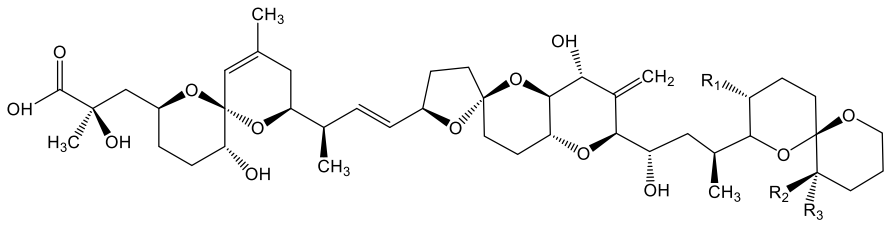
1.2 TOXINAS REGULADAS: TOXINAS DEL GRUPO DEL ÁCIDO OKADAICO

Las toxinas del grupo del ácido okadaico son producidas por algunas especies de dinoflagelados de los géneros *Dinophysis* spp. y *Prorocentrum* spp. (EFSA 2008; Berdalet et al. 2015). No obstante, esta toxina obtiene su nombre de la esponja negra *Halichondria okadai*, ya que fue en extractos metanólicos de esta especie donde se aisló la toxina. Se demostró que causa toxicidad en ratones tras su adminsitración intraperitoneal (Tachibana et al. 1981). En el verano de los años 1976 y 1977 en Japón ocurrieron intoxicaciones que afectaron a 42 y 122 personas, respectivamente (Yasumoto et al. 1978). El consumo de mejillones (*Mytilus edulis*) y vieiras (*Patinopecten yessoensis* y *Chlamys nipponensis akazara*) fue el desencadenante de las intoxicaciones. Los pacientes sufrieron de diarrea, vómitos, náuseas y dolor abdominal que comenzaba en los casos más graves a los 30 min, sin embargo, se recuperaron en 3 días con independencia del tratamiento. Se investigó si los síntomas podrían tener un origen infeccioso, pero no se encontraron agentes patógenos y además las intoxicaciones se habían producido después de cocinar los moluscos. Asimismo, se descartaron la intoxicación por toxinas paralíticas así como la intoxicación por ciguatera (Yasumoto et al. 1978). Se identificó que el microorganismo causante fue *Dinophysis fortii* (Yasumoto et al. 1980) y más adelante se aisla la dinofisistoxina 1 como causante (Murata et al. 1982). Cabe destacar que en ese momento se sospechaba que intoxicaciones por consumo de moluscos con una sintomatología gastrointestinal podrían haber sido producidos por dinoflagelados del género *Dinophysis* spp. en Holanda y en Chile (Murata et al. 1982; Reguera et al. 2014). No fue hasta 1992 cuando se aisló la dinofisistoxina 2 en mejillones recogidos en Irlanda (Hu et al. 1992). Esta información permitió documentar casos de intoxicación diarreica (DSP) en Chile, Argentina, México, Norte América, Irlanda, Gran Bretaña, España, Portugal, Italia, Grecia, India, Tailandia, Australia y Nueva Zelanda (Reguera et al. 2014; Lassus et al. 2015). Con el fin de preoteger al consumidor de estas toxinas, se establecieron programas de monitorización de toxinas en moluscos para proteger al consumidor de estas toxinas. Esto es de especial importancia en la Comunidad Autónoma de Galicia donde las floraciones algales nocivas recurrentes durante cada año vetan la producción de moluscos en ocasiones durante el 80% del año (Rodriguez et al. 2015). Galicia produjo 217999 Tn de mejillón en el año 2015, lo que representa el 95,7% del mejillón del país (Biodiversidad 2017). Por consiguiente, el cierre de las áreas de producción puede causar pérdidas económicas de gran importancia en el sector.

1.2.1 Estuctura química y análogos

Las toxinas del grupo del AO son poliéteres lipofílicos que son termoestables, inoloros y no alteran el sabor del alimento contaminado (Yasumoto et al. 1978). El compuesto de referencia es AO. Los principales análogos identificados en moluscos son las dinofisistoxinas 1 (DTX1) y 2 (DTX2) (Figura 2). La primera tiene un grupo metilo en la posición C35, mientras que DTX2 es un isómero del AO. Derivados esterificados de AO, DTX1 y DTX2 con ácidos grasos se agrupan bajo el nombre de DTX3 (Yasumoto et al. 1984; FAO/WHO 2016). Por otro lado, también se han aislado los precursores de estas toxinas en los dinoflagelados productores: DTX4, DTX5 y DTX6 que son diésteres sulfatados (Lee et al. 2016; Hu et al. 2017). Se cree

que estos compuestos se degradan en los organismos filtradores a causa de su metabolismo (FAO/WHO 2016).



Análogo	R_1	R_2	R_3
OA	CH_3	H	H
DTX1	CH_3	CH_3	H
DTX2	H	H	CH_3

Figura 2. Estructura química del ácido okadaico y los análogos dinofisistoxinas 1 y 2. La estructura química se dibujó utilizando el software ChemDraw 21.0.0.

1.2.2 Toxicidad *in vivo*

La intoxicacidón por toxinas del grupo del ácido okadaico causa diarrea, vómitos, náuseas y dolor abdominal a partir de 30 min tras el consumo de moluscos contaminados (Yasumoto et al. 1978). La EFSA llevó a cabo un análisis de riesgos para determinar límites y parámetros de seguridad (EFSA 2008). En el informe resultante indican que no se pudo establecer una ingesta tolerable diaria (*Tolerable Dose Intake*, TDI) por la falta de estudios crónicos de administración de las toxinas. Basándose en datos de episodios de DSP, determinaron que la dosis más baja a la que se observan efectos adversos *(Lowest-Observed-Adverse-Effect-Level*, LOAEL) es de 50 µg equivalentes de AO/persona, a partir del que extrapolan una dosis de referencia aguda (*Acute Reference Dose*, ARfD) de 0,3 µg equivalentes de AO/kg de peso corporal (EFSA 2008). La Unión Europea estableció el límite legal de las toxinas del grupo del ácido okadaico en 160 µg equivalentes de AO/kg de molusco (EU 2004).

La toxicidad de las toxinas del ácido okadaico se ha evaluado en base a la dosis letal 50 (DL_{50}) tras la administración intraperitoneal de la toxina. Por un lado, la DL_{50} del AO en ratones varía desde 185-225 µg/kg, siendo la mortalidad del 100% al inyectar una dosis de 375 µg/kg (Tachibana et al. 1981; Dickey et al. 1990; Ito and Terao 1994; Tubaro et al. 2003; Aune et al. 2012; FAO/WHO 2016; Suzuki and Okada 2018). La DL_{50} para DTX1 es de 150-160 µg/kg y para DTX2 es de 352 µg/kg (Aune et al. 2007; Dominguez et al. 2010; Suzuki and Okada 2018). Según estos resultados, DTX1 es algo más potente que el AO y más todavía que DTX2. También hay datos comparando la toxicidad de AO, DTX1 y DTX3 en el que una dosis de 375 µg/kg causaron la muerte de todos los animales (Ito and Terao 1994).

Por otro lado, la administración por vía oral muestra más discrepancias entre la potencia de los análogos. Se han documentado DL_{50} que varían de 400 µg/kg a 880 µg/kg (Ito et al. 2002; Aune et al. 2012; Abal et al. 2018). En algunos casos, se reportan dosis que causan la muerte, aunque no necesariamente especifiquen la DL_{50}. Por ejemplo, Tubaro et al. (2003) administra dosis 1000 y 2000 µg/kg AO, sin embargo, sólo la última causa la muerte del animal. En cuanto a los análogos DTX1 y DTX2, los datos son escasos. Para la DTX1 se ha reportado una DL_{50} de 300 µg/kg (Ogino et al. 1997) y de 487 µg/kg (Abal et al. 2018).

1.2.3 Mecanismo de acción

Las toxinas del ácido okadaico inhiben las protein fosfatasas de serina/treonina (PP), especialmente la PP2A, PP1, PP4, PP5 y la PP6 (Takai et al. 1992b; Brewis et al. 1993; Chen et al. 1994; Prickett and Brautigan 2006). Las PPs no sólo se expresa en múltiples tejidos y tipos celulares, sino que la alteración de su funcionamiento puede afectar a múltiples rutas de metabolismo celular (Yadav et al. 2017). Se ha relacionalo la función de las PPs con el ciclo celular, los canales iónicos, apoptosis y organización del citoesqueleto (Herzig and Neumann 2000; Klumpp and Krieglstein 2002; Margolis et al. 2003; Virshup and Shenolikar 2009; Hoffman et al. 2017).

Se ha postulado la posibilidad de que el mecanismo de acción de estas toxinas sea que la inhibición de protein fosfatasas aumente la fosforilación de las proteínas responsables de la secreción de sodio al lumen intestinal lo que desencadenaría la diarrea (Cohen et al. 1990; Munday 2013; Tripuraneni et al. 1997). Sin embargo, esta hipótesis no se ha cofirmado. De hecho, algunos estudios sugieren que algunos efectos del AO y las DTXs no podrían ser explicados exclusivamente por la inhibición de las PPs (Kikuchi et al. 1999; Espina et al. 2010). A nivel celular se ha descrito que tanto AO como DTXs inducen apoptosis e inhibición del crecimiento en algunos modelos celulares intestinales, neuronales, hepáticos, pulmonares y sanguíneos (Valdiglesias et al. 2011). Esto puede ser resultado de alteraciones en la expresión génica, desequilibrios en el potencial de membrana de mitocondrias, activación de múltiples isoformas de caspasas, liberación del citrocromo c al citosol desde la membrana interna mitocondrial, inhibición de la síntesis de proteínas y, alteraciones en el citoesqueleto (Leira et al. 2001; Lago et al. 2005; Cabado et al. 2004; Espina et al. 2010).

Aunque no está clasificada como neurotoxina, diversos estudios han probado los efectos neurotóxicos del AO generalmente relacionados con apoptosis neuronal, alteraciones morfológicas debido a modificaciones en genes relacionados con citoesqueleto y neurotransmisión e incluso hiperfosforilación de la proteína tau (implicada en Alzheimer), tanto en cultivos in vitro como en modelos animales *in vivo* (Kamat et al. 2013). Esto último, hace que el AO y sus análogos sean herramientas útiles para el estudio del Alzheimer y otras enfermedades neurodegenerativas ya que se ha demostrado que la exposición crónica de ciertos animales (ratas principalmente) a estas toxinas puede inducir síntomas similares a los de las enfermedades mencionadas (Kamat et al. 2013). Además, se ha estudiado que esta toxina también afecta al neurotransmidor Neuropéptido Y (Valdiglesias et al. 2012; Louzao et al. 2015)

A las DSPs se les ha atribuido propiedades genotóxicas tras realizarse ensayos *in vitro* (Valdiglesias et al. 2011). Además, se han realizado ensayos *in vivo* que apoyan que las moléculas del grupo del AO son protumorales. Se han evaluado además los efectos que pueden tener otros inhibidores de PPs como las microcistinas. No obstante, la tautomicina que también inhibe estas protein fosfatasas, no actúa como promotor tumoral. Esto apoya la premisa de que el AO puede tener otras dianas de relevancia en su mecanismo de acción. Por lo tanto, es de interés evaluar cómo puede este grupo de toxinas causar la sintomatología gastrointestinal.

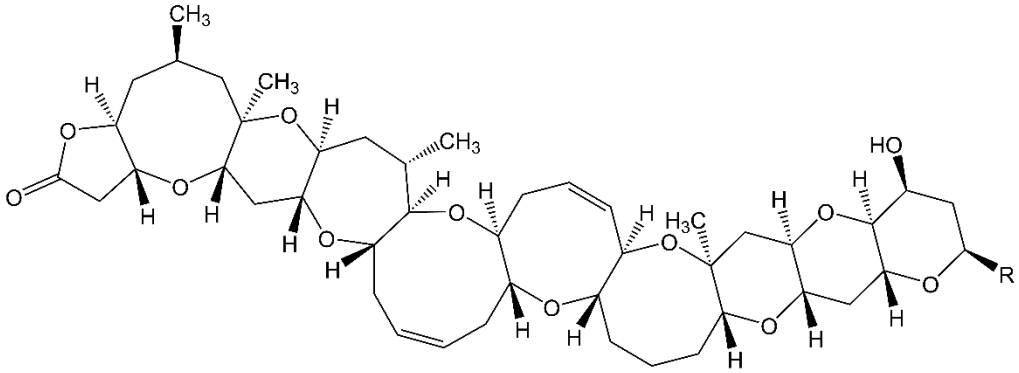
1.3 TOXINAS NO REGULADAS: BREVETOXINAS

Las brevetoxinas (PbTxs) son ficotoxinas producidas por dinoflagelados del género *Karenia* spp. y por algunas rafidófitas del género *Chattonella* spp. (Bourdelais et al. 2002; Lassus et al.

2015). Se describieron eventos de mortalidad de fauna marina que afectaron a las costas de Florida en los años de 1940 que se asociaron con cambios en el color del agua (Gunter et al. 1947; Davis 1948). Además describen la irritación y la tos causada por un "gas" inodoro que afectó a los residentes de la costa (Gunter et al. 1947). Se asociaron estos eventos con el dinoflagelado *Karenia brevis* (anteriormente conocido como *Gymnodinium breve* y *Ptychodiscus brevis*) (Gunter et al. 1947; Ramsdell 2008). Sin embargo, no fue hasta 1962 que se asoció el consumo de ostras (*Crassostrea virginica*) y almejas (*Venus mercenaria campechiensis*) con la intoxicación con una sintomatología comparable a la causada por las ciguatoxinas (McFarren et al. 1965). Los afectados desarrollaron sensación de hormigueo en la boca, las extremidades y la cara, así como sensación de embriaguez, de frío o calor, reducción en el pulso cardíaco y pupilas dilatadas. Se recuperaron tras 24-48 h (McFarren et al. 1965). Con el tiempo se purificaron y se elucidó la estructura química de la PbTx1 y la PbTx2 (Risk et al. 1979; Lin et al. 1981; Shimizu et al. 1986).

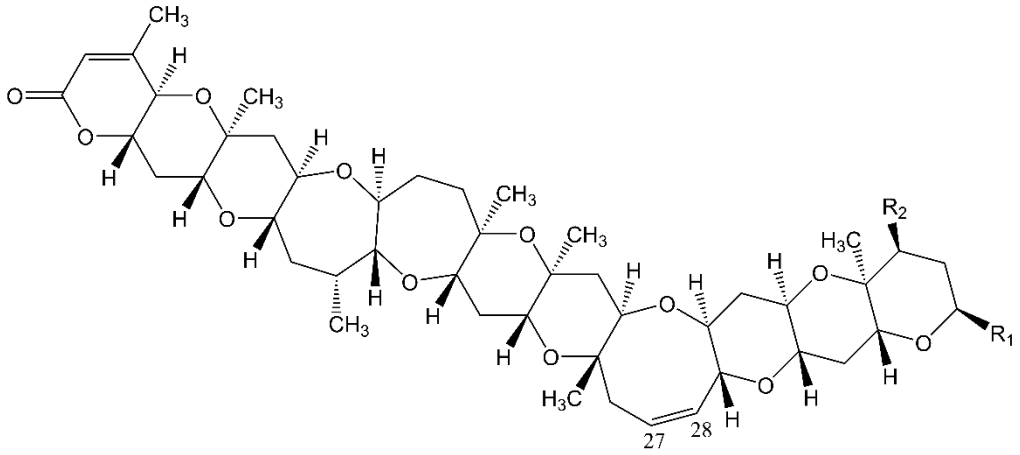
1.3.1 Estructura química y análogos

Las brevetoxinas son biotoxinas lipofílicas que se dividen en dos grupos en función de su estructura química en las PbTxs de tipo A y de tipo B (Figura 3) ((EFSA) 2010b; Lassus et al. 2015). Son poliéteres termoestables que se diferencian por el número de anillos (Poli 1988). Las PbTxs de tipo A tienen 10 anillos y a este grupo pertenecen PbTx1, PbTx7 y PbTx10. Por otro lado, las brevetoxinas de tipo 2 tienen 11 anillos y está contituido por PbTx2, PbTx3, PbTx5, PbTx6 y PbTx9 (Poli et al. 1986; Ramsdell 2008).



Brevetoxinas tipo A

Análogo	R
PbTx1	CH_2 (=CH_2).CHO
PbTx7	CH_2(=CH_2).CH_2OH
PbTx10	$CH_2CH(CH_3).CH_2OH$



Brevetoxinas tipo B

Análogo	R_1	R_2	C27-C28
PbTx2	CH_2 (=CH_2).CHO	OH	CH=CH
PbTx3	CH_2(=CH_2).CH_2OH	OH	CH=CH
PbTx5	CH_2 (=CH_2).CHO	O-CO-CH_3	CH=CH
PbTx6	CH_2 (=CH_2).CHO	OH	HC-CH (O)
PbTx9	$CH_2CH(CH_3).CH_2OH$	OH	CH=CH

Figura 3. Estructura química de las brevetoxinas tipo A y tipo B. También se muestra la estructura de algunos de sus análogos. La estructura química se dibujó utilizando el software ChemDraw 21.0.0.

Se considera a las PbTx1 y PbTx2 are como los compuestos a partir de los que derivan el resto de análogos ((EFSA) 2010b). Su metabolismo puede resultar de numerosas reacciones como oxidación, hidrólisis y reducción, entre otras (Ramsdell 2008). Estas ficotoxinas también pueden metabolizarse mediante la conjugación a cisteína o a taurina en algunos organismos filtradores. En consecuencia, el número de análogos se incrementa hasta 40 aproximadamente. Es importante comentar que no todos ellos se han encontrado en moluscos, como es el caso de la PbTx1, mientras que la PbTx3 se ha detectado en variedad de especies de moluscos ((EFSA) 2010b). Además, el perfil tóxico varía en función de la especie de molusco (Ishida et al. 2004a).

1.3.2 Toxicidad *in vivo*

La intoxicación neurotóxica (NSP) se caracteriza por la aparición de sensación de hormigueo (parestesias), mareos, alteraciones musculares y pérdida de la coordinación, entre otros. Síntomas gastrointestinales como diarrea o náuseas también se han detectado con frecuencia. En raras ocasiones se han descrito alteraciones cardiovasculares. Los afectados se suelen recuperar en las primeras 48 h (McFarren et al. 1965; Morris et al. 1991; Poli et al. 2000; Watkins et al. 2008). El análisis de riesgos de la EFSA determinó que debido a la falta de datos de intoxicación en humanos e *in vivo* no pudieron proponer una dosis de referencia agua. En esta línea, la ingesta tolerable diaria no se pudo establecer por no haber ensayos de administración repetida con brevetoxinas.

Unos pocos estudios han evaluado la toxicidad intraperitoneal tanto de las PbTx1, PbTx2 y la PbTx3 como de otros análogos (FAO/WHO 2016). La DL_{50} de la PbTx1 es de 100 μg/kg (Dechraoui et al. 1999), mientras que para la PbTx2 es de 200 μg/kg (Baden and Mende 1982)

y para la PbTx3 se han obtenido valores de 170 μg/kg (Baden and Mende 1982) y 250 μg/kg con la toxina semisintética en este último caso (Selwood et al. 2008). También se conoce la DL_{50} para los análogos brevetoxina B2 y su derivado la S-deoxi-brevetoxina B2, siendo estos valores de 400 y 211 μg/kg, respectivamente (Selwood et al. 2008). Comparando estos estudios, la PbTx1 es la que tiene mayor potencia, seguida de las PbTx2 y PbTx3. Los datos de la toxicidad oral se reducen a un solo estudio en el que evalúa la DL_{50} de la PbTx2 y la PbTx3. Como resultado obtienen una DL_{50} de 6600 μg/kg PbTx2 y de 520 μg/kg PbTx3, mostrando no sólo que la toxicidad oral es más baja que la intraperitoneal, sino también que la PbTx2 resulta mucho menos tóxica (Baden and Mende 1982). Basándonos en esta información se infiere la necesidad de evaluar la toxicidad de las brevetoxinas.

1.3.3 Mecanismo de acción

Las brevetoxinas se unen al sitio 5 de los canales de sodio dependientes de voltaje (Na_V) (Catterall and Risk 1981). Esta toxina se une a los segmentos S5 y S6 de los dominios DI y DIV, respectivamente (Wang and Wang 2003). Se ha caracterizado que las PbTxs tienen la capacidad de desplazar el voltaje de activación, inhibir la inactivación y aumentar el tiempo de apertura medio (Ramsdell 2008). Esto implica que se desencadenen señales de transmisión neuronal y/o muscular en condiciones próximas a las fisiológicas, cuando los canales de sodio dependientes de voltaje no se activarían. Es decir, el desplazamiento del voltaje de activación a potenciales más negativos conlleva la apertura de los canales en condiciones en las que no se abrirían normalmente. Además, al inhibir la inactivación el canal permanece más tiempo abierto, por lo tanto, la señal es más sostenida en el tiempo. Estos efectos han sido evaluados tanto en ensayos *in vitro* como *ex vivo*. Los efectos en estos canales explican en su mayoría la sintomatología neurológica correspondiente a la intoxicación por brevetoxinas.

2 HIPÓTESIS Y OBJETIVOS GENERALES Y ESPECÍFICOS

Las toxinas diarreicas aparecen con frecuencia en las costas europeas, además de ser las principales causantes de cierres de bateas y bancos marisqueros durante gran parte del año en Galicia. Se pueden acumular en moluscos bivalvos, causando intoxicación alimenticia con síntomas gastrointestinales. El conocer la relacion de la diana de la toxina con los síntomas de la intoxicación, así como sus características farmacocinéticas aportará datos que contribuyan a la protección del consumidor. Por otro lado, las brevetoxinas son prevalentes en el Golfo de México, pero de nueva aparición en Europa. Por lo tanto, se consideran toxinas emergentes no reguladas en europa planteando un nuevo reto alimentario y de salud pública. Ante la escasez de información toxicológica los estudios de toxicidad aguda aportarán datos fundamentales de cara a seguridad a limentaria y salud pública.

El objetivo principal de esta tesis doctoral es evaluar y caracterizar la toxicidad oral de algunas toxinas marinas y su relación con el mecanismo de acción.

Con la finalidad de alcanzar este objetivo se plantean los siguientes objetivos específicos:

- Realizar una evaluación farmacocinética *in vivo* de las toxinas diarreicas (ácido okadaico, dinofisistoxina 1 y dinofisistoxina 2) estudiando su distribución en los distintos órganos y fluidos del ratón.

- Determinar la toxicidad aguda *in vivo* de las toxinas diarreicas (ácido okadaico, dinofisistoxina 1 y dinofisistoxina 2) por vía oral, valorando la sintomatología y analizando los efectos ultraestructurales.

- Relacionar el mecanismo de acción de las toxinas diarreicas con los efectos *in vivo*.

- Caracterizar la neurotoxicidad de las brevetoxinas por vía oral e intraperitoneal y evaluar sus implicaciones y determinar los índices de toxicidad.

3 MATERIAL Y MÉTODOS

En la presente sección se recoge la metodología empleada durante el desarrollo de esta tesis doctoral. Para alcanzar los objetivos planteados, se parte de toxinas con un alto grado de pureza (≥ 95% PbTx3; >95% AO; >95% DTX1) o de material de referencia certificado (≥ 99% AO, >98% DTX2). Tanto la brevetoxina 3 como las toxinas del grupo del AO son lipofílicas, por lo que, se disuelven en etanol. Cuando se van a administrar por vía oral se preparan el mismo día de la administración en solución salina (0,9% NaCl).

3.1 ANIMALES

Los animales utilizados son ratones hembra de 4 semanas de edad que se mantienen a una temperatura de 23 ºC, con una humedad de 60-70% y en ciclos de luz/oscuridad de 12 h/12 h. Los animales se aclimatan a estas condiciones durante una semana antes del inicio del experimento. La guía de la Organización para la Cooperación y el Desarrollo Económicos para estudios de toxicidad aguda indica que un sexo es suficiente y además ensayos anteriores muestran que generalmente cuando hay diferencias entre sexos, las hembras suelen ser ligeramente más sensibles (OECD/OCDE 2002). Por estos motivos, trabajamos con ratones hembra. Los animales, de 4 semanas (18-26 g), procedían de las colonias propias de la Universidade de Santiago de Comostela. Los ensayos en los que se utilizan animales de experimentación se realizan conforme lo recogido por la Directiva Europea 2010/63/UE (EU 2010), el Real Decreto 53/2013 (Presidencia 2013) y por el Decreto 296/2008 sobre la experimentación animal. Asimismo, los procedimientos que se llevan a cabo fueron aprovados por el Comité de Bioética de la Universidade de Santiago de Compostela (01/17/LU-002).

3.2 ENSAYOS *IN VIVO*

Los animales se meten en jaulas metabólicas el día anterior a los ensayos, en ayunas y con acceso a suero glucosado. Esto evita que diferencias en el estado del contenido gastrointestinal lleven a mayor variabilidad en la absorción de las toxinas. Debido a que los casos de intoxicación con ficotoxinas marinas se producen por consumo de moluscos bivalvos contaminados, la administración de todas las toxinas contempladas en este trabajo se realiza por sonda gástrica. La sonda empleada es de acero inoxidable, rígida con el extremo redondeado. De este modo se tratan de minimizar los posibles daños causados en los epitelios esofágico y gástrico, así como garantiza la administración completa de la dosis indicada. Por otro lado, el volumen administrado por vía oral es de 10 mL/kg de peso del animal y el administrado por vía intraperitoneal de 1% del peso. Los animales se asignan aleatoriamente a cada grupo de ensayo. Una vez finalizados los tiempos de experimentación, las eutanasias se realizan mediante inhalación de dióxido de carbono acorde los métodos indicados en la directiva europea 2010/63/UE (EU 2010) y al Real Decreto 53/2013 (Presidencia 2013) que la recoge.

3.2.1 Estudios de toxicidad aguda

Los ensayos de toxicidad aguda implican el registro de los síntomas de forma constante hasta el tiempo final de estudio. Para poder observar diferencias a lo largo del tiempo, se calcula una suma del número de síntomas a cada hora, de tal forma que la presencia de un síntoma suma 1, mientras que la ausencia no suma. En la evaluación de síntomas incluimos un sistema de puntuación de la diarrea, para poder definir y comparar la gravedad de la misma (Tabla 2). Se le asigna una puntuación de 0 a las heces normales, una puntuación de 1 a las heces blandas con forma, una puntuación de 2 a las heces blandas sin forma, una puntuación de 3 a la diarrea acuosa y una puntuación de 4 a los casos donde el animal desarrollaba diarrea en más de una ocasión. Para que se considere que el animal tiene diarrea en repetidas ocasiones se estable un tiempo mínimo de 20 min tras la anterior deposición. Se considera diarrea con una puntuación de 2 o más.

Tabla 2. Energía de colisión y fragmentos iónicos de las toxinas diarreicas.

Puntuación	**Definición**
0	Heces normales
1	Heces blandas con forma
2	Heces blandas sin forma
3	Diarrea acuosa
4	Diarrea que aparece en repetidas ocasiones

Gracias al sistema de las jaulas metabólicas, se recogen las muestras de orina y heces a distintos tiempos tras la administración del tratamiento. Además, presentan la ventaja de poder medir el consumo de comida y agua durante el tiempo experimental.

3.2.1.1 Procedimiento de *Up & Down*

Se lleva a cabo un procedimiento de *Up & Down* siguiendo la Guía nº 425 de toxicidad aguda c (OECD 2008; OECD 2022). Este protocolo consiste en 4 niveles experimentales, en los que el número de animales aumenta con el nivel y cada uno se corresponde con una dosis de toxina diferente (Figura 4). Inicialmente, a 3 animales se les administra una dosis de 1000 µg/kg en el caso del AO, DTX1 y la PbT3, mientras que se adminsitran 2000 µg/kg para el ensayo con DTX2. Si más del 50% de animales sobrevive, se aumenta la dosis en el siguiente nivel. Si por el contrario menos del 50% sobrevive, la dosis del siguiente nivel se reduce. Estos animales se individualizan en jaulas metabólicas y se mantienen en ayunas con acceso a suero glucosado durante la noche. A la mañana siguiente se les adminsitra por sonda gástrica la toxina DSP correspondiente preparada en solución salina y se les da acceso a comida y agua *ad libitum*. La toxina PbTx3 que se administra por vía oral e intraperitoneal. Al alcanzar las 24 h, los animales que sobrevivien se somenten a eutanasia y según el porcentaje de supervivientes a este tiempo, se aumenta o disminuye la dosis en el siguiente nivel.

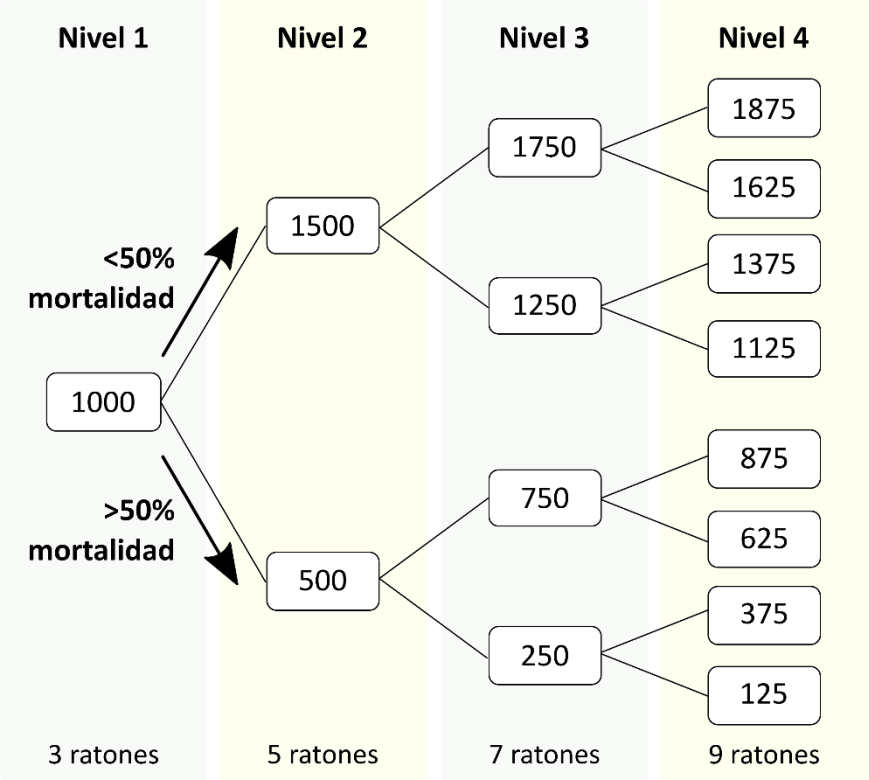


Figura 4. Representación del procedimiento de *Up & Down* comenzando por una dosis de 1000 µg/kg de toxina. Si la mortalidad es superior al 50%, la siguiente dosis que se va a administrar será más baja. Si la mortalidad es superior al 50%, la dosis que se administra en el siguiente nivel será mayor. Modificado de Costas et al. (2023).

3.2.1.2 Ensayo Dosis-respuesta: Diarrea inducida por el ácido okadaico

Para seleccionar una dosis que nos permitiese estudiar la diarrea en los ratones durante un periodo de 24 h sin que se produjese la muerte del animal, se realizó un ensayo *in vivo* en el que se administraron dosis crecientes de ácido okadaico. Se registraron los tiempos de aparición de la diarrea, se le asignó una puntuación en base a su aspecto, así como se anotaron los síntomas que presentaban a distintos tiempos: 1, 3, 6, 9, 12 y 24 h. Al finalizar el experimento se realizaron las necropsias y la evaluación anatomopatológica, así como se recogieron los intestinos delgado y grueso.

3.2.1.3 Efecto del pretratamiento farmacológico

Se llevaron a cabo una serie de ensayos en los que se administraron neuropéptido Y (NPY), péptido YY(3-36) [PYY(3-36)] o ciproheptadina (CPH) por vía intraperitoneal antes del tratamiento con ácido okadaico. Esto constituye tres bloques de experimentos independientes. El primero consistió en evaluar si el NPY podía interferir con la diarrea causada por el ácido okadaico. Para ello se diluyó el NPY en agua milliQ y se preparó en solución salina a una dosis de 107 µg/kg NPY que se adminsitró por vía intraperitoneal. Por otro lado, se prepararon 550 µg/kg AO. Se establecieron otros cuatro grupos experimentales de ratones: el grupo control al se administraba por vía intraperitoneal el vehículo correspondiente al NPY (agua milliQ) y 15 min después se adminsitraba por vía oral el vehículo correspondiente a la

toxina (etanol); el grupo pretratado con NPY; el grupo tratado con AO por vía oral, y el grupo pretratado con NPY por vía intraperitoneal y 15 min después tratado con AO por vía oral.

El segundo bloque consistió en el estudio de las posibles alteraciones que pudiera causar la administración del PYY(3-36) antes del tratamiento con AO. El PYY(3-36) se diluyó primeramente en agua milliQ y se preparó el mismo día de la administración en suero fisiológico a una dosis de 1 mg/kg PYY(3-36). De foma similar al ensayo anterior, los animales se dividieron en cuatro grupos: (i) control, (ii) PYY(3-36), (iii) 550 μg/kg AO y (iv) PYY(3-36) y AO. El PYY(3-36) se administró por vía intraperitoneal 15 min antes del tratamiento con AO.

El tercer bloque consistió en la evaluación de los efectos de la administración de 3 mg/kg CPH antes del tratamiento con AO (250 μg/kg). La CPH se diluyó en etanol y se preparó en suero fisiológico para su adminsitración intraperitoneal. En línea con los bloques anteriores, se dividieron los animales en cuatro grupos. Esto es, un grupo control, un grupo al que se le administró CPH, otro grupo al que se administró AO por vía oral y un último grupo que recibió CPH y AO.

3.2.1.4 Efecto de la ciproheptadina en un ensayo dosis-respuesta

Los resultados previos nos llevaron a evaluar los efectos de la administración de varias dosis ciproheptadina (CPH) antes del tratamiento con dosis inferiores de AO (250 μg/kg). Dosis de CPH de 0,1, 1, 3 y 10 mg/kg se administraron 30 min antes del tratamiento con la toxina. Durante las 2 h de estudio se registraron los síntomas, incluyendo el tiempo de aparición de la diarrea. Se recogieron las heces y se puntuaron y se evaluó la presencia de alteraciones morfológicas en la cavidad abdominal durante la necropsia. Finalmente, se tomaron muestras de los intestinos delgado y grueso.

Por otro lado, se llevó a cabo una dosis-respuesta de CPH finalizando el estudio en el tiempo de aparición de la diarrea en los ratones tratados con 250 μg/kg AO por vía oral. Los animales que recibían solo el AO marcaban el tiempo del estudio. El resto de animales se agruparon en control, pretratamiento con CPH, la combinación del pretratamiento de CPH y el AO. Las dosis de CPH que se utilizaron fueron de 0,1, 1, 6 mg/kg CPH. Al igual que en el caso anterior, se registraron los síntomas, se recogieron las heces y se les asignó una puntuación, así como se llevó a cabo una evaluación anatomopatológica de la cavidad abdominal y se recogieron los intestinos delgado y grueso.

3.2.2 Test funcionales

En los estudios *in vivo* realizados con la toxina PbTx3, se llevaron a cabo tres test para valorar la fuerza muscular, la actividad motora y la sensibilidad al frío de los ratones (Figura 5). Estas pruebas se realizaron el día anterior a la administración del tratamiento para establecer el control del propio animal y posteriormente se repitieron transcurridas 6 y 24 h tras la administración de la PbTx3.

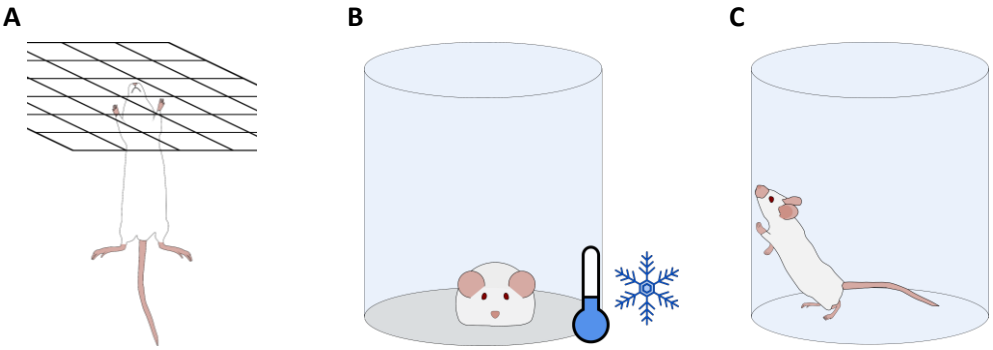


Figura 5. Representación de los tests funcionales. (A) Test de la rejilla, (B) test de la placa fría, (C) test del vaso.

- Test de la rejilla para evaluar la debilidad muscular modificado de Asorey et al. (2018). El principio de este test es que el animal tiene fuerza suficiente como para agarrarse y moverse por la rejilla estando boca abajo a una altura de 23 cm. Se mide el tiempo que tarda el animal en caerse. Si el animal no se suelta de la rejilla, el tiempo máximo del ensayo es 1 min y es este el tiempo que se asigna.
- Test del vaso para evaluar la actividad motora. Para ello, se utilizó un vaso de precipitados en el que se introdujo al animal, se grabó en vídeo durante 1 min. De este modo, se pudo contabilizar las veces que mostraba el comportamiento exploratorio que se conoce como "*rearing*" en inglés. Este consiste en que el animal se estira para apoyarse sobre la pared del vaso y es un reflejo de comportamiento y actividad normales.
- Test de la placa fría para evaluar cambios en la sensibilidad al frío (Ruan et al. 2018; Salat et al. 2019). Se utilizó un vaso de precipitados que en este caso se situaba sobre una superficie fría (4 ºC). Se introducía al animal en el vaso y se grababa en vídeo durante 30 segundos. A posteriori, se cuantificaban las veces que alzaba las patas traseras, ya que es una respuesta habitual para evitar el frío (Ruan et al. 2018).

3.3 Detección de toxinas en distintos órganos de ratón

Para poder determinar la concentración de toxina en los distintos órganos y materiales biológicos del ratón, se realiza la extracción de las muestras y se cuantifican posteriormente mediante cromatografía líquida acoplada a espectrometría de masas en tándem.

3.3.1 Extracción de toxinas

Tras la recogida de muestras biológicas de animales tratados con toxinas diarreicas, se realizaron distintos procedimientos de extracción para poder evaluar la concentración de toxinas en cada muestra.

3.3.1.1 Sangre

Las muestras de sangre se extrajeron siguiendo el protocolo descrito en Abal et al. (Abal et al. 2018). En una proporción 1:4 se mezcló la sangre con metanol (75%) y se homogenizó en un vórtex durante 1 minuto. A continuación, se filtraron por columnas de 0,45

μm que se centrifugaron a 3000 rpm durante 30 minutos a temperatura ambiente. La solución resultante se evaporó en un rotavapor y posteriormente la muestra se resuspendió en 200 μL de metanol (100%).

3.3.1.2 Orina y contenido gastrointestinal

Las muestras de orina y de contenidos estomacales e intestinales se extrajeron siguiendo el protocolo descrito en (Abal et al. 2017). Por cada 100 μL de orina se añadieron 40 μL de metanol (100%) y se homogenizaron durante 30 segundos mediante agitación en un vórtex. A continuación, se añadieron 10 μL de ácido tricloroacético (10%) y se volvió a agitar la muestra en las mismas condiciones. Para finalizar la extracción, se añadieron 50 μL de acetonitrilo (100%) a la disolución y se agitó durante 1 minuto. Por último, se pasó la muestra por un filtro de 0,22 μm centrifugándose a 14500 x g 10 minutos a temperatura ambiente.

3.3.1.3 Heces y órganos

El procesamiento de las muestras de heces y de las muestras de tejidos es similar al de orina con algunos pasos más debido a la complejidad de la muestra. En esta ocasión, por cada 100 mg de heces se añaden 400 μL de metanol, se homogeniza durante un minuto y se somete a sonicación durante 30 segundos para disgregar la muestra. Se centrifuga la muestra a 10000 x g durante 10 minutos a temperatura ambiente. El sobrenadante resultante se separa, mientras que el precipitado se vuelve a extraer. La muestra se extrae un total de tres veces. Los sobrenadantes de cada centrifugado se juntan y se evaporan en un rotavapor. Se disuelve la muestra en metanol en 100 μL de metanol para concentrarla, a la cual se le añadieron 40 μL metanol, 10 μL de ácido tricloroacético (10%) y 50 μL de acetonitrilo. Tras añadir cada disolución, se agita la muestra durante 30 segundos o 1 minuto tras el último paso. Por último, se pasó la muestra por un filtro de 0,22 μm centrifugándose a 14500 x g 10 minutos a temperatura ambiente.

3.3.2 Cuantificación de las toxinas

Para la detección de la concentración de toxinas presente en las muestras procesadas se utilizó un equipo de cromatografía líquida de ultra-alta resolución acoplado a un espectrómetro de masas triple cuadrupolo (UHPLC-MS/MS). Se utilizó una cromatografía de fase reversa con una columna de tipo C18 de 2,1 mm (diámetro) x 100 mm (largo) y un tamaño de poro de 1,7 μm. Se utilizaron dos fases móviles:

- Fase móvil A: 50 mM de ácido fórmico y 2 mM de formiato de amonio disueltos en agua MilliQ.
- Fase móvil B: 50 mM de ácido fórmico y 2 mM de formiato de amonio disueltos en acetonitrilo:agua (95:5 v/v).

La columa se mantuvo a una temperatura de 40 ºC durante todo el proceso y la fase móvil se injectaba a un flujo de 0,4 mL/min. Las muestras preparadas se mantenían a 4 ºC en el módulo desde donde se tomaban automáticamente 5 μL para la cromatografía. Esta comienza con un 30% de fase móvil B durante 3 min, aumenta al 90% durante 1,5 min y se vuelve a rebajar al 30% hasta los 6,5 min. El eluyente se pulveriza mediante ionización por electrospray (ESI) y se utiliza el monitoreo de reacciones múltiples (MRM) en modo negativo. Esto permite identificar el ion parental y su producto, así se distinguen los tres análogos pese a sus similitudes estructurales. Si bien las condiciones del módulo de aceleración de voltaje (CAV; 4 V) y las del fragmentador (320 V) fueron las mismas para detectar los análogos de las toxinas diarreicas, la

energía de colisión fue diferente para cada toxina y cada fragmento (Tabla 3). Asimismo, se utilizaron dos transiciones por cada toxina: una para confirmar la identificación del análogo y otra para cuantificar la concentración de la misma.

Tabla 3. Energía de colisión y fragmentos iónicos de las toxinas diarreicas.

	Transición (m/z)	Energía de colisión
Ácido okadaico	803,5>255,2	50 V
	803,5>113,2	66 V
Dinophysistoxina 1	817,5>255,2	54 V
	817,5>113,0	70 V
Dinophysistoxina 2	803,5>255,1	56 V
	803,5>151,0	56 V

También se utilizaron curvas patrón para poder determinar la concentración de las toxinas. Los límites de cuantificación fueron de 1,3 ng/mL para el AO y la DTX1, mientras que para la DTX2 fue de 2,33 ng/mL.

3.4 DETERMINACIÓN DE NEUROMODULADORES EN MUESTRAS DE INTESTINO

3.4.1 Extracción de muestras

La determinación de distintos neurotransmisores se realiza en muestras de intestino delgado e intestino grueso. El procesamiento es el mismo en ambos tejidos. Fragmentos de íleo y colon proximal se lavan en una disolución de tampón fosfato salino (PBS) previamente refrigerado. A continuación, se pesan y se añade PBS en una proporción 1:9 (p/v). Los tejidos se homogenizan con una varilla de vidrio y se sonican para disgregar la muestra. Finalmente, las muestras se centrifugan a 10000 x g durante 5 min a 4 ºC. La concentración de NPY, PYY y serotonina se mide en el sobrenadante.

3.4.2 Cuantificación de los neuromoduladores

Se utilizan kits comerciales de ELISA (ensayo inmunoadsorbente ligado a enzimas) para cuantificar la concentración de neuropéptido Y (NPY), péptido YY (PYY) y serotonina en tejido de intestinos delgado y grueso. Los tres kits empleados tenían en común que se trata de ELISA competitivos y que la determinación se hace por colorimetría. Para cuantificar NPY y PYY (Figura 6) se utilizaron kits de la casa comercial Cloud Clone Corp., con un rango de detección de 2,47-200 pg/mL y de 12,35-1000 pg/mL, respectivamente. Ambos utilizan el 3, 3', 5, 5'-tetrametilbencidina (TMB) como sustrato que oxida la peroxidasa de rábano otorgando un color azul de intensidad inversa a la concentración del péptido. La reacción se frena añadiendo ácido sulfúrico a baja concentración, lo que vira el color a amarillo. Este color absorbe a una longitud de onda de 450 nm.

En la detección de serotonina se utilizaron kits de Enzo Life Sciences con un rango de detección de 0,49-500 ng/mL. En este caso, el sustrato es el p-nitrofenil fosfato disódico (pNpp) que es catalizado por la fosfatasa alcalina conjugada a la serotonina generando un color amarillo pálido que absorbe la luz a 405 nm. La serotonina conjugada con la fosfatasa alcalina compite

con la serotonina de la muestra por unirse a los anticuerpos primarios, por lo tanto, cuanta menor sea la concentración de serotonina en la muestra, mayor será la absorbancia.

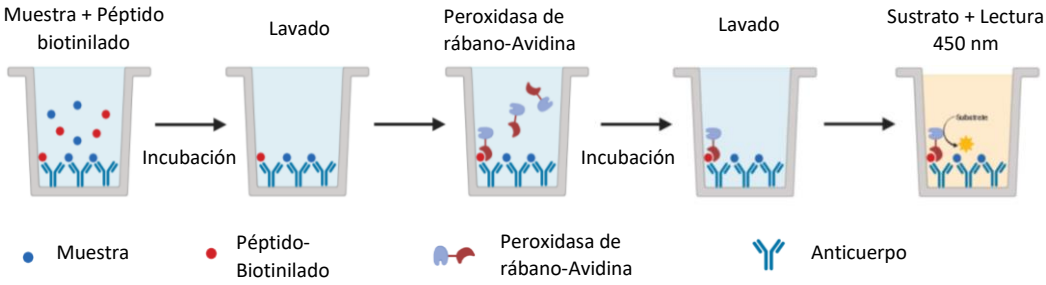


Figura 6. Esquema del protocolo de ELISA competitivo del NPY y del PYY.

3.5 DETERMINACIÓN DE ELECTROLITOS EN HECES

La diarrea es el síntoma principal causado por el ácido okadaico en roedores. No obstante, para poder caracterizar el tipo de diarrea, estudiamos la concentración de electrolitos en heces.

3.5.1 Extracción de electrolitos fecales

Las concentraciones de electrolitos se analizaron en heces (Figura 7). El procedimiento de extracción comienza con la dilución de al menos 50 mg de heces se diluyen en agua MilliQ previamente atemperada a 37 ºC en una proporción 1:7 (p/v). A continuación, las muestras se agitan primero en un vórtex durante 30 segundos y después 1 h en agitación vertical a 37 ºC. Para garantizar la disgregación de las heces, se realizaron dos ciclos de sonicación 5 min y agitación 1 min en vórtex. Las muestras se centrifugaron a 10000 x g durante 20 min. El sobrenadante se vuelve a centrifugar en dos ocasiones más en las mismas condiciones para asegurar la eliminación de desechos. Finalmente, las muestras se filtran mediante centrifugación a 10000 x g 10 min utilizando filtros de 0,22 µm.

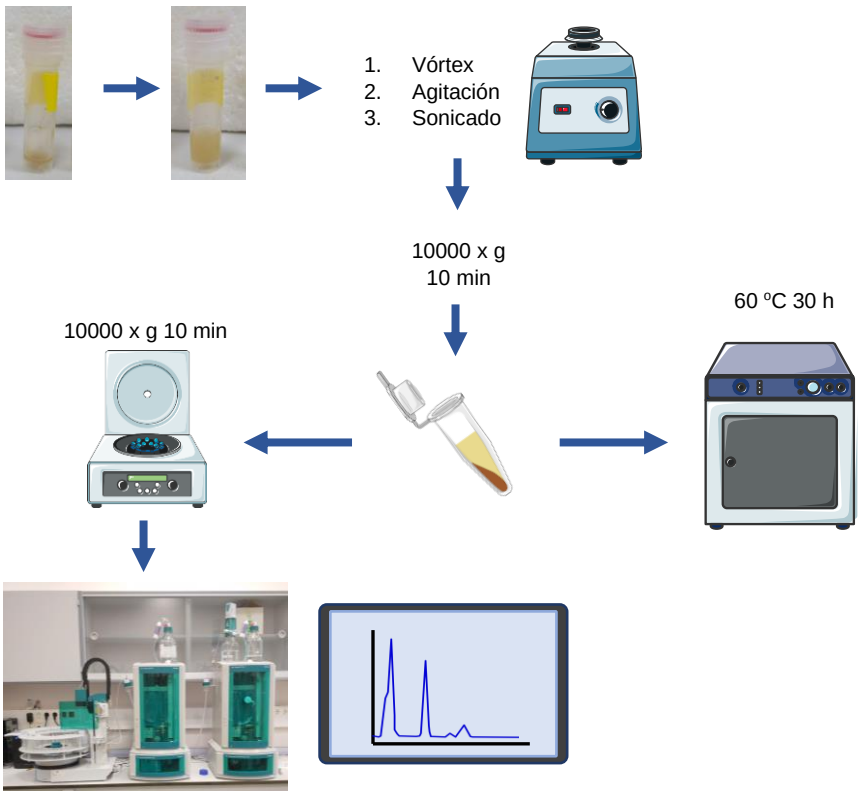


Figura 7. Diagrama de la extracción y detección de electrolitos en muestras fecales.

3.5.2 Cuantificación de electrolitos en heces

Se cuantifican los iones cloruro, sodio, potasio y calcio en muestras fecales mediante cromatografía iónica acoplada a un detector de conductividad y un supresor químico. El método de detección es distinto según si se trata de aniones o cationes. En el caso del ion cloruro, se emplea una columna cromatográfica de alcohol polivinílico con grupos de amonio cuaternario. La temperatura de la columna se mantiene a 45 ºC y la fase móvil es carbonato de sodio (3,6 mM) a un flujo de 0,8 mL/min. Por el contrario, la columna cromatográfica para la separación de cationes es de sílica gel recubierta de grupos carboxilo. La temperatura de la columna se mantiene a 30 ºC, mientras que el fujo es de 1,1 mL/min. La fase móvil está compuesta de 1,7 mM de ácido nítrico y 1,7 de ácido dipicolínico. Los límites de detección son de 0,00048, 0,00944, 0,00350 y 0,00436 mM para cloro, sodio, potasio y calcio respectivamente. Los límites de cuantificación son de 0,00159, 0,03146, 0,01168 y 0,01452 mM para cloro, sodio, potasio y calcio respectivamente. Estos ensayos se realizan el área de Analisis Instrumental de *Rede de Infraestruturas de Apoio á Investigación e ao Desenvolvemento Tecnolóxico* (RIAIDT) de la Universidad de Santiago de Compostela.

3.5.3 Cálculo del gap osmótico fecal

Se calcula el gap osmótico fecal para las muestras de heces de las que se mide la concentración de electrolitos. El gap osmótico fecal se basa en una osmolalidad similar entre el suero y las heces (Eherer and Fordtran 1992; Fokam Tagne et al. 2019). La medición de la osmolalidad directamente de la muestra puede alterarse rápidamente a causa del metabolismo bacteriano. Por ello se emplea el gap osmótico fecal. El cálculo (Ecuación 1) consiste en restarle

a 290 mOsm/kg (la osmolalilad del suero) el doble de la suma de la concentración de sodio y de potasio (Rao 2019).

$$\text{Ecuación 1} \qquad 290\ \text{mOsm/kg} - 2\ x([Na^+] + [K^+])$$

Si la diferencia es mayor que 125 mOsm/kg, se considera uan diarrea de tipo osmótica. Si el valor resultante se encuentra entre 50 y 125 mOsm/kg, se puede considerar una diarrea de tipo mixto. Por último, si el resultado es inferior a 50 mOsm/kg se puede considerar una diarrea secretora (Eherer and Fordtran 1992; Rao 2019).

3.6 ANÁLISIS BIOQUÍMICO EN SANGRE

Se obtiene el plasma de las muestras de sangre intracardiaca recogida en los ratones al finalizar los estudios *in vivo*, durante la necropsia. Para medir los parámetros bioquímicos, se utiliza un equipo con un sistema de microfluidos que distribuye 100 µL de plasma en un panel con forma de disco que realiza distintas reacciones colorimétricas para cada parámetro. El equipo Chemistry Analyzer Pointcare® V3 lee las longitudes de onda correspondientes a cada parámetro.

3.7 ESTUDIO DE LA ACCION DE LA PBTX3 EN LOS CANALES DE SODIO DEPENDIENTES DE VOLTAJE

Utilizamos la técnica de patch-clamp en modo célula completa para estudiar la acción de la PbTx3 en los canales de sodio dependientes de voltaje. Concretamente evaluamos los cambios en el voltaje de activación y en la intensidad máxima de la corriente de entrada de sodio en los canales de sodio dependientes de voltaje $Na_V1.6$ expresados en células embrionarias de riñón humano HEK293. Las células se encuentran en una disolución que mimetiza las condiciones extracelulares y se mantienen en una cámara de registro que permite su visualización por microscopía óptica. El sistema consta de dos electrodos: un electrodo de referencia y un electrodo de registro. El primero está en contacto con la disolución extracelular en la cámara. El electrodo de registro se encuentra en el interior de un microcapilar que contiene una solución recreando las condiciones del medio intracelular. Cuando el microcapilar entra en contacto con la membrana de una célula, al realizar presión negativa se crea un sello de alta resistencia. A continuación, se aplica de nuevo presión negativa para romper la membrana celular que se encuentra adherida a la pipepta. De este modo, se genera un continuo entre la membrana plasmática y el microcapilar. Proporcionando así acceso a la célula completa. Durante varios minutos, se deja que el medio intracelular y la disolución del microcapilar entren en equilibrio. La diferencia entre las señales de los electrodos de referencia y de registro permiten obtener la diferencia de potencial. La célula se mantiene a un voltaje de reposo de -55 mV. Entonces, se añaden concentraciones crecientes de PbTx3 en la cámara de registro hasta alcanzar los 100 nM. Tras añadir cada concentración de PbTx3, se mantienen las células con la toxina 5 min y se aplican pulsos desde -80 a +80 mV en intervalos de 10 mV. El electrodo de registro detecta si hay un cambio en la intensidad de corriente, las señales se amplifican (Multiclamp 700B), se filtran y convierten de analógicas a digitales (sistema de adquisición Digidata 1440A). Las resistencias se compensaron con el circuito de compensación interna en un 70-80%. Se analizan

los cambios de las corrientes con el software pClamp 10. Se obtienen de este análisis el voltaje de activación de los canales $Na_V1.6$, así como la intensidad de corriente máxima.

3.8 ESTUDIO DE LAS ALTERACIONES TISULARES Y CITOMORFOLOGICAS OCASIONADA POR LAS TOXINAS

Durante la realización de las necropsias se evalua la presencia de alteraciones macroscópicas. Para la evaluación de daño tisular ocasionado por la toxina en ratón se realiza mediante la observación de las muestras de tejido por microscopía óptica. Las aleraciones citomorfológico se estudian por microscopía electrónica de transmisión.

3.8.1 Evaluación del daño tisular

Las muestras de los distintos tejidos que se van a observar por microscopía óptica, se fijan en solución de Bouin en el momento de recogida durante la necropsia de los ratones al finalizar el estudio *in vivo*. Para poder realizar cortes de las muestras es necesario incluir el tejido en parafina. Debido a la hidrofobia de la parafina se comienza con un proceso de deshidratación con una disolución de etanol al 70% hasta una concentración del 100%. Se sumerge la muestra en xilol para facilitar la inclusión en parafina. Con un microtomo se corta el tejido en secciones finas (3-4 μm) que se colocan en un portaobjetos. Para poder teñir las muestras, se retira la parafina y se hidratan los cortes mediante su inmersión en xilol y en etanol a concentraciones decrecientes. Se realiza una tinción de hematoxilina-eosina, ya que, estos colorantes tiñen distintamente las estructuras básicas y ácidas del tejido.

3.8.2 Estudio de las alteraciones citomirfológicas

En el procesado de las muestras que se observaron por microscopía electrónica de transmisión, primeramente, las muestras se fijaron en glutaraldehído al 2,5% preparado en 0,1 M cacodilato de sodio trihidrato (pH 7,4). Posteriormente, transcurridas 4 y 24 h las muestras se lavaron con 0,1 M cacodilato de sodio trihidrato a las 4 y 24 h. Durante este tiempo se mantuvieron a 4 ^{o}C. La pos-fijación se llevó a cabo en 1% OsO4 preparado en 0,1 M cacodilato de sodio trihidrato durante 1 h. Tras dos lavados, las muestras se fueron deshidratando en soluciones con concentraciones crecientes de etanol, lo que incluyó un paso de inmersión en 0,5% de acetato de uranilo disuelto en etanol al 70%. En este punto, se lavaron los tejidos en óxido de propileno para facilitar la inclusión en resina epoxi. Se realizan cortes ultrafinos que se contrastan con citrato de plomo y acetato de uranilo. La afinidad de los componentes celulares a estos reactivos permite diferenciar las estructuras celulares según su opacidad haz de electrones del microscopio. Las fotos de las muestras se realizaron en el microscopio electrónico de transmisión modelo JEOL JEM-1011 de las instalaciones de la *Rede de Infraestruturas de Apoio á Investigación e ao Desenvolvemento Tecnolóxico* (RIAIDT) de la Universidad de Santiago de Compostela. El análisis de las alteraciones citomorfológicas se centró en el estado de las mitocondrias, las microvellosidades, las uniones estrechas y uniones adherentes (Figura 8). El ancho de las uniones estrechas y las uniones adherentes se midió en el punto de mayor separación utilizando el programa ImageJ (Fiji). Solo se midieron las uniones de cortes longitudinales (Martinez et al. 2013).

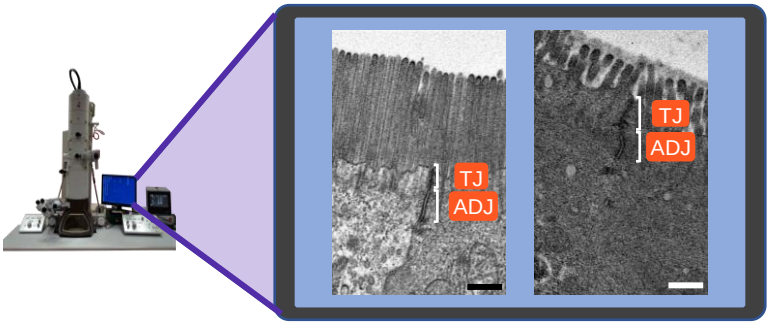


Figura 8. Esquema con el modelo de microscopio electrónico de transmisión junto con dos imágenes de yeyuno y colon indicando las uniones estrechas (TJ) y las uniones adherentes (ADJ)(escala de 500 nm).

3.9 ANÁLISIS ESTADÍSTICO

Los análsis estadísticos se llevan a cabo utilizando los programas R y GraphPad Prism. En todos los casos, se estudia si el conjunto de datos sigue una distribución normal y se trata de una muestra homocedástica. De cumplirse estas condiciones, se realiza o bien una prueba t de Student para comparar dos grupos o bien un análisis estadístico de varianzas en una dirección (ANOVA). En ambos casos, se realiza un análisis *post hoc* con el test de comparación múltiple de Bonferroni. Cuando la muestra no sigue una distribución normal o las varianzas no son homogéneas, se realizan tests no paramétricos como el test de Mann-Whitney o el test de Kruskal-Wallis. Como test *post hoc* se utiliza el test de Bonferroni o el de Dunn. Para el análisis de los tests funcionales, se tiene en cuenta que se trata de muestras apareadas, es decir, se corresponden con el mismo sujeto a distintos tiempos. Al igual que para el resto de conjunto de datos se estudia si siguen una distribución normal y si la varianza es homogénea. Si se cumplen estas condiciones, se ejecuta un ANOVA en dos sentidos para muestras apareadas. Si estas condiciones no se cumplen, se realiza un test de Friedman. En todos los análisis se establece un valor p de 0,05 para considerar la diferencia entre grupos significativa.

4 RESULTADOS Y PUBLICACIONES

En esta sección se recopilan los resultados obtenidos durante el desarrollo de la presente tesis doctoral. El conjunto de datos se encuentra recogido en los siguientes cuatro artículos publicados en revistas internacionales incluidas en el JCR.

- *DSP toxin organ distribution across organs in mice after acute oral administration*.
- *Serotonin involvement in okadaic acid-induced diarrhoea in vivo.*
- *Intestinal secretory mechanisms in okadaic acid induced diarrhoea*.
- *Acute toxicology report of the emerging marine biotoxin brevetoxin 3 in mice: Food safety implications*.

4.1 PUBLICACIÓN 1: DSP TOXIN ORGAN DISTRIBUTION ACROSS ORGANS IN MICE AFTER ACUTE ORAL ADMINISTRATION.

Año de la publicación: 2021

Autores y filiación en orden: M Carmen Louzao [1], Paula Abal [1], Celia Costas [1], Toshiyuki Suzuki [2], Ryuichi Watanabe [2], Natalia Vilariño [1], Ana M Botana [3], Mercedes R Vieytes [4], Luis M Botana [1]

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Resumen: Las toxinas del grupo del ácido okadaico (AO) son ficotoxinas lipofílicas de distribución mundial que se pueden acumular en bivalvos y causar la denominada intoxicación diarreica por consumo de marisco (DSP). Este grupo de toxinas engloba al ácido okadaico (compuesto de referencia) y sus análogos dinofisistoxinas 1 (DTX1) y 2 (DTX2). En este estudio evaluamos su toxicocinética administrando distintas dosis de OA, DTX1 y DTX2 por vía oral a ratones en los que registramos los síntomas de la toxicidad durante 24 h. La

distribución de las toxinas en los principales órganos del tracto gastrointestinal se evalúa por cromatografía líquida de ultra-alta resolución acoplada a espectrometría de masas en tándem. Los resultados indican una absorción gastrointestinal de estas toxinas dependiente de la dosis. Además, se distingue un comportamiento toxicocinético distinto entre los distintos análogos. Transcurridas 24 h de la administración, la mayor concentración de toxinas se detectó en el estómago, seguida del intestino grueso, intestino delgado e hígado. No obstante, la concentración de DTX2 en estos órganos es muy inferior a la del AO y la DTX1. La escasa cantidad de DTX2 detectada en sangre y orina, unida a la mayor eliminación en heces indica una baja absorción de toxina lo que podría contribuir a la menor toxicidad de este análogo. Estos resultados aportan información en relación con la toxicidad y toxicocinética de los compuestos del grupo del ácido okadaico de aplicación en la evaluación de la seguridad alimentaria y protección de la salud del consumidor.

A referencia completa da publicación: Louzao, M.C.; Abal, P.; Costas, C.; Suzuki, T.; Watanabe, R.; Vilariño, N.; Botana, A.M.; R. Vieytes, M.; Botana, L.M. DSP Toxin Distribution across Organs in Mice after Acute Oral Administration. *Mar. Drugs* **2021**, *19*, 23.

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La contribución de la doctoranda en la publicación: la doctoranda contribuyó en el análisis de los datos, en el desarrollo de la investigación y en la preparación y escritura del borrador original del artículo.

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Article

DSP Toxin Distribution across Organs in Mice after Acute Oral Administration

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Abstract: Okadaic acid (OA) and its main structural analogs dinophysistoxin-1 (DTX1) and dinophysistoxin-2 (DTX2) are marine lipophilic phycotoxins distributed worldwide that can be accumulated by edible shellfish and can cause diarrheic shellfish poisoning (DSP). In order to study their toxicokinetics, mice were treated with different doses of OA, DTX1, or DTX2 and signs of toxicity were recorded up to 24 h. Toxin distribution in the main organs from the gastrointestinal tract was assessed by liquid chromatography-mass spectrometry (LC/MS/MS) analysis. Our results indicate a dose-dependency in gastrointestinal absorption of these toxins. Twenty-four hours post-administration, the highest concentration of toxin was detected in the stomach and, in descending order, in the large intestine, small intestine, and liver. There was also a different toxicokinetic pathway between OA, DTX1, and DTX2. When the same toxin doses are compared, more OA than DTX1 is detected in the small intestine. OA and DTX1 showed similar concentrations in the stomach, liver, and large intestine tissues, but the amount of DTX2 is much lower in all these organs, providing information on DSP toxicokinetics for human safety assessment.

Keywords: dinophysistoxin-1; dinophysistoxin-2; LC/MS/MS; okadaic acid; toxicokinetic

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1. Introduction

Okadaic acid (OA) and dinophysistoxins (DTXs) are marine lipophilic phycotoxins globally distributed and produced by benthic and planktonic dinoflagellates of the genera *Prorocentrum* and *Dinophysis* [1,2], with the species of the genus *Dinophysis* being the main source of toxins in the marine trophic chain [3,4]. This group of toxins includes a wide range of molecules, with dinophysistoxin-1 (DTX1) [1] and dinophysistoxin-2 (DTX2) [5] being the main structural analogs of OA [6]. Harmful Algal Blooms (HABs) cause the accumulation of these toxins in edible filter-feeding shellfish, the ingestion of which by human consumers leads to diarrheic shellfish poisoning (DSP) [7]. This gastrointestinal illness is characterized by symptoms such as diarrhea, nausea, and vomiting that begin from 30 min to a few hours after ingestion of the toxic shellfish [8]. OA and DTX2 are routinely found in mussels, clams, and oysters in the Atlantic coast [9,10], while DTX1 is commonly found in Japan [11]. The presence of these toxins is also associated with important economic consequences for producing areas [12].

OA and DTXs are inhibitors of protein phosphatases (PP), mainly PP2A [13], with different potency [6,14,15]. PPs are important modulators of enzyme activity and cell signaling pathways [16]; however, the OA-dependent molecular mechanisms leading to

diarrhea are not fully elucidated [15,17,18]. Some studies have indicated that the target organ of OA and DTXs is the small intestine [19–21], but the mode of toxicity seems complex and diverse [22]. OA could have targets, other than PPs, involved in the diarrhetic process [23]. Some studies revealed that modulation of neuropeptide levels induced by OA may be the key triggers of diarrhea [24].

Acute Refence Dose (ARfD) and Lowest Observed Adverse Effect Level (LOAEL) have been established in humans, for which the values are 0.3 and 0.8 µg OA equivalents/kg body weight (bw) for adults, respectively [25]. Nevertheless, the toxicological database for the OA group of toxins is limited and comprises mostly studies on their intraperitoneal acute toxicity in mice. A recent study based on the oral lethal doses 50 (LD_{50}) in mice showed that DTX1 is the most toxic analog and that DTX2 is the least toxic one [15], suggesting a reevaluation of the Toxicity Equivalency Factor (TEF) values previously established by the European Food Safety Authority (EFSA) considering intraperitoneal LD_{50} [25].

It has been seen that these toxins are easily absorbed orally in a short period of time [15,19,26,27]. However, what happens to OA and its analogs in the body remain to be determined. As human exposure to OA and DTXs is exclusively by ingestion, the aim of this study was to characterize their kinetics following oral administration to mice. Thus, we perform the first comparative toxicokinetic study of OA, DTX1, and DTX2 measuring the toxins in gastrointestinal tissues by LC/MS/MS analysis, the official method for detection of the lipophilic toxins group, where OA-group toxins are also included [28].

2. Results

2.1. Lethality and Symptoms

In vivo studies were performed following an optimized 4-level up and down procedure where the toxins were administered individually by oral gavage to female mice. The lethality 24 h after oral gavage administration was 67% in mice treated with 1000 µg/kg OA or DTX1. At the same time, point lethality was 0% in mice treated with 1000 µg/kg DTX2. This clearly confirms that DTX2 is less toxic than OA or DTX1. Moreover, DTX1 was more toxic than OA since lethality was 60% in mice treated with 500 µg/kg DTX1 and 40% in mice treated with the same dose of OA (Table 1). These data agree with the previously determined LD_{50} for DTX1 (487 µg/kg), OA (760 µg/kg), and DTX2 (2262 µg/kg) [15,27].

Table 1. Lethality (%) of mice after Okadaic acid (OA), dinophysistoxin-1 (DTX1), or dinophysistoxin-2 (DTX2) administration by oral gavage.

Toxin	Dose (µg/kg bw)	Lethality (%)	Number Mice
OA	1000	67	3
	875	67	9
	750	43	7
	500	40	5
DTX1	1000	67	3
	500	60	5
	375	0	9
	250	0	7
DTX2	3000	100	5
	2500	86	7
	2250	44	9
	2000	33	3
	1000	0	3

The mice were observed during the whole experiment, and toxicity signs were recorded. Diarrhea and nonspecific symptoms were quickly evident in both OA and DTX1 treated mice (Table 2). However, mice administered with the less toxic compound DTX2 showed individual variability, and nonspecific symptoms such as piloerection, squint-eyes, spasms, and posture on hind legs were less common.

Table 2. Symptoms registered in mice after OA, DTX1, or DTX2 administration by oral gavage.

	Appearance of Symptoms (%)												
	OA (μg/kg bw)				DTX1 (μg/kg bw)				DTX2 (μg/kg bw)				
Symptom	1000	875	750	500	1000	500	375	250	3000	2500	2250	2000	1000
diarrhea	66.67	100	57.14	100	66.67	80	66.67	71.43	100	100	33.33	66.67	66.67
apathy	100	88.89	100	100	100	100	88.89	85.71	100	100	44.44	66.67	66.67
piloerection	100	55.56	42.86	20	33.33	40	44.44	28.57	40	14.29	11.11	33.33	66.67
squint-eyes	100	77.78	57.14	60	100	80	77.78	57.14	20	0	0	0	33.33
spasms	33.33	22.22	28.57	0	33.33	20	22.22	28.57	0	0	11.11	33.33	33.33
cyanosis	66.67	88.89	57.14	0	66.67	80	66.67	0	60	85.71	33.33	66.67	0
on hind legs	0	44.44	14.29	60	0	20	22.22	14.29	0	14.29	0	0	33.33
dyspnea	0	0	14.29	0	33.33	0	0	0	40	14.29	0	0	0

2.2. LC/MS/MS Analysis

In those kinds of studies, after mice death or euthanasia, organs from the gastrointestinal tract were collected to quantify DSP toxins by LC/MS/MS, both in the tissue itself and in the content of some of the organs (contents of the stomach and the small and large intestines). The screening of OA, DTX1, and DTX2 across different organs is presented in Figure 1.

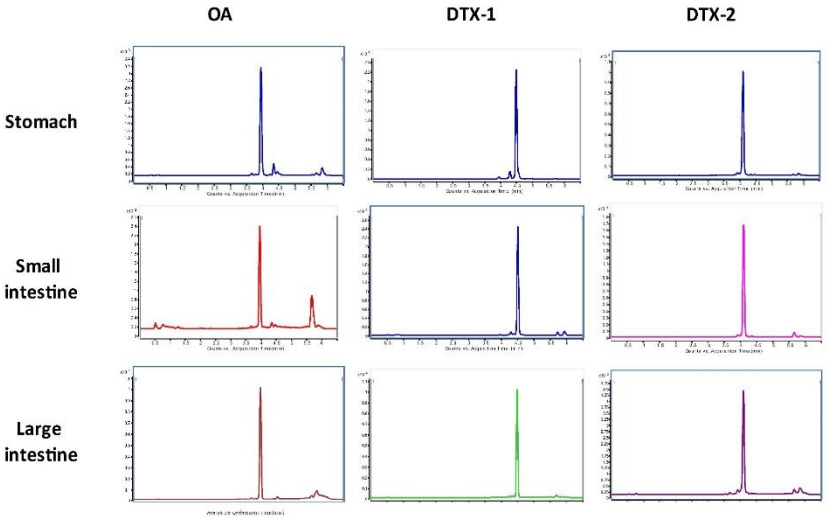

Figure 1. Total ion chromatogram of OA, DTX1, and DTX2 of the stomach, small intestine, and large intestine samples.

The organs belonging to the digestive tract showed a dose-dependent toxin concentration in most cases. Toxins were more concentrated in stomach tissue and in descending order in the large intestine, small intestine, and liver.

The highest amount of toxins was found in the stomach (Figure 2). The less toxic compound DTX2 quantified in this organ encompassed much lower values at the doses of 1000 and 2000 µg/kg of DTX2 bw but reached very high concentrations in tissue at the highest doses (2250–3000 µg/kg) (Figure 2C). Statistically significant differences were obtained between OA and DTX2.

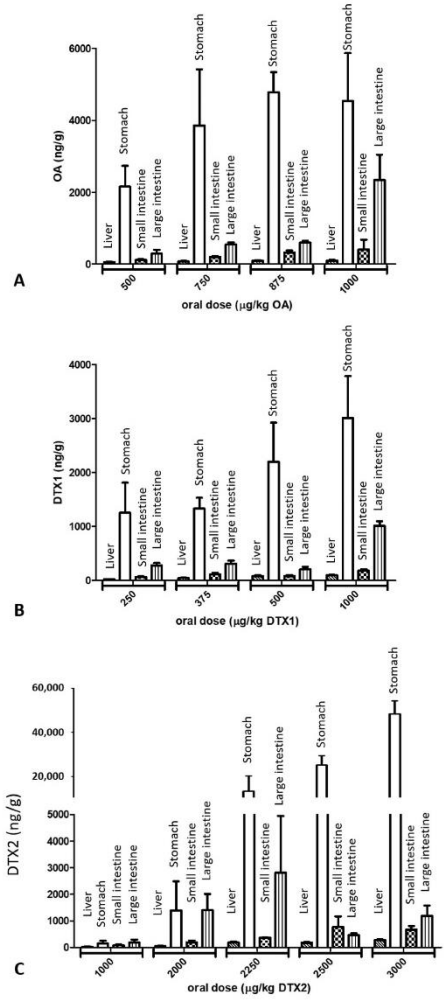

Figure 2. Toxin concentration in different tissues of the digestive organs (ng/g) according to oral dose administered to mice: (**A**) okadaic acid, (**B**) dinophysistoxin-1, and (**C**) dinophysistoxin-2, reported as mean ± SEM.

The large intestine was another organ with high toxin content, especially OA. The concentration of DTX2 was low, even at the highest doses administered (Figure 2C), and was statistically different from OA. It should be noted that the contents of the three toxins in liver were very low.

When we compared the results of the same dose, we found that mice treated with OA and DTX1 had similar concentrations of toxins in stomach tissue while the amount of DTX2 was much lower (Table 3). Similar results were registered in the liver and large intestine. It is interesting that, in the small intestine, the concentration of the most toxic compound DTX1 was markedly inferior to OA, around 2 times less.

Table 3. Concentration of toxin (ng/g tissue) in gastrointestinal organs from mice treated with 1000 µg/kg body weight (bw) doses (mean ± SEM): 1-way ANOVA–Newman–Keuls Multiple Comparison Test was used, and * $p \leq 0.05$ was considered statistically significant.

	Liver	Stomach	Small Intestine	Large Intestine
OA	96 ± 23	4540 ± 1326	408 ± 271	2340 ± 706
DTX1	93 ± 6	3006 ± 782	182 ± 27	1009 ± 83
DTX2	21 ± 11 *	142 ± 105 *	75 ± 38	176 ± 120 *

The contents of the stomach, small intestine, and large intestine were collected from mice treated with each of the three toxins 24 h after administration (Figure 3). The highest amount of OA and DTX1 was found in the stomach content followed by the large intestine and small intestine contents. However, mice treated with DTX2 showed almost a lack of toxin in large intestine content (Figure 3C).

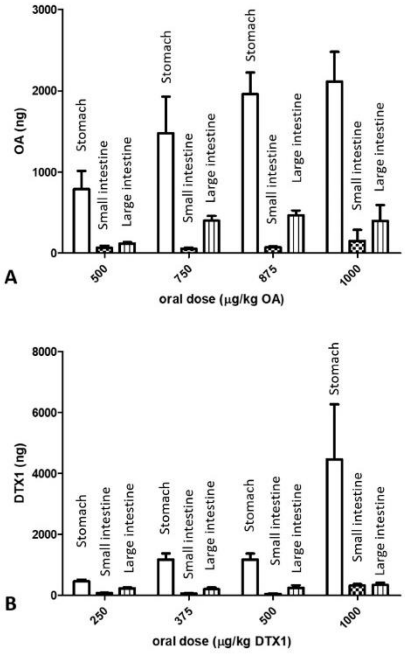

Figure 3. *Cont.*

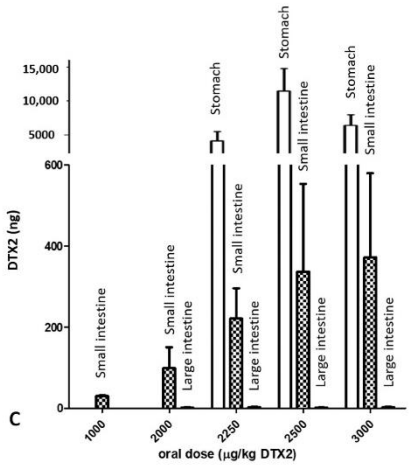

Figure 3. Total toxin (ng) detected in different contents of the digestive organs according to oral dose administered to mice: (**A**) okadaic acid, (**B**) dinophysistoxin-1, and (**C**) dinophysistoxin-2, reported as mean ± SEM.

The comparison between toxins (Table 4) indicated that 24 h after administration of 1000 µg/kg of toxin, the amount of DTX1 in the stomach content and small intestine fluids is twofold higher compared to OA. Additionally, DTX1 is tenfold higher compared to DTX2 in the small intestine content, although these differences were not significant. There was a small difference in OA and DTX1 data in the large intestine content. Nevertheless, DTX2 was almost absent in fluids collected form the large intestine. Significant differences between DTX1 and DTX2 were registered.

Table 4. Toxin content (ng) quantified in fluids collected from the stomachs and the small and large intestines of mice treated with 1000 µg/kg bw doses (mean ± SEM): 1-way ANOVA–Newman–Keuls Multiple Comparison Test showed no significant differences between OA and DTX1. $p \leq 0.05$ was considered statistically significant.

	Stomach Content	Small Intestine Content	Large Intestine Content
OA	2112 ± 365	151 ± 135	397 ± 193
DTX1	4468 ± 1802	321 ± 50	341 ± 72
DTX2	-	30 ± 1.3	0.19 ± 0.092

The cumulative toxin excreted in urine and feces after doses of 1000 µg/kg OA, DTX1, or DTX2 in mice are summarized in Figure 3. In urine, OA increased with time up to 24 h (Figure 4A). However, the main excretion of toxins was in feces. OA was excreted with the first diarrheic feces 1 and 3 h after toxin administration. Meanwhile, DTX2 was detected in samples of feces collected 6, 12, and 24 h after toxin administration.

Twenty-four hours following gavage administration of OA and DTX1, measurable concentrations of toxins were found in the blood (Figure 5).

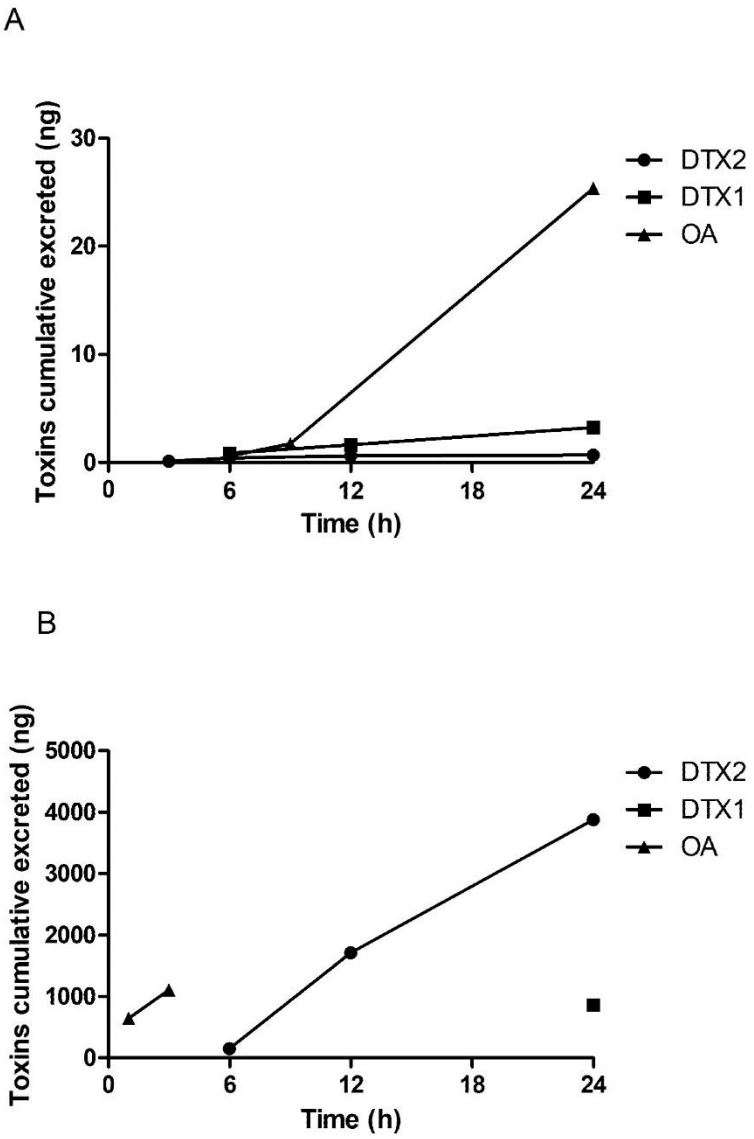

Figure 4. Time course of OA, DTX1, or DTX2 cumulatively excreted (ng) in urine (**A**) and feces (**B**): mice received a dose of 1000 µg/kg of each toxin.

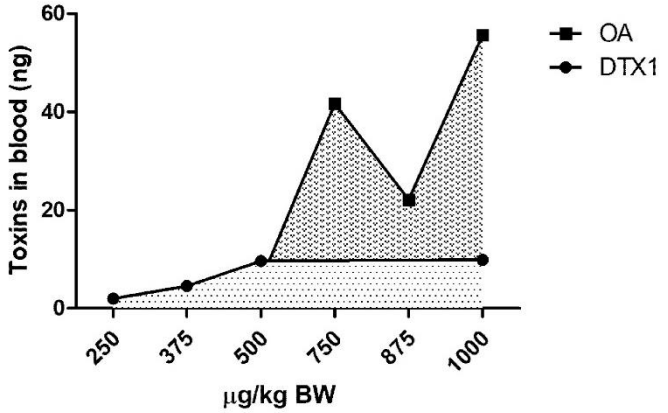

Figure 5. Plasma amount of toxins in mice 24 h after oral gavage administration of each dose of OA or DTX1.

As depicted in Figure 6, around 20% of the dose was recovered at 24 h after a single dose administration of OA. This percentage is similar for DTX1, but it should be highlighted that a recovery of 32% was found for the dose of 1000 µg/kg. In contrast, following a single dose of DTX2, the mean recovery of toxins accounted for 2% of the total dosages 1000 and 2000 µg/kg. The recoveries from the other DTX2 doses were higher and reached 35% (2500 µg/kg).

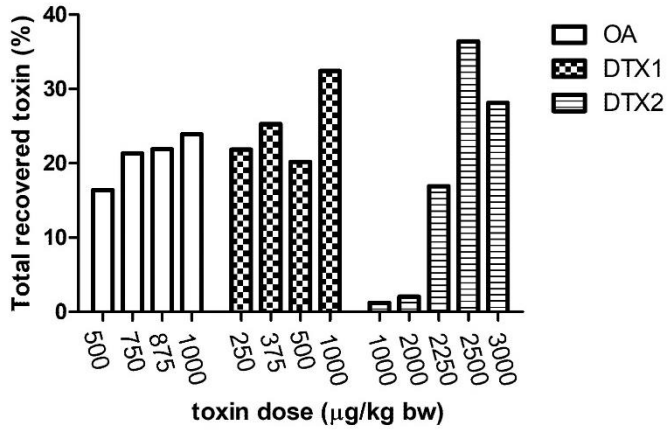

Figure 6. Toxin recovery: % OA, DTX1, or DTX2 detected 24 h after administration of the dose indicated.

3. Discussion

The worldwide incidence of diarrheic shellfish toxins poses a threat to public health, with a consequent marine environment effect and great economic impact on the seafood industry [10,29,30]. In vivo toxicity differences were observed for the toxins of this group, both intraperitoneally [14,31] and orally [15]. In toxicity studies, it is important that the

route of administration of the toxins is appropriate to the human situation; therefore, oral administration has been recently proposed as the most suitable for the study of these aquatic toxins and others naturally acquired by this route [14]. Besides, toxins' tissue distribution could be a starting point to understand their behavior in the organism. For this purpose, different doses of OA, DTX1, and DTX2 were administered by oral route in mice and they were detected by LC/MS/MS analysis in gastrointestinal organs and different fluids.

Our results show that, after acute oral administration in mice, symptoms were observed after 1 h, with completely recovery within 24 h [19,20]. OA, DTX1, and DTX2 passed through the gastrointestinal barrier, are distributed across organs, accumulate in the stomach and the small and large intestines, and are eliminated in feces and in stomach and intestine contents. After each toxin administration, the recorded nonspecific systemic signs and symptoms included apathy, piloerection, squint-eyes, spams, cyanosis, on hind legs, and dyspnea, which was in accordance with previous findings after acute oral OA administration. [32]. One hundred percent of mortality was only reached with 3000 μg/kg DTX2, while all mice survived with the lowest concentrations of DTX1 (250 and 375 μg/kg) and DTX2 (1000 μg/kg). However, the representative symptom of DSP is diarrhea, which appeared soon after oral administration of toxins such as OA and DTX1 (30 min–2 h). This indicates the rapid effect of the toxins that was previously associated with fast absorption [15,19,26,27]. Related to that, pathological changes by OA were previously detected within 2 h [20]. In an in vivo situation, intestinal peristalsis prevents long exposure times within the same intestine section [33]. However, DSP toxins cause an alteration in intestinal motility that enables their intestinal absorption and enterohepatic circulation [34].

Therefore, DSP toxins pass through the gastrointestinal barrier to the bloodstream [18,20,32,34]. An analysis of the passage of these toxins through the gut barrier indicates that their absorption could be related to the ability to modify cellular structures such as the cytoskeleton or tight junctions. These changes were previously evaluated by electron and confocal microscopy, confirming modification in the intestinal microvilli as well as the redistribution of occludins, an important protein of tight junctions [15,27]. These alterations affect the barrier function of the intestinal epithelium and therefore could be involved in the changes in absorption of the different analogs. In our hands, these toxins were detected in blood 24 h after oral administration, with higher plasma levels of OA than those of DTX1. However, the rates of gastrointestinal transfer to the bloodstream orally appear to be low compared to blood levels attained by i.p. Similar differences between oral and i.p. administration were recently reported using other toxins such as mycrocystin [35].

Via blood circulation, the toxins are able to reach a variety of organs. Nevertheless, DSP toxin biodistribution was not uniform for all tissues. Analogs of OA, DTX1, and DTX2 vary in C31 and C35 methylation and stereochemistries [36,37]. The structure–activity relationship in the OA toxin group was previously studied by Twiner et al. [6]. In our experiments, small variations in OA and DTX1 toxin structures, specifically methylation at C35, only mildly affected body tissue accumulation whereas a lack of methylation at C31 along with methylation at C35 provided structural bases not only for the reduced toxicity but also for changes in tissue accumulation of DTX2. We focused on gastrointestinal organs in which OA-induced morphological changes were already described [38]. Within 24 h posttreatment, the three toxins were detected by LC-MS/MS analysis in all of the examined organs (liver, stomach, and small and large intestines). Even though the amount of compound depends on the administered dose, the highest concentration of OA and DTX1 were found in stomach with results similar to those reported by nearby doses of OA [32], while lower concentrations were found in liver. Therefore, these toxins could be retained in the stomach, which may explain the lesions noted in some studies at the stomach mucosa and submucosa in mice treated with OA [38,39]. In addition, OA and DTX1 seem to be accumulated in the intestine wall, which could be in connection with their gastrointestinal injuries [6,15,40]. The direct or local action of OA and DTX1 in the intestine may be the trigger for hypersecretion, inflammation, and diarrhea [20,23,41]. Our

data revealed toxicokinetic differences between OA and DTX1 since, at the same dose of toxin (1000 µg/kg), the level of OA was higher (408 ng/g and 2340 ng/g) than DTX1 (182 ng/g and 1009 ng/g) in both the small and large intestines. Interestingly, the amount of DTX2 (142 ng/g) in the stomach was very low compared to OA (4540 ng/g) or DTX1 (3006 ng/g). This behavior was the same in the tissues of the liver and the small and large intestines and could indicate relatively low absorption of DTX2 from the gastrointestinal tract, as was previously suggested [20]. In agreement with that, studies of transepithelial permeability using an in vitro intestinal model demonstrated the very low ability of DTX2 to cross the intestinal barrier [42]. Besides, DTX2 was found in feces up to 24 h, with this excretion being important for the toxin. High fractions of the administered doses of OA and DTX1 were found in feces and were also recovered from the stomach content. Particularly, OA was detected in feces 2–3 h after administration, suggesting that this excretion was a fast and predominant route of toxin elimination from the organism. The recovery of the administered doses up to 1000 µg/kg OA (18–23%) and DTX1 (20–32%) was higher than that of DTX2 (1–2%). Only with doses of DTX2 higher than 2250 µg/kg was the total recovery around 30%, indicating that the absorption rate of DTX2 is low [27].

The present experiments confirmed some data about organ distribution and excretion of OA previously published [32,34], indicating that, 24 h after oral administration, OA was detected in urine, gastrointestinal contents, and gastrointestinal tissues. However, so far, relatively little information has been collected about the organ distribution of DTX1 and DTX2. Our data suggest a different toxicokinetic pathway between OA, DTX1, and DTX2 and incomplete absorption of the toxins. This could be due to the rapid induction of diarrhea and the consequent elimination of a considerable quantity of OA and analogs by feces or intestinal content [12,14,15,27]. DSP toxins could also change to other metabolites. Recent papers focused on the rapid esterification of DSP toxins with fatty acids in mollusks [43,44]. The presence of diol esters of DSP toxins in dinoflagellates was also recently evaluated; moreover, a different intraperitoneal toxicity of esters compared with free toxins was suggested [45]. The metabolism of OA by NADPH-dependent enzymes present in human or rat liver S9 fractions was already reported and resulted in different toxic effects [46]. Nevertheless, in previous studies, excreted OA and DTX1 were on the free form, not esters, and toxins were particularly in connection with injuries [15,20,34,38]. Therefore, this study did not include toxin ester analysis. A more detailed investigation will be required to confirm any toxin biotransformation in mice.

It should be noted that the amount of DTX2 in the small intestine was higher than in large intestine, opposite to OA and DTX1. This information is interesting but partial since the toxins could be excreted or accumulated in other organs.

Then, the in vivo differences in the toxicity of DTX2 versus OA and DTX1 lie in the less pharmacological potency [6,7,27,47] but could also be associated with scarce gastrointestinal absorption and low accumulation in intestinal tissues.

4. Materials and Methods

The toxins OA and DTX1 were provided by the National Research Institute of Fisheries Science (NRIFS) from the Fisheries Research and Education Agency (Yokohama, Japan). OA and DTX1 isolated from toxic dinoflagellate *Prorocentrum lima* [48] were quantified by the PULCON method [49] on a quantitative NMR with external standards. The purities (purity > 95%) of both toxins were also confirmed by NMR spectroscopy. DTX2 (purity > 98%) was a certified reference material (CRM) supplied by Laboratorio CIFGA S.A. (Lugo, Spain). Stock solutions of the toxins were diluted to target doses in 0.9% saline solution and administered orally at 10 mL/kg bw in mice.

All chemicals employed were HPLC or analytical grade from Sigma-Aldrich Quimica S.A. (Madrid, Spain).

4.1. In Vivo Assays and Animal Conditions

In vivo assays were performed according to the Organisation for Economic Cooperration and Development (OECD) standardized method 4-level up and down procedure, which includes the reduction of the number of animals in the three Rs principle (replace, reduce, and refine) [50]. In all cases, the starting dose was 1000 µg/kg bw. The dose of the next level in the design depended on the toxicity in the previous level, and the number of mice was increased at each dosage level, as was previously described [27].

Briefly, four-week-old female C57BL/6J mice weighing 20 g were fasted overnight and, at 9 a.m., were weighed again. Then, they received a dose of one of the toxins (OA, DTX1, or DTX2) by oral gavage at the moment in which food and drink were provided ad libitum. The experiment concluded 24 h after toxin administration with euthanasia of the surviving animals.

For the urine and fecal excretion studies, urine and feces were collected at time points of 1, 3, 6, 9, 12, and 24 h after toxin administration. All samples were stored frozen at −20 °C until analysis.

The whole blood samples were collected in heparinized tubes at the end of the experiment and centrifuged at 3000× *g*. Plasma was separated and stored frozen at −20 °C until analysis.

All animal procedures described in the manuscript were carried out in conformity to European legislation (EU directive 2010/63/EU) and Spanish legislation (Real Decreto 53/2013, Decreto 296/2008) and to the principles approved by the Institutional Animal Care Committee of the Universidad de Santiago de Compostela under the procedure code: 01/17/LU-002 (approved on 22 September 2017).

4.2. LC/MS/MS Analysis of Mice Organs

All animals in the study were subjected to a full necropsy. Organs from the gastrointestinal tract were collected after mice death or euthanasia to evaluate toxin distribution. Organs were stored at −80 °C until LC/MS/MS analysis. Then, the organs were weighed and extracted with methanol, as was previously described [27]. Briefly, 0.1 g of homogenized sample was extracted by adding 400 µL of methanol, and after 60 s of vortex mixing and 30 s of sonication, the mixture was centrifuged at 10,000× *g* for 10 min at room temperature. The supernatant was transferred to a microtube, and the remaining pellet was extracted two more times. Then, the combined supernatants were evaporated and reconstituted in 100 µL of methanol to finally be mixed with 40 µL of methanol (vortex-mixed for 30 s) and 10 µL of trichloroacetic acid 10% for protein precipitation (vortex-mixed for 30 s). Then, 50 µL of CH_3CN was added (vortex-mixed for 1 min), and after centrifugation at 14,500× *g* for 10 min at room temperature, the mixture was filtered through 0.22 µm into HPLC vials for analysis by LC/MS/MS, with two replicates of the same sample.

The urine sample extraction protocol was performed according to Guada et al. (2013) [51] and modified by Abal et al. (2017) [27]. Briefly, 40 mL of methanol was added to 100 mL of urine homogenized samples and vortex-mixed for 30 s. Then, for protein precipitation, the samples were mixed with 10 mL of 10% trichloroacetic acid for 30 s. Finally, 50 mL of CH_3CN was added and vortex-mixed for 1 min. Samples were then centrifuged at 14,500× *g* for 10 min at room temperature, and the extract was filtered (0.22-mm centrifugal filter, Merk Millipore, Billerica, MA, USA). Five microliters of this sample were injected into the LC/MS system, with two replicates of the same sample. Stomach, small intestine, and large intestine content extractions were performed following the urine sample extraction protocol.

Fecal sample extraction was performed according to Abal et al. (2017) [27]. Feces were weighed and extracted by adding 400 mL of methanol to 0.1 g of the homogenized sample. After 60 s of vortex mixing and 30 s of sonication, samples were centrifuged at 10,000× *g* for 10 min at room temperature, and the supernatant was transferred to an eppendorf. After three extraction procedures, the total supernatant was evaporated and reconstituted in 100 mL of methanol. The subsequent steps of the extraction protocol were common to urine sample extraction.

The blood extraction protocol was performed according to Abal et al. (2018) [15]. Briefly, 800 µL of 75% methanol was added to 200 µL of the intracardiac blood sample and vortexed for 1 min. The mixture was transferred to an ultrafiltration spin column and centrifuged at 3000 rpm for 30 min. Then, the ultrafiltered solution was evaporated and reconstituted with 200 µL of methanol 100%. Finally, samples were filtered by 0.22 µm for 10 min at 14,500× *g* at room temperature, and 5 µL was subjected to LC-MS/MS, with two replicates of the same sample.

LC/MS/MS Conditions

Analysis of the organ extracts was performed on a 1290 Infinity ultra-high performance liquid chromatography system coupled to a 6460 Triple Quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany), as previously described [15,27]. The mass spectrometer was operated in Multiple Reaction Monitoring (MRM) in negative mode, analyzing all OA, DTX1, and DTX2 transitions known, using the highest intensity transition for quantification (m/z 803.5 > 255.2 OA, m/z 817.5 > 255.2 DTX1, and m/z 803.5 > 255.1 DTX2) and one transition for confirmatory purposes (m/z 803.5 > 113.2 OA; m/z 817.5 > 113.0 DTX1, and m/z 803.5 > 151.0 DTX2).

All parameters were optimized with accurate well-characterized OA, DTX1, and DTX2 standards in order to achieve the maximum level of sensitivity. Cell accelerator voltage (CAV) was 4 V, and the fragmentor was 320 V. Furthermore, collision energy (CE) value was optimized for each transition: m/z 803.5 > 255.2 (CE = 50 V) and 803.5 > 113.2 (CE = 66 V) for OA, m/z 817.5 > 255.2 (CE = 54 V) and 817.5 > 113.0 (CE = 70 V) for DTX1, and m/z 803.5 > 255.1 (CE = 56 V) and 803.5 > 151.0 (CE = 56 V) for DTX2.

Toxin standards were used for toxin calibration in the range 0.19–100 ng/mL. The estimated limit of detection (LOD) based on a signal-to-noise ratio of 3 (S/N = 3) and the limit of quantification (LOQ) considering a signal-to-noise ratio of 10 (S/N = 10) were 0.2 ng/mL and 1.3 ng/mL, respectively, for both OA and DTX1, and 0.7 ng/mL (LOD) and 2.33 ng/mL (LOQ) for DTX2.

4.3. Statistical Analysis

The results were analyzed by 1-way ANOVA complemented with Newman–Keuls Multiple Comparison Test. $p \leq 0.05$ was considered statistically significant.

5. Conclusions

The results help us understand the different distribution pattern of DSTs in gastrointestinal organs. Absorption of DSP toxins from the gastrointestinal tract and accumulation in the organs were different and dose-dependent, suggesting a distinct toxicokinetic pathway between OA, DTX1, and DTX2. It can be speculated that the low absorption of DTX2 may reduce its in vivo effects. From a toxicological point of view, it is important to highlight that OA and DTX1 are quickly absorbed orally and can accumulate in the stomach and the small and large intestines, which is associated with its rapid and acute effects, even though the toxic potency cannot be excluded. This preliminary study provides useful information to better assess human health risks associated with DSP toxin-contaminated seafood.

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Informed Consent Statement: Not applicable.

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4.2 PUBLICACIÓN 2: SEROTONIN INVOLVEMENT IN OKADAIC ACID-INDUCED DIARRHOEA IN VIVO.

Año de la publicación: 2021

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Resumen: El consumo de bivalvos con toxinas del grupo del ácido okadaico (AO) causa la intoxicación diarreica por consumo de marisco (DSP) al ingerir estos productos contaminados. Se caracteriza por una sintomatología gastrointestinal, causando vómitos, náuseas, dolor abdominal y diarrea, siendo este último el síntoma con mayor prevalencia. El ácido okadaico se considera un inhibidor de las protein fosfatasas de serina/treonina, lo que causa un estado de hiperfosforilación en las células. No obstante, la inhibición de estas enzimas no explica por completo la sintomatología gastrointestinal, por lo que otras dianas o rutas de señalización podrían ser relevantes en el mecanismo de acción de estas toxinas. Hay una gran variedad de mecanismos fisiopatológicos que producen diarrea en los que participa el sistema nervioso entérico y están involucradas hormonas intestinales y neurotransmisores. Estudios previos involucraban al sistema nervioso en la toxicidad del AO. Realizamos una aproximación *in vivo* para evaluar si el neuropéptido Y (NPY), péptido YY (PYY) o la serotonina pudieran estar implicadas en la diarrea producida por el ácido okadaico. Para ello, pretratamos a ratones por vía intraperitoneal con NPY, PYY(3-36) o ciproheptadina (CPH) antes de administrarles por vía oral ácido okadaico. La administración de NPY (agonista de los receptores Y1) o de PYY(3-36) (agonista de los receptores Y2) producía un retraso no significativo en la aparición de la diarrea inducida por el ácido okadaico. Por el contrario, el pretratamiento con CPH (antagonista/agonista inverso de los receptores de serotonina 1 y 2) suprimía o retrasaba la diarrea causada por el ácido okadaico, dependiendo de la dosis del fármaco. Durante las evaluaciones anatomopatológicas, se observó que el contenido del intestino grueso de los animales pretratados con CPH era similar al del control y distinto del contenido diarreico que se observó en los animales que recibieron el ácido okadaico. Estos resultados sugieren que la serotonina juega un papel importante en la diarrea causada por el ácido okadaico. Esta es la primera vez que se indica que la serotonina podría estar involucrada en la intoxicación desencadenada por el AO.

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ORGAN TOXICITY AND MECHANISMS

Serotonin involvement in okadaic acid-induced diarrhoea in vivo

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Abstract
The consumption of contaminated shellfish with okadaic acid (OA) group of toxins leads to diarrhoeic shellfish poisoning (DSP) characterized by a set of symptoms including nausea, vomiting and diarrhoea. These phycotoxins are Ser/Thr phosphatase inhibitors, which produce hyperphosphorylation in cellular proteins. However, this inhibition does not fully explain the symptomatology reported and other targets could be relevant to the toxicity. Previous studies have indicated a feasible involvement of the nervous system. We performed a set of in vivo approaches to elucidate whether neuropeptide Y (NPY), Peptide YY (PYY) or serotonin (5-HT) was implicated in the early OA-induced diarrhoea. Fasted Swiss female mice were administered NPY, PYY(3–36) or cyproheptadine intraperitoneal prior to oral OA treatment (250 µg/kg). A non-significant delay in diarrhoea onset was observed for NPY (107 µg/kg) and PYY(3–36) (1 mg/kg) pre-treatment. On the contrary, the serotonin antagonist cyproheptadine was able to block (10 mg/kg) or delay (0.1 and 1 mg/kg) diarrhoea onset suggesting a role of 5-HT. This is the first report of the possible involvement of serotonin in OA-induced poisoning.

Keywords Okadaic acid · Diarrhoeic shellfish poisoning (DSP) · 5-Hydroxytryptamine · Neuropeptide Y · Peptide YY

Introduction

Okadaic acid (OA) group of toxins comprise polyether fatty acids synthetized by dinoflagellates of the genera *Prorocentrum* and *Dinophysis*. Bivalves may accumulate the toxins following the consumption of this toxic phytoplankton. Therefore, OA and related compounds enter the food chain reaching humans through toxin-containing seafood ingestion causing diarrhoeic shellfish poisoning (DSP) (Yasumoto et al. 1978). DSP can be developed fast, between 30 min and a few hours afterwards. Symptomatology includes nausea, vomiting, diarrhoea and abdominal pain, achieving full recovery after 3 days (Yasumoto et al. 1978; EFSA 2008). Exposure to DSP has been frequently reported in various countries (Young et al. 2019; Vale 2020), representing the primary cause of bans on the harvesting of aquaculture in Japan and Europe (Reguera et al. 2014).

Previous studies have revealed that OA inhibits serine/threonine protein phosphatases (PPs) 1, 2A, 4, 5 and 6 activity (Bialojan and Takai 1988; Brewis et al. 1993; Chen et al. 1994; Prickett and Brautigan 2006). PPs remove a phosphate group from the phosphorylated amino acid residue of a wide variety of proteins (Yadav et al. 2017), meaning disturbance in their activity can modify downstream cellular pathways. OA in vitro has been described to induce cytoskeleton reorganization (Espina et al. 2010; Opsahl et al. 2013; Louzao et al. 2015), cell death (Dietrich et al. 2020) and cell cycle alteration (Feng et al. 2018). However, during the last decade, it has been discussed whether OA-exerted effects are fully explained by its PP inhibition (Espina et al. 2010; Munday 2013).

Diarrhoea is defined as reduced stool consistency, increased water content and number of evacuations. A wide array of causes and pathophysiological mechanisms have been proposed for both infectious and non-infectious

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diarrhoea (Thiagarajah et al. 2015; Anand et al. 2016; Camilleri et al. 2017). A considerable number of those mechanisms involve neuronal activation of the Enteric Nervous System (ENS). The ENS together with parasympathetic and sympathetic innervation throughout the gastrointestinal tract coordinate and regulate essential functions regarding pancreatic secretion, gut motility, fluid secretion and nutrient absorption among others (Li et al. 2000; Hu and Spencer 2018). Within components of the ENS some members of the Neuropeptide Y (NPY) family have been closely related to functions such as fluid absorption and gastric emptying (Saria and Beubler 1985; Wang et al. 2010). These 36-aa peptides' location include neural and endocrine components (Ekblad and Sundler 2002; Mongardi Fantaguzzi et al. 2009). For instance, NPY is expressed in different regions of the brain, but also in sympathetic neurons and in the ENS (e.g., secretomotor neurons) (Cox 2007; Mongardi Fantaguzzi et al. 2009). On the contrary, enteroendocrine L cells are the mayor contributors of Peptide YY (PYY) in the body, though it has been likewise detected in myenteric neurons and in some brain areas (Ekblad and Sundler 2002; Morimoto et al. 2008). In vitro, the DSP toxin OA downregulated NPY content and release of SH-SY5Y neuroblastoma cell line (Valdiglesias et al. 2012; Louzao et al. 2015).

Another key signalling molecule in the gut is serotonin (5-HT), a bioamine mainly expressed along the digestive tract (Erspamer and Testini 1959; Erspamer 1966; Savelieva et al. 2008; Mawe and Hoffman 2013), whose physiological functions comprise intestine fluid secretion and motility (El-Salhy et al. 2013; Mawe and Hoffman 2013; Coates et al. 2017; Camilleri et al. 2017; Hu and Spencer 2018). 5-HT is present in serotoninergic enteric neurons (Okamoto et al. 2014), though enterochromaffin cells (ECCs) are its major producers which are scattered distributed along the epithelia (Sjolund et al. 1983). ECCs act as chemosensors (Braun et al. 2007; Lund et al. 2018) and mechanosensors (Fujimiya et al. 1997; Alcaino et al. 2018), triggering a response in the underlying nerve terminals and the surrounding cells via neurotransmitters' or hormones' signalling (Bertrand et al. 2000; Reynaud et al. 2016; Fazio Coles et al. 2020).

Gathering the variety of pathophysiologic mechanisms resulting in diarrhoea and the important role of the ENS, we studied if OA-caused diarrhoea involves alteration of intestinal hormones (PYY) and/or neurotransmitters (5-HT and NPY). To elucidate this premise, we firstly performed an in vivo approach to determinate the dose–response of OA doses on diarrhoea outcome. Second, we assessed the effect of exogenous NPY or PYY(3–36) on OA-induced diarrhoea. Furthermore, we evaluated 5-HT implication in DSP in mice using the 5-HT_1 and 5-HT_2 antagonist cyproheptadine (CPH) prior to OA treatment.

Materials and methods

Animal model

Mouse bioassay had been an accepted method for marine biotoxins detection, though nowadays has been replaced by analytical methods on behalf of NC3R's principles (Union 2011). Based on the Organization for Economic Cooperation and Development guidelines for acute oral toxicity studies, we decided to use female mice as an animal model (OECD/OCDE 2002). One-month-old Swiss female mice weighing between 18 and 22 g from the colonies of the University of Santiago de Compostela were employed for all the experiments described. They were kept in controlled conditions of temperature (23 ± 2 °C), humidity (60–70%) and light/dark cycles (12 h/ 12 h). Mice were placed individually on metabolic cages and fasted overnight with access to 5% glucose serum. Animals were randomly assigned to each treatment. Mice received the toxin by oral gavage at 9 a.m. (10 mL/kg body weight), moment at which food and drink were provided ad libitum. When any pre-treatment was studied, it was given by intraperitoneal injection (1% body weight) prior to the toxin. Note that the assays described hereafter were preceded by these conditions. At the end of each experiment, euthanasia by CO_2 inhalation was conducted. All animal procedures were carried out in conformity to the European (EU directive 2010/63/EU), the Spanish legislation (Real Decreto 53/2013, Decreto 296/2008) and to the principles approved by the Institutional Animal Care Committee of the University of Santiago de Compostela under the procedure Code: 01/17/LU-002 (approved on 22 September 2017).

Materials

Okadaic acid employed in this study was kindly provided by the National Research Institute of Fisheries Science (NRIFS) from the Fisheries Research and Education Agency (Yokohama, Japan). OA isolated from toxic dinoflagellate *Prorocentrum lima* (Suzuki et al. 2014) was quantified by PULCON method (Watanabe et al. 2016) on the quantitative NMR with an external standard. Purities (purity > 95%) were also confirmed by the NMR spectroscopy. Neuropeptide Y and Peptide YY(3–36) were purchased from TOCRIS, cyproheptadine hydrochloride sesquihydrate from Sigma-Aldrich. All chemicals employed were analytical grade from Sigma-Aldrich Quimica S.A. (Madrid, Spain).

Dose–response of okadaic acid on diarrhoea

OA was previously reconstituted with ethanol. For administration, OA doses were prepared by serial dilutions in 0.9%

saline solution. OA was given by oral gavage at 10, 50, 100, 250 and 400 µg/kg doses. Control mice received the vehicle alone. Diarrhoea onset time and diarrhoea score were registered along with the symptoms presented at 1, 3, 6, 9, 12 and 24 h. Anatomopathological examination took place when the necropsy was performed. Small and large intestines were removed and stored at − 20 °C.

Pre-treatment in vivo studies at 6 h

NPY was previously reconstituted with milliQ water. Doses of 550 µg/kg OA and 107 µg/kg NPY were prepared by dilution of each compound in physiological solution. Four treatment groups were set: (i) control, (ii) NPY, (iii) OA and (iv) NPY plus OA. Each group was performed in duplicate. In this last case, intraperitoneal injection of NPY was performed 15 min prior to OA administration by oral gavage. The time of diarrhoea outbreak, diarrhoea score and symptomatology were registered and stools collected at 1, 3 and 6 h of treatment. At the end of the experiment, animals were subjected to necropsy. Small and large intestines were sampled and kept at − 20 °C.

A similar approach was performed for PYY(3–36) pre-treatment studies. PYY(3–36) was also reconstituted with milliQ water. The peptide was diluted in physiological solution to reach 1 mg/kg PYY(3–36). Mice were split into four groups comprising control, PYY(3–36), OA and PYY(3–36) plus OA. In this last group, PYY(3–36) was given by intraperitoneal injection 15 min before oral administration of 550 µg/kg OA. Same performance and data as in the previous experiment were obtained, ending the experiment at 6 h post-toxin administration.

CPH pre-treatment approach was in line with the previous ones. CPH was first reconstituted with ethanol. CPH was diluted in physiological solution to prepare the dose 3 mg/kg. Different sets of animals were given vehicles, CPH or OA each alone or CPH plus OA. Mice were first injected CPH intraperitoneally 30 min prior to 250 µg/kg OA by oral gavage. Same data as in the previous approaches were also collected.

NPY or PYY(3–36) pre-treatment of mice in 2 h experiments

Preparation of treatments and administration were performed as described in the preceding assessment. The peptides were each diluted in physiological solution to obtain 107 µg/kg NPY and 1 mg/kg PYY(3–36). NPY or PYY(3–36) was given intraperitoneal 15 min before oral administration of 250 µg/kg OA. During the following 2 h, the same data as detailed in the above experiment were obtained.

Dose–response of cyproheptadine in vivo at 2 h

Doses of 0.1, 1, 3 and 10 mg/kg CPH were tested as a pre-treatment to 250 µg/kg OA for 2 h. The experimental development was as detailed above. Dosages were diluted in physiological solution. OA was given by oral gavage 30 min after CPH dose was injected via intraperitoneal. Mice were observed, stools were collected, diarrhoea score and time of onset were measured 2 h of treatment. Then necropsy was performed and the gut was removed and stored at −20 °C.

Short-time exposure CPH dose–response

CPH and OA doses were prepared as described in previous sections. In this case, animals were split in six groups: (i) control, (ii) CPH, (iii) OA, (iv) 0.1 mg/kg CPH plus OA, (v) 1 mg/kg CPH plus OA and (vi) 6 mg/kg CPH plus OA. CPH was given via intraperitoneal 30 min before administering 250 µg/kg OA by oral gavage. Mice receiving the toxin alone were first treated, the time of diarrhoea onset was set as the end of the experiment for the remaining treatments. Same performance and data as in the previous experiment were obtained, but for 30 min post-toxin administration.

Diarrhoea score

To assess the differences in terms of how severe the diarrhoea was, we designed a scoring system (Table 1), meaning 0 normal faeces; 1 soft faeces; 2 shapeless soft faeces; 3 watery diarrhoea; 4 having for more than once diarrhoea. To be considered a different time of diarrhoea, it was required to be at least 20 min past the last defecation.

Neuromodulators' detection

Small intestine (ileum) and large intestine (proximal colon) were first extracted. Samples were cleansed in ice-cold PBS and weighted immediately afterwards. PBS was added (1:9 w/v) and tissues were homogenized and sonicated. Finally, they were centrifuged for 5 min at 10,000×*g* at 4 °C and

Table 1 Diarrhoea scoring system criteria

0	Normal faeces
1	Soft faeces
2	Shapeless soft faeces
3	Watery diarrhoea
4	Watery diarrhoea repeatedly (taking into account 20 min between each time)

[a]A score equal or higher than 2 is considered diarrhoea

stored at − 20 °C. When required, extracts were diluted for the compounds' concentration to fall within the linear range of the standard solutions. In all cases, absorbance was measured in a Multi-mode Microplate Reader Synergy 4 (Biotek).

NPY was measured in samples from OA dose–response at 24 h and from 6 h NPY pre-treatment studies. Enzyme-linked Immunosorbent Assay (ELISA) Kit for Neuropeptide Y from Cloud Clone Corp. was employed. The range of detection was 2.47–200 pg/mL and absorbance was measured at 450 nm.

PYY was analysed in mice intestines from OA dose–response at 24 h and from 6 h PYY(3–36) pre-treatment experiments. ELISA Kit for Peptide YY (Cloud-Clone Corp.) was used. The detection range was 12.35–1000 pg/mL and absorbance was read at 450 nm.

5-HT was determined in samples stored from CPH pre-treatment (6 h), dose–response of CPH (2 h) and short time exposure CPH dose–response experiments. The Serotonin ELISA kit from Enzo Life Sciences was used for 5-HT detection. The range of detection was 0.49–500 ng/mL and absorbance was read at 405 nm.

Statistical analysis

Graphpad Prism and RStudio were employed to perform the statistical analyses. First, the distribution and homoscedasticity of the data set were tested. If it followed a normal distribution, *t* test was performed to compare two treatments or one-way ANOVA plus Bonferroni multiple comparison test in the case more groups were analysed. Conversely, under no normal distribution of data, Mann–Whitney test or Kruskal–Wallis test followed by Bonferroni multiple comparison test was conducted. The significance threshold was set at $P < 0.05$.

Results

Dose–response of okadaic acid

To determine at which concentration OA is able to trigger diarrhoea, several doses of the toxin (10, 50, 100, 250 and 400 µg/kg) were administered to mice that were observed for 24 h. During the experiment, the symptomatology was monitored in detail at 1, 3, 6, 9, 12 and 24 h of OA treatment. No symptoms were detected in 10 µg/kg treated mice throughout the experiment. In the case of 50 µg/kg dose, these animals did not present any symptoms, but one mouse alone had soft faeces at 12 h, being normal at 24 h. Treatments of 100, 250 and 400 µg/kg induced squint-eyes, piloerection, spasms, cyanosis and even death for the highest dose (Table 2). This approach allowed to register the time at which mice recovered, i.e., had no symptoms. Clinical signs were noted at 1 h for 100 µg/kg OA treatment, being absent at 3 h. None of the reported symptoms were identified at 9 h for 250 µg/kg OA. Finally, no mice that received 400 µg/kg OA reached the 24 h of treatment.

Variations in body weight after 24 h of OA treatment were measured, as well as food and water consumption during the experiment (Fig. 1a). Both body weight variation and food intake display a similar pattern.

At the end of the experiment, mice were subjected to necroscopic analysis, focusing on anatomopathological evaluation of the gastrointestinal tract (Fig. 1b). While 10, 50 and 100 µg/kg OA examination revealed no differences with control, 250 µg/kg dose was featured by a swollen stomach and fluid accumulation in the small intestine. In the case of 400 µg/kg OA-treated mice, swollen stomach accompanied by moderate to strong fluid accumulation in the small intestine were observed for all animals.

Neuropeptides like NPY and PYY are involved in the regulation of nutrients absorption and exert a protective role (Moriya et al. 2010; Tough et al. 2011). Thus, we aimed to elucidate how OA affects NPY and PYY along the gut in vivo after 24 h treatments. OA reduces NPY in the small

Table 2 Symptomatology of 24 h OA-treated animals (%)

Symptoms	Control	OA (µg/kg)				
		10	50	100	250	400
Apathy	0	0	0	33.3	100	50
Piloerection	0	0	0	33.3	66.7	50
Cyanosis	0	0	0	0	0	50
Spasms	0	0	0	33.3	66.7	50
On hind legs	0	0	0	66.7	33.3	0
Squint-eyes	0	0	0	66.7	66.7	50
Diarrhoea	0	0	0	100	100	100
Mortality	0	0	0	0	0	100

intestine and in large intestine (Fig. 1c). Only 250 µg/kg OA treatment decreases PYY in small intestine while large intestine's PYY is not affected by OA at any of the given doses at 24 h (Fig. 1d).

The evaluation of clinical signs was focused on diarrhoea (percent of mice with this symptom, diarrhoea onset time and diarrhoea score). To assess the severity of diarrhoea, faeces from each mouse were scored as described above (Table 1). Based on diarrhoea score criteria, there is a dose-dependent increase up to the maximal punctuation (Fig. 1e). Control, 10 µg/kg and 50 µg/kg OA had normal faeces, except for one mouse that received the latter dose. Then the lowest dose tested for developing diarrhoea was 100 µg/kg, with several defecations. Since neither control, 10 nor 50 µg/kg treated mice had diarrhoea, no onset is represented regarding these treatments (Fig. 1f).

To further assure diarrhoea in the following approaches, we also included the dose: 550 µg/kg OA (Fig. 1f). We found no significant differences in diarrhoea onset between any of the OA doses that triggers the symptom, showing an all-or-none response.

NPY effect on OA poisoning in 6 h experiments

Based on how OA affected in vitro NPY expression in addition to the pro-absorptive role of the neuropeptide, we designed a 6 h experiment in which mice were intraperitoneally administered NPY prior to OA treatment. Due to the lack of differences between doses in diarrhoea onset (Fig. 1f) along with the evaluation of a dose closer to the previously described oral LD_{50} for OA (760 µg/kg) (Abal et al. 2018), we considered 550 µg/kg to be suitable to perform this assessment. The time of the experiment, 6 h, was selected to assure not just the inhibition of diarrhoea detection, but any delay in diarrhoea onset. Symptomatology of the animals was recorded during the experiment (Table S1). Most animals exhibited a variety of symptoms such as piloerection and squint-eyes. Symptoms were still observed in mice treated with OA alone or NPY-OA at the end of the experiment. It should be remarked the fact that all mice treated with the toxin or with the combination of both, OA and NPY (NPY-OA), developed diarrhoea.

Body weight variations (Fig. S1a), food (Fig. S1b) and water intake (Fig. S1c) were measured. Necropsy of all animals revealed that the toxin induced fluid accumulation along the small intestine, being modestly improved by NPY (Fig. S1d).

The type of diarrhoea was evaluated with the diarrhoea score (Fig. 2a). Pre-treatment with NPY did not modify OA-induced diarrhoea nor supressed it (Fig. 2a). Diarrhoea onset time displays a short, non-significant, delay with NPY pre-treatment (OA 33 ± 3.4 min; NPY-OA 43 ± 4.9 min) (Fig. 2b).

NPY was measured in the intestine of these animals 6 h after toxin administration (Fig. 2c, d). OA reduces NPY concentration in the small intestine; meanwhile, in NPY-OA-treated animals' NPY resembles control concentration (Fig. 2c). Conversely, the same treatment seems to induce a modest rise of NPY in the large bowel (Fig. 2d).

PYY(3–36) effect on OA poisoning in 6 h experiments

The enteric nervous system plays a vital role in the response to various gastrointestinal stimuli. The peptides of this nervous system regulate gastrointestinal movement, secretion, absorption and other complex functions through endocrine, paracrine and neuronal actions. Both NPY and Peptide YY are important enteric peptides. The observation of a soft effect of NPY over OA intoxication led us to study PYY. Y_2 receptors are not only in nerve terminals around myenteric neurons, but also in mucosa and muscle layers and its agonist PYY(3–36) has additionally been related to a clear anti-diarrhetic effect (Moriya et al. 2010; Tough et al. 2011). Thus, we aimed to check whether this agonist had the ability to relieve OA-induced diarrhoea. PYY(3–36) (1 mg/kg) was administered prior to OA (550 µg/kg) was given. Symptomatology was monitored along 6 h (Table S2). Apathy, piloerection and squint-eyes were developed following toxin treatment, alone or in combination with PYY(3–36) [PYY(3–36)-OA]. Both OA and PYY(3–36)-OA-treated mice still presented symptoms at the end of the experiment.

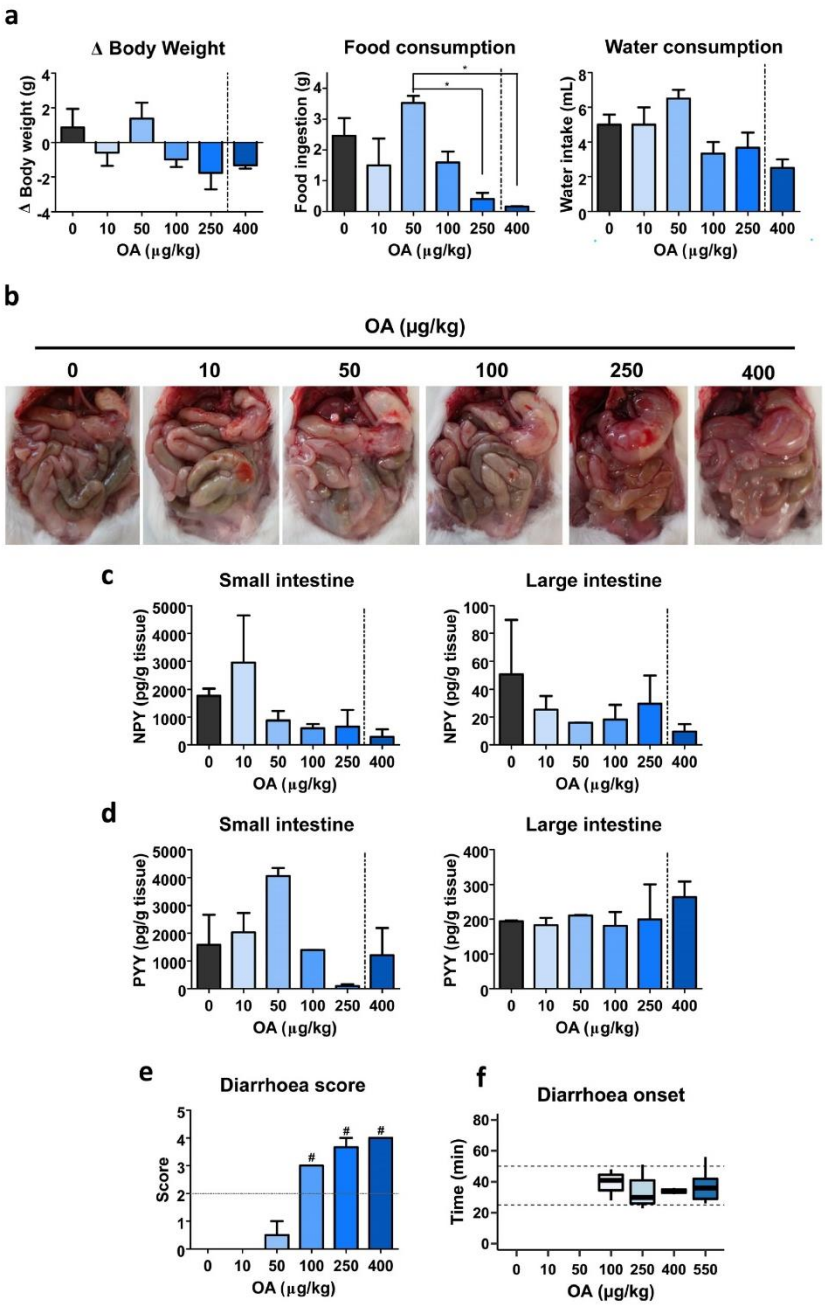
Subsequently, the balance of body weight (Fig. S2a), food (Fig. S2b) and water consumption (Fig. S2c) were measured. Anatomopathological evaluation revealed swollen stomachs and small intestine fluid accumulation of the toxin-treated animals, with or without PYY(3–36) (Fig. S2d).

Diarrhoea score shows no differences between OA and PYY(3–36)-OA (Fig. 3a). Diarrhoea outbreak of PYY(3–36)-OA-treated mice displays a slight time delay when compared to OA alone [OA 43 ± 7.8 min; PYY(3–36)-OA 49 ± 4.2 min] (Fig. 3b). Still, it is a remarkable fact that 100% of OA-treated mice presented diarrhoea; meanwhile, 85.7% of PYY(3–36)-OA-treated mice showed this symptom (Table S2 and Fig. 3a).

It was then of interest to analyse the effect of the toxin on PYY concentration (Fig. 3c, d). The amount of PYY measured in small intestine has a remarkable non-significant increase in PYY(3–36)-OA-treated mice (Fig. 3c). In the large intestine, PYY(3–36)-OA treatment induces a minor rise compared to control (Fig. 3d).

NPY and PYY(3–36) pre-treatment effect on OA poisoning in 2 h experiments

Neither NPY nor PYY(3–36) modified OA poisoning, still it was of interest to assure that this was independent of OA



◂**Fig. 1** Dose–response for diarrhoea induced by doses from 10 to 400 µg/kg okadaic acid in a 24 h study period. **a** Variation on mice body weight, food and water intake. **b** Representative images of the mice abdominal cavity at the end of the experiment. Image corresponding to 400 µg/kg OA was taken 11:45 h after toxin administration, time at which the animal died. **c** Neuropeptide Y measured in small and large intestines. **d** Peptide YY detected in small and large intestines. **e** Diarrhoea score. **f** Diarrhoea onset time. Inner box line indicates median and dashed lines are set at 25 and 50 min. Graphs **a**, **c**, **d** and **e** display mean ± SEM ($n = 3$). Since no animal treated with 400 µg/kg accomplished the experimental time, data for this dose are separated by dotted line. Statistical analysis was conducted with one-way ANOVA–Bonferroni multiple comparison test. In **e**, treatments with '#' over the bar are significantly different ($P < 0.001$) from those with a '+'. Significance is indicated with asterisks over the line between treatments, so that $*P < 0.05$, otherwise non-significant

dose. Subsequently, 250 µg/kg OA was chosen to both assure diarrhoea (Fig. 1e–g) and avoid mortality (Table 2). Since diarrhoea outbreak appears in less than 2 h, this was set as the endpoint of the experiment. During this time, monitoring of clinical signs identified symptoms such as piloerection or squint-eyes in all treatments involving the toxin (Table S3).

Body weight variation (Fig. S3a) and food intake (Fig. S3b) were measured showing no significant differences between treatments. At the end of the experiment, macroscopic evaluation of the abdominal cavity was performed (Fig. S3c). Animals treated with OA, NPY-OA and PYY(3–36)-OA revealed fluid accumulation mainly along the intestine even some mice that had no stools. Large intestines were also removed and examined separately (Fig. S3c). Diarrhoeic content was observed in OA-treated mice alone or in combination with NPY or PYY(3–36).

Diarrhoea was also evaluated (Fig. 4). The type of diarrhoea developed by animals pre-treated with either NPY or PYY(3–36) was not different from that induced by OA alone (Fig. 4a). Note that animals treated with control, NPY or PYY(3–36) had no stools during the experimental time, so the score is 0 (Fig. 4a) and no diarrhoea onset is shown for these treatments (Fig. 4b). Regarding onset time of diarrhoea, only a slight non-significant delay is observed in PYY(3–36) pre-treatment [OA 35 ± 2.1 min; NPY-OA 38 ± 8.1 min; PYY(3–36)-OA 45 ± 5.5 min] (Fig. 4b).

CPH effect on OA poisoning

The lack of a robust reaction to Y receptor ligands led us to study secretory pathways instead of pro-absorptive mechanisms. Serotonin is a key signalling molecule that mediates physiological processes in the gut and its release is stimulated by diarrhoeagenic compounds. Cyproheptadine (CPH), an inverse agonist/antagonist of 5-HT receptors 1 and 2, has been described to elicit a response at the level of other antisecretory drugs (Meddah et al. 2014). Consequently, to assess the role of 5-HT on OA mechanism to induce diarrhoea, we administered 3 mg/kg CPH before OA treatment. The set of symptoms developed were observed for 6 h and were similar in mice that received OA and OA with CPH (CPH-OA) (Table S4). Diarrhoea was the representative symptom and it should be highlighted that CPH pre-treatment reduced the prevalence from 100% of the toxin alone to 61.5% (Table S4 and Fig. 5a).

As in previous approaches, body weight variations (Fig. S4a) as well as food (Fig. S4b) and water intake (Fig. S4c) were measured. Food intake was reduced in groups of mice that received the toxin or CPH-OA (Fig. S4b). Macroscopic evaluation of the abdominal cavity revealed mild fluid accumulation in the small intestine of animals that received OA, resembling that of CPH-OA treated mice (Fig. S4d). Large intestines were removed and examined (Fig. S4d). OA large intestines were featured by diarrhetic content; meanwhile, CPH pre-treatment helped nearly restore normal intestinal content.

With regard to diarrhoea measured parameters, no significant difference in diarrhoea score was detected between OA and CPH-OA treated mice (Fig. 5a). Conversely, onset of diarrhoea is significantly delayed by CPH administration [OA 34 ± 3.7 min; CPH-OA 69.1 ± 3.2 min] (Fig. 5b). OA induced a stark increase in faeces wet weight that decreased significantly with CPH pre-treatment (Fig. 5c).

Intestine's 5-HT was quantified in mice samples (Fig. 5d, e). OA and CPH-OA induced 5-HT increase in the small intestine (Fig. 5d). Although in large intestine, CPH-OA treatment shows a non-significant increase over the other treatments (Fig. 5e).

Dose–response of CPH in OA poisoning

The suppression of OA-triggered diarrhoea led us to perform a dose–response study. Since CPH effect occurs within 2 h after toxin administration, experimental time was reduced to 2 h. Here we assessed 0.1, 1, 3 and 10 mg/kg CPH as a pre-treatment for 250 µg/kg OA. Clinical signs developed by each group of treatment were monitored (Table S5). It is noteworthy that diarrhoea was present in all mice administered with OA, but was absent in those pre-treated with 3 or 10 mg/kg CPH.

Body weight variation (Fig. S5a) as well as food ingestion (Fig. S5b) were measured. It was observed a tendency in weight lost and reduced food intake. On the contrary, anatomopathological examination provides information regarding the gastrointestinal tract at macroscopic level (Fig. S5c). Fluid accumulation in the stomach and intestine was observed for OA-treated mice. In animals pre-treated with 0.1, 1 and 3 mg/kg CPH, OA still induced fluid accumulation in the intestine and stomach. However, mice with 10 mg/kg CPH pre-treatment displayed an ameliorated fluid content in the intestine and a degree of solid content

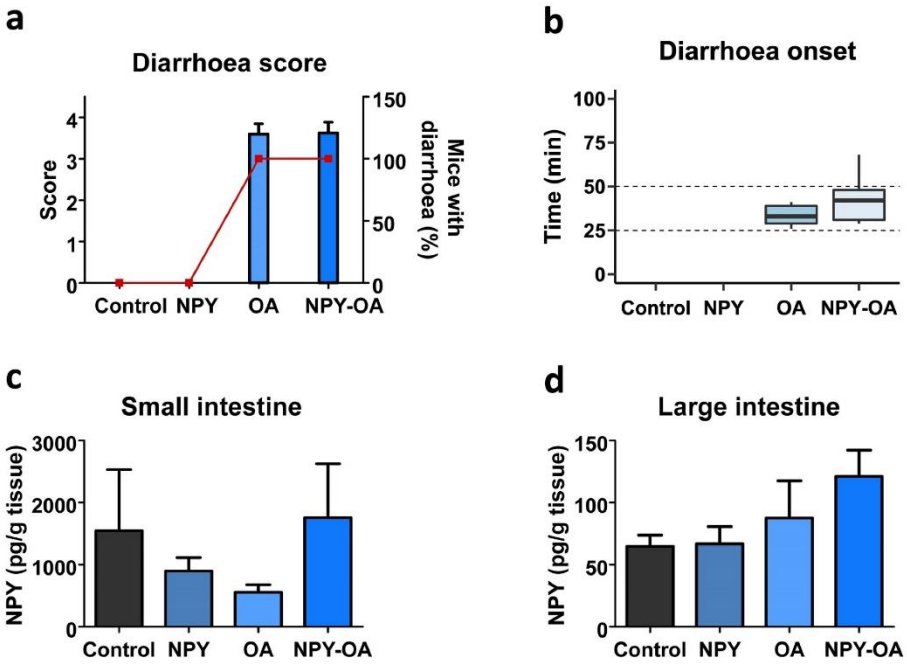


Fig. 2 NPY effect on OA-induced diarrhoea and changes in NPY in the gut at 6 h experiment. Animals were given 107 µg/kg NPY 15 min prior to 550 µg/kg OA administration. **a** Diarrhoea score (bars) and percent of mice that developed diarrhoea (closed squares). **b** Time of diarrhoea outbreak, values are expressed as boxplot showing the median. **c–d** NPY concentration in small (**c**) and large intestines (**d**). Data are mean ± SEM ($n = 4$ performed in duplicate). Student t test resulted in no significant differences (**b**). For **c** and **d**, statistical analyses were performed by one-way ANOVA and Bonferroni multiple comparison test with no significant differences

in the stomach. Complementary, large intestine state was evaluated (Fig. S5c), diarrhetic content is appreciated in mice administered the toxin alone or in combination with 0.1 mg/kg CPH. A mild improvement can be appreciated in the intestine of mice pre-treated with 1 mg/kg CPH, being back to normal with 3 and 10 mg/kg CPH.

In a further evaluation of diarrhoea, score and onset were studied (Fig. 6a, b). Diarrhoea score varies between 2.7 and 4 (Fig. 6a). There was a delay in diarrhoea onset when mice were pre-treated with 0.1 or 1 mg/kg CPH (Fig. 6b). In opposite, 3 and 10 mg/kg CPH pre-treatment blocked OA-induced diarrhoea (Fig. 6a). It should be taken into consideration the fact that control mice, or mice treated with CPH or OA plus 3 or 10 mg/kg CPH had no stools along the experiment (Fig. 6b).

We examined if different doses of CPH had any effect on 5-HT concentration (Fig. 6c, d). Overall, no significant variations were observed in small intestine's 5-HT (Fig. 6c). In the large intestine, there was a dose-dependent no significant decrease in 5-HT (Fig. 6d).

Evaluation of CPH doses effect when OA induced diarrhoea

Based on the previous results, it was interesting to assess the effect of CPH when diarrhoea was triggered by OA. To elucidate this, first animals were pre-treated with different doses of CPH and 30 min later treated with 250 µg/kg OA and euthanised at the time when diarrhoea should appear. Average of OA-triggered diarrhoea outbreak was 33 ± 2.3 min (Fig. 7b); therefore, this time was set as the end of the experiment. Notice the fact that only OA-treated mice had diarrhoea as shown by diarrhoea score (Fig. 7a).

During the brief experimental time, symptoms developed were observed (Table S6). No mice treated with any dose of CPH plus OA developed diarrhoea.

Both mice body weight balance (Fig. S6a) and food consumption (Fig. S6b) display subtle variations due to the shortened experimental time.

Determination of 5-HT in the small (Fig. 7c) and large intestine (Fig. 7d) was then conducted. OA increased 5-HT

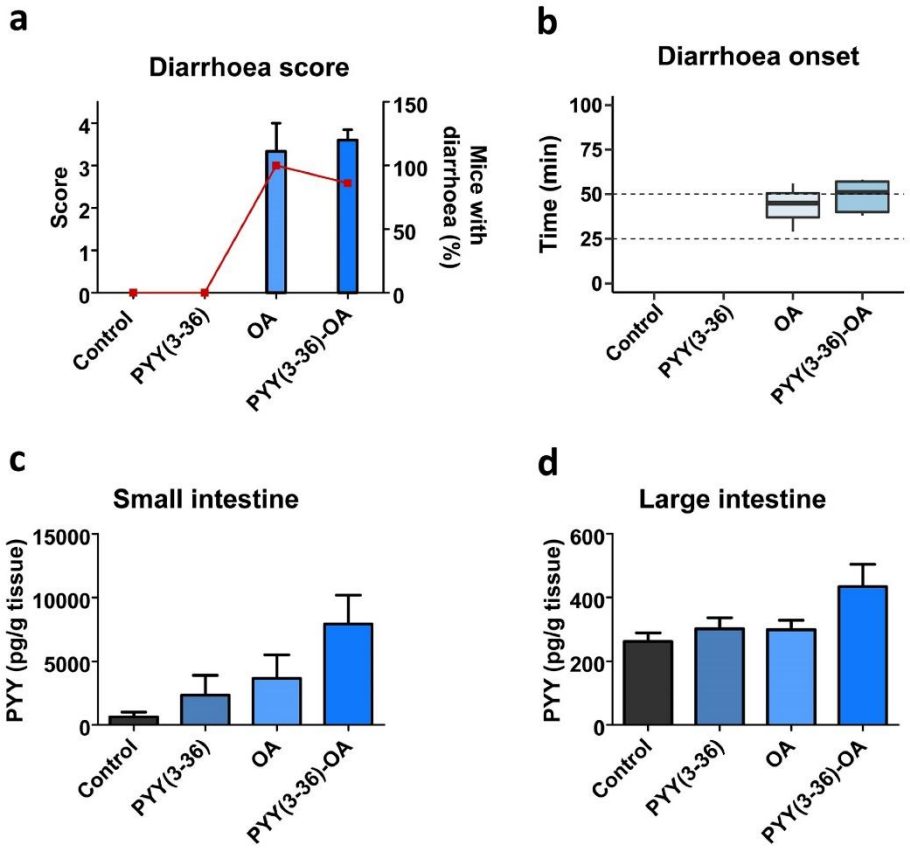


Fig. 3 PYY(3–36) effect on OA-induced diarrhoea and changes in PYY in the gut at 6 h. PYY(3–36) (1 mg/kg) was given to mice 15 min previous to OA (550 µg/kg). **a** Diarrhoea score (bars) along with percent of mice with diarrhoea (closed squares). **b** Time of diarrhoea onset; median is shown within each box. **c**–**d** PYY detected in small (**c**) and large (**d**) intestine of mice 6 h after treatments. Data are presented as mean ± SEM ($n = 3$ performed in duplicate). Student t test (**b**) or one-way ANOVA (**a**, **c** and **d**) were performed with no statistical significance detected in either case

in the small intestine (non-significant) that decreased with CPH pre-treatments (Fig. 7c). Conversely, the toxin induced a modest 5-HT rise not observed in the presence of 1 nor 6 mg/kg CPH in the large intestine (Fig. 7d).

Necroscopy of mice allowed the evaluation of the OA-induced effects along the gastrointestinal tract (Fig. 7e). No clear differences between administration of OA alone or in combination with 0.1 or 1 mg/kg CPH were detected. Yet, pre-treatment with 6 mg/kg CPH did improve the gastrointestinal tract aspect bringing it closer to that of control. In the large intestine, diarrhetic content was noticed in mice treated with OA and OA plus 0.1 mg/kg CPH, which was partially reversed by 1 mg/kg CPH (Fig. 7e). As described for the small intestine, 6 mg/kg CPH large intestines resembled those of control.

Discussion

Microalgae of the genera *Dinophysis* and *Prorocentrum* produce OA and form hazardous blooms leading to adverse environmental consequences associated with the declines of zooplankton populations (Gong et al. 2021). Besides, the consumption of seafood contaminated by OA or their structural derivatives, dinophysis toxins, causes DSP (Yasumoto et al. 1978). Due to the human health concerns associated

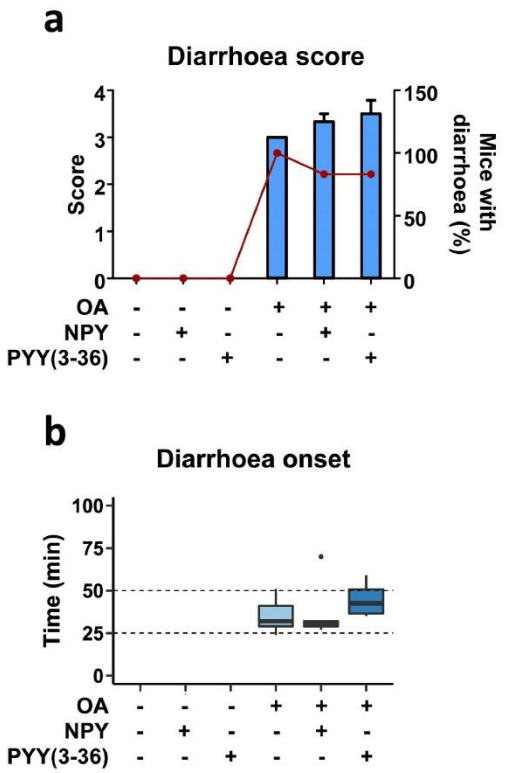


Fig. 4 NPY or PYY(3–36) pre-treatment effects on OA diarrhoea during 2 h experiments. NPY (107 µg/kg) or PYY(3–36) (1 mg/kg) were administered 15 min prior to receiving OA (250 µg/kg). **a** Diarrhoea score (bars) and percent of mice that developed diarrhoea (closed circles). **b** Diarrhoea onset time, boxes show data distribution, indicating the median as the line within each box. Data are expressed as mean ± SEM ($n = 3$ with duplicates). One-way ANOVA was conducted resulting in no significant differences in either case

with DSP, OA group of phycotoxins are tightly regulated by European Union legislation (Union 2011). Even though many in vitro and in vivo studies have been performed with OA, there are still many gaps about the targets involved in its acute oral toxicity (Louzao et al. 2021; Huguet et al. 2020; Dietrich et al. 2019; Reale et al. 2019; Tripuraneni et al. 1997; Ferron et al. 2014; Vilarino et al. 2018). It is stated in the literature that okadaic acid group of toxins are inhibitors of Ser/Thr protein phosphatases 1 (PP1) and 2A (PP2A) which play many roles in the cell (Takai et al. 1992). However, some challenging reports arise the possibility of different action mechanisms triggering gastrointestinal symptoms (Vilarino et al. 2008; Espina et al. 2010; Munday 2013). However, taking into account the rapid onset of this main symptom, the involvement of the enteric nervous system should not be ruled out. Thus, it is of great interest to elucidate the specific signalling pathway resulting in OA-induced diarrhoea.

We designed an OA dose–response study to characterize, among others, effects caused by the toxin for 24 h and particularly diarrhoea onset. In some mice, OA induced various symptoms such as on hind legs, squint-eyes, apathy, piloerection or spasms, but all animals that showed clinical signs of intoxication developed diarrhoea. The toxic effects of OA included fluid accumulation in the gastrointestinal tract, and even death at high doses. To perform a risk assessment, parameters as No-Observed-Adverse-Effect-Level (NOAEL) derived from the estimated exposures have been used to define Acute reference dose (ARfD) for humans. In our hands, 50 µg/kg OA was the highest administered dose at which no symptom or clinical sign was observed. This NOAEL agrees with the one proposed by EFSA in mice (2008). Besides our study revealed that 100 µg/kg OA was the lowest dose developing symptoms according to the LOAEL (Lowest-Observed-Adverse-Effect-Level) previously indicated in humans (Toyofuku 2006). Administration of doses equal or higher than 100 µg/kg OA triggers diarrhoea reaching the score of 3 or 4, with no differences in time onset indicating an all-or-none response. This could suggest a neuronal pathway in OA pathophysiology as was previously published in relation to the intestine water absorption-secretion balance (Delbro and Lange 1997). Recently, OA has also been involved in oxidative stress and inflammation pathways activation in enteric glial cell culture (Reale et al. 2019).

Diarrhoea represents an increase in water content of the stool and in the frequency of evacuation and mainly results from dysregulation of either intestinal secretory function or colonic motor function (Moriya et al. 2010). These intestinal activities are regulated by the enteric nervous system and implicate the Neuropeptide Y family as mediators (Vona-Davis and McFadden 2007). This family includes Neuropeptide Y (NPY) and Peptide YY (PYY) that act as hormone and/or neurotransmitters/neuromodulators. They exert their functions through binding to Y-receptor subtypes of transmembrane-domain G-protein-coupled receptors (El-Salhy et al. 2020). PYY and NPY have similar biological effects and bind to and activate receptors Y_1 and Y_2 localized in epithelial cells and submucosal and myenteric plexus neurons of the small intestine and colon (Mao et al. 1996; Cox et al. 2001; Wang et al. 2010). They delay gastric emptying and are mediators of the ileal break, also inhibit gastric and pancreatic secretion and stimulate the absorption of water and electrolytes. In some ways, they provide an integrated functional defence against luminal harmful factors including toxins.

Some diarrhetic agents have been proven to alter NPY and PYY expression (Moriya et al. 2010). Previous studies

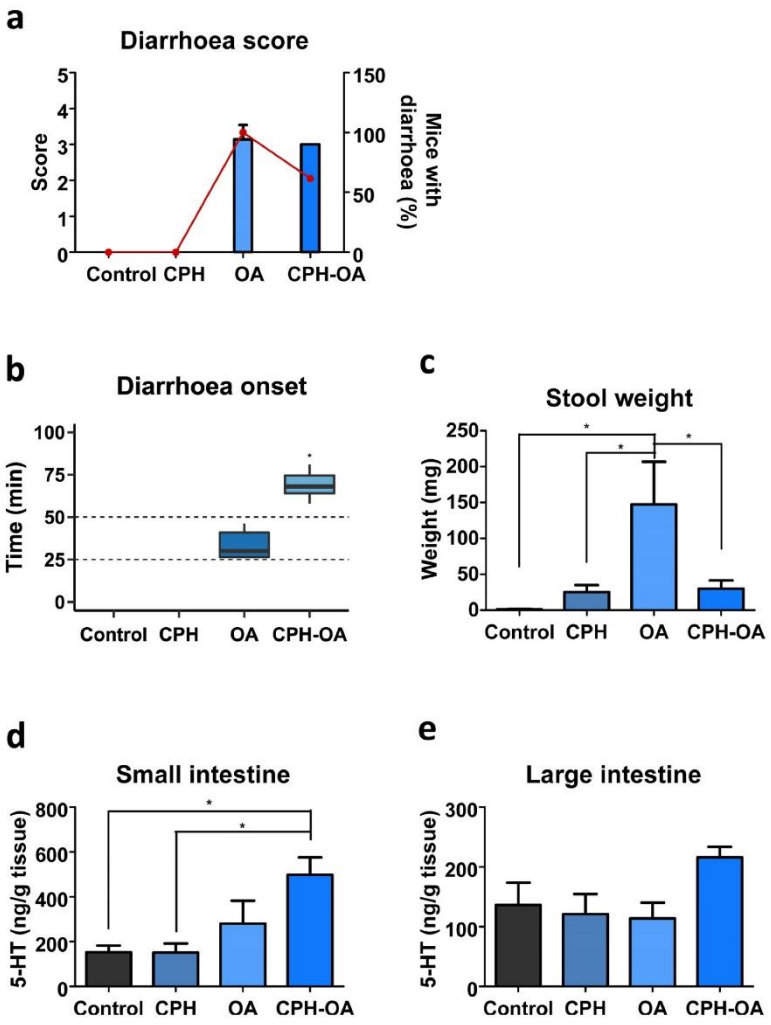


Fig. 5 CPH pre-treatment effect on OA-induced diarrhoea (6 h). Mice were treated with CPH (3 mg/kg) 30 min before OA (250 µg/kg) administration. **a** Diarrhoea score (bars) along with the percent of animals developing diarrhoea (closed circles). **b** Time of diarrhoea outbreak. Inside boxes median is indicated. **c** Faeces wet weight. **d**–**e** 5-HT concentration measured in small (**d**) and large intestines (**e**) of mice. Mean ± SEM ($n = 7$ of duplicates) are presented. One-way ANOVA (**a**, **c**) or Kruskal–Wallis (**d**–**e**) followed by Bonferroni multiple comparison test was performed. Significance is indicated by asterisks over the line between groups, such as $^*P < 0.05$. Student t test comparing OA with CPH-OA was conducted to study diarrhoea onset (**b**), resulting in $^*P < 0.05$

(Valdiglesias et al. 2012; Louzao et al. 2015) have also shown an impairment of NPY production on SH-SY5Y neuroblastoma cell line when treated with OA. In agreement with these results, in vivo, we found a decrease in small intestine NPY concentration mainly after 24 h of oral OA administration. This could be related to the increase in intestinal fluid secretion observed in necropsy. Other diarrhoeic compounds, for example, Cholera toxin, induce hyperexcitability of secretomotor neurons in enteric pathways (Gwynne et al. 2009), while intraarterial infusion of the neuropeptide notably reduced this enterotoxin-evoked fluid secretion in cats (Sjoqvist et al. 1988). However, pre-treatment with NPY did not reduce OA-induced diarrhoea and intestinal fluid accumulation was only modestly improved. This was further supported by similar results obtained at a shorter time of exposure and with lower OA dose.

PYY exerts multiple physiological effects on the gastrointestinal tract (El-Salhy et al. 2020). PYY inhibited Prostaglandin E_2 (PGE_2) and Vasoactive Intestinal Peptide that stimulated intestinal water secretion in the human small intestine being a defence against diarrhoea (Moriya et al. 2010; Roze et al. 1997). We detected a reduction in PYY in the small intestine of mice 24 h after receiving OA but this decrease was not clear after 6 h of treatment. Besides, Y_2 agonist PYY(3–36) administration had no effect on OA-induced diarrhoea although it was previously reported that

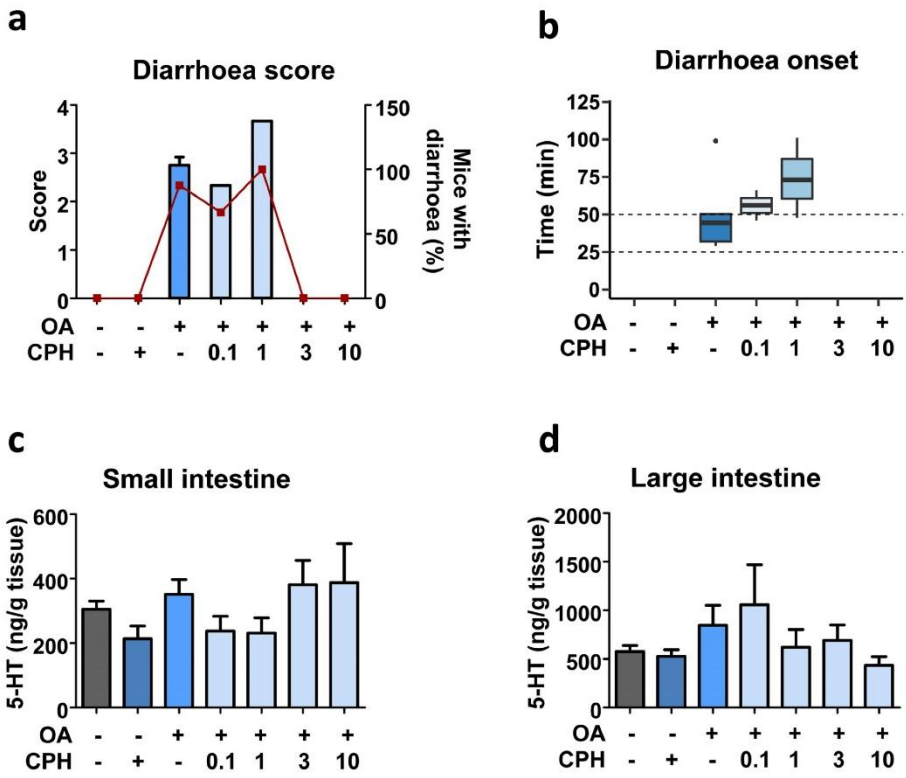


Fig. 6 Dose-dependent effect of CPH on OA-induced diarrhoea (2 h). Mice received 0.1, 1, 3 or 10 mg/kg CPH 30 min before the administration of 250 µg/kg OA. **a** Diarrhoea score (bars) and percent of mice developing diarrhoea (closed squares). **b** Time of diarrhoea onset; line within each box represents the median. **c–d** 5-HT concentration measured in small (**c**) and large (**d**) intestines. Data are shown as mean ± SEM (n = 3 of duplicates). One-way ANOVA (**a–b**) or Kruskal–Wallis (**c–d**) and Bonferroni multiple comparison tests were performed as statistical analyses. No significant differences were detected

PYY prevented faecal pellet output caused by dimethyl-PGE_2 (Moriya et al. 2010) or inhibited propulsive colonic motor function through Y_2 receptor in conscious mice (Wang et al. 2010).

Therefore, the addition of Y receptor agonist NPY (Y_1 and Y_2 receptors) or PYY(3–36) (Y_2 receptor) induced almost no improvement on intestinal and stomach fluid accumulation even in mice that had no faeces. Besides, the lack of a robust delay or prevention of OA-induced diarrhoea by targeting pro-absorptive peptides suggests that other enteric nervous pathways should be involved.

Serotonin is an endogenous signalling molecule involved in the regulation of fluid and mucus secretion as well as regulation of ion transport in gastrointestinal tract (Banskota et al. 2019) capable of altering intestinal motility and implicated in diarrhoea outcome (Thiagarajah et al. 2015; Camilleri et al. 2017; Hu and Spencer 2018). In fact, several diarrhoeagenic agents have been strongly related to this molecule (Ha et al. 2021; Westerberg et al. 2018; Singhal et al. 2017). Serotonin effects are achieved through the action on epithelial 5-HT_2 receptor and neuronal 5-HT_1, 5-HT_3 and 5-HT_4 receptors (Fidalgo et al. 2013). Cyproheptadine (CPH) a 5-HT_1 and 5-HT_2 receptor antagonist/inverse agonist has potent antiserotoninergic effects decreasing contraction of longitudinal smooth muscles of small intestine in mice (Fida et al. 2000). Our experiments are the first to evaluate the effects of 5-HT receptor antagonist during OA intoxication in vivo. In our CPH dose–response study at 2 h, the highest dose (10 mg/kg CPH) prevented the phycotoxin effects regarding diarrhoea, even lower doses (0.1 and 1 mg/kg CPH) delayed OA-induced diarrhoea onset. Average of OA-triggered diarrhoea outbreak was 33 ± 2.3 min. At this time, serotonin measured in large intestine was slightly elevated in OA-treated mice and

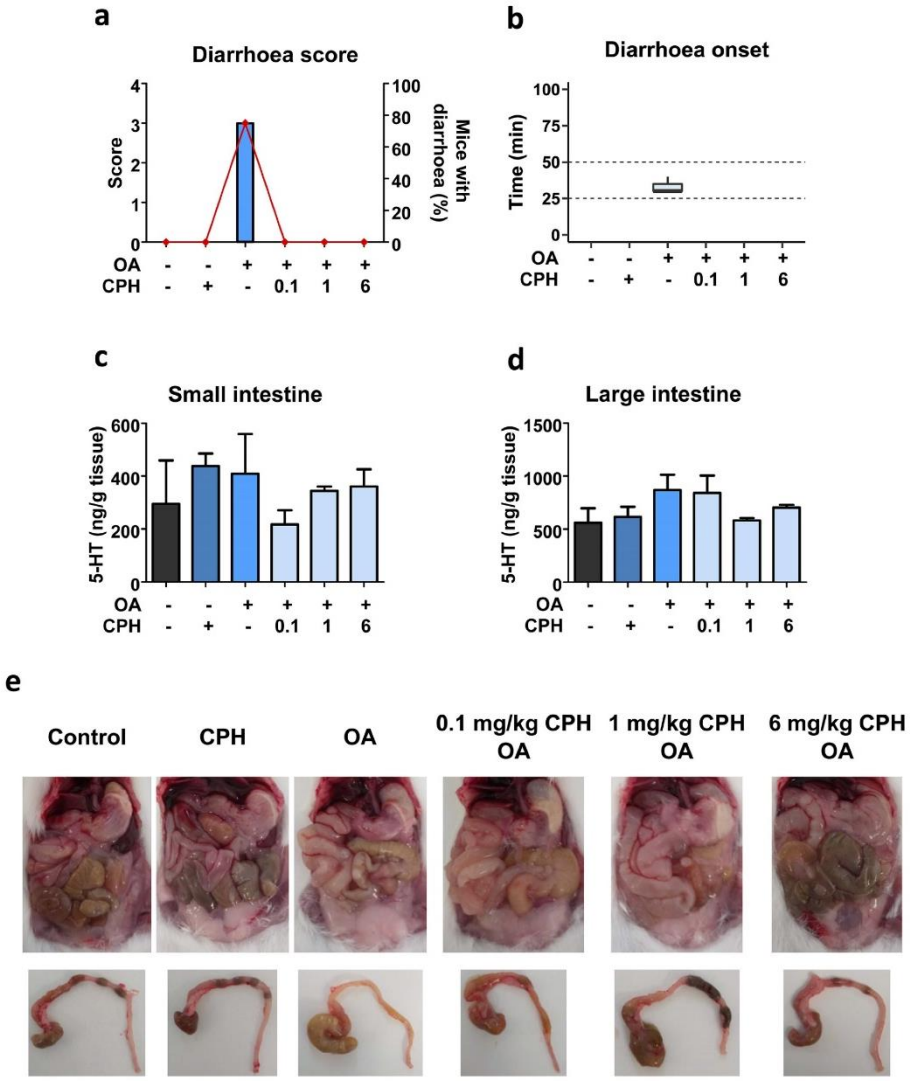


Fig. 7 CPH pre-treatment effect on OA-induced diarrhoea onset. Mice were pre-treated with 0.1, 1 or 6 mg/kg CPH and 30 min later treated with 250 µg/kg OA. The end of the experiment was set at the time OA triggered diarrhoea. **a** Diarrhoea score (bars) and percent of mice having diarrhoea (closed diamonds). All animals had normal or no stools (score of 0), but for those treated with OA. **b** Time of diarrhoea onset presented as a boxplot indicating the median inside the box. **c–d** 5-HT concentration detected on small (**c**) and large (**d**) intestines of mice. **e** Representative images of abdominal cavity and large intestine. Mean ± SEM ($n = 3$ of duplicates) is presented. Kruskal–Wallis (**c**) or one-way ANOVA (**d**) resulted in no significant differences

remains high in CPH pre-treated mice. Interestingly, our results showed no diarrhoea in mice pre-treated with CPH at any dose; meanwhile, OA-treated mice have this symptom 30 min after receiving the toxin. Other potent diarrhoeagenic compounds, such as Cholera toxin, cause the symptom by increasing the secretion of water into intestinal lumen. It can modify gastrointestinal motility by stimulating secretomotor neurons leading to the release of serotonin from enteroendocrine cells (Spencer and Hu 2020). This enterotoxin has been reported to prompt hypersecretion via 5-HT release in

human (Bearcroft et al. 1996) and rat jejunum (Beubler et al. 1989) as well as in vitro primary enterochromaffin tumour cells (Hagbom et al. 2011). In accordance, 5-HT_2 and 5-HT_3 receptors have been described to mediate this toxin-induced fluid secretion in rat jejunum (Beubler et al. 1989; Beubler and Horina 1990) while the antagonist of 5-HT_2 receptor ketanserin ameliorated fluid secretion evoked by the compound in rats (Harville and Dreyfus 1995). Our results showed that an increase in fluid secretion occurs within 30 min exposure of OA. This early secretion can be partially inhibited by CPH, making the contents of the large intestine normal (this was not achieved with pre-treatments with NPY or PYY), suggesting a role for serotonin as a mediator during this stage. In relation to this, it was reported that CPH has a direct effect on the inhibition of electrogenic ion secretion in the intestinal epithelium (Meddah et al. 2014). This effect could also explain the clear improvement of clinical signs and major gross findings of dilation of the large bowel appreciated during necropsy in CPH pre-treated mice. Therefore, CPH inhibited the OA-induced diarrhoea by blocking serotonin activity on 5-HT receptors. All these findings entail an indication of neuronal signalling mediation in the pathophysiology of DSP in mice, mainly involving 5-HT activity.

Conclusions

The fast symptoms OA causes during shellfish poisoning in humans (diarrhoea, nausea, vomiting and abdominal pain) suggested a neurogenic component. We determined that diarrhoea onset is an all-or-none response independent from the given OA dose. Moreover, we showed the inhibitory effect of cyproheptadine on OA-induced diarrhoea, involving serotonin in the toxicity mechanism. This work evidences OA effect mainly on serotonin action and leads to gain further insight into the mechanism triggering diarrhoea. Also, it opens the possibility to further research the OA effect in the enteric nervous system and the enteroendocrine cross-talk.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00204-021-03095-z.

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Author contributions Conceptualization: MCL; methodology: MCL, CC, PA, TS and RW; investigation: CC, MCL, PA, NV and CCR; formal analysis: CC, ABJ and CV; resources: LMB and MCL; writing-original draft: CC, ABJ and CV; writing-review and editing: MCL, CC and LMB; visualization: CC, ABJ and CV; supervision: MCL and MRV; funding acquisition: MCL and LMB.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Code availability Graphpad Prism and RStudio were the software used to perform statistical analysis.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethics approval All animal procedures were carried out in compliance with the European (EU directive 2010/63/EU), the Spanish legislation (Real Decreto 53/2013, Decreto 296/2008) and with the principles approved by the Institutional Animal Care Committee of the University of Santiago de Compostela under the procedure Code: 01/17/LU-002 (approved on 22 September 2017).

Consent to participate Not applicable.

Consent for publication Not applicable.

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Material suplementario:

Serotonin involvement in okadaic induced diarrhoea *in vivo*

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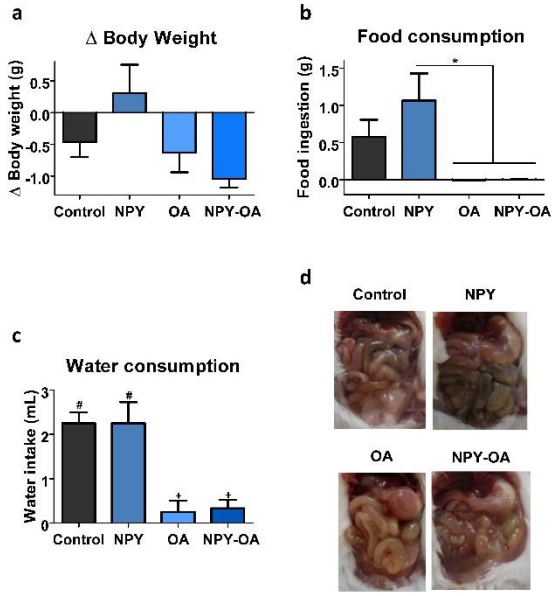

Fig. S1 NPY effect on OA poisoning at 6 h of treatment. Animals received 107 µg/kg NPY 15 min prior to 550 µg/kg OA administration. (**a**) Body weight variation, (**b**) food intake and (**c**) water intake during the assessment. (**d**) Representative images of the abdominal cavity. Graphs (**a**, **b** and **c**) show mean ± SEM (n=4 performed in duplicate). ANOVA followed by Bonferroni Multiple Comparison Test were executed. Statistical significances are indicated by asterisks set over the line between treatments, so that *P<0.05 is indicated in (**b**). In (**c**), different sign indicates differences between groups of *P<0.05

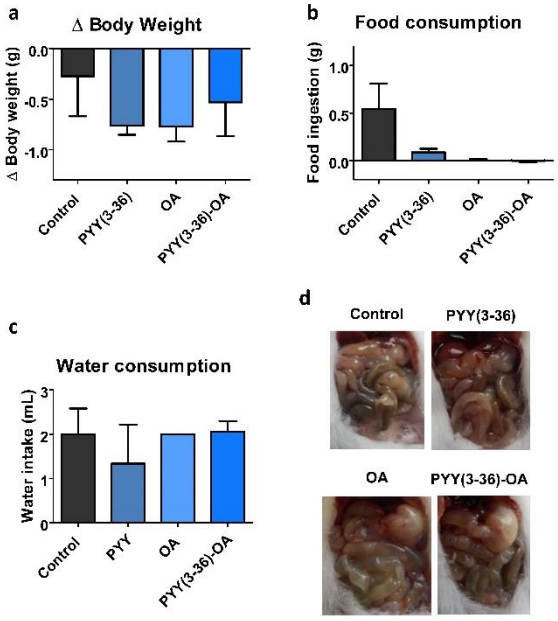

Fig. S2 PYY(3-36) pre-treatment effect on OA at 6 h. *In vivo* assay testing 1 mg/kg PYY(3-36) and 550 µg/kg OA on mice. (**a**) Body weight variation, (**b**) food intake and (**c**) water intake along the experiment. (**d**) Macroscopic examination representative photos of treatments. Mean ± SEM (n=3 performed in duplicate) is presented. No statistical significance was detected by one-way ANOVA

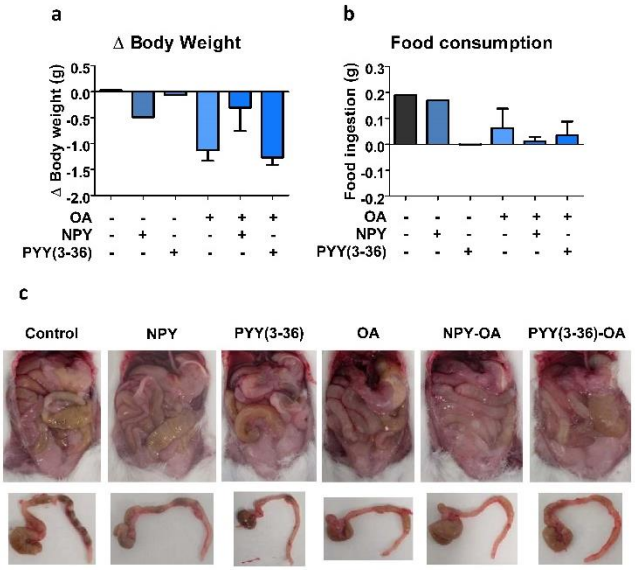

Fig. S3 NPY or PYY(3-36) pre-treatment effects on OA after 2 h of toxin administration. NPY (107 µg/kg) or PYY(3-36) (1 mg/kg) were injected 15 min prior to OA (250 µg/kg) oral treatment. (**a**) Body weight variance and (**b**) food intake during the experiment were measured. (**c**) Representative images of the abdominal cavity and the large intestine at the end of the treatment. Data are presented as mean ± SEM (n=3 of duplicates). Kruskal-Wallis analysis resulted in no significant differences between groups

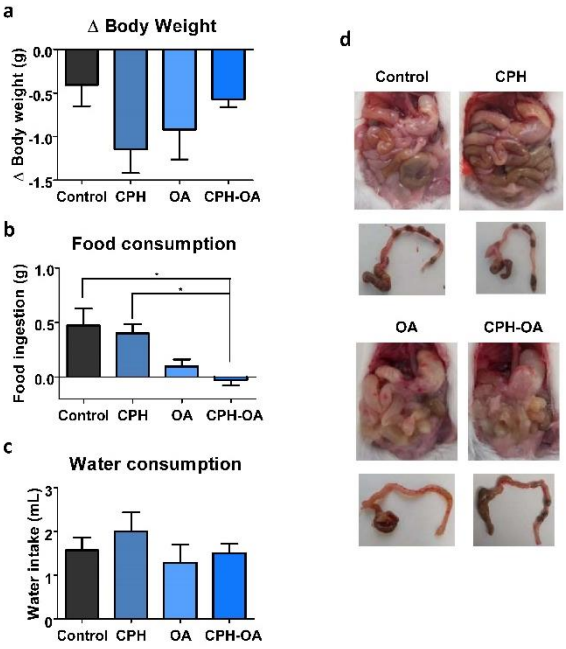

Fig. S4 CPH effect on OA intoxication after 6 h of treatment. Animals were treated with 3 mg/kg CPH 30 min before 250 µg/kg OA administration for 6 h. (**a**) Body weight balance, (**b**) food intake and (**c**) water intake during the assessment were measured. (**d**) Representative images of abdominal cavity and large intestine at the end of the experiment. Mean ± SEM (n=7 of duplicates) is presented. Data were analysed by ANOVA and Bonferroni Multiple Comparison Test. Significant differences are indicated by asterisks centred over the line between treatments as *P<0.05

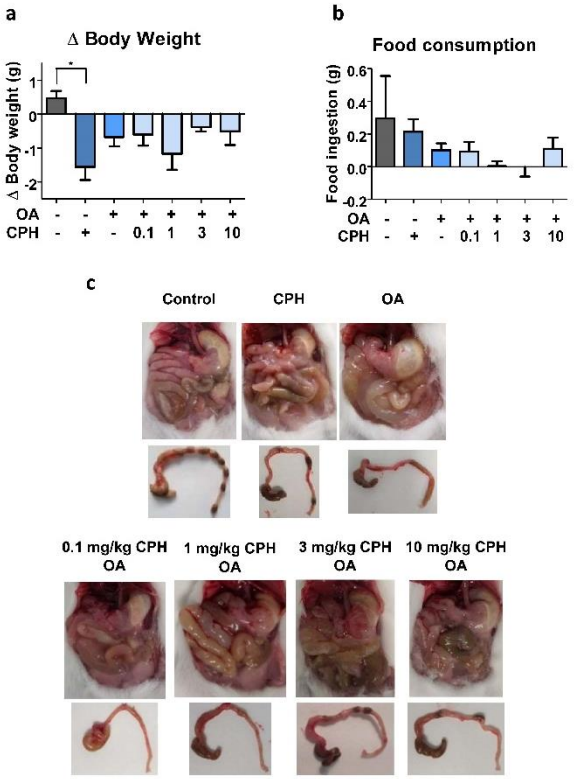

Fig. S5 Dose-response of CPH assessment on OA treatment for 2 h. Mice received 0.1, 1, 3 or 10 mg/kg CPH and 30 min later 250 µg/kg OA. Effects of treatments on (**a**) body weight and (**b**) food intake along the experiment. Values are expressed as mean ± SEM (n=3 of duplicates). (**c**) Representative images of mice stomach and small intestine as well as large intestine at the end of the experiment. One-way ANOVA (**a**) or Kruskal-Wallis (**b**) were conducted, significant differences between groups are stated as *P<0.05

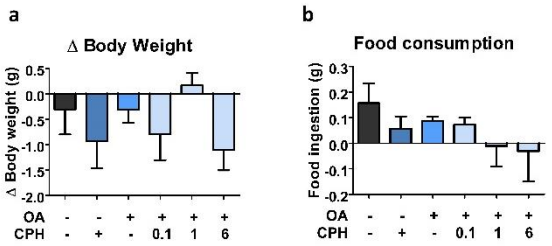

Fig. S6 CPH dose-response effect on OA-induced diarrhoea at the time it occurred. Mice were pre-treated with 0.1, 1 or 6 mg/kg CPH and 30 min later with 250 µg/kg OA. The end of the experiment was set at the time OA triggered diarrhoea. Effects of treatments on (**a**) body weight modification and (**b**) food intake in mice. Mean ± SEM (n=3 of duplicates) are presented. ANOVA followed by Bonferroni Multiple Comparison, resulting in no significant differences

Table S1 Effect of NPY pre-treatment on symptomatology of OA-treated animals for 6 h (%)

Symptoms	Control	NPY 107 µg/kg	OA 550 µg/kg	107 µg/kg NPY 550 µg/kg OA
Apathy	0	0	40	54.5
Piloerection	0	25	80	63.6
Cyanosis	0	25	100	63.6
Spasms	0	0	40	27.3
On hind legs	0	0	60	9.1
Squint eyes	0	25	80	81.8
Dyspnea	0	0	0	9.1
Diarrhoea	0	0	100	100
Mortality	0	0	20	9.1

Table S2 Effect of PYY pre-treatment on clinical signs of mice treated with OA for 6 h (%)

Symptoms	Control	PYY(3-36) 1 mg/kg	OA 550 µg/kg	1 mg/kg PYY(3-36) 550 µg/kg OA
Apathy	0	0	66.7	71.4
Piloerection	0	0	100	71.4
Cyanosis	0	0	33.3	57.1
Spasms	0	0	33.3	0
On-hind legs	0	0	100	0
Squint eyes	0	0	100	85.7
Dyspnea	0	0	0	14.3
Diarrhoea	0	0	100	85.7
Mortality	0	0	0	14.3

Table S3 NPY or PYY(3-36) effect on the symptomatology induced by OA at 2 h of treatment (%)

Symptoms	Control	NPY 107 µg/kg	PYY(3-36) 1 mg/kg	OA 250 µg/kg	107 µg/kg NPY + 250 µg/kg OA	1 mg/kg PYY(3-36) + 250 µg/kg OA
Apathy	0	0	0	66.6	100	83.3
Piloerection	0	0	0	100	83.3	50
Cyanosis	0	0	0	0	0	16.7
Spasms	0	0	0	0	0	16.7
On-hind legs	0	0	0	33.3	50	33.3
Squint eyes	0	0	0	100	100	83.3
Dyspnea	0	0	0	0	0	0
Hunched position	0	0	0	33.3	33.3	33.3
Diarrhoea	0	0	0	100	83.3	83.3
Mortality	0	0	0	0	0	0

Table S4 Effect of CPH pre-treatment on mice symptomatology after treatment with OA for 6 h (%)

Symptoms	Control	CPH 3 mg/kg	OA 250 µg/kg	3 mg/kg CPH 250 µg/kg OA
Apathy	0	0	71.4	92.3
Piloerection	0	0	28.6	46.1
Cyanosis	0	0	14.3	7.7
Spasms	0	0	14.3	7.7
On-hind legs	0	0	57.1	15.4
Squint eyes	0	0	57.1	76.9
Dyspnea	0	0	0	15.4
Hunched position	0	0	57.1	30.8
Diarrhoea	0	0	100	61.5
Mortality	0	0	0	0

Table S5 Effect of pre-treatment with different doses of CPH on symptomatology monitored in OA treated mice until 2 h (%)

Symptoms	Control	CPH (mg/kg)	OA 250 µg/kg	CPH (mg/kg) + 250 µg/kg OA			
				0.1	1	3	10
Apathy	0	0	37.5	33.3	66.7	14.3	66.7
Piloerection	33.3	0	37.5	100	100	0	33.3
Cyanosis	0	0	25	33.3	66.7	0	33.3
Spasms	0	0	50	0	0	28.6	0
On-hind legs	0	0	50	33.3	0	0	0
Squint eyes	16.7	0	25	33.3	33.3	0	100
Dyspnea	0	0	0	0	0	0	0
Hunched position	0	0	50	66.7	66.7	0	33.3
Diarrhoea	0	0	87.5	66.7	100	0	0
Mortality	0	0	0	0	0	0	0

Table S6 Effect of pre-treatment with different doses of CPH on clinical signs exhibited by short exposure to OA (%)

Symptoms	Control	CPH (mg/kg)	OA 250 µg/kg	CPH (mg/kg) + 250 µg/kg OA		
				0.1	1	6
Apathy	0	0	75	33.3	33.3	33.3
Piloerection	0	0	50	33.3	0	0
Cyanosis	0	0	0	0	0	0
Spasms	0	0	0	33.3	0	0
On-hind legs	0	0	0	0	0	0
Squint eyes	0	0	50	33.3	0	33.3
Dyspnea	0	0	25	0	0	0
Hunched position	0	0	0	0	0	0
Diarrhoea	0	0	75	0	0	0
Mortality	0	0	0	0	0	0

4.3 PUBLICACIÓN 3: INTESTINAL SECRETORY MECHANISMS IN OKADAIC ACID INDUCED DIARRHOEA.

Año de la publicación: 2022

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Resumen: El ácido okadaico (AO) es una toxina lipofílica de origen marino sintetizado por dinoflagelados de los géneros *Prorocentrum* spp. y *Dinophysis* spp. responsable de la intoxicación diarreica por consumo de marisco (DSP). El ácido okadaico inhibe las protein fosfatasas de serina/treonina 1 y 2A, no obstante, la acción en estas enzimas no explica por completo la sintomatología de la intoxicación. La pared intestinal actúa como una barrera de defensa frente a agentes externos y potencialmente dañinos. El funcionamiento normal del tracto gastrointestinal implica una regulación estrecha de los mecanismos de absorción-secreción de fluidos y electrolitos, por lo que un desajuste en alguno de los mecanismos regulatorios podría causar diarrea. En este artículo evaluamos la diarrea en ratones a los que les administramos AO por vía oral, así como en aquellos pretratados con diferentes dosis de ciproheptadina (CPH) y después tratados con varias dosis de AO. Estudiamos la concentración fecal de electrolitos en heces, contenido de agua, así como, las alteraciones a nivel ultraestructual de los intestinos de ratones a distintos tiempos experimentales. La concentración de sodio y cloro fue mayor en las heces de ratones tratados con AO que en los controles, sugiriendo una diarrea secretora. Sin embargo, la concentración de cloro en los animales que recibieron el pretratamiento con ciproheptadina y posteriormente el AO se aproxima a los valores del control. En concordancia, la administración de AO causó lesiones a nivel celular a las 2 h en yeyuno y colon proximal que fueron de menor gravedad en los animales que recibieron el pretratamiento con ciproheptadina. La alteración de las uniones estrechas sólo se observó en yeyuno a los 30 min de recibir el AO, tiempo al que apareció la diarrea. Estos resultados involucran a varios mecanismos celulares en la diarrea inducida por AO poniendo en evidencia la complejidad de la toxicidad de este compuesto.

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Intestinal secretory mechanisms in Okadaic acid induced diarrhoea

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ABSTRACT

Okadaic acid (OA) is an important marine lipophilic phycotoxin responsible for diarrhetic shellfish poisoning (DSP). This toxin inhibits protein phosphatases (PPs) like PP2A and PP1, though, this action does not explain OA-induced toxicity and symptoms. Intestinal epithelia comprise the defence barrier against external agents where transport of fluid and electrolytes from and to the lumen is a tightly regulated process. In some intoxications this balance becomes dysregulated appearing diarrhoea. Therefore, we evaluated diarrhoea in orally OA-treated mice as well as in mice pre-treated with several doses of cyproheptadine (CPH) and then treated with OA at different times. We assessed stools electrolytes and ultrastructural alteration of the intestine, particularly evaluating tight and adherens junctions. We detected increased chloride and sodium faecal concentrations in the OA-exposed group, suggesting a secretory diarrhoea. Pre-treatment with CPH maintains chloride concentration in values similar to control mice. Intestinal cytomorphological alterations were observed for OA mice, whereas CPH pre-treatment attenuated OA-induced damage in proximal colon and jejunum at 2 h. Conversely, tight junctions' distance was only affected by OA in jejunum at the moment diarrhoea occurred. In this study we found cellular mechanisms by which OA induced diarrhoea revealing the complex toxicity of this compound.

1. Introduction

Phycotoxins of marine origin are a source of foodborne intoxications. Okadaic acid (OA) group of toxins outstand as lipophilic molecules that typically accumulate in filter-feeding organisms like bivalve molluscs (Yasumoto et al., 1984). They are distributed worldwide and pose increasing threat to humans (Chen et al., 2018; Taylor et al., 2013; Reguera et al., 2012). No death cases have been reported but severe gastrointestinal symptoms develop between 30 min and hours following their ingestion (Yasumoto et al., 1984). Diarrhoea, nauseas, vomiting and abdominal cramps are the main symptomatology of these intoxications known as Diarrhetic Shellfish Poisoning (DSP) (Bresnan et al., 2021; Yasumoto et al., 1984).

Diarrhoea can be defined as an increase faecal pellet output accompanied by loss of stools' consistency (Who. Diarrhoeal disease, 2017). Several mechanisms that involve enhanced fluid secretion and motility, diminished absorption and activation of inflammatory responses can result in watery diarrhoea (Rao, 2019; Camilleri et al., 2017). These processes can co-occur in the diarrhoea triggered by the same toxicant. Intestinal epithelia comprise the defence barrier against external agents, along which are distributed enterochromaffin cells. Enterochromaffin cells are the major producers of serotonin (5-HT) in the intestine, though it is also synthetised in serotoninergic myenteric neurons (Mawe and Hoffman, 2013; Okamoto et al., 2014). Its function in physiological regulation of fluid secretion, and intestinal motility have been described (Banskota et al., 2019; Mawe and Hoffman, 2013; Hu and Spencer, 2018). Serotonin has been involved in the pathophysiology of diarrhoeas with variable origins (Rao, 2019). Upon toxicants' stimuli, 5-HT can act on nerve terminals and neighbouring cells, promoting diarrhoea (Rao, 2019).

Intestinal barrier integrity is essential in secretion/absorption balance. Intercellular junctions in the apical side of epithelial cells have a pivotal role in maintaining this function (Campbell et al., 2017). They are composed of tight junctions (TJs), adherens junctions (AJs), and desmosomes (Hollander and Kaunitz, 2020). These structures allow for passive water, ion and small molecules passage, while impeding larger entities entrance (Hollander and Kaunitz, 2020). Subsequently, they modulate intestinal permeability, hence, their alteration has also been related to diarrhoea (Martinez et al., 2013). Permeability is gradually

Abbreviations: OA, Okadaic acid.

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Abbreviations

5-HT	Serotonin
AJs	Adherens Junctions
CPH	Cyproheptadine
DSP	Diarrhetic Shellfish Poisoning
OA	Okadaic acid
PP	Protein phosphatase
TJs	Tight Junctions

reduced throughout the gut, being the small intestine more permeable than colon (Rao, 2019). Jejunum accounts for nutrient absorption and coupled electrolytes and water transport (Engevik and Engevik, 2021). Moreover, electrolytes and water reabsorption occur in proximal colon (Engevik and Engevik, 2021). Alteration of these processes can result in ions and water accumulation in lumen, leading to diarrhoea. OA has been reported to induce fluid accumulation in small intestine, markedly in jejunum (Abal et al., 2018; Ito et al., 2000; Tubaro et al., 2003; Aune et al., 2012). Epithelial erosion, shortened villi, and oedema have been described in small intestine (Ito et al., 2002; Aune et al., 2012). This could reflect disrupted electrolytes' secretion/absorption balance. However, these histological observations do not necessary develop in diarrhetic animals (Ito et al., 2002; Aune et al., 2012). Conversely, colonic impairment after OA treatment *in vivo* seems limited at any given dose and time of exposure (Ito et al., 2002). This does not discard the notion that OA could alter ion transport, resulting in increased water and electrolytes presence in stools.

In vivo experiments indicated that OA could pass the intestinal barrier and distributed in several organs, like intestine and liver (Louzao et al., 2021a). Some studies have suggested that OA may be converted into other metabolites in gut which reveal the complex toxicity of DSP toxins (Liu et al., 2022). These toxins have been associated with cytotoxicity, genotoxicity, neurotoxicity and tumour promotion (Le Hegarat et al., 2020; Amonruttanapun et al., 2022; Fujiki et al., 2018). Additionally, OA effects on cytoskeleton and TJs have been described in a variety of cell lines (Leira et al., 2001; Dietrich et al., 2019). Alterations on TJs vary depending on cell line, dose and time (Dietrich et al., 2019; Leira et al., 2001). Yet, a common feature in intestinal models is the elevated sensibility to OA when administered via basolateral rather than apical. Conversely, the relation between these effects and the symptomatology observed *in vivo* has not been studied in depth. The main action mechanism proposed for them is the inhibition of protein phosphatases (PPs) like PP2A and PP1 (Takai et al., 1992). They are ubiquitously distributed and, thus, interplay in an array of pathways (Yadav et al., 2017). Still, it has been discussed whether this inhibition is responsible for the diarrheagenic effects (Munday, 2013; Espina et al., 2010).

Therefore, to provide new information about OA toxicity on mammals, we aimed to assess mechanism by which OA induced diarrhoea *in vivo* (the main intoxication symptom). We performed fecal electrolyte testing for evaluation of diarrhoea in OA treated mice. Besides we studied cytomorphological alterations in the intestine by transmission electron microscopy. Moreover, since pathophysiology of OA has been related to 5-HT (Louzao et al., 2021b), we also evaluated whether the pre-treatment with cyproheptadine, an antagonist of serotonin receptors 1 and 2, modified this OA-induced diarrhoea and gut cytomorphology.

2. Materials and methods

2.1. Materials

Okadaic acid (OA) was a Certified Reference Material (CRM) (CAS number 209266-80-8) with a purity ≥99% supplied by Laboratorios CIFGA S.A (Lugo, Spain). OA stock concentration was of 5.77 μg/μL in methanol. Cyproheptadine hydrochloride sesquihydrate (CAS number 41354-29-4) was from Sigma-Aldrich (Madrid, Spain). Stock solution was prepared in ethanol to reach a concentration of 30 mg/mL. Physiological solution was from Grifols (Barcelona, Spain). All other reagents were form Sigma-Aldrich (Madrid, Spain).

2.2. Animals

Four-weeks old female Swiss mice of University of Santiago de Compostela colonies were used. Animals were kept in controlled conditions of humidity (60–70%), temperature (23 ± 2 °C) and 12 h light/dark cycles.

They were individually placed in metabolic cages with 5% glucose serum *ad libitum* for 15 h until treatment administration. Then, mice were weighed (18–22 g). Treatments were given by oral gavage (10 mL/kg body weight) or by intraperitoneal injection (1% body weight). Free access to food and water was then provided. At the end of the experiment animals were euthanised by CO_2 inhalation (EU, 2010).These conditions were shared by all *in vivo* assessments described below.

All mice experimentation was in accordance with the European (EU directive 2010/63/EU) and the Spanish legislation (Real Decreto 53/2013, Decreto 296/2008). All procedures were approved by the Institutional Animal Care Committee of the University of Santiago de Compostela (01/17/LU-002).

2.3. Okadaic acid treatment

Immediately before administration OA (in ethanol) was diluted in 0.9% saline solution. Serial dilutions were prepared to reach 100, 175, 250 and 500 μg/kg body weight. Control animals received vehicle alone (10 mL/kg saline solution containing 2.5% ethanol). Animals were randomly assigned to each treatment or control group. Treatments were administered by oral gavage. Faecal pellets were collected at 1 h.

2.4. Cyproheptadine treatment

Cyproheptadine (CPH) was first dissolved in ethanol, then prepared in physiological solution to reach 0.1, 1 and 10 mg/kg body weight. Animals were randomly assigned control, CPH, OA or CPH plus OA. First, CPH was administered via intraperitoneal 30 min before 250 μg/kg OA was given by oral gavage. Control mice received vehicle via intraperitoneal and oral. Stools were collected at 1 h of treatment. OA induces diarrhoea in mice within the first hour of treatment, while prior CPH administration can suppress or delay its outcome. In the latter case, diarrhoea onset was consistently within 2 h after toxin administration. Ultrastructural alterations following diarrhoea were evaluated, thus, the endpoint was set at 2 h, when animals were euthanised and necropsy was performed. Samples from jejunum and proximal colon were taken for Transmission Electron Microscopy.

A different set of experiments with a similar protocol were performed. Similarly, animals were randomly assigned to control, CPH, OA or CPH plus OA. For this case, the endpoint was set at the onset time of diarrhoea for OA treated mice. In this way, cytomorphological disturbances could be assessed when diarrhoea occurred. During the necropsy, jejunum and proximal colon samples were collected for Transmission Electron Microscopy.

2.5. Faecal electrolytes' extraction

Faecal samples were weighed, requiring a minimum of 50 mg stools. Pellets were dissolved 1:7 in previously heated milliQ water (37 °C). Samples were vortexed (30 s), then they were in vertical agitation for 1 h at 37 °C. Sonication 5 min plus 1 min vortex was performed twice. Samples were centrifuged 20 min 10000×g. Supernatants were transferred to a different tube, meanwhile pellets were left to dry at 60 °C for

Table 1
Faecal pellets outcome evaluation in OA-induced diarrhoea (mean ± SEM).

Treatment	Number of mice that had diarrhoea	Diarrhoea score	Diarrhoea onset (min)
Control	0/6	0 ± 0	–
OA (μg/kg)			
100	6/6	3 ± 0	36 ± 4.5
175	6/6	3 ± 0	38 ± 0.7
250	6/6s	3.7 ± 0.3	26 ± 1.3
500	6/6	3.9 ± 0.05	38 ± 2.1

30 h. Supernatants were further centrifuged twice at 10000×*g* 10 min to remove all debris. Finally, samples were filtered through 0.22 μm filters at 10000×*g* 10 min.

2.6. Electrolytes' measurement

Ions were detected by ion chromatography coupled to conductivity detector and chemical suppression (Compact IC Flex, Metrohm) (Chapp et al., 2018). A calibration curve with known concentrations of mix standards for each ion was prepared. Samples were diluted in milliQ water and placed in the autosampler, from which were injected into the ion chromatography. To measure chloride, a column Metrosep A Supp 7–250/4.0 was used to separate the sample at 45 °C. Mobile phase was 3.6 mM sodium carbonate and it was eluted at 0.8 mL/min flow rate. To measure cations (sodium, potassium and calcium), separation in a column Metrosep C 6–250/4.0 at 30 °C was performed. Mobile phase was 1.7 mM nitric acid and 1.7 mM dipicolinic acid at a flow rate of 1.1 mL/min. Retention times for chloride, sodium, potassium and calcium were of 9.7, 10, 17.3 and 25 min, respectively.

2.7. Osmotic gap

Osmotic gap is a tool that facilitates to distinguish between osmotic and secretory diarrhoeas. The calculation for this gap is based on the fact that faecal osmolality is similar to that of serum (Eherer and Fordtran, 1992; Fokam Tagne et al., 2019). Thus, this equation enables to obtain the difference from sodium and potassium concentration, taking into account anions (Eq. (1)).

$$290\ \text{mOsm/kg} - 2 \times [Na^{+}] - 2 \times [K^{+}] \quad (1)$$

If the difference is above 125 mOsm/kg, it is considered osmotic diarrhoea. If it is between 50 and 125 mOsm/kg, then it is a mixed type of diarrhoea. Finally, if the difference is below 50 mOsm/kg, it would be classified as secretory diarrhoea (Eherer and Fordtran, 1992).

2.8. Transmission electron microscopy

Jejunum and proximal colon samples from CPH treatment assays were processed as described before (Abal et al., 2017; Mascorro and Bozzola, 2007). Briefly, tissues (1 mm^3) were immersed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate trihydrate (pH 7.4) for fixation. After 4 h at 4 °C, samples were cleaned with 0.1 M sodium cacodylate trihydrate buffer and once more at 24 h. Post-fixation with

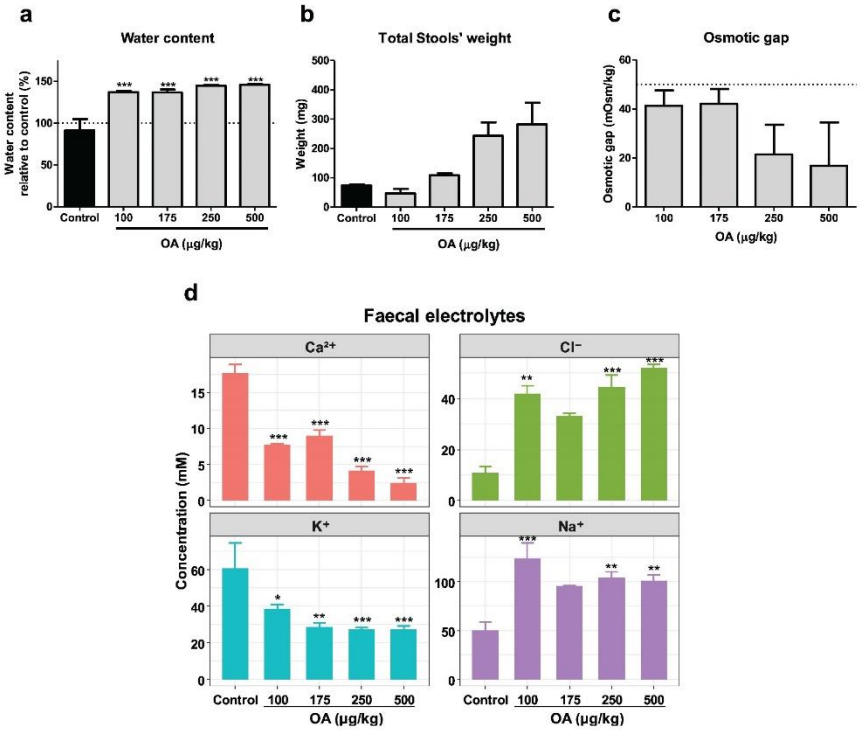

Fig. 1. Stools collected at 1 h after OA treatment at different doses. **a** Stools water content *versus* control. **b** Total faeces wet weight for each treatment. **c** Stools' osmotic gap, dotted line is at 50 mOsm/kg which indicates control value. **d** Concentrations of Ca^{2-}, Cl^{-}, K^{+} and Na^{+} in stools samples. Mean ± SEM (n = 6) is displayed. Statistical significance differences to control are $P < 0.05$*, $P < 0.01$** and $P < 0.001$***.

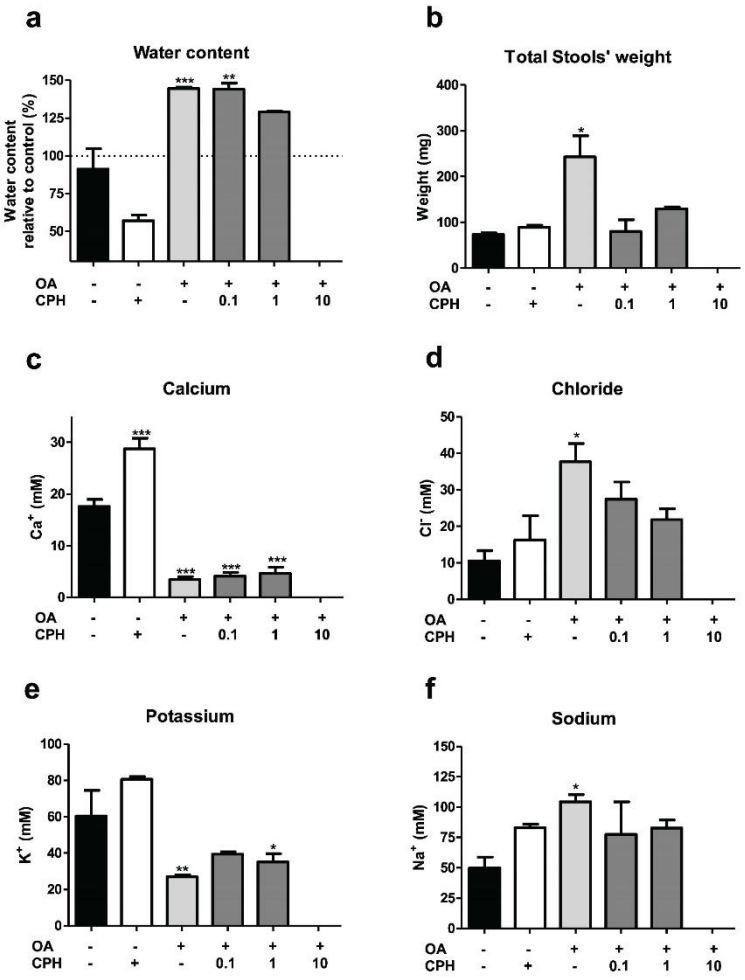

Fig. 2. Stools of mice pre-treated with CPH (doses expressed in mg/kg) and collected at 1 h after OA treatment. **a** Stools water content as percent of control. **b** Total faecal wet weight for each treatment. Concentration of **c** calcium, **d** chloride, **e** potassium and **f** sodium present in faeces. Note that data from 10 mg/kg CPH plus 250 μg/kg OA animals are lacking because they did not defecate for the first hour of treatment. Mean ± SEM (n = 6) is shown. Statistically significant differences to control are $P < 0.05$*, $P < 0.01$** and $P < 0.001$***.

1% OsO_4 in 0.1 M cacodylate trihydrate for 1 h was performed. Followed by washing twice and dehydration with increasingly graded ethanol solutions. During dehydration, an immersion in 0.5% uranyl acetate in 70% ethanol was performed. Then, tissues were washed with propylene oxide and embedded in epoxy resin. Ultrathin sections were obtained and counterstained with lead citrate and uranyl acetate. Images were taken from an JEOL JEM-1011 Transmission Electron Microscopy. Intestinal epithelial ultrastructure was assessed for disturbances focusing on microvilli, mitochondria and TJs and AJs. TJs and AJs' width were measured at its maximum width point by ImageJ (Fiji) software. Only longitudinal orientated borders and only junctions between enterocytes were evaluated (Martinez et al., 2013).

2.9. Statistical analysis

Analysis was performed using GraphPad Prism and R. When homoscedasticity (Bartlett test) and normal distribution (Kolmogorov-Smirnov test) are met by the dataset, one-way analysis of variance (ANOVA) along with Bonferroni Multiple Comparison Test is performed. Meanwhile, when the criteria are not met the non-parametric Kruskal-Wallis test along with Dunn's Multiple Comparison post-hoc test. Dunn's Multiple Comparison post-hoc test were performed alpha value was of 0.05, so that it minimises to 5% the probability of commuting false-positive results. Significance was considered when $P < 0.05$, otherwise non-significant.

3. Results

3.1. Disturbances in normal faecal parameters: OA treatment

OA induced poisoning is featured by diarrhoea as the main symptom developed in DSP in mice (Louzao et al., 2021b). Although extensive studies have evaluated OA effects *in vivo*, characterization of diarrhoea has not received such efforts. Therefore, we performed an *in vivo*

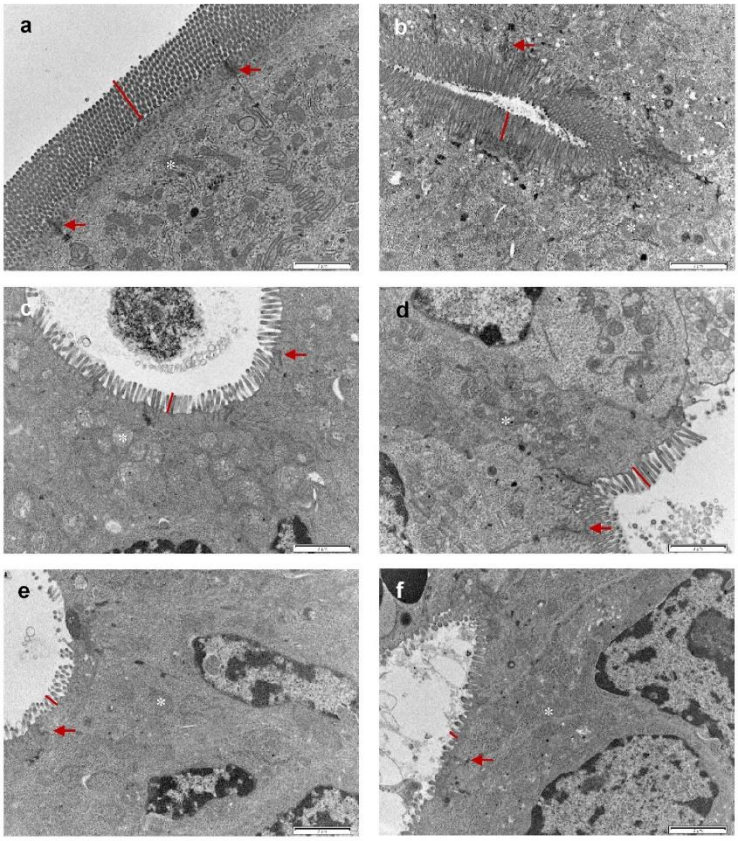

Fig. 3. Ultrastructural and cell junctions' evaluation of jejunum at 2 h of treatment. Jejunum representative images of **a** control and mice treated with, **b** 1 mg/kg CPH, **c** 250 μg/kg OA, **d** 0.1 mg/kg CPH-250 μg/kg OA, **e** 1 mg/kg CPH-250 μg/kg OA and **f** 10 mg/kg CPH-250 μg/kg OA. Scale at 2 μm. Microvilli (red line), apical junctions (red arrow) and mitochondria (white asterisk) are indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

experiment treating mice with different doses of OA from 100 to 500 μg/kg chosen based on previous study (Louzao et al., 2021b). These toxin doses correspond to OA concentration over the legal limit (EU, 2004), though they are found in seafood products (Contreras and Garcia, 2019; Blanco et al., 2019; Bresnan et al., 2021). A scoring system was described to assess severity of diarrhoea. Normal faeces were considered those with normal (score of 0) or soft consistency, but maintaining a defined shape (score of 1). Stools with non-defined shape and watery were considered diarrhetic samples and a score of 2 and 3 were given, respectively. Finally, a score of 4 was assigned when diarrhoea was developed in several occasions, i.e., at least 20 min should pass between times. All OA-treated animals developed diarrhoea, whereas control mice had normal pellets (Table 1). Diarrhoea develops between 25 and 40 min after toxin administration (Table 1).

Further characterization of stools was performed (Fig. 1). Firstly, we evaluated the water content of all samples from the first hour of treatment (Fig. 1a). All OA tested doses induced similar faecal water content that was significantly increased when compared to control (Fig. 1a). Total stools weight for the first hour was also measured. In this case a non-significant dose-dependent pellet outcome is observed (Fig. 1b). Fluid secretion resulting in diarrhoea can be triggered by a variety of agents, thus, there is not only one responsible mechanism. Watery diarrhoea can be classified into osmotic or secretory based on the driving force for water movement (Fine and Schiller, 1999; Camilleri et al., 2017). Osmotic diarrhoea is driven by an osmotically active poorly-absorbed compound that causes fluid secretion (Rao, 2019). Conversely, secretory diarrhoea is driven by augmented ions concentration into the lumen (Fine and Schiller, 1999; Rao, 2019). The calculation of the osmotic gap facilitates the differentiation between these two types of diarrhoea. OA-treated mice had a faecal osmotic gap below 50 mOsm/kg (Fig. 1c), indicating secretory diarrhoea. To further assess how the electrolytes were responsible for this gap, electrolytes concentration of stool samples was measured (Fig. 1d). Both calcium and potassium concentrations were significantly diminished in stools samples from OA-treated mice (Fig. 1d). Meanwhile, the opposite pattern is observed in chloride and sodium concentrations. All tested OA doses induced a significant elevation of chloride and sodium concentrations in faeces (Fig. 1d).

3.2. Disturbances in normal faecal parameters: CPH pre-treatment

Based on previous reports (Louzao et al., 2021b), it was of interest to evaluate whether cyproheptadine modified OA-induced diarrhoea. Faeces from control and CPH treated mice had normal consistency and shape, while stools of mice that received CPH-OA are only of animals that developed diarrhoea within the experiment time. It should be noted that 10 mg/kg CPH plus OA treated mice had no pellets along the experiment. Water content as well as stools weight were measured (Fig. 2a and b). Faeces from all animals that had diarrhoea display an elevated water content when compared to control, being significant only

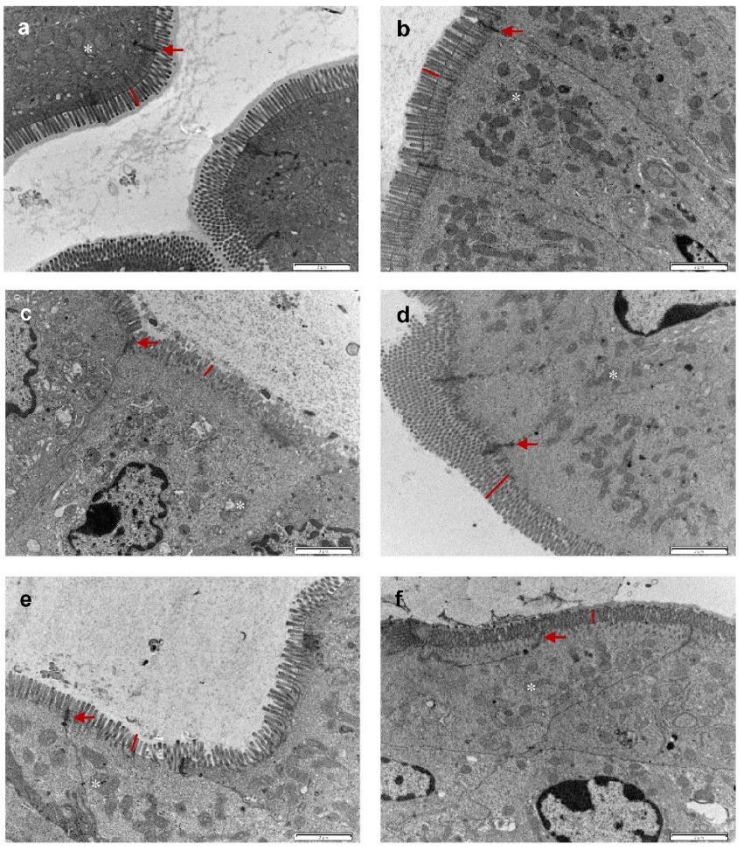

Fig. 4. Proximal colon cytomorphological and cell junctions' evaluation at 2 h of treatment. Proximal colon representative images of **a** control, and mice treated with **b** 1 mg/kg CPH, **c** 250 μg/kg OA, **d** 0.1 mg/kg CPH-250 μg/kg OA, **e** 1 mg/kg CPH-250 μg/kg OA and **f** 10 mg/kg CPH-250 μg/kg OA. Scale at 2 μm. Brush border (red line), apical junctions (red arrows), and mitochondria (white asterisk) are highlighted. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

for OA and the lowest CPH pre-treatment dose in combination with the toxin (Fig. 2a). On the contrary, faecal weight for CPH-OA treated mice is similar to that of control and considerably lessened than that of OA, though non-significant compared to the toxin alone (Fig. 2b). Electrolytic profile was also evaluated. Calcium concentration was significantly reduced in all OA-treated mice stools (Fig. 2c). Conversely, chloride was significantly elevated in OA treated mice, though CPH pre-treatment seemingly avoid the increase though non-significant compared to the toxin (Fig. 2d). Potassium concentration shows a significant decrease from all OA given mice when compared to control (Fig. 2e). Still, a slight non-significant increase is observed for CPH-OA treated animals (Fig. 2e). Regarding sodium concentration, faeces from OA-treated mice are significantly above those of control (Fig. 2e). This profile is slightly modified by CPH pre-treatment in a non-significant fashion compared to OA (Fig. 2f).

3.3. *Effect of CPH on the intestine of OA treated mice at 2 h: ultrastructural assessment*

Based on electrolytes profile and previous results of CPH decreasing or blocking OA-induced diarrhoea (Louzao et al., 2021b), it was of interest to assess whether CPH modified OA-induced damage along the intestine at 2 h. Jejunum from control and CPH group show normal microvilli, mitochondria and TJs (Fig. 3a and b). Intestine of OA-treated mice display disorganised microvilli, and swollen mitochondria (Fig. 3c). Pre-treatment with 0.1 mg/kg CPH does not alleviate OA-induced mitochondria swelling or microvilli disturbance (Fig. 3d). In the case of 1 mg/kg CPH (Figs. 3e) and 10 mg/kg CPH (Fig. 3f) pre-treatments, microvilli are shortened. To assess the effects of OA on TJs and AJs, their width was measured at their widest point. TJs' width was not different for either OA or any CPH pre-treatment (Fig. S1a). Similarly, no statistical significance was measured in the case of AJs' width between any treatment (Fig. S1b). Overall, CPH pre-treatment at any of the given doses did not avoid OA-induced damage at 2 h in the jejunum.

Proximal colon was also evaluated (Fig. 4). For control and CPH treatments normal brush border, with normal enterocyte structure were observed (Fig. 4a and b). In OA treatment, microvilli were disorganised and shortened, mitochondria were swollen or with electron-dense deposits (Fig. 4c). Even with pre-treatment with the lowest dose of CPH (0.1 mg/kg), a modest improvement can be observed: microvilli resemble control while mitochondria still present with some electron-dense granules but few are swollen (Fig. 4d). A similar aspect is shown for 1 mg/kg CPH pre-treatment, with rather normal microvilli and slightly swollen mitochondria are depicted (Fig. 4e). Finally, 10 mg/kg CPH pre-treatment proximal colon are normal but for shortened microvilli (Fig. 4f). TJs and AJs' width was also evaluated. CPH pre-treatment at all doses seemed to displayed tighter junctions, though no significant differences were detected (Fig. S1c). AJs' width is not affected by OA or any of CPH pre-treatments (Fig. S1d). In proximal

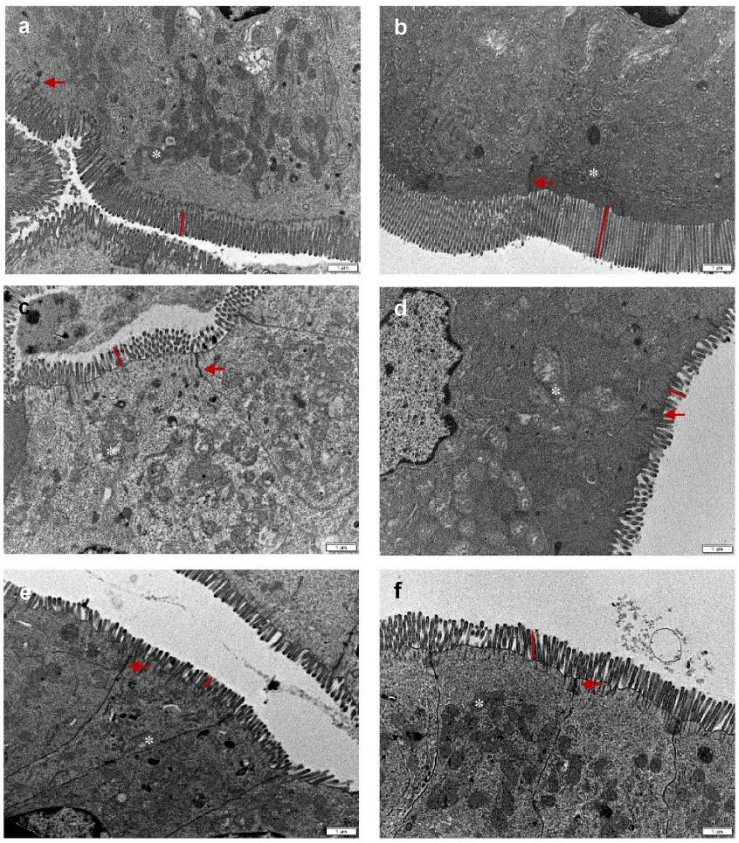

Fig. 5. Jejunum ultrastructural assessment and apical cell junctions' evaluation at 30 min of treatment. Jejunum representative images of **a** control, and mice treated with **b** 6 mg/kg CPH, **c** 250 μg/kg OA, **d** 0.1 mg/kg CPH-250 μg/kg OA, **e** 1 mg/kg CPH-250 μg/kg OA and **f** 6 mg/kg CPH-250 μg/kg OA. Scale at 1 μm. Microvilli (red line), apical junctions (red arrow) and mitochondria (white asterisk) are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

colon, pre-treatment with CPH alleviated OA-induced damage in intestinal epithelia.

3.4. Effect of CPH on the intestine of OA treated mice at the moment of diarrhoea: ultrastructural assessment

Since apical junctions were not affected after 2 h of treatment, it was of interest to observe if OA induced cytomorphological alterations at the moment of diarrhoea. To do so, animals were pre-treated with different doses of CPH and experiments ended when diarrhoea was detected around 33 min after OA administration. It should be noted that only mice treated with OA developed diarrhoea, meanwhile none of animals pre-treated with CPH had it. Ultrastructural evaluation for jejunum (Fig. 5) and proximal colon (Fig. 6) was assessed. Jejunum from control and CPH display normal microvilli and mitochondria (Fig. 5a and b). Conversely, jejunum from mice treated with OA show swollen mitochondria and disorganised microvilli (Fig. 5c). Similar features are observed in 0.1 mg/kg CPH-OA-treated mice (Fig. 5d). However, pre-treatment with 1 mg/kg (Fig. 5e) or 6 mg/kg CPH (Fig. 5f) present mild alterations like disorganised brush border, though similar height and mitochondria resembling those of control are observed. TJs and AJs' width was also assessed. OA treatment induced narrowed TJs (Fig. S1e) when compared to control. In CPH pre-treated mice width TJs are similar to controls (Fig. S1e). Regarding AJs, no significant differences were detected between treatments, though all OA-treated mice seemed to display tighter junctions (Fig. S1f).

Proximal colon's ultrastructure was also evaluated (Fig. 6). Microvilli and mitochondria of control (Fig. 6a) and CPH (Fig. 6b) display normal morphology. On the contrary, OA damages microvilli normal structure and minor mitochondrial alterations are also observed (Fig. 6c). This is slightly alleviated by 0.1 mg/kg CPH pre-treatment, where a brush border more similar to control is depicted (Fig. 6d). Conversely, mitochondria disturbances are like those present in OA treatment (Fig. 6d). In 1 mg/kg (Fig. 6e) and 6 mg/kg (Fig. 6f) cyproheptadine pre-treatments brush border structure resembles that of control, while mitochondria are similarly altered to OA treatment. In the evaluation of TJs (Fig. S1g) and AJs (Fig. S1h), no modifications are observed either for treatments of OA alone or either for any of CPH pre-treatments.

4. Discussion

Diarrhetic Shellfish Poisoning is characterised by severe gastrointestinal symptoms, being diarrhoea its hallmark (Yasumoto et al., 1978). It is defined as presence of low consistency stools and increased number of depositions (Rao, 2019; Who. Diarrhoeal disease, 2017). Due to the complexity of the gastrointestinal tract, pathophysiology of diarrhoea can vary depending on the causative agent. A number of *in vivo* assays have evaluated OA toxicity after its oral administration, reporting presence of diarrhoea (Ito et al. 2000, 2002; Tubaro et al., 2003; Aune

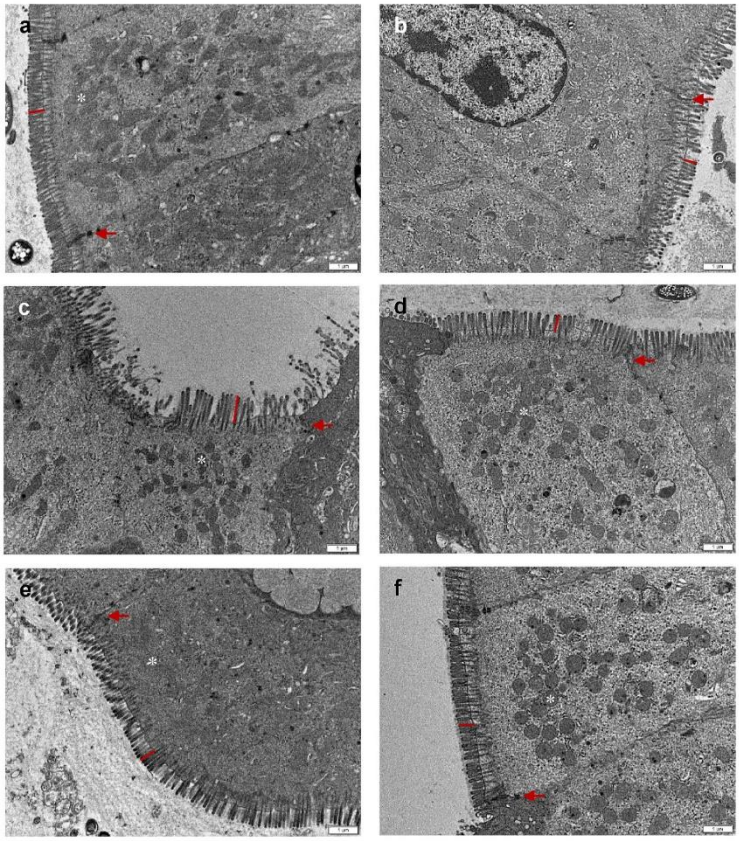

Fig. 6. Cytomorphological assessment and apical cell junctions' evaluation of proximal colon at 30 min of treatment. Proximal colon representative images of **a** control and mice treated with **b** 6 mg/kg CPH, **c** 250 μg/kg OA, **d** 0.1 mg/kg CPH-250 μg/kg OA, **e** 1 mg/kg CPH-250 μg/kg OA and **f** 6 mg/kg CPH-250 μg/kg OA. Scale at 1 μm. Intestinal epithelial cells morphology including microvilli (red line), apical junctions (red arrow) and mitochondria (white asterisk) are displayed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

et al., 2012). However, detailed studies describing diarrhoea to better define DSP toxicity are scarce. Therefore, we assessed OA-induced diarrhoea features. This allowed to determine a consistent water content increase supported by elevated faecal chloride and sodium concentrations. This could be an indicative of active fluid secretion pathways and/or inhibition of absorptive mechanisms. To elucidate this premise, the osmotic gap was calculated. This formula enables to distinguish between osmotic and secretory diarrhoeas (Fine and Schiller, 1999; Camilleri et al., 2017). The former is characterized by a poorly-absorbed substance with osmotic activity, enhancing water accumulation in the lumen (Camilleri et al., 2017). Secretory diarrhoea is the result of elevated luminal electrolyte concentration (Rao, 2019). The osmotic gap is based on the fact that osmolality between plasma and lumen at the end of the gastrointestinal tract are equal (Fine and Schiller, 1999; Parakh et al., 2017; Fokam Tagne et al., 2019). We calculated an osmotic gap of <50 mOsm/kg for OA, therefore an indicative of secretory diarrhoea (Camilleri et al., 2017). This is consistent with calcium and potassium concentrations reduction in all OA tested doses. Diminished potassium concentration is a feature found in other secretory diarrhoeas such as that induced by cholera toxin (Sorensen et al., 2010; van Dinter et al., 2005). This could be explained by an increase in water content, thus diluting the normal concentration. Likewise, active absorption of potassium mainly occurs at distal colon (Sorensen et al., 2010; Rao, 2019) which could further decrease its concentration. Therefore, this reduction could be secondary to a main secretory pathway. Accordingly, elevated faecal chloride and sodium concentrations were measured in OA-induced diarrhoea. Active chloride secretion in the intestine has been directly related with diarrhoea caused by enterotoxins like cholera toxin and rotaviral non-structural protein 4 (Das et al., 2018). Chloride channels are expressed in apical and basolateral membranes of enterocytes throughout the intestine and can be activated by different pathways (Das et al., 2018). Second messenger released directly in enterocytes or via activation of immune cells, enteroendocrine cells or nerve signalling can also result in profuse chloride luminal secretion (Rao, 2019). Sodium efflux along the gastrointestinal tract mainly occurs through passive paracellular transport (Tamura et al., 2011). Hence, sodium luminal concentration can increase driven by active chloride secretion (Rao, 2019). As stated above, absorption could also be suppressed leading to electrolytes' accumulation in the intestine resulting in diarrhoea. Chloride absorption can occur via paracellular or via transcellular (Rao, 2019; Engevik and Engevik, 2021). On the other hand, sodium can be absorbed by cotransport with nutrients, channels or in exchange with other ions (Engevik and Engevik, 2021). Thus, secretion and/or absorption could be affected by OA and reflected in stools electrolyte concentration. Effects of OA on short circuit currents have been studied in models of intestinal epithelia *in vitro* (Tripuraneni et al., 1997; Chow and Barrett, 2007) and *ex vivo* (Hosokawa et al., 1998). These studies show that no direct increase in currents were elicited by OA (Tripuraneni et al., 1997; Chow and Barrett, 2007; Hosokawa et al., 1998), which could possibly

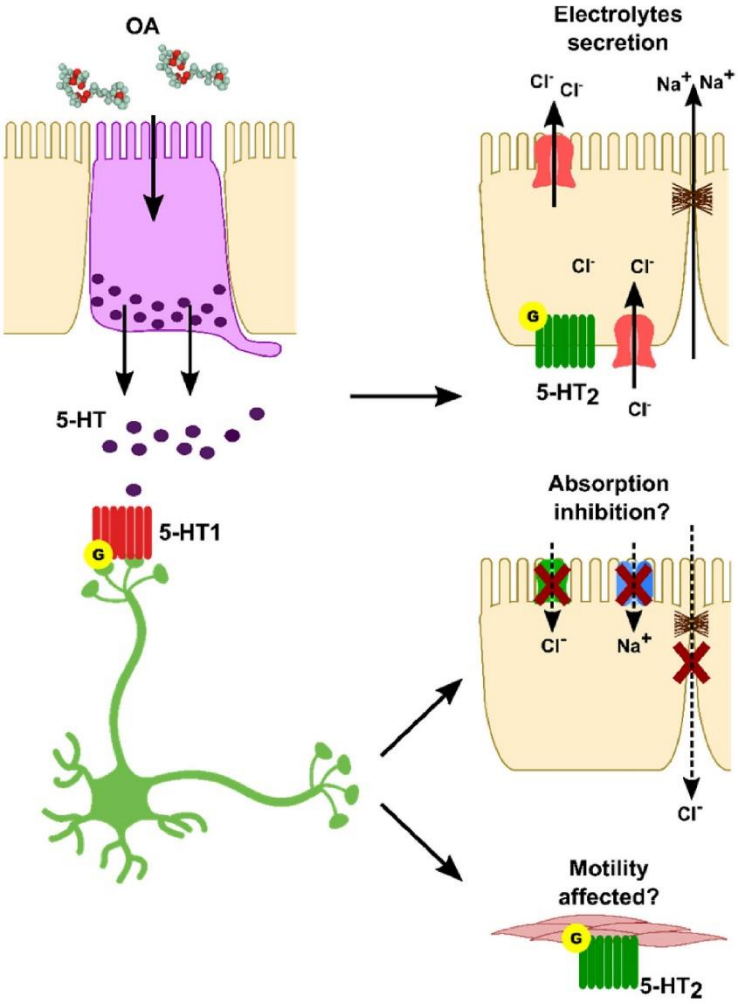

Fig. 7. Representation of the possible OA mechanism of action based on the results here presented and the literature. OA may act on enterochromaffin cells leading to serotonin release to the submucosal side. Then serotonin can act in a paracrine manner on enterocytes 5-HT2 receptors leading to chloride secretion into gut lumen, followed by sodium and water. On the other hand, serotonin can also trigger neuronal pathways via 5-HT1 receptors. This signalling cascade amplifies the response which can also lead to suppression of electrolytes absorption for instance by acting on other serotonin receptors. It should be noted that serotoninergic pathways are of great complexity, meaning other neurotransmitters may be involved in OA induced diarrhoea and may alter other mechanisms such as intestinal motility patterns.

be due to inhibition of electroneutral absorption of chloride and sodium (Creydt et al., 2004; Das et al., 2018) and increase paracellular permeability. Accordingly, it was reported that OA did enhance sodium paracellular flux *in vitro* (Tripuraneni et al., 1997) and enhanced conductance in rat colon *ex vivo* (Hosokawa et al., 1998). Still, some of these responses should be taken with caution since the toxin was applied either at the basolateral/serosa side or both apical and basolateral. Conversely, other studies loading OA to the apical chamber of cell monolayers do not reveal variations in monolayers resistance when exposed for a comparable period (Ehlers et al., 2011; Fernandez et al., 2014; Louzao et al., 2015). Some authors have suggested that OA induces diarrhoea by PPs inhibition of dephosphorylation of sodium secretion proteins (Cohen et al., 1990). However, no evidence is in support of or dismisses this hypothesis (Munday, 2013; Espina et al., 2010). Moreover, it should be taken into account the fact that alterations of motility patterns could also have a role (Camilleri et al., 2017).

Serotonin (5-HT) is a biogenic amine which has been studied for its roles in intestinal motility and fluid secretion, as well as its function under pathologic conditions, like diarrhoea (Das et al., 2018; Rao, 2019). Serotonin can induce fluid secretion by acting on 5-HT2 receptors in enterocytes and by triggering secretomotor responses through receptors 5-HT1, 5-HT3 and 5-HT4 on nerve terminals (Shajib et al., 2017). This can ultimately lead to chloride secretion and inhibition of absorption (Rao, 2019; Mawe and Hoffman, 2013; Meddah et al., 2014) as well as to enhance propagating motility patterns (Fida et al., 2000). Recently we have reported the possible involvement of serotonin in OA-induced diarrhoea (Louzao et al., 2021b). The pre-treatment with the antagonist of receptors 5-HT1 and 5-HT2 cyproheptadine alleviated and even blocked OA-induced diarrhoea (Louzao et al., 2021b). Thus, we assessed whether this effect was translated to diarrhetic parameters. Water content and faecal weight indicate that CPH attenuates the copious diarrhoea caused by OA. This could be explained in part by the reduction of chloride concentration in stools with CPH pre-treatment. Conversely, this effect was not reflected in cytomorphological alterations we observed in jejunum. OA induced ultrastructural alterations characterised by cytotoxic effects like microvilli disorganization and

mitochondria swelling in both jejunum and proximal colon. These alterations persisted in jejunum of CPH-OA treated mice at 2 h after toxin administration, whereas they were mildly improved at 30 min. Likewise, TJs' width was only affected by OA at 30 min, being similar to control at all CPH-OA treatments. TJs can be unaffected or even tighter in other types of diarrhoeas like that caused by rotavirus (Hagbom et al., 2020). Even though we cannot discard the possibility of OA affecting permeability, we observe normal structure of apical intercellular junctions. OA damage in small intestine at a few hours of treatment has been previously reported, i.e., epithelial erosion, microvilli and mitochondria impairment (Ito et al. 2000, 2002; Wang et al., 2012). Still, not only have these alterations been detected in diarrhetic animals, but also in OA-treated mice that do not develop diarrhoea (Wang et al., 2012; Ito et al. 2000, 2002). On the contrary, previous large intestine findings report from none to mild epithelia alterations (Hosokawa et al., 1998; Ito et al. 2000, 2002). We show that OA induces epithelial damage at 2 h and 30 min of exposure, whereas overall ultrastructure of CPH-OA treated proximal colon resembled that of control mice. Regarding TJs and AJs' width, it was not affected by OA. These ultrastructural alterations are consistent with other secretory diarrhoeas in which TJs and AJs maintain their structure and microvilli are disorganised (Markov et al., 2019). OA could be acting via complex mechanisms involving serotoninergic pathways leading to chloride plus fluid secretion (Fig. 7). However, it should be taken into consideration the fact that serotonin has also a role in intestinal motility by acting on receptors 5-HT2 (Mawe and Hoffman, 2013) that is avoided by CPH in colonic strips (Fida et al., 2000). Therefore, it should not be ruled out the alteration of motility patterns as part of OA mechanism of action. Our findings are in support of OA triggering secretory diarrhoea involving serotoninergic pathways.

CRediT authorship contribution statement

Celia Costas: Data curation, Formal analysis, Writing – original draft. **M Carmen Louzao:** Conceptualization, Data curation, Formal analysis, Writing – review & editing. **Sandra Raposo-García:** Formal analysis, Writing – original draft. **Carmen Vale:** Formal analysis, Writing – original draft. **Luis M. Botana:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2022.113449.

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Material Suplementario:

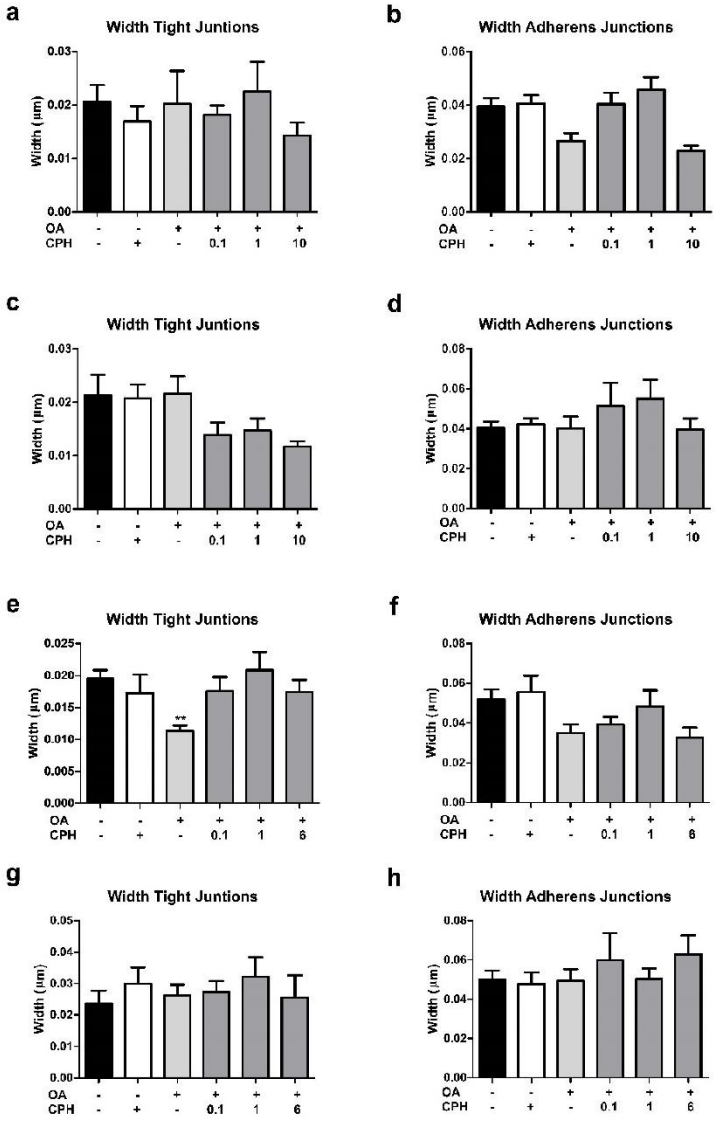

Fig. S1 Width of tight junctions (a, c, e, g) and adherens junctions (b, d, f, h) in the gut of mice pre-treated with CPH (doses expressed in mg/kg) and OA (250 µg/kg) at different times. Jejunum (a, b) and proximal colon (c, d) junctions' width of animals treated for 2 h. Jejunum (e, f) and proximal colon (g, h) junctions' width of mice at 30 min of treatment.

Mean ± SEM. Statistical significance compared to control is indicated by an asterisk when

$P < 0.05$

4.4 PUBLICACIÓN 4: ACUTE TOXICOLOGY REPORT OF THE EMERGING MARINE BIOTOXIN BREVETOXIN 3 IN MICE: FOOD SAFETY IMPLICATIONS.

Año de la publicación: 2023

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Resumen: Las brevetoxinas (PbTxs) son toxinas marinas sintetizadas por el dinoflagelado desnudo *Karenia* spp. y algunas rafidófitas. Esta toxina se puede acumular en moluscos bivalvos que al ser consumidos causan la intoxicación neurotóxica por consumo de marisco (NSP). Se han descrito una amplia variedad de síntomas que comprenden mayormente una afectación neuromuscular y gastrointestinal. Recientemente se han detectado PbTxs en marisco de regiones donde no se había detectado previamente como en las costas europeas donde se considera una toxina emergente. Los datos de toxicidad oral, actualmente escasos para las PbTxs, son necesarios para garantizar la seguridad alimentaria del marisco y la salud de los consumidores. Realizamos un estudio comparativo de la toxicidad oral e intraperitoneal de la brevetoxina 3 (PbTxs 3) en ratón siguiendo el protocolo de administración *Up & Down*. Se registraron los síntomas, se realizaron tests funcionales a lo largo del ensayo de 24 h de duración. Tras la necropsia se extrajeron muestras de órganos para la evaluación histopatológica y sangre para el análisis bioquímico. La PbTx3 a las dosis más altas causó síntomas neuromusculares como ataxia y convulsiones que fueron similares en todos los ratones independientemente de la ruta de administración de la toxina. Además, tras 6 h de tratamiento tenían debilidad muscular. También se observó una mejoría y reducción de los síntomas a las 24 h, tiempo al que no se detectaron daños en los estudios de histopatología ni en el análisis bioquímico en sangre. La administración oral de la PbTx3 causó una menor toxicidad que la administración intraperitoneal, ya que sólo la dosis de 4000 μg/kg PbTx3 por vía oral causó la muerte de los animales, mientras que la dosis letal 50 fue de 875 μg/kg PbTx3 por inyección intraperitoneal. Sin embargo se detectaron signos clínicos de toxicidad oral a dosis bajas de toxnia y pudimos establecer un NOAEL de 10 μg/kg PbTx3 y un LOAEL de 100 μg/kg PbTx3 para la administración oral en ratón. Estos datos aportan una importante información en las evaluaciones de riesgo de las PbTxs para el consumidor.

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Acute toxicology report of the emerging marine biotoxin Brevetoxin 3 in mice: Food safety implications

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ABSTRACT

Brevetoxins (PbTxs) are emerging marine toxins that can lead to Neurotoxic Shellfish Poisoning in humans by the ingestion of contaminated seafood. Recent reports on brevetoxin detection in shellfish in regions where it has not been described before, arise the need of updated guidelines to ensure seafood consumers safety. Our aim was to provide toxicological data for brevetoxin 3 (PbTx3) by assessing oral toxicity in mice and comparing it with intraperitoneal administration. We followed an Up-and-Down procedure administering PbTx3 to mice and registering clinical signs, neuromuscular function, histopathology, and blood changes. Neuromuscular dysfunction like seizures and ataxia, as well as loss of limb strength were observed at 6 h. Performance and clinical signs largely improved at 24 h, time at which no blood biochemical or histological alterations were detected independently of the administration route. However, PbTx3 oral administration results in lower toxicity than intraperitoneal administration. Mortality was only observed at 4000 μg/kg bw PbTx3 administered via oral, but we still found toxicity clinical signs at low toxin doses. We could stablish an oral Lowest-Observable-Adverse-Effect-Level for PbTx3 of 100 μg/kg bw and an oral No-Observable-Adverse-Effect-Level of 10 μg/kg bw in mice. The data here reported should be considered in the evaluation of risks of PbTxs for human health.

1. Introduction

Brevetoxins (PbTxs) are polyether lipophilic marine biotoxins synthetised by some dinoflagellates of the genus *Karenia* spp. Under certain environmental conditions, these microorganisms can exponentially proliferate causing negative economic and ecosystem impacts ((EFSA) 2010a). Blooms often result in wildlife mortality, through exposure to PbTxs (Flewelling et al., 2005). As algal blooms grow and persist, *Karenia* spp. cells rupture and PbTxs concentration increase in the surrounding water and air. PbTxs can accumulate in filter-feeding organisms like bivalve molluscs without altering their organoleptic properties and these compounds are heat-stable and are not modified in the cooking process (Vilarino et al., 2018). It is important to note that molluscs can have toxic levels of PbTxs for weeks post bloom depletion (Plakas et al., 2008). PbTxs are responsible of Neurotoxic Shellfish Poisoning (NSP), commonly occurring through the ingestion of contaminated seafood. However, poisoning through inhalation of aerosols and dermal contact can also occur ((EFSA) 2010a). PbTxs bind to site 5 of voltage-gated sodium channels (Na_v) which is between Segment 5 of Domain IV and Segment 6 of Domain I of the α subunit (Mackieh et al., 2021). As a consequence, the activation voltage threshold shifts towards more negative potentials, i.e., voltage potentials closer to resting potential will trigger Na_v opening (Wang and Wang, 2003). Moreover, they slowdown inactivation time of Na_v resulting in increased Na^+ influx time (Jeglitsch et al., 1998). Consequently, symptoms of intoxication include paraesthesia, dizziness, nausea, vomiting, ataxia, muscle weakness, asthenia as well as seizures and coma in more severe cases, though no fatalities have been reported (Arnich et al., 2021). These clinical signs can co-occur with gastrointestinal and cardiovascular signs. NSP outbreaks have been reported in U.S.A., Mexico, and New Zealand, but climate change is leading to the geographical expansion of PbTx-producing microalgae in other marine

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environments. Thereby, potentially toxic dinoflagellates and raphidophytas' distribution along the world has been described in the past decade (Turner et al., 2015; Yamaguchi et al., 2016; Patel et al., 2020; Bresnan et al., 2021; Zingone et al., 2021). Countries where *Karenia* spp. presence have been documented, as well as fauna mortality events registered, lack a legislation framework enabling consumers health protection (Turner et al., 2015; Zingone et al., 2021). Such has been the case of France: a recent study reported PbTxs detection in shellfish samples from Diana Lagoon (France) as early as 2015 (Amzil et al., 2021).

PbTxs are a group of marine cyclic polyether compounds subdivided in PbTxs type A and type B depending on the backbone structure ((EFSA) 2010a; Mackieh et al., 2021). Type A PbTxs have 10 ether rings and are comprised by PbTxs 1, 7 and 10, whereas type B have 11 rings and is constituted by PbTxs 2, 3, 5, 6, 9 ((EFSA) 2010a; Hort et al., 2021). PbTx1 and PbTx2 are considered the parent compounds that can undergo oxidation, hydrolysis, and reduction, among other chemical modifications. Altogether, roughly 40 derivatives have been described in seafood ((EFSA) 2010a; FAO/WHO, 2016; Hort et al., 2021). Different molluscs display various toxin profiles due to different metabolization of PbTxs (Ishida et al., 2004). Among all of them, PbTx3 has been detected in shellfish with higher concentrations than counterparts in several molluscs (Hort et al., 2021; Abraham et al., 2021). Regarding the considerable number of PbTx metabolites, monitoring programs have been stablished. Mouse Bioassay had been in monitoring programs to detect PbTxs presence in some countries where animals received the toxin or contaminated extract via intraperitoneal injection (FAO/WHO, 2016). Due to ethical, legal and technical concerns this method is being replaced by approaches like ELISA and liquid chromatography coupled to mass spectrometry (FAO/WHO, 2016; FDA, 2019. PbTx2 has been the reference toxin regarding regulatory limits due to its concentration in *Karenia* spp. and the fact that it is considered the toxin from which the analogues derive ((EFSA) 2010a). However, PbTx3 has been suggested as reference toxin to monitor PbTxs level in seafood since high concentration was reported in shellfish and greater toxicity than PbTx2 has been showed (Baden and Mende, 1982). Therefore, a guidance level of 180 μg PbTx3 equivalents/kg shellfish has been proposed (ANSES, 2021) However, oral toxicity information is scarce, though it is essential in health risk assessments performed to protect the consumer. More research in this field is needed in order to set the legal limits (Arnich et al., 2021).

The aim of this study is to provide data on oral toxicity of PbTx3 in mice for the possible establishment of an adequate framework on food safety regarding Neurotoxic Shellfish Poisoning.

2. Material and methods

2.1. Materials

Brevetoxin 3 with 95% purity determined through high performance liquid chromatography was from Latoxan (L8902; Portes lès Valence, France). Ethanol was from PanReac (Castellar del Vallès, Spain), 5% glucose serum was from B. BRAUN VetCare (Barcelona, Spain), and physiological solution was from Grifols (Parets del Vallès, Spain). All reagents used were from Sigma-Aldrich (Madrid, Spain), unless stated otherwise.

2.2. Animals

Female Swiss (ICR background) 4-week-old mice of initial body weight 20–24 g were used in all experiments as stated by the Organization for Economic Cooperation and Development (OECD) guideline n° 425 (OECD, 2022). Animals were weighed and individually placed in metabolic cages. After fasting for 15 h with free access to 5% glucose serum, mice were treated with PbTx3. Then, animals were provided with standard chow and water *ad libitum* throughout the experimental period. At the end of the experiment (24 h after treatment), mice were euthanised by CO_2 inhalation and necropsy was performed. All procedures were performed according to the European (EU directive 2010/63/EU) and the Spanish legislation (Real Decreto 53/2013, Decreto 296/2008). All procedures were approved by the Institutional Animal Care Committee of the University of Santiago de Compostela (01/17/LU-002).

2.3. Acute oral and intraperitoneal PbTx3 toxicity: Up-and-Down procedure

PbTx3 was firstly dissolved in ethanol, and diluted in physiological solution before administration via oral gavage or intraperitoneal injection. Up-and-Down procedure was performed according to the OECD guideline n° 425 (OECD, 2022) in order to determine the PbTx3 median lethal dose 50 (LD_{50}). Four dosages of toxins were administered and animals were monitored for 24 h. Each dose was tested in a different number of animals as indicated in the Up-and-Down procedure guidelines. At the first dosage, 3 animals were treated. At the second one, 5 mice were dosed, followed by 7 and 9 mice at third and fourth doses, respectively. Due to previous report of a LD_{50} of 520 μg/kg bw PbTx3 by oral administration (Baden and Mende, 1982), we chose 1000 μg/kg bw PbTx3 (3 mice) as the starting dose. If more than half of them survived, the dose was increased, otherwise was reduced (Fig. 1). Ethanol was prepared as the corresponding toxin dose for control mice. There was one control mouse per dose. Moreover, clinical signs were recorded closely after administration and at 1, 2, 3, 4, 6, 9, 12 and 24 h. Overall animal welfare was evaluated by a summatory score approach in which the presence of any clinical sign is counted as 1 whereas 0 is assigned if the clinical sign is not observed. Functional tests were performed the day prior treatment administration, after 6 h and 24 h of treatment.

2.4. Oral administration of medium-low PbTx3 doses

To assess Lowest-Observed-Adverse-Effect-Level (LOAEL) and No-Observed-Adverse-Effect-Level (NOAEL). Doses of 10, 100, 500 and 750 μg/kg bw PbTx3 were given to mice by oral gavage (3 mice per dose). Clinical signs were recorded up to 24 h. Tests were performed the day prior to treatment, at 6 and 24 h after administration, time at which

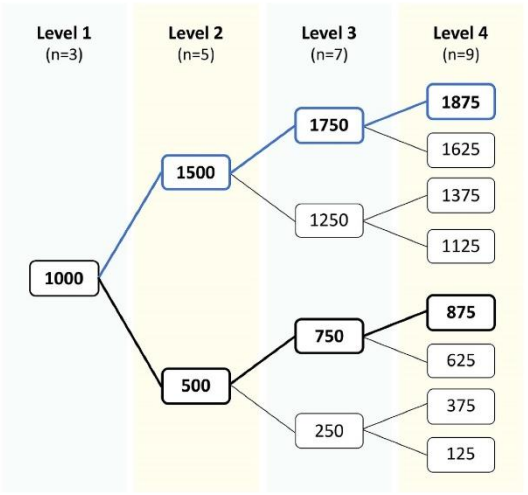

Fig. 1. Up-and-Down scheme followed by PbTx3 oral (blue) and intraperitoneal (black thick line) treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ended the assay.

2.5. Functional tests

In order to evaluate muscular strength, the hanging grid test was performed (Asorey et al., 2018). Mice were placed over a grid, gently shaken to ensure that they grabbed the grid. Then, the grid was turned upside down and latency to fall from 23 cm height was recorded. In case animal didn't fall, the cut-off was 60 s.

To improve the evaluation of mice clinical signs along with motor behaviour, the cylinder test was performed. Mice were placed in a transparent 2 L beaker for 60 s and were recorded by video. Afterwards, videos were assessed and the number of rearing events were counted.

To assess sensitivity alterations, even when mice were treated at low PbTx3 doses, a modification of cold plate test was performed (Ruan et al., 2018; Salat et al., 2019). A 2 L beaker was set on a cold aluminium covered plate, with ice below to keep the temperature at 5 °C. After the temperature was reached, animal was placed on the beaker and recorded for 30 s. As a respond to cold, times of hindpaws' lifting were measured (Ruan et al., 2018; Salat et al., 2019).

2.6. Blood analysis

Blood samples collected in heparin coated tubes were analysed for biochemical parameters. Only samples from animals that survived for the 24 h experiment were analysed. Following centrifugation, 100 μL were loaded into Health Checking Profile and measured in Chemistry Analyzer Pointcare® cV3 MNCHIP device. Health Checking Profile included blood urea nitrogen (BUN), creatinine, ratio BUN/creatinine, Alkaline Phosphatase, Alanine Transaminase (ALT), total bilirubin, calcium, phosphorus and creatin kinase (CK).

2.7. Histopathology

Brain, liver, spleen, heart, lungs, stomach, intestine and kidney of mice were collected at the end of the *in vivo* experiments. They were fixed with Bouin's fixative for 24 h. After that organs were transferred to 70% ethanol, dehydrated and paraffin wax embedded. Sections of 3–4 μm thick were stained with Haematoxylin and Eosin.

2.8. Human cell cultures

Human embryonic kidney cell line (HEK293) expressing the human voltage gated sodium channel alpha subunit 1.6 ($Na_v1.6$) was kindly provided, under a material transfer agreement, by Dr Andrew Powell (GlaxoSmithKline R&D, Stevenage, U.K.). Cells were cultured in DMEM/F12 medium supplemented with glutamax, MEM non-essential amino acids solution (Gibco, 1% w/v), 10% foetal bovine serum and 0.4 mg/mL geneticin (G418, Gibco). Cells were maintained at 37 °C with 95% humidity and 95% O_2/5% CO_2 atmosphere. Medium was replaced every 2–3 days and passage was performed weekly. Cells were placed at 30 °C to improve sodium channel expression one or two days before electrophysiological recordings.

2.9. Electrophysiology

Cells of passages 8–12 previously seeded on glass coverslips, were placed in a recording chamber with 0.5 mL extracellular solution containing (in mM): 154 NaCl, 5.6 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 5.6 glucose (pH was adjusted to 7.4 prior to use). Recording electrodes were fabricated with borosilicate glass microcapillaries (1.5 outer diameter) and had resistances ranging from 5 to 10 MΩ, filled with an intracellular pipette solution that contained (in mM): 140 CsF, 10 EGTA, 10 HEPES, 5 NaCl, 2 $MgCl_2$, pH adjusted to 7.3 with CsOH. Cells were maintained at a holding potential (V_{hold}) of −55 mV. Experiments were initiated 5–10 min after establishing the whole-cell configuration to ensure adequate equilibration between the internal pipet solution and the cell interior. Voltage-gated sodium currents were recorded in HEK293 $Na_v1.6$ cells using the whole-cell voltage-clamp mode, at room temperature (20–24 °C) in a computer-controlled current and voltage clamp amplifier (Multiclamp 700B, Molecular Devices) and the Digidata 1440A data acquisition system (from Axon Instruments, California, U.S.A.). Signals were sampled at 50 kHz after low pass Bessel filtering at 10 kHz and analysed offline, using the pClamp 10 software (Axon Instruments). Compensation circuitry was used to reduce the series resistance error by at least 70%. PbTx3 was firstly diluted in ethanol, then intermediate concentrations were prepared in the extracellular solution. Afterwards, it was added to the chamber to reach the final concentration in a cumulative manner. The concentrations tested were: 0.1, 1, 5, 10, 15, 35, 50 and 100 nM PbTx3. The effect of PbTx3 on sodium channels was evaluated after 5 min exposure of the cells to the toxin. Control consisted on the solvent (ethanol) being prepared and tested in the same way as PbTx3. To record the activation of voltage-gated sodium currents (I_{Na}), maintaining the holding potential at −55 mV, voltage steps from −80 to 80 mV were applied.

2.10. Statistical analysis

Data analysis was performed using Graphpad Prism 5.0. Data was firstly analysed to assess homoscedasticity and normal distribution of the data set. When these conditions were met, one-way ANOVA and Bonferroni multiple comparison tests were conducted, whereas, Kruskal-Wallis non-parametric test along with Dunn's multiple comparison test were performed when the conditions were not met. Hanging grid test, cylinder test and cold plate test data were also analysed for normal distribution, if that was the case, two-way ANOVA for repeated measures was performed, whereas Friedman test was conducted otherwise. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Clinical observations of PbTx3 treatment in mice

Up-and-Down procedure was performed for PbTx3 administered via oral and via intraperitoneal injection to mice (Fig. 1).

It should be noted that instant irritability was triggered immediately after PbTx3 intraperitoneal administration at all tested doses. Conversely, this behaviour was not developed in any control mice. Aside from this fact, first clinical signs were observed within the first 5 min and 30 min of administration intraperitoneal and oral, respectively. Orbital tightening and pilocrection as general indicators of animal discomfort were observed independently of the toxin administration route and in most of the doses tested (Table 1). In oral and intraperitoneal administrations of PbTx3, neuromuscular manifestations of intoxication comprised ataxia, tremors, Straub tail, seizures, convulsions, involuntary muscle contraction, and different degrees of limb paralysis. It should be noted that stereotypies (i.e., licking cage's walls) and/or abnormal behaviours (i.e., vocalization) were commonly observed. More severe cases also presented with mouth foaming and dyspnoea. Doses of 4000 μg/kg bw oral and 935 μg/kg bw PbTx3 intraperitoneal were additionally tested to complete the assay with high doses of toxin. It should be noted that two animals were tested for the 4000 μg/kg bw oral, from which one died. Despite the severity of the clinical signs, mortality was only observed at the 4000 μg/kg bw PbTx3 administered by oral gavage and at the highest doses tested via intraperitoneal (875, 935 and 1000 μg/kg bw).

Even though, the clinical signs were similar between groups, there is a clear difference in recovery (Fig. 2). Score, obtained from the number of clinical signs present, varied between 3 and 5 during the first hours, which gradually improved up to 24 h and recovery was achieved for 1000 μg/kg bw PbTx3 oral treatment. Conversely, intraperitoneal treatment reached its peak within 2 h after toxin administration,

Table 1
Clinical signs of mice up to 24 h from PbTx3 oral administration.

	Control	PbTx3 (μg/kg bw)							
		10	100	500	750	1000	1500	1750	1875	4000
Orbital tightening	1/7	2/3	2/3	3/3	3/3	3/3	5/5	6/7	9/9	1/2
Pilocrection	2/7	2/3	3/3	3/3	3/3	3/3	5/5	6/7	9/9	1/2
Lordosis	2/7	2/3	3/3	3/3	3/3	3/3	4/5	6/7	9/9	1/2
Ataxia	0/7	0/3	1/3	3/3	3/3	3/3	5/5	7/7	9/9	2/2
Seizures	0/7	0/3	0/3	0/3	1/3	2/3	4/5	4/7	5/9	1/2
Stereotypies and/or abnormal behaviour	0/7	0/3	0/3	0/3	2/3	2/3	3/5	6/7	4/9	1/2
Tremors	0/7	0/3	0/3	0/3	0/3	1/3	0/5	4/7	2/9	1/2
Convulsions	0/7	0/3	0/3	0/3	0/3	0/3	2/5	1/7	1/9	1/2
Apathy	0/7	0/3	0/3	0/3	1/3	3/3	2/5	6/7	8/9	2/2
Staub tail	0/7	0/3	0/3	0/3	0/3	2/3	1/5	2/7	3/9	1/2
Dyspnoea	0/7	0/3	0/3	0/3	0/3	1/3	3/5	2/7	0/9	1/2
Abnormal movement	0/7	0/3	0/3	3/3	3/3	3/3	5/5	7/7	8/9	2/2
Mortality	0/7	0/3	0/3	0/3	0/3	0/3	0/5	0/7	0/9	1/2

Number of mice that presented the clinical sign per total animals is shown.

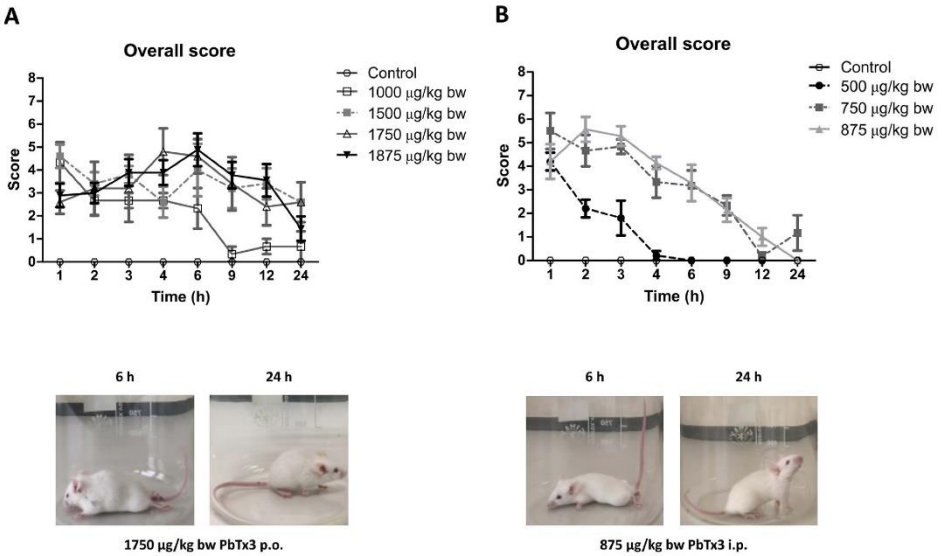

Fig. 2. Clinical signs' assessment in mice treated via oral (A) and intraperitoneal (B) with PbTx3. The overall score is obtained from the number of clinical signs that were observed at each timepoint. This includes the following clinical signs: convulsions, seizures, Straub tail, tremors, ataxia, dyspnoea, abnormal movement, apathy and stereotypies and/or abnormal behaviour. Mean ± SEM is displayed for each dose and time point. No score is presented for 935 and 1000 μg/kg bw PbTx3 intraperitoneal since animals did not survive for the first hour. Representative images of mice treated with 1750 μg/kg bw oral (A) and 875 μg/kg bw intraperitoneal PbTx3 (B) at 6 and 24 h are displayed in the bottom line.

followed by a steep improvement until 24 h with recovery of mice treated with the lowest doses. PbTx3 highest doses (935 and 1000 μg/kg bw intraperitoneal) did not survived for the first hour of treatment.

It was of interest to evaluate whether PbTx3 poisoning caused altered physiological functions in mice. Subsequently, biochemical blood testing was performed (Table 2). Note the fact that only blood of animals that survived the 24 h assay was analysed. This resulted in no significant differences between PbTx3 treated mice (either by oral or intraperitoneal administration) and controls in any of the parameters measured, suggesting no liver, renal or severe muscular damage at this time in any of the treatments and administration routes used. Even though blood concentration of alanine aminotransferase (ALT) is elevated at oral 1875 μg/kg bw PbTx3, it might suggest that PbTx3 could induce some degree of alteration in the liver.

Likewise, histopathological evaluation of cerebellum, spleen, liver, stomach, small intestine and kidneys of mice treated with PbTx3 orally (Fig. 3) or i.p. (Fig. 4) revealed no morphological alterations. No inflammation, necrotic areas, nor abnormal sizes or loss of normal structure was observed in any case.

3.2. Functional testing in PbTx3 treated mice

Taking into consideration the severity of the neuromuscular symptoms reported in NSP outbreaks, we decided to assess the muscular strength of PbTx3 treated mice performing the functional tests (Fig. 5). Before PbTx3 administration, basal response in the hanging grid test resulted in mice not falling off the grid within 60 s, i.e., the latency to fall is the cut-off threshold: 60 s. We recorded a decreased latency to fall in mice at 6 h after toxin administration in all oral (Fig. 5A) and intraperitoneal tested doses (Fig. 5B). Some differences could be highlighted

Table 2
Blood parameters of oral or intraperitoneal PbTx3 treatment harvested at 24 h.

Blood parameter	Control (oral) (n = 6)	Oral PbTx3 (μg/kg bw)					Control (i.p.) (n = 6)	Intraperitoneal PbTx3 (μg/kg bw)		
		1000 (n = 2)	1500 (n = 5)	1750 (n = 4)	1875 (n = 9)	4000 (n = 1)		500 (n = 5)	750 (n = 6)	875 (n = 7)
Hepatic										
Alkaline phosphatase (IU/L)	145 ± 12.2	164	129 ± 44.7	172 ± 32.0	149 ± 8.6	195	149 ± 18.6	179 ± 27.0	186 ± 23.7	156 ± 20.8
ALT (IU/L)	52.0 ± 9.7	28.5	38.6 ± 9.9	45 ± 13.1	221 ± 160	34	56.5 ± 7.91	365 ± 284	88.3 ± 18.6	60.0 ± 13.6
Total bilirubin (mg/dL)	0.37 ± 0.12	0.17	0.36 ± 0.07	0.22 ± 0.02	0.45 ± 0.09	0.22	0.32 ± 0.10	0.50 ± 0.14	0.3 ± 0.1	0.44 ± 0.05
Renal										
BUN (mg/dL)	16.2 ± 0.8	15.0	17.6 ± 3.7	18.4 ± 4.0	19.5 ± 1.6	13	17.8 ± 2.0	21.2 ± 3.1	17.3 ± 2.0	18.2 ± 2.3
Creatinine (mg/dL)	0.52 ± 0.07	0.59	0.58 ± 0.09	0.73 ± 0.14	0.59 ± 0.06	0.35	0.66 ± 0.11	0.63 ± 0.10	0.70 ± 0.1	0.64 ± 0.07
Ratio BUN/creatinine	34.3 ± 5.2	33.0	30.4 ± 6.7	27.2 ± 5.9	36.3 ± 4.6	37	30.0 ± 6.1	37.0 ± 6.2	27.7 ± 4.5	29.7 ± 4.6
Calcium (mg/dL)	10.5 ± 0.31	10.5	8.5 ± 2.08	10.1 ± 0.23	9.8 ± 0.38	10.1	10.7 ± 0.25	9.6 ± 0.26	10.4 ± 0.2	9.4 ± 1.21
Phosphorus (mg/dL)	11.0 ± 0.98	10.4	7.1 ± 2.38	12.0 ± 0.95	9.9 ± 0.78	7.2	8.5 ± 0.59	8.0 ± 0.29	9.7 ± 1.2	8.6 ± 0.53
Muscular and hepatic										
CK	934 ± 508	334	702 ± 138	774 ± 318	393 ± 82	293	446 ± 214	408 ± 182	864 ± 216	236 ± 47

ALT: alanine aminotransferase; PbTx3: brevetoxin 3; BUN: blood urea nitrogen; CK: creatine kinase. Mean ± SEM is displayed.

between the two routes of administration at the end of the experiment. High variability was observed at 23 h of treatment in oral administration displaying no recovery of basal performance in the hanging grid test (Fig. 5A). However, at this time mice that received 500 or 750 μg/kg bw PbTx3 i.p. did not fall off the grid whereas mice with 875 μg/kg bw PbTx3 resulted in increased latency to fall (Fig. 5B). In order to assess the activity of mice, the cylinder test was carried out (Fig. 5C and D). In this case, we measured the times the animal would stay on its rear limbs. The results obtained were similar to those of the hanging grid test: after 6 h of oral (Fig. 5C) and intraperitoneal (Fig. 5D) treatment there is a sharp reduction in the number of rearing, which improves by the end of the assay. An oral dose as high as 4000 μg/kg bw PbTx3 is needed to completely suppress the ability to rear at 6 h (Fig. 5C), whereas, 750 μg/kg bw PbTx3 intraperitoneal is enough to obtain similar outcome (Fig. 5D).

3.3. Oral administration of medium-low PbTx3 doses

Taking into consideration the high doses achieved to induce death by oral administration of PbTx3, it was of interest to assess the Lowest-Observed-Adverse-Effect-Level (LOAEL) and No-Observed-Adverse-Effect-Level (NOAEL). To do so a 24 h medium-low dose response assay was performed, testing 10, 100, 500 and 750 μg/kg bw PbTx3 (Fig. 6). No deaths were recorded in this assay, but doses of 100 μg/kg bw PbTx3 or higher still caused poisoning clinical signs (Fig. 6A). Mice that received 10 μg/kg bw PbTx3 displayed clinical signs that could indicate general discomfort but not poisoning since they were also detected in some controls (Table 1). This was also reflected in the functional tests. In hanging grid test, significant differences were only observed for 750 μg/kg bw at 6 h, whereas all animals performed as in basal conditions at the end of the experiment (Fig. 6B). More variability is shown for the number of rearing in the cylinder test: no differences between treatments were observed as well as no changes were detected in different times of testing (Fig. 6C). With the cold plate test we detected sensitivity alterations, significantly lower times of hindpaw lifting were recorded for 750 μg/kg bw at 6 h when compared to baseline, though it was recovered at 24 h (Fig. 6D).

3.4. Characterization of PbTx3 effect on $Na_v1.6$

PbTx3 binds to voltage-gated sodium channels (Na_v) leading to NSP. However, few studies have evaluated PbTx3 effects on Na_v α isoforms (Finol-Urdaneta et al., 2023). Since seizures, ataxia and muscular dysfunction has been related to gain-of-function of $Na_v1.6$ (Zybura et al., 2021), we evaluated the effects PbTx3 could induce in this isoform (Fig. 7). Electrophysiological recording in $Na_v1.6$ transfected cells was performed. PbTx3 distribution in rats after oral treatment show that around 0.25% of the given dose was detected in brain at 24 h (Cattet and Geraci, 1993). Thus, we estimated that 1000 μg/kg bw PbTx3 oral would roughly correspond to 0.05 μg PbTx3 (0.056 nmol) in the brain. This dose elicited clinical signs though no death in mice, so 100 nM was the maximum concentration tested in whole-cell patch clamp. PbTx3 exerts a mild effect on $Na_v1.6$ currents (Fig. 7A), whereas there are significant differences in activation voltage (Fig. 7B). Even at low doses, PbTx3 shifts the activation voltage towards more negative potentials.

3.5. Brevetoxin 3 oral and intraperitoneal acute toxicity

Acute toxicity was assessed from the Up-and-Down procedure. No animal lethality was observed with oral administration of PbTx3 up to 1875 μg/kg bw PbTx3 (level 4). Consequently, we evaluated the effects of the high dose of 4000 μg/kg bw PbTx3 from which half of the animals did not survive the end of the assay (Table 3). As indicated by the clinical signs, intraperitoneal administration displays higher toxicity than oral treatment. In the acute toxicity study, single intraperitoneal administration of 1000 μg/kg PbTx3 produced 100% mortality, meanwhile all 500 or 750 μg/kg bw treated-mice survived. An additional level with a dose of 935 μg/kg bw PbTx3 was performed in order to increase the robustness of the assay and found 100% mortality. The OECD guidelines state that (i) when only one dose in which some animals died and others live; (ii) when all animals tested with higher doses died, and (iii) when all animals tested with lower doses were alive; then the LD_{50} is the common dose (OECD, 2022). Since 875 μg/kg bw PbTx3 was the only common dose of dead and live animals, this is the LD_{50} (Table 3).

4. Discussion

On average, worldwide shellfish consumption is estimated to be of

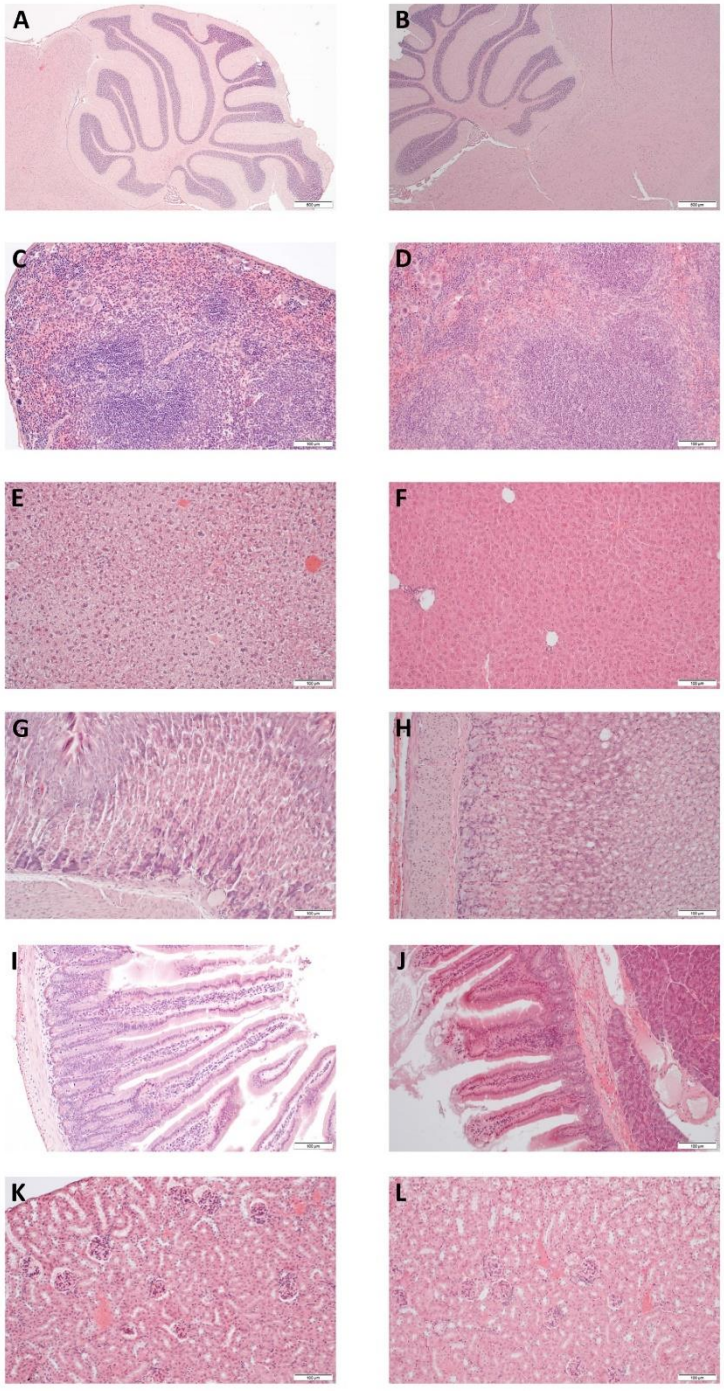

Fig. 3. Representative images of histology of cerebellum (A,B), spleen (C,D), liver (E,F), stomach (G,H), small intestine (I,J) and kidney (K,L) of control (A,C,E,G,I,K) and 4000 μg/kg bw PbTx3 treated-mice (B,D,F,H,J,L) by oral gavage.

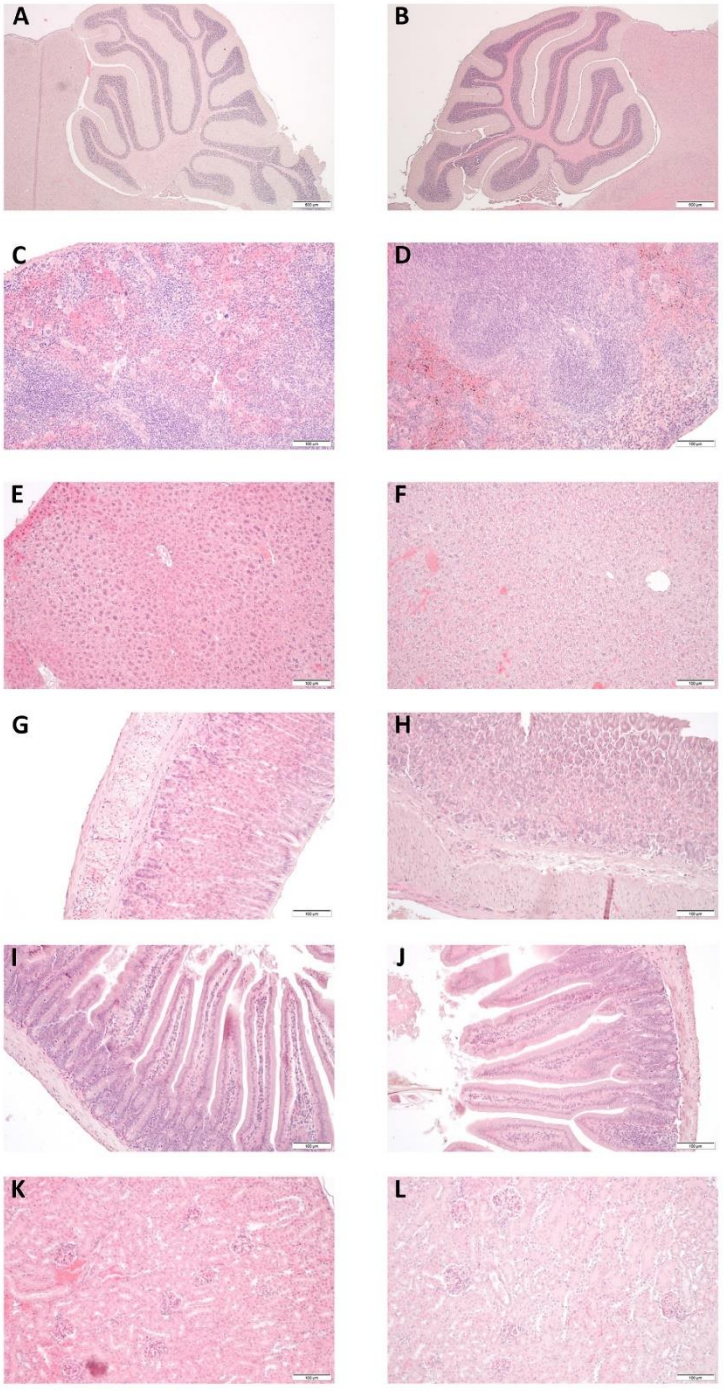

Fig. 4. Representative images of cerebellum (A,B), spleen (C,D), liver (E,F), stomach (G,H), small intestine (I,J) and kidney (K,L) of control (A,C,E,G,I,K) and 875 μg/kg bw PbTx3 treated-mice (B,D,F,H,J,L) by intraperitoneal injection.

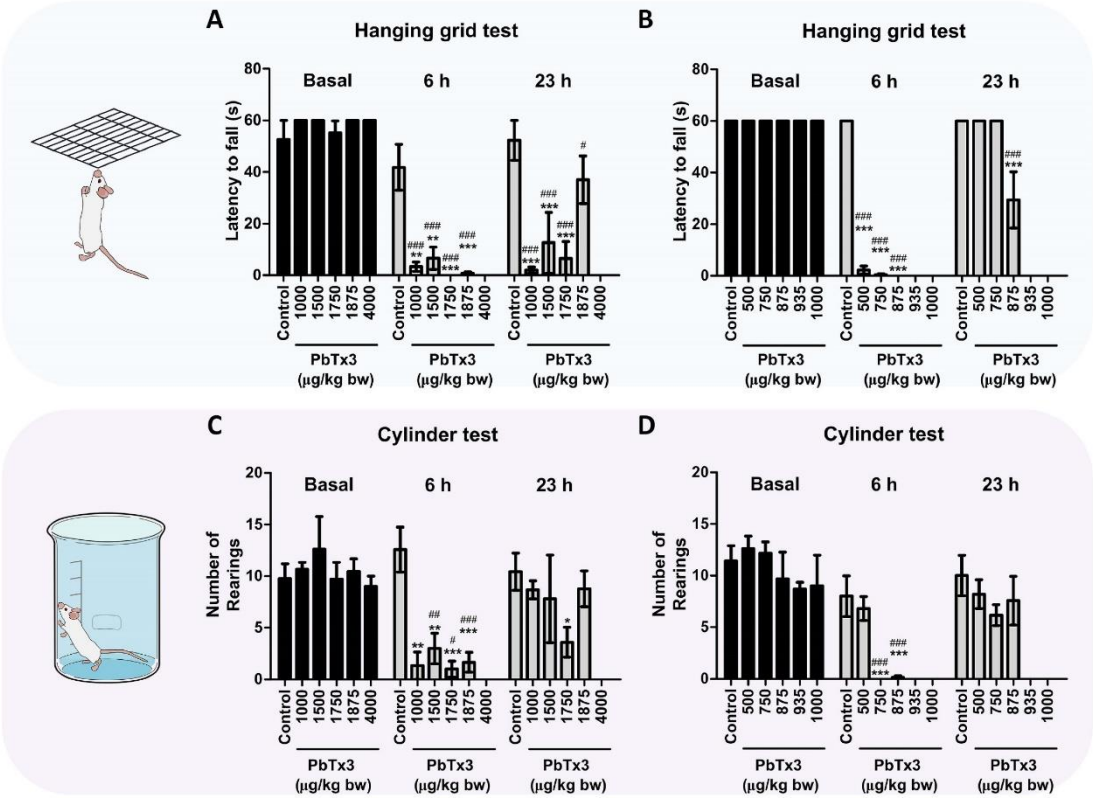

Fig. 5. Functional tests (hanging grid and cylinder test) performed in mice treated with PbTx3 administered orally or intraperitoneally. Latency time to fall (in seconds) of mice in the hanging grid test for (A) oral and (B) intraperitoneal treatments. Number of times the animal would stay on its rear limbs (number of rearing) in the cylinder test in (C) oral and (D) intraperitoneal administration. In both test mice were recorded before PbTx3 administration (basal), at 6 h and 23 h after PbTx3 administration. Mean ± SEM is displayed. Statistical analysis was performed by two-way ANOVA for repeated measures and Bonferroni Multiple Comparison test. Significant differences with basal conditions are indicated like $\#P < 0.05$, $\#\#P < 0.01$, or $\#\#\#P < 0.0001$. Significancy when compared to control of the same time is indicated like ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). No data is displayed for 935 and 1000 µg/kg bw PbTx3 intraperitoneal at 6 or 23 h since they did not survive for the first hour.

2.53 kg per capita annually. Globally, Asia has been the largest mollusc consumer with an estimation of 3.62 kg per capita in 2020 (FAO, 2020). In the European Union, consumption of in-between 200 g and 4 kg per capita annually has been estimated (Hough, 2022). Not only has the consumption of shellfish raised since late twentieth century, but it is also expected to increase in the following years (FAO, 2022). On the other hand, the Intergovernmental Panel on Climate Change expects a growing trend in the frequency of harmful algal blooms (Bindoff et al., 2019). For these reasons, monitoring of hazardous compounds is essential to protect seafood consumers health. Currently, toxins like PbTxs are not regulated in countries where potentially toxic dinoflagellates and raphidophytes are present such as Greece or France (Zingone et al., 2021; Liang et al., 2022). Conversely, legal limits are set in countries where NSP outbreaks have occurred like U.S.A. or New Zealand (FDA, 2022; Hough, 2022). The value of 20 Mouse Units/100 g shellfish meat or 800 µg PbTx2 equivalents/kg shellfish is the regulatory limit in the U.S.A, Mexico, New Zealand and Australia (FAO/WHO, 2016). Due to the fact that NSP is caused by the consumption of brevetoxin-contaminated shellfish *in vivo* toxicological data of oral administration is essential in performing risk assessment. Still, scarce toxicology data have been reported about PbTxs. The doses we tested in mice, would roughly correspond to a range from a consumption of 7–56 µg PbTx3 in a large portion of 400 g shellfish ((EFSA) 2010b; Lawrence et al., 2011).

Our results provide an in-depth assessment of the clinical signs developed and neuromuscular functioning after oral and intraperitoneal treatments in mice. Not only this enables the capability to characterise PbTx toxicology, but also highlights the fast recovery even at elevated doses. It should be noted that when the toxin was administered via oral, only the very high dose of 4000 µg/kg bw PbTx3 was able to induce death, whereas lower doses resulted in death for intraperitoneal administration. This indicate that PbTx3 administered intraperitoneal to mice is more than 4.5 times more toxic than via oral. Although the clinical signs observed are similar independently of the administration route (mainly comprising neuromuscular malfunction/dysfunction such as ataxia, stereotypies, Straub tail, or convulsions) their onset and duration are different. Intraperitoneal administration of PbTx3 resulted in a peak of clinical signs within the first 2 h, whereas after that time a constant recovery started. Oral administration induced rather steady severity for the first 6 h, though slow recovery also happens for medium dosages. More severe clinical signs like convulsions were not present in mice treated with doses below 1000 µg/kg bw, whereas 100 µg/kg bw

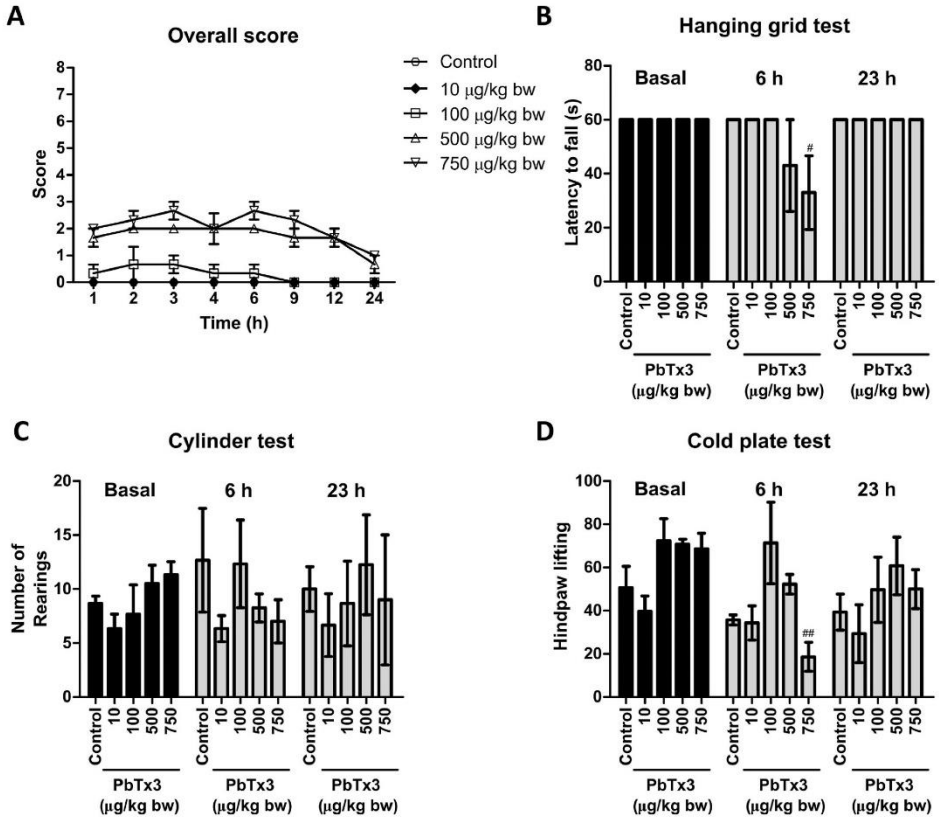

Fig. 6. Dose-response of oral medium-low PbTx3 for 24 h. (A) Clinical signs' overall score obtained from the number of clinical signs that were observed: convulsions, seizures, Straub tail, tremors, ataxia, dyspnoea, abnormal movement, apathy and stereotypies and/or abnormal behaviour. (B) Latency time to fall in the hanging grid test. (C) Number of rearing counted in the cylinder test. (D) Times of hindpaws lifting in the cold plate test. Mean ± SEM is shown. Two-way ANOVA for repeated measures and Bonferroni Multiple Comparison test was performed. Significant differences with basal conditions are indicated like ##$P < 0.01$. Significancy when compared to control of the same time is indicated like *$P < 0.05$.

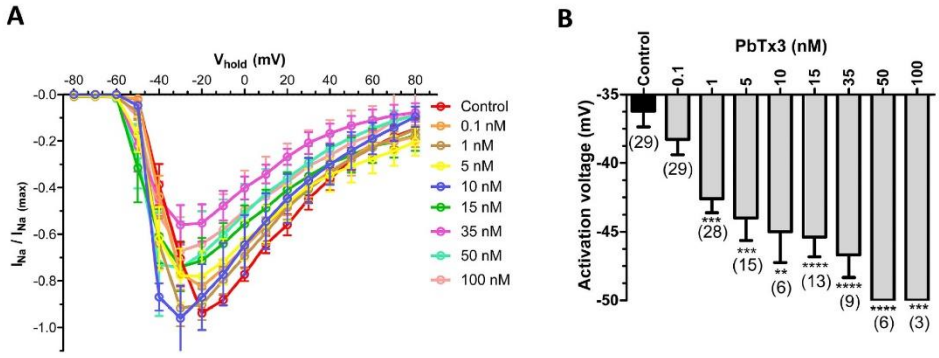

Fig. 7. Characterization of PbTx3 effects on voltage-gated sodium channels 1.6. (A) Relative sodium current at different holding voltage. (B) Activation voltage at increasing concentrations of PbTx3. Mean ± SEM is displayed. Sample size is indicated in within brackets. Statistically significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$) with control is stated by asterisks. I_{Na}: sodium current; V_{hold}: holding voltage.

Table 3
Mice mortality after PbTx3 administered via oral and intraperitoneal.

PbTx3 (μg/kg bw)	Mortality
Oral administration	
1000	0/3
1500	0/5
1750	0/7
1875	0/9
4000	1/2
Intraperitoneal administration	
500	0/5
750	0/7
875	2/9
935	3/3
1000	3/3

still displayed poisoning clinical signs like ataxia. However, same dose performs as baseline at 6 h of treatment in the functional tests. It is worth mentioning that changes in cold sensitivity were only significant for 750 μg/kg bw at this time. Still, we report decreased strength (hanging grid test) and rearing (cylinder test) at 6 h when compared to near normal results at 24 h for intraperitoneal treatment. Even though similar performance in the hanging grid test and the cylinder test was observed at 6 h after oral administration, strength was not regained by the end of the experiment. This is accordance with Neurotoxic Shellfish Poisoning symptomatology and no-reported fatalities registered during outbreaks (Arnich et al., 2021) even though several reports have documented mass mortality in marine mammals and fish populations due to brevetoxicosis (Twiner et al., 2012). This could be due to the combination of several toxins rather than one analogue.

Previous studies assessing radiolabelled PbTx3 toxicokinetic in rats reported that at 24 h the liver accounted for the organ that accumulated most PbTx3, followed by gastrointestinal tract, heart and kidney (Cattet and Geraci, 1993). However, we show that biochemical blood parameters analysis for liver, kidney and muscular function revealed no significant alteration even at high doses. Besides, histological evaluation showed no organ damage at 24 h of treatment. Previous histopathological findings vary depending on the animal, dose, route of administration and time of exposure. For instance, PbTx3 inhalation in rats resulted in lung damage with no alteration in other organs after 22 days (Benson et al., 2005), while at 5 days no lesions in lung were reported (Benson et al., 2004). In this line, lesions detected in freshwater turtles could not be attributed to brevetoxin 3 due to presence of these lesions also in controls (Cocilova et al., 2017). On the contrary, mild lesions in liver and spleen have been reported in mice after i.p. administration of PbTx2 and PbTx6 at 8, 24 and 72 h (Walsh et al., 2003). A *Karenia brevis* bloom associated with mass mortality of manatees in Florida revealed microscopic damage in upper respiratory tract, lung and cerebellum (Bossart et al., 1998). Longer exposure to the toxin after oral administration might be needed to exert histopathological alterations.

NSP characteristic clinical signs are related to PbTxs mode of action: PbTxs bind to the site 5 of voltage-gated sodium channels (Na_v) resulting in the shift of the voltage activation threshold towards more negative potentials leading to channel opening and Na^+ entrance as well as to a slowdown of channel inactivation (Wu and Narahashi, 1988; Mackieh et al., 2021). There are 9 isoforms of the α subunit which are expressed differently throughout the body. Among them, $Na_v1.6$ gain-of-function and hyperexcitability have been related to epilepsy, ataxia and dystonia (Zybura et al., 2021). Our results show that even if sodium currents were only mildly modulated by PbTx3, voltage activation was shifted towards more negative potential in $Na_v1.6$ channels. In accordance, previous reports have evaluated PbTx binding to $Na_v1.2$, $Na_v1.4$, $Na_v1.5$ subtypes (Konoki et al., 2019). $Na_v1.2$ is expressed in central and peripheral nervous systems,; whereas expression of $Na_v1.4$, and $Na_v1.5$ is localized to skeletal muscle, and heart, respectively (Konoki et al., 2019; Wang et al., 2017). Increased dissociation constant for $Na_v1.5$ when compared to $Na_v1.2$ and $Na_v1.4$ could explain the severeness of mobility and seizures observed *in vivo*, as well as the high doses needed to cause mortality following oral treatment. Similarly, fish and rat preparations of brain, skeletal and cardiac muscles show that rat heart muscle preparations are resistant to PbTx3 (Dechraoui et al., 2006). Likewise, mice with severely impaired mobility that achieve full recovery at 24 h could be the result of PbTx3 metabolism and/or dissociation from these channels.

Based on the Up-and-Down procedure results, we determined a LD_{50} of 875 μg/kg bw PbTx3 intraperitoneal. This value is well above previous studies, where an LD_{50} of 170 μg/kg bw PbTx3 (Baden and Mende, 1982) and 250 μg/kg bw PbTx3 (Selwood et al., 2008) have been described. Likewise, due to the high doses needed to cause death after PbTx3 oral administration, only intraperitoneal LD_{50} can be align to mouse units (MU). MU is the amount of extract that kills 50 % of mice (20 g) in 930 min. Since LD_{50} is of 875 μg/kg bw PbTx3, 43.7 μg PbTx3 would be equivalent to one MU. This also reflects the variation with prior studies (Baden and Mende, 1982; Arnich et al., 2021). The use of an extract (Baden and Mende, 1982) or a semisynthetic product (Selwood et al., 2008) to perform the assessment compared to the purified toxin (purity 95%) that we used could account for the discrepancies observed. It should be noted that during harmful algal blooms, not only one analogue is produced. Instead, a combination of several compounds causes the human and faunal intoxication episodes (Ishida et al., 2004; Hort et al., 2021). Even though, one analogue *per se* might not be as toxic as PbTx3, lower concentrations of other compounds can have a synergistic effect. However, there are no studies assessing the combination of at least two PbTx analogues. On the other hand, the solvent used in each study could also be partly accounted for some degree of variation (Bartsch et al., 1976). We dissolved the toxin first in ethanol and then in saline solution, whereas Baden and Mende (1982) dissolved PbTx3 in corn oil instead of ethanol, and Selwood et al. (2008) used the same solvents with the addition of 1% Tween-60 in the saline solution. Moreover, still found toxicity clinical signs at low PbTx3 doses, therefore we could stablish an oral LOAEL for PbTx3 of 100 μg/kg bw in mice and an oral NOAEL of 10 μg/kg bw. Food and Agriculture Organization, ANSES and European Food Safety Authority stated that to confirm toxicity values longer exposure time as well as the effect on male mice should be evaluated ((EFSA) 2010a; FAO/WHO, 2016; ANSES, 2021.

5. Conclusions

The present study provides novel toxicology data on the emerging toxin PbTx3 with a purity of 95%. Oral and intraperitoneal administration results in neuromuscular clinical signs like ataxia, seizures and loss of limb's strength. Moreover, fast recovery can be observed, as well as no blood biochemical alterations or histopathological changes at the end of the experiment. Based on these results, we could stablish an oral LOAEL for PbTx3 of 100 μg/kg bw and a NOAEL of 10 μg/kg bw, which can aid policy makers when faced with important decisions regarding the risks of PbTxs for human health.

CRediT authorship contribution statement

Celia Costas: Formal analysis, Investigation, Methodology, Visualization, Roles, Writing – original draft, Writing – review & editing. **M. Carmen Louzao:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Roles, Writing – original draft, Writing – review & editing. **Sandra Raposo-García:** Investigation, Roles, Writing – original draft, Writing – review & editing. **Carmen Vale:** Formal analysis, Roles, Writing – original draft, Writing – review & editing. **Almudena Graña:** Investigation. **Cristina Carrera:** Formal analysis, Resources. **José Manuel Cifuentes:** Investigation, Resources. **Natalia Vilariño:** Formal analysis. **Mercedes R. Vieytes:** Conceptualization, Supervision. **Luis M. Botana:** Conceptualization, Funding acquisition, Supervision, Writing –

review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Glossary

ALT: Alanine Transaminase
BUN: blood urea nitrogen
bw: body weight
CK: creatin kinase
LOAEL: Lowest-Observed-Adverse-Effect-Level
NOAEL: No-Observed-Adverse-Effect-Level
NSP: Neurotoxic Shellfish Poisoning; PbTx, brevetoxin

5 DISCUSIÓN

La presencia y distribución global de ficotoxinas de origen marino representa una constante amenaza para la salud pública. En consecuencia, las autoridades competentes han establecido programas de monitorización de microalgas productoras de toxinas marinas en el agua, así como de la concentración de toxinas en moluscos bivalvos para garantizar la protección del consumidor (EU 2004; EU 2021). Los límites legales permitidos en moluscos bivalvos están recogidos en las directivas de la Unión Europea (EU 2004; EU 2021), para las toxinas diarreicas actualmente son de 160 µg equivalentes de AO/kg de molusco. Dentro del grupo de las toxinas diarreicas se incluyen el ácido okadaico y sus análogos: las dinofisistoxinas 1 y 2, así como sus derivados acil-ésteres que se agrupan bajo el nombre de dinofisistoxina 3 (EFSA 2008). Los perfiles de toxinas encontrados en moluscos varían según la especie productora y la región. En el caso de las rías gallegas, se encuentran con mayor frecuencia el ácido okadaico y la dinofisistoxina 2, mientras que la dinofisistoxina 1 se detecta de forma anecdótica (Blanco et al. 2019). De hecho, en el periodo 2014-2019 el ácido okadaico fue la toxina que causó más cierres de zonas de cultivo en batea (Blanco et al. 2019; INTECMAR 2021). Aunque es difícil evaluar el impacto económico que tienen estos cierres, sí es importante destacar que hay zonas de cultivo que se mantienen cerradas durante 200-270 días al año (Blanco et al. 2019; INTECMAR 2021).

Pese a que la estructura del AO, DTX1 y DTX2 sólo difiere en la presencia de un grupo metilo en las posiciones 31 y 35, se han documentado importantes diferencias en cuanto a la potencia de los distintos análogos. Esta divergencia se refleja en los Factores de Equivalencia Tóxica (TEF), que se definen como "el ratio de toxicidad de un compuesto químico que comparte mecanismo de acción con el compuesto de referencia de ese mismo grupo" (FAO/WHO 2016). En las DSPs, el ácido okadaico es el compuesto de referencia. La Autoridad Europea en Seguridad Alimentaria (*European Food Safety Authority*, EFSA) se basó en la Dosis Letal 50 (DL_{50}) obtenida al administrar cada análogo por vía intraperitoneal en ratón para establecer los factores de equivalencia tóxica de las DSPs. Como resultado se obtuvieron unos Factores de Equivalencia Tóxica que indicaban una toxicidad similar entre el AO y la DTX1, mientras que la DTX2 resultaba ser un 40% menos tóxica (EFSA 2008). Es decir, los Factores de Equivalencia Tóxica fueron de 1 para el AO y la DTX1 y de 0,6 para la DTX2 (EFSA 2008). No obstante, debido a que la vía de intoxicación por DSPs en personas es por consumo de moluscos bivalvos, hay estudios de toxicidad oral resultan cruciales. Recientemente se obtuvieron los valores de DL_{50} por vía oral para las 3 toxinas y se propusieron unos TEF de 1 para el AO, 1,5 para la DTX1 y de 0,3 para la DTX2 (Abal et al. 2017; Abal et al. 2018). Esta información toxicológica facilita la determinación de los riesgos sanitarios que supone la ingestión de cada análogo y tiene relevancia de cara a establecer límites que protejan al consumidor. Sin embargo, también es importante conocer su toxicocinética.

En la presente tesis doctoral se compara la distribución AO, DTX1 y DTX2 en el organismo en un estudio de toxicidad aguda por vía oral utilizando un procedimiento de *Up & Down* de 4 niveles. Durante las primeras horas, síntomas indicativos de dolor y malestar genéricos como piloerección y apatía se observaron con elevada incidencia en el caso de las tres toxinas diarreicas. De todos los síntomas, la diarrea resultó el más distintivo, mientras que los animales tratados con dosis más altas (por encima de 750 μg/kg AO, 275 μg/kg DTX1 y 2000 μg/kg DTX2) también llegaban a desarrollar cianosis y disnea. Estudios previos sugieren una pronta aparición de síntomas que depende de la dosis administrada, aunque la sintomatología varía con el ensayo. Por ejemplo, se han documentado casos en los que una dosis oral de 750 μg/kg AO en ratones ICR macho les produce apatía y diarrea en las primeras 3 h (Wang et al. 2012). En otro estudio, donde se evaluaron un rango de dosis entre 600-1140 μg/kg AO administrado por vía oral describen síntomas a partir de las 2 h de apatía y tasa respiratoria elevada, mientras que especifican que no han observado diarrea (Aune et al. 2012). Por el contrario, se ha reportado que en ensayos en los que se administran desde 115 μg/kg hasta 1341 μg/kg AO por vía oral, la dosis más baja que desarrolla diarrea es a 435 μg/kg (Le Hegarat et al. 2006). En cuanto a dosis inferiores, se ha observado que 90 μg/kg AO induce diarrea en ratón, aunque aparece transcurridas 8 h de la administración (Matias et al. 1999). Si bien parece haber una cierta consistencia en cuanto a que el ácido okadaico cause la diarrea en roedores, hay mucha variabilidad entre estudios, lo que puede deberse los distintos grados de pureza de la toxina, así como del modelo animal y de las dosis administradas. Además, se debe tener en cuenta que, en ocasiones, los síntomas que causa la toxina generalmente se han descrito con brevedad en estudios previos de toxicidad oral (Ito et al. 2000; Ito et al. 2002; Tubaro et al. 2003; Le Hegarat et al. 2006; Aune et al. 2012).

Una vez finalizado el estudio de toxicidad oral aguda se realizan las necropsias de los ratones y la extracción de los distintos órganos y fluidos. El órgano en el que se detectó una mayor cantidad de toxina fue el estómago para los tres análogos, pese a que las dosis no eran las mismas en todas las ficotoxinas. Estos resultados son consistentes con un estudio previo en el que se reportó que el estómago del ratón es el órgano que más AO acumula transcurridas 24 h de la administración oral (Aune et al. 2012). Por el contrario, en otro estudio en el que administran dosis más bajas (50 y 90 μg/kg AO) se indica que el contenido intestinal es el que concentra mayor cantidad de toxina (Matias et al. 1999). Los resultados obtenidos en esta tesis permiten comparar la distribución de AO, DTX1 y DTX2, lo que resulta novedoso. Cuando se compara la misma dosis (1000 μg/kg), la cantidad que se detecta en el estómago de los animales tratados con DTX2 es muy inferior a la cuantificada en aquellos tratados con AO o DTX1. Este patrón se repite para los intestinos delgado y grueso, así como para el hígado, en todo caso las toxinas se acumulan en mayor cantidad en el intestino grueso que en el intestino delgado. En cuanto a la cantidad de toxina en los contenidos de estómago e intestinos delgado y grueso, sigue siendo el contenido estomacal el que concentra mayor cantidad de AO y DTX1. No obstante, si bien la cantidad de DTX1 es similar entre los contenidos de los intestinos delgado y grueso, la cantidad de AO es superior en el contenido del intestino grueso cuando se compara con el intestino delgado. Por el contrario, DTX2 sólo se detectó en el contenido estomacal de animales tratados con dosis por encima de 2250 μg/kg, mientras que a dosis inferiores sí que se detectó toxina en el contenido de intestino delgado. En cuanto al análisis de orina y heces recogidas durante el periodo experimental a distintos tiempos hasta las 24 h, permitió evaluar

la absorción y excreción de las toxinas. Continuando con la comparación de la misma dosis de toxina (1000 μg/kg), la cantidad de DTX2 eliminada en heces es de 4 veces la excretada de AO y DTX1, lo cual sugiere una eliminación más rápida de DTX2. Por otro lado, la poca cantidad de toxina detectada en orina indica una absorción limitada, aunque tampoco se puede descartar que no sea una ruta de eliminación principal. En consonancia, la cantidad detectada en sangre de AO y DTX1 se encuentra por debajo del 0,5% de la dosis administrada, lo que apoya una absorción limitada.

Teniendo en cuenta el porcentaje de toxina que se detectó en las muestras respecto a la dosis administrada, la variación entre toxinas DSPs fue notable. En el caso del AO, se detectó entre un 23% de la dosis de 1000 μg/kg. Por el contrario, el porcentaje de recuperación fue de un 32% en el caso de DTX1. Finalmente, el mayor contraste proviene de DTX2, donde sólo se recuperó un 2% en esa misma dosis. Esto indica diferencias en cuanto a la absorción de cada análogo. Un estudio previo en un modelo *in vitro* de epitelio intestinal, muestra que, tras 24 h, la absorción de DTX2 es limitada en comparación con la de DTX1 o AO (Fernandez et al. 2014). Por otro lado, en un estudio con dosis comparables de AO también observan que el tracto gastrointestinal concentra la mayor parte de la toxina, así como unas concentraciones muy bajas en sangre (Aune et al. 2012). La absorción limitada puede deberse a mecanismos de detoxificación en el epitelio intestinal que devuelvan la toxina al lumen (Ehlers et al. 2011). Además, concuerda con que a las 24 h se detecte en heces una concentración más alta de DTX2 que de AO o DTX1. Los resultados obtenidos en la presente tesis doctoral son especialmente relevantes ya que la información de toxicidad *in vivo* y datos farmacocinéticos reportados hasta ahora se centran en el AO como compuesto de referencia, mientras que la información sobre las toxinas DTX1 y DTX2 era escasa.

El AO inhibe las protein fosfatasas de Ser/Thr (PP) PP1, PP2A, PP4, PP5 y PP6 (Takai et al. 1992b; Brewis et al. 1993; Chen et al. 1994; Prickett and Brautigan 2006). Estas enzimas tienen una distribución ubicua, por lo que su inhibición lleva a un estado de hiperfosforilación en las células, que desencadena la alteración de una considerable variedad de rutas de señalización (Yadav et al. 2017). No obstante, se ha reportado que esta actividad no explica el efecto diarreico de estas ficotoxinas (Vilarino et al. 2008; Espina et al. 2010; Munday 2013). Así por ejemplo el metil okadato que tiene una afinidad 10 veces menor que el AO por la PP2A presenta una mayor citotoxicidad en hepatocitos primarios de rata que el AO (Espina et al. 2010). Si toxinas que tienen poca afinidad por las PP tienen un efecto tóxico mayor que el AO, la inhibición de las PP no explicaría los efectos tóxicos del AO en su totalidad. Otro argumento que sostendría esta hipótesis es que compuestos que inhiben a las PPs (microcistina, nodularina, tautomicina, caliculina, etc.) no desencadenan diarrea, un síntoma específico de la intoxicación por AO (Swingle and Honkanen 2019).

La Organización Mundial de la Salud define la diarrea como una pérdida de consistencia en las heces, así como un aumento en la frecuencia de las deposiciones (WHO 2017). Esto puede ser consecuencia de un desequilibrio de los mecanismos de absorción-secreción (reduciéndose la capacidad absortiva y/o aumentando la secreción al lumen intestinal) y/o una alteración en los patrones de motilidad intestinal (Moriya et al. 2010). La homeostasis de la absorción y secreción intestinal está estrechamente regulada por las células enteroendocrinas presentes en el epitelio intestinal, las células del sistema inmune, el sistema nervioso entérico y su comunicación con

el sistema nervioso central (Rao 2019). La rápida aparición de diarrea que observamos en nuestos estudios con las DSPs que coincidía con una detección de las toxinas en heces podría estar relacionado con una señalización nerviosa, lo que nos llevó a profundizar en la relación del mecanismo de acción del AO con su efecto *in vivo*.

Se han realizado algunos estudios previos con AO en los que el registro de la diarrea se limita a su presencia o ausencia (Ito et al. 2000; Ito et al. 2002; Tubaro et al. 2003; Le Hegarat et al. 2006; Aune et al. 2012), en la presente tesis doctoral caracterizamos en profundidad la diarrea causada por esta toxina. Así, administramos la toxina por vía oral al ratón y nos centramos en la diarrea como síntoma característico de las DSPs. Determinamos la dosis de AO a la que no se observan efectos adversos (*No-Observed-Adverse-Effect-Level*, NOAEL) en nuestro caso es de 50 μg/kg AO, lo que coincide con el NOAEL propuesto por la EFSA (EFSA 2008). Además, también reportamos 100 μg/kg AO como la dosis más baja a la que se observan efectos adversos *(Lowest-Observed-Adverse-Effect-Level* LOAEL), lo que está en línea con el LOAEL descrito en personas (Toyofuku 2006). Dosis iguales o superiores a 100 μg/kg AO indujeron una diarrea acuosa que podía darse en más de una ocasión. En un estudio en el que utilizan antagonistas de los receptores nicotínicos de acetilcolina para inhibir la secreción en el yeyuno del AO, muestran que efectivamente la secreción neta se reduce (Delbro and Lange 1997). Asimismo, el AO activa vías de señalización que inducen estrés oxidativo e inflamación en cultivos de células gliales entéricas (Reale et al. 2019). La familia del Neuropéptido Y (NPY) juega un papel relevante en la mediación de estos procesos (Vona-Davis and McFadden 2007). Esta familia incluye tanto al NPY como al Péptido YY (PYY) y ambos pueden actuar como hormonas o neuromoduladores. Son agonistas de los receptores tipo Y, que son receptores acoplados a proteínas G (El-Salhy et al. 2020). Debido a que la homología entre ambos neuropéptidos es alta, los dos pueden unirse y activar los receptores Y1 e Y2 que se expresan en células epiteliales y neuronas de los plexos submucoso y mientérico de los intestinos delgado y grueso (Mao et al. 1996; Cox et al. 2001; Wang et al. 2010). Tienen una gran importancia en el retraso del vaciado gástrico y son mediadores del freno ileal, así como inhiben las secreciones gástrica y pancreática. Además, estimulan la absorción de agua y electrolitos. Se ha demostrado que algunos fármacos que producen diarrea pueden alterar la expresión del NPY y del PYY (Moriya et al. 2010). Estudios con la toxina del cólera muestran que causa la hiperexcitabilidad de neuronas secretomotoras en el intestino, y que la infusión intraarterial del NPY reducía considerablemente la acumulación de fluido intestinal en gatos (Sjoqvist et al. 1988; Gwynne et al. 2009). Además, estudios *in vitro* muestran una reducción en la expresión del NPY en la línea celular de neuroblastoma SH-SY5Y tras el tratamiento con AO (Valdiglesias et al. 2012; Louzao et al. 2015). En consonancia con estos estudios *in vitro*, observamos en estudios *in vivo* que la concentración de NPY en intestino delgado se reduce después de 24 h de tratamiento, lo que podría explicar también la mayor acumulación de fluido en el intestino delgado. Sin embrago, cuando realizamos el pretratamiento con NPY, no hay una mejoría en cuando al tiempo de aparición de la diarrea causada por el AO, ni en la puntuación de la diarrea que nos indicaría la gravedad del síntoma y la acumulación de fluido en el intestino sólo se reduce ligeramente. Por otro lado, está descrito que el PYY inhibe la acumulación de agua en el intestino causada por la prostaglandina E2 y por el péptido intestinal vasoactivo en intestino humano (Roze et al. 1997; Moriya et al. 2010). Al igual que ocurría para el NPY, también observamos una reducción de la concentración del PYY en intestino delgado tras 24 h de la

administración oral del AO. Sin embargo, esta disminución del PYY no se detecta transcurridas las primeras 6 h. Estudios previos señalan que la administración de PYY redujo la cantidad de heces inducida por la dimetil-prostaglandina E2 (Moriya et al. 2010) y disminuyó los movimientos de propagación del colon en ratones conscientes (Wang et al. 2010). Por el contrario, nuestros estudios muestran que el pretratamiento con la forma truncada y agonista de los receptores Y2 PYY(3-36) no modificó la diarrea causada por el AO. En resumen, hemos observado que la administración de los agonistas de NPY o PYY(3-36) apenas mejoran la acumulación de fluido en estómago e intestino. Además de la ausencia de cambios en los parámetros relacionados con la diarrea causada por el ácido okadaico. Por tanto, este planteamiento de ensayos *in vivo* utilizando péptidos que promueven la absorción intestinal nos proporcionó unos resultados que indican que otras posibles vías de señalización entéricas podrían estar implicadas en la aparición de la diarrea.

La serotonina (5-HT) es una molécula que puede actuar de forma autocrina, luminal, paracrina, inmunológica, neural y endocrina en el aparato digestivo (Rao 2019). Está implicada en procesos de regulación de secreción de fluido y moco, en el transporte de iones en el tracto gastrointestinal, así como en procesos de motilidad intestinal capaz de inducir diarrea (Hu and Spencer 2018; Rao 2019; Banskota et al. 2019). Se ha relacionado la serotonina con agentes causantes de diarrea como *Escherichia coli* enteropatogénica (Singhal et al. 2017) y el adenovirus humano 41 (Westerberg et al. 2018). En el tracto gastrointestinal se expresan los receptores 5-HT2 en células epiteliales y los receptores 5-HT1, 5-HT3 y 5-HT4 en neuronas (Fidalgo et al. 2013). La ciproheptadina es antagonista también categorizado como agonista inverso de los receptores 5-HT1 y 5-HT2. El tratamiento del músculo liso longitudinal del intestino delgado de ratón con CPH reduce la contracción muscular (Fida et al. 2000). Nosotros observamos que el pretratamiento con CPH bloqueaba o bien retrasaba la aparición de la diarrea causada por el ácido okadaico. Realizamos un ensayo con una dosis-respuesta de pretratamiento con CPH y posterior administración de AO que finalizaba cuando los animales tratados sólo con AO desarrollaban diarrea (media de 33 ± 2,3 min). La concentración de serotonina en el intestino grueso de animales tratados con AO y pretratados con CPH (0,1 mg/kg) era ligeramente superior al control. No obstante, los animales a los que se les administró CPH y AO no tuvieron diarrea, cuando los ratones tratados sólo con AO sí la desarrollaron. En esta línea, durante la evaluación de la cavidad abdominal al realizar la necropsia, el contenido del intestino grueso de los ratones pretratados con CPH tenía una apariencia más similar al de los controles que al de los animales tratados con AO. Esto nos indica que la serotonina puede estar implicada en la diarrea causada por el ácido okadaico.

La relación de la serotonina con distintos tipos de diarrea se ha estudiado en profundidad. Por ejemplo, la toxina del cólera estimula las neuronas secretomotoras lo que causa la liberación de serotonina de las células enterocromafines alterando así la motilidad intestinal (Spencer and Hu 2020). En consonancia, se ha determinado que los receptores 5-HT2 y 5-HT3 median la secreción de fluido en yeyuno de rata, así como que el antagonista del receptor 5-HT2 ketanserina reduce la secreción causada por la toxina del cólera (Beubler et al. 1989; Beubler and Horina 1990; Harville and Dreyfus 1995). Cuando evaluamos el contenido electrolítico de las heces de ratones tratados con distintas dosis de AO, nuestros resultados muestran un elevado contenido de agua que concuerda con una mayor concentración de cloro y sodio. Esto es coherente con los efectos prosecretores de la serotonina en el intestino. Aún así, aplicamos el

gap osmótico fecal para poder discernir el tipo de diarrea inducida por el AO (Fine and Schiller 1999; Camilleri et al. 2017). La diarrea osmótica es causada por compuestos que apenas se absorben causando el movimiento de agua al lumen intestinal por ósmosis (Camilleri et al. 2017). La mayor concentración de toxina la detectamos en el tracto gastrointestinal, lo que sugiere una absorción limitada que podría ser coherente con este tipo de diarrea. Por el contrario, la diarrea secretora implica un aumento en la concentración de electrolitos (Rao 2019). La serotonina se ha relacionado con este último tipo de diarrea. El trabajo presentado en esta tesis muestra que el gap osmótico fecal de heces de animales tratados con AO fue inferior a 50 mOsm/kg, lo que sugiere una diarrea secretora (Camilleri et al. 2017). Las heces de estos animales tenían una concentración elevada de sodio y cloro, que se correspondía con una concentración baja de potasio y calcio. Esto apoya que la diarrea causada por el AO sea secretora. Es más, las diarreas secretoras no sólo se han relacionado directamente con el transporte activo del cloro al lumen intestinal, sino también con una concentración baja de potasio (van Dinter et al. 2005; Sorensen et al. 2010). La combinación de un aumento en la cantidad de agua en el lumen con que una absorción de potasio ocurre mayormente en el colon distal pueden explicar esta reducción (Sorensen et al. 2010; Rao 2019). La importancia del equilibrio entre la absorción y secreción de agua y electrolitos, conlleva una regulación muy estrecha de estos procesos. Es decir, distintas rutas de señalización pueden provocar la apertura de canales de cloro y la secreción al lumen intestinal (Das et al. 2018; Rao 2019). Por el contrario, la secreción del sodio al lumen es por vía paracelular (Tamura et al. 2011). Esto implica que el flujo de sodio al lumen puede ocurrir como consecuencia de la secreción de cloro además de por la inhibición de la absorción de estos electrolitos. Sin embargo, estudios previos sobre los efectos del AO en transporte iónico sugieren que no causa un aumento de corrientes iónicas en modelos *in vitro* y *ex vivo* (Chow and Barrett 2007; Hosokawa et al. 1998; Tripuraneni et al. 1997). Aunque estos efectos también podrían deberse a un bloqueo de corrientes electroneutras de sodio y cloro, así como a un aumento en la permeabilidad. El AO actúa sobre la permeabilidad de la membrana en enterocitos (Tripuraneni et al. 1997), teniendo un efecto polar, es decir, la aplicación basal del AO promueve el aumento de la permeabilidad de la monocapa a concentraciones más bajas que su aplicación en la cara apical (Ehlers et al. 2011; Fernandez et al. 2014; Louzao et al. 2015). También hay que considerar que los efectos del AO sobre la motilidad pueden formar parte de la fisiopatología del AO.

El AO induce por tanto una diarrea secretora que relacionaría su acción con la serotonina. Se ha demostrado que la CPH inhibe la secreción de electrolitos en epitelio intestinal (Meddah et al. 2014). Nosotros comprobamos que las heces de los animales que recibieron un pretratamiento con CPH tenían un contenido de agua menor y una ligera reducción de la concentración de cloro con respecto a las heces de los ratones tratados sólo con AO. Estos resultados son coherentes con el aspecto del contenido del intestino grueso de los ratones pretratados con CPH. No obstante, la prevención del daño macroscópico no se refleja al evaluar las alteraciones citomorfológicas. Tanto en yeyuno como en colon proximal se observó una desorganización del microvilli y la inflamación de las mitocondrias. En esta ocasión no pudimos evaluar cambios en la permeabilidad, pero no observamos cambios en las estructuras de las uniones estrechas ni en las uniones adherentes. Aunque las uniones estrechas pueden aparecer sin cambios morfológicos en algunos tipos de diarrea como la causada por el rotavirus (Hagbom et al. 2020). Nuestros resultados son coherentes con diarreas que no alteran estas uniones, pero

sí causan desorganización del microvilli (Markov et al. 2019). En estudios previos de las alteraciones ultraestructurales ocasionadas por AO se han descrito erosiones epiteliales acompañadas de alteraciones del microvilli y de la mitocondria en intestino delgado transcurridas unas horas de la administración de la toxina (Ito et al. 2000; Ito et al. 2002; Wang et al. 2012). Es interesante destacar que estas alteraciones se observaron tanto en animales que tuvieron diarrea como animales que no la desarrollaron (Ito et al. 2000; Ito et al. 2002; Wang et al. 2012). Por el contrario, en el intestino grueso los daños causados por el ácido okadaico se han descrito como ligeros o nulos (Hosokawa et al. 1998; Ito et al. 2000; Ito et al. 2002). Sin embargo, nuestros resultados muestran que el colon de los animales tratados con la toxina muestra lesiones a tiempos cortos, mientras que el intestino grueso de los ratones tratados con CPH presentaban una morfología más parecida al control. Por consiguiente, el AO puede estar actuando mediante mecanismos complejos que involucran rutas de señalización serotoninérgicas que desencadenen la acumulación de fluido en el intestino, aunque no podemos descartar que esté afectando también la motilidad intestinal.

Como se ha descrito anteriormente, las toxinas diarreicas del grupo del ácido okadaico se han estudiado extensivamente debido a la distribución global de los dinoflagelados productores, la frecuencia de las floraciones algales nocivas en las costas europeas y el riesgo de intoxicaciones (Tamele et al. 2019; Bresnan et al. 2021; Hallegraeff et al. 2021; Zingone et al. 2021; Sunesen et al. 2021; Sakamoto et al. 2021; Liang et al. 2022). Esto conlleva una regulación estricta, con límites máximos de AO en marisco, en muchos países donde se han detectado y, por ende, unos programas de monitorización que implican el cierre de las áreas de producción de bivalvos con el fin de proteger al consumidor de la intoxicación diarreica por consumo de marisco (*Diarrhetic Shellfish Poisoning*; DSP). En consecuencia, estas toxinas no sólo están reguladas, sino que forman parte de los programas de monitorización en la Unión Europea desde el 2004 (EU 2004). Un contexto y situación contrarias ocurre con el grupo de otras toxinas marinas, las brevetoxinas (PbTxs). Al contrario que las toxinas diarreicas, las brevetoxinas se consideran toxinas emergentes, ya que los dinoflagelados que las producen como el género *Karenia* spp. tienen una distribución tropical y la detección de brevetoxinas está geográficamente limitada a estas zonas (Watkins et al. 2008). Lo mismo sucede con las intoxicaciones: los casos de NSP se han concentrado en el Golfo de México al que se suman algunos brotes en Nueva Zelanda (Arnich et al. 2021). En consecuencia, se encuentran reguladas en Estados Unidos, México, Australia y Nueva Zelanda (FSANZ 2016; FDA 2019; COFEPRIS 2022; FDA 2022; New-Zealand 2022), no así en la Unión Europea. Las brevetoxinas se han detectado recientemente en moluscos recogidos del Estanque de Diana (Córcega) (Amzil et al. 2021). Además se ha registrado también un evento de mortalidad en delfines en las Islas Canarias en cuyos contenidos estomacales se han detectado brevetoxinas 2 y 3 (Fernández et al. 2022). A esta situación reciente que indica que se deben tomar medidas preventivas para proteger al consumidor de estas toxinas consideradas emergentes en Europa, se añade la falta de ensayos toxicológicos *in vivo* que sirvan de base para establecer un marco legal adecuado ((EFSA) 2010b; Vilarino et al. 2018; ANSES 2021).

Las PbTxs son ficotoxinas marinas lipofílicas que engloba un elevado número de análogos (Hort et al. 2021). Es importante comentar que la PbTx3 es un derivado de la PbTx2 por reducción del grupo aldehído del carbono C42. La PbTx3 se ha encontrado tanto en un número considerable de moluscos, como en peces y mamíferos marinos (Hort et al. 2021). Dado que en

un estudio comparativo entre las brevetoxinas 2 y 3, esta última resultó ser más tóxica, se seleccionó este análogo para realizar los estudios del a presente tesis doctoral (Baden and Mende 1982). Uno de los métodos que se ha utilizado para la detección de estas toxinas es el Bioensayo en Ratón (*Mouse Bioassay*, MBA) que consiste en la administración al roedor por vía intraperitoneal de un extracto de moluscos que contiene la toxina y la posterior observación del número de animales que muere en 15,5 h (FAO/WHO 2016). Si bien el bioensayo en ratón es un método que se ha utilizado para determinar la presencia de toxinas en bivalvos, la intoxicación en personas se produce por la ingestión de marisco con toxinas. Por eso resulta interesante comparar la toxicidad de PbTx3 por las 2 vías de administración, oral e intraperitoneal. Nuestros resultados muestran una clara diferencia, no sólo en cuanto a la mayor toxicidad intraperitoneal, sino a la diferencia en la dinámica del desarrollo y recuperación de la intoxicación. Fue necesario aumentar la dosis hasta los 4000 μg/kg PbTx3 por vía oral para que la toxina indujera la muerte del animal, mientras que la inyección intraperitoneal de PbTx3 causaba la muerte de los animales a 875 μg/kg PbTx3. Esto está en línea con ensayos previos, en los que también se reporta mayor toxicidad intraperitoneal que oral (Baden and Mende 1982). Asimismo, la aparición de los síntomas fue más rápida cuando la toxina se administró por vía intraperitoneal (5 min), no obstante, también se desarrollaban pronto tras la administración oral (30 min). Aparecían afectaciones neurológicas como ataxia, estereotipias, cola de Straub o convulsiones. Sin embargo, la duración de los síntomas fue muy distinta según la ruta de administración. Los animales tratados por vía intraperitoneal presentaban el pico de síntomas en las primeras 2 h y a partir de ese momento comenzaba su recuperación. Por el contrario, basándonos en la puntuación de los síntomas, los animales tratados con sonda gástrica mostraban un grado de intoxicación estable durante las primeras 6 h, tiempo a partir del que comenzaba una recuperación más lenta. Esto se vio reflejado también en los tests funcionales. En cuanto a la sensibilidad al frío, solo observamos cambios en la dosis 750 μg/kg PbTx3 oral a 6 h, mientras que la sensibilidad era normal transcurridas 23 h. En los animales tratados vía intraperitoneal, el test de la rejilla pone de manifiesto que los animales tratados con PbTx3 por vía intraperitoneal aguantan menos tiempo agarrados a la rejilla indicando que tienen menor fuerza que los controles transcurridas 6 h desde la administración de la toxina. Asimismo, el comportamiento exploratorio que se evalúa en el test del vaso se reduce cuando se compara a las condiciones basales y al control a las 6 h, mientras que al finalizar el ensayo hay una clara mejoría de los animales tratados con 500-875 μg/kg PbTx3 intraperitoneal. Los ratones que recibieron la PbTx3 por vía oral tuvieron un comportamiento similar en los tests a excepción de la fuerza muscular que no se recupera con dosis por encima de 1000 μg/kg PbTx3, aunque transcurran 24 h. La intoxicación oral en ratón muestra una sintomatología y un desarrollo similar a los casos de NSP descritos en humanos (Arnich et al. 2021). En casos de intoxicación humana se han reportado una mayor variedad de síntomas tales como náuseas, diarrea, parestesia, disestesia o vértigo. Pese a ello, algunas características como la ataxia y la pérdida de fuerza muscular coinciden con los síntomas que reportamos. Del mismo modo, en las personas afectadas se ha descrito una recuperación a los pocos días (Arnich et al. 2021). Nosotros reportamos una tendencia a la recuperación tras las 6 h de administración en ratones. Estas circunstancias contrastan con los eventos de mortalidad faunal causados por las floraciones algales nocivas causadas por *Karenia brevis* (Bossart et al. 1998; Twiner et al. 2012). No obstante, las condiciones de exposición a las brevetoxinas, así como el perfil toxicológico durante un episodio de floración varía ampliamente. Por ejemplo, los animales

están constantemente expuestos a las PbTxs durante los periodos de floraciones algales nocivas mientras que en nuestro estudio los ratones están expuestos a una única administración de la toxina. Además, los dinoflagelados del género *Karenia* spp. pueden producir otros metabolitos que también podrían estar implicados en estos eventos (Hort et al. 2021).

Si bien se ha demostrado en rata que la brevetoxina se concentra en mayor medida en el hígado, seguida del tracto gastrointestinal, corazón y riñones (Cattet and Geraci 1993), en nuestro estudio no detectamos alteraciones en parámetros bioquímicos en la sangre que pudieran indicar daño hepático, renal o muscular. Incluso en la evaluación histopatológica de estos órganos, así como del cerebelo, no encontramos lesiones a las dosis más altas. Estudios previos en distintos organismos indican variación en daños histológicos según el tipo de exposición y tiempo. En ratas la administración por inhalación durante 5 días no causó daños histológicos (Benson et al. 2004), mientras que tras 22 días de administración repetida por inhalación sólo se reportaron lesiones en los pulmones (Benson et al. 2005). Las tortugas tratadas con PbTx3 por vía oral mostraron daños en tráquea, pulmón, intestino, meninges y corazón, sin embargo, cabe destacar que en algunos animales control también se detectaron lesiones similares. Por lo tanto, no se puede discernir qué alteraciones son debidas a la brevetoxina 3 (Cocilova et al. 2017). En otro estudio con ratón donde se administran las brevetoxinas de tipo B PbTx2 y PbTx6 por vía intraperitoneal observan lesiones leves en hígado y bazo tras 8, 24 y 72 h (Walsh et al. 2003). En un caso de mortalidad de manatíes expuestos por vía inhalatoria y/o por ingesta relacionado con la floración de *Karenia brevis* en Florida se detectaron daños severos en las vías respiratorias, pulmón y cerebelo (Bossart et al. 1998). La exposición continuada de estos animales a la toxina durante las floraciones podría ser responsable de las diferencias en los daños histológicos.

El mecanismo de acción de las brevetoxinas explica en gran medida el cuadro clínico característico de la intoxicación neurotóxica por consumo de marisco (*Neurotoxic Shellfish Poisoning*; NSP). Las PbTxs se unen al sitio 5 de los canales de sodio dependientes de voltaje que se encuentra entre los segmentos S5 del dominio IV y S6 del dominio I, actuando como agonistas parciales (Mackieh et al. 2021; Raposo-Garcia et al. 2023). Esto causa la hiperpolarización del voltaje de activación de los canales de sodio dependientes de voltaje (es decir, una activación de los canales a potenciales más negativos y próximos al potencial de membrana en reposo), así como la inhibición de la inactivación, un aumento en el tiempo de apertura del canal y múltiples subniveles de conductancia (Wu and Narahashi 1988; Ramsdell 2008; Mackieh et al. 2021). Los estudios iniciales de la caracterización farmacológica de las brevetoxinas se llevaron a cabo en sistemas complejos como el axón gigante de calamar (Westerfield et al. 1977) o el cordón nervioso ventral de cangrejo de agua dulce (Parmentier et al. 1978). Si bien estos estudios han sido de especial relevancia para elucidar el mecanismo de acción de estas neurotoxinas, los sistemas empleados expresan distintas isoformas de los canales de sodio dependientes de voltaje. Estos canales se conforman por una subunidad α que conforma el poro y una o dos subunidades β importantes para la regulación de los canales (Mackieh et al. 2021). Hay 9 isoformas de la subunidad α que se expresan distintamente en función del órgano. La isoforma 1.6 ($Na_V1.6$) se localiza tanto en el sistema nervioso central como en el periférico (Tzoumaka et al. 2000; Wang et al. 2017). La sobreactivación de estos canales se ha asociado a la epilepsia, ataxia y distonía (Zybura et al. 2021). Hemos podido estudiar *in vitro* la toxicidad de la PbTx3 mediante la modulación de los canales de sodio

dependientes de voltaje $Na_V1.6$ expresados en la línea celular HEK293. La expresión heteróloga de algunas isoformas en esta misma línea celular ha servido con anterioridad para el estudio farmacológico de las brevetoxinas, ciguatoxinas y saxitoxina (Bottein Dechraoui and Ramsdell 2003; Alonso et al. 2016). En consonancia con ensayos previos (Ramsdell 2008), observamos que los efectos de la PbTx3 sobre la intensidad máxima de la corriente de sodio son leves, mientras que sí detectamos un desplazamiento del voltaje de activación a potenciales más negativos. Esto conlleva que los canales de sodio dependientes de voltaje se abran a potenciales de membrana próximos al potencial de reposo. Las brevetoxinas muestran diferente afinidad a las isoformas $Na_V1.2$, $Na_V1.4$ y $Na_V1.5$. Los $Na_V1.2$ se expresan en los sistemas nerviosos central y periférico, mientras que $Na_V1.4$ es predominante en músculo esquelético y $Na_V1.5$ se expresa en el corazón (Wang et al. 2017; Konoki et al. 2019). La PbTx3 tiene una constante de disociación más elevada para la isoforma $Na_V1.5$, que para las isoformas $Na_V1.2$ y $Na_V1.4$ (Konoki et al. 2019). En la misma línea, en preparaciones de cerebro, corazón y músculo esquelético de rata y pez han observado que la afinidad de la PbTx3 por el músculo cardíaco de rata era muy baja (Dechraoui et al. 2006). Unos resultados similares se obtuvieron en un ensayo competitivo en cerebro de rata y las isoformas $Na_V1.4$ y $Na_V1.5$ (Bottein Dechraoui and Ramsdell 2003). En este estudio la afinidad de la PbTx3 por $Na_V1.5$ era 6 veces menor que por la preparación de cerebro de rata y 3 veces menor que por la isoforma $Na_V1.4$. La sobreactivación de la isoforma $Na_V1.6$ está en línea con los estudios publicados por otros autores. Si bien la intensidad de corriente apenas se vio afectada por el tratamiento con PbTx3, sí reportamos un desplazamiento del voltaje hacia potenciales más negativos. La hiperexcitabilidad de la isoforma estudiada en esta tesis, está en concordancia con algunos de los síntomas observados como la ataxia.

La farmacocinética descrita por otros autores podría explicar la rápida aparición de síntomas y la recuperación parcial o total de los ratones tratados a las 24 h. Tras la administración oral de PbTx3 a ratas, la toxina se ha distribuido por todo el cuerpo a las 6 h, tiempo en el que la concentración es mayor mientras que a las 24 h el porcentaje de toxina eliminado es del 40% aproximadamente (Cattet and Geraci 1993). Más de la mitad de la toxina administrada se encuentra en el cuerpo tras las 24 h. Sin embargo, en otro estudio en el que se administra PbTx3 por vía intraperitoneal detectan metabolitos de la toxina en la orina recogida transcurridas entre 4 y 8 h tras la administración (Radwan et al. 2005). Lo cual sugiere que el metabolismo de la PbTx3 empieza pronto y que los signos de toxicidad aguda que observamos pueden reducirse a lo largo del tiempo debido a la transformación en análogos menos tóxicos.

En esta tesis se han obtenido resultados importantes para la caracterización de la toxicidad aguda de PbTx. Pudimos determinar una DL_{50} de 875 μg/kg PbTx por vía intraperitoneal. Así como reportamos que hay que alcanzar dosis muy altas en la administración oral para que causen la muerte del animal. Sin embargo, estudios previos donde administran PbTx3 por vía oral reportan una DL_{50} de 170 μg/kg PbTx3 (Baden and Mende 1982) y de 250 μg/kg PbTx3 (Selwood et al. 2008). Estas diferencias podrían explicarse en parte por el origen de la toxina, ya que utilizan un extracto (Baden and Mende 1982) y un producto semisintético (Selwood et al. 2008), así como por los distintos disolventes (Bartsch et al. 1976). Es importante destacar que normalmente las intoxicaciones en humanos no son debidas a un solo análogo, sino que se trata de una combinación de distintos análogos con diferentes potencias y toxicidad (Vale and Sampayo 1999; Ishida et al. 2004b; Hort et al. 2021). Estimamos una LOAEL de 100 μg/kg

PbTx3 y una NOAEL de 10 μg/kg PbTx3 por administración oral en ratones. Estos parámetros pueden servir de base para establecer la dosis de referencia aguda en personas, así como el límite máximo de brevetoxinas en marisco. Esto es de especial relevancia debido a que son toxinas que no actualmente no están reguladas en Europa ((EFSA) 2010b; FAO/WHO 2016; ANSES 2021).

6 CONCLUSIONES

1. Okadaic acid and dinophysistoxin 1 are absorbed quicker than dinophysistoxin 2, and the latter is fastly excreted in feaces, which may limit its toxic effect.
2. Oral administration of the toxins okadaic acid, dinophysistoxins 1 and 2 results in gastrointestinal distribution with greater detection in the stomach and, in decreasing order, large intestine, small intestine and liver of mice.
3. Clinical signs caused by okadaic acid poisoning are due to the toxin triggering signalling routes that involve serotoninergic pathways and the neuropeptide Y system with limited relevance.
4. Okadaic acid induced secretory diarrhoea with water accumulation in the intestinal lumen and increased sodium and chloride concentration, without disrupting intercellular unions morphology in the intestinal barrier.
5. Brevetoxin 3 is a partial agonist of voltage-gated sodium channels that causes neuromuscular alterations with fast recovery, which may be due to the effect on voltage-gated sodium channels with the toxicicty index of NOAEL 10 μg/kg PbTx3 and LOAEL of 100 μg/kg PbTx3.

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Las ficotoxinas marinas se pueden acumular en moluscos bivalvos y causar intoxicaciones alimentarias al consumirlos. Algunas de ellas están reguladas y son frecuentes en las costas gallegas, como las del grupo del ácido okadaico. Otras, como las brevetoxinas, no están reguladas en Europa, pero los datos de toxicidad que permitan establecer un marco regulatorio escasean. La presente tesis doctoral se estudia la toxicocinética del ácido okadaico, las dinofisistoxinas 1 y 2 in vivo, y el mecanismo de acción del ácido okadaico. Esto ha resultado en la implicación de vías de señalización serotoninérgicas. Por otro lado, también se abordó un estudio de toxicidad oral e intraperitoneal de la brevetoxina 3, esto permitió obtener parámetros de toxicidad que pueden servir de base a las autoridades competentes.