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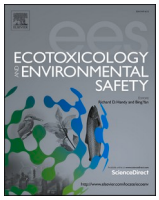

Changes at small intestine induced by food-fish contaminated with ciguatoxins

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ABSTRACT

Ciguateric syndrome is a food poisoning associated with the consumption of some species of fish that have accumulated ciguatoxins (CTXs) in their tissues. The effects of the syndrome occur with nervous imbalances which have been described for quite some time, and mentioned in sailing literature for centuries. In the last decade, research has been focused on the implementation of analytical methods for toxin identification and the study of action modes of CTXs to design effective treatments. However, an important aspect is to determine the damage that CTXs caused in the organs of affected individuals. In this work, the damages observed in tissues of mice, mainly in the small intestine, were analyzed. The animals were fed with CTX-contaminated fish muscle at concentrations 10-times below the median lethal dose (LD_{50}) for 10 weeks. The analysis of tissues derived from the oral treatment resulted in an increased occurrence of Paneth cells, presence of lymphoid tissue infiltrating the mucosa and fibrous lesions in the mucosal layer of the small intestine. A decreasing weight in animals fed with toxic muscle was observed.

1. Introduction

Ciguatera Poisoning (CP) syndrome results from the ingestion of seafood that has accumulated marine biotoxins known as ciguatoxins (CTXs) (Yasumoto and Murata, 1993; Soliño and Costa, 2020; Costa et al., 2023). CTXs constitute a complex family of lipophilic polyether compounds produced by dinoflagellates of genus *Gambierdiscus* and *Fukuyoa*. These thermostable neurotoxins bioaccumulate and transform throughout the trophic chain of predatory fish and affect humans when fish tissues are consumed. CTXs are present at very low levels in contaminated fish and seafood, usually below the range of μg/kg. The lowest concentration measured in fish associated with symptoms in humans was determined at about 0.02 μg/kg equiv. CTX1B in fish flesh from the Caribbean (Soliño and Costa, 2018; 2020; FAO and WHO, 2020). CTXs are neurotoxins that act on voltage-sensitive sodium channels on excitatory membranes of nerves and muscles resulting in an excess intestinal fluid secretion, diarrhea, cramping, vomiting and paresthesia, muscle weakness, myalgia, arthralgia, fatigue, pruritus, and numbness of lips and limbs (Soliño and Costa, 2020). The neuromuscular signs may persist for weeks or months, and in severe cases, death can occur. In general, there is abundant information about the symptoms caused by ciguateric syndrome; however, data on the damage that toxins produce on the different organs of affected individuals are scarce (Soliño and Costa, 2018; Chinain et al., 2021).

Terao et al. (1991) observed marked swelling and focal necrosis of cardiac muscle cells as well as effusion into the interstitial space of the heart of mice after intraperitoneal (ip) or oral administration of 0.7 μg/kg of Pacific CTX (also CTX1B) and CTX4C (Satake et al., 1996). These experiments did not e exceed 24 h. Other effects detected were degeneration of cells in the medulla of the adrenal glands and

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continuous erection of the penis in about 15 % of the mice suffering from ciguatoxicosis. Although severe diarrhea was produced by the administration of these phycotoxins, no morphological alterations were observed in the mucosa and muscle layers of the small intestine except in autonomic nerve fibers and synapses (Terao et al., 1991). In a subsequent study, the use of a repeated oral and ip (0.1 μg/kg) dose of CTX and CTX4C for 15 days resulted in marked swelling of cardiac cells, and endothelial lining cells of blood capillaries in the heart of mice. Damage of capillaries, and effusion of serum and erythrocytes into the interstitial spaces of the myocardium occurred. Furthermore, swelling of the endothelial lining cells of capillaries caused narrowing of the lumen and accumulation of blood platelets in capillaries triggering multiple single cell necroses of cardiac muscle cells. In the period of one month after the treatments with the toxins, myocytes and capillaries appeared to be normal. Nonetheless, the effusion in the interstitial spaces produced formation of bundles of dense collagen, which persisted for 14 months (Terao et al., 1992).

Also, toxicokinetic evaluation of CTX1B in rats after an oral or ip treatment confirmed the rapid absorption capacity of CTXs, and their ability to cross the blood–brain barrier, revealing that biologically active CTXs are detectable in blood, liver, muscle, and brain (Bottein et al., 2011; FAO and WHO, 2020).

On the other hand, a recent study by Raposo-García et al. evaluated the effects of both acute and subchronic oral toxicity of Pacific CTX3C and other CTX-related analogues in mice. The most relevant effect of oral acute exposure to CTX3C at a dose of 1 μg/kg was a decreased urine production in 24 h by about 85 % suggesting that either activation or inhibition of voltage-gated sodium channels can alter renal function, however no effects were reported after a 28-day oral administration period (Satake et al., 1993; Raposo-García et al., 2022).

The European regulations (EU) 2017/625 and (EU) 2019/627 ensure that fishery products containing biotoxins and other toxins such as ciguatera are not placed on the market (Eur-lex.europa.eu). Thus, the safety levels of these environmental contaminants are well regulated to prevent acute symptoms of CP.

In this study, we intend to establish a correlation between environmental toxicology, food web interactions and public health risks through the possible tissue alterations that might occur in the different organs of individuals when a continuous consumption of fish with low levels of CTXs contamination escape the usual sanitary controls. This research describes the alterations observed in tissues, mainly in the small intestine, of mice exposed to low doses of CTXs (10-times below the median lethal dose (LD_{50})). The experiment approaches the oral administration of CTX-contaminated fish muscle throughout 10 weeks.

2. Materials and methods

2.1. Toxic amberjack muscle for oral treatment

Fish sample of an amberjack from the Canary Islands (Spain), screened as positive for CTX monitoring control at the University Institute of Animal Health and Food Safety (IUSA), Universidad de Las Palmas de Gran Canaria (SG3 of EuroCigua project), as described in Section 2.2, was used for oral feeding of mice. The fish tissue was stored at −20 °C prior to use and preparation as toxic flour.

2.2. Quantification of CTXs of experimental food-fish muscle

The experimental diet consisted of flesh from a naturally contaminated amberjack. The presence of C-CTX1 (0.27 ± 0.02 μg/kg) was previously confirmed by liquid chromatography mass spectrometry (LC-MS/MS) in the framework of the interlaboratory collaborative analysis of EuroCigua project. Furthermore, the presence of CTX-like toxicity was also determined by N2a-MTT assay at IUSA laboratory, resulting in an effect of multiple CTXs analogues, such as 1.10 μg/kg equiv. CTX1B (Pacific type 1 CTX (CTX1B) (Sanchez-Henao et al., 2021; Ramos-Sosa et al., 2023). In fact, this value reflects the overall toxicity of the flesh in terms of CTX activity, and therefore, this concentration was used to calculate the toxic meat intake doses for experimental mice.

CTXs extraction from fish for the Cell-Based Assay (CBA) analysis was performed following the Lewis protocol (Lewis, 2003) with some modifications, according to the needs of the IUSA laboratory. For the cytotoxicity assay, neuroblastoma cells (N2a line, CCL-131, from ATCC, LGC Standards SLU, Barcelona, Spain) were maintained in Roswell Park Memorial Institute medium (RPMI)-1640 with 5–10 % of fetal bovine serum at 37 °C in a 5 % CO_2 atmosphere. The Pacific type 1 CTX (CTX1B) provided by Prof. Richard J. Lewis (Queensland University, Australia) (Lewis et al., 1991) was used as a standard (STD) for the assessment of CTX-like toxicity. The CBA was conducted as described by Caillaud et al. (2012) with minor adaptations; 96-well flat bottom plates were seeded with $4{\cdot}10^4$ cells per well. Dilutions of both, STD and flesh extract, were performed to expose cells with and without Ouabain (0.1 mM) and veratridine (0.01 mM) pre-incubation. Cell viability was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] and DMSO solutions and the corresponding absorbances were read at 570 nm by a multiwell spectrophotometer scanner (iMark Microplate Reader, Bio-Rad). The toxicity of the sample expressed as CTX1B equiv. was calculated according to the 50 % inhibition concentration (IC_{50}) from the dose-response curves of the sample and the STD.

2.3. Oral treatment with toxic fish muscle

A feeding experiment using CTX-toxic fish muscle was proposed to analyze tissue damage of Swiss albino male mice, using fresh fish samples of toxic amberjack (50.8 g, 1.10 μg/kg equiv. CTX1B). Control group was fed with non-toxic sole (42.6 g). Animals were fed by oral gavage to assure that the exact dose was administered.

Fish samples were cut into small pieces, frozen at –80°C for 30 min, and lyophilized (Spielmeyer et al., 2021). Next, the samples were powdered and sieved to obtain fish flour. The flours were homogenized, resuspended with physiological serum (NaCl 0.9 %) and distributed in doses.

Toxic amberjack flour was distributed in 10 doses of 800 μL at 1/10× of the lethal dose of CTX1B equiv. (0.50 ng equiv. CTX1B/dose). Each dose was administered in 4 × 200 μL oral sub-doses (0.125 ng equiv. CTX1B/sub-dose) in day 1 (Wednesday: 2 × 200 μL) and day 2 (Thursday: 2 × 200 μL) every week. A total dose of 5.00 ng equiv. CTX1B/mouse was administered at the end of the experiment (Fig. 1). Similar procedure was developed for control group (UBC ACC TECH 09a Oral Dosing, 2021).

Two experimental groups of 20 g Swiss albino male mice, control and toxic, were fed by oral gavage through a gastric catheter for 10 weeks. Group 1 (n=10) (non-Contaminated fish; NC): Ten mice received equivalent sole meal in physiological serum (NaCl 0.9 %). Group 2 (n=10) (Contaminated fish; C): Ten mice were given amberjack flour (0.5 ng equiv. CTX1B/doses·week) suspended in physiological serum (NaCl 0.9 %). All animals were subjected to weight control during the experiment (Fig. 1). Weight control was performed once a week, every Wednesday, before feeding. Moreover, everyday throughout the experiment, the animals were supervised in order to assure welfare and to detect possible behavioral alterations.

2.4. Necropsies and histological samples preparation using fish flesh containing CTXs

At the end of the experiments, the animals were sacrificed using the rules of animal protection and ethic protocols of Universidad de La Laguna (ULL) [Reference number ULL-CEIBA2020–0397]. To analyze the organs' toxicity, liver, intestines, kidney, lung, spleen, heart and brain samples were extracted and prepared for histological analysis. The samples were fixed in 10 % formalin solution (pH 7.4), dehydrated in a graded series of ethanol, and embedded in Paraplast® highly purified

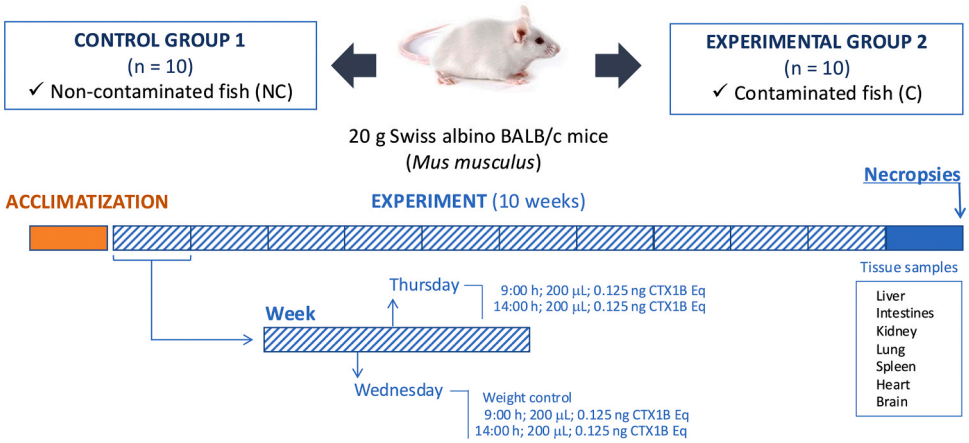

Fig. 1. Scheme of the experimental design.

paraffin for tissue embedding. Longitudinal, transversal, and sagittal microtome (Shandon Finesse 325) sections of 5 µm thickness were prepared. The sections were stained with haematoxylin–erythrosine (H-Er), Cleveland–Rucker–Wolfe (CRW) and Masson's trichrome to identify tissue and cell structure. The sections were inspected with a Leica DM 4000B light microscope, and the images were captured with a Leica DFC 300 FX camera. For histomorphometrical analysis all sections of the small intestine were evaluated using computer-based image analysis software (Q-win V3 Pro-image analysis system, Leica, Barcelona, Spain). Fifteen randomly selected microscopic fields were counted at 400x magnification in which Paneth cells, identified by their morphological characteristics, were quantified.

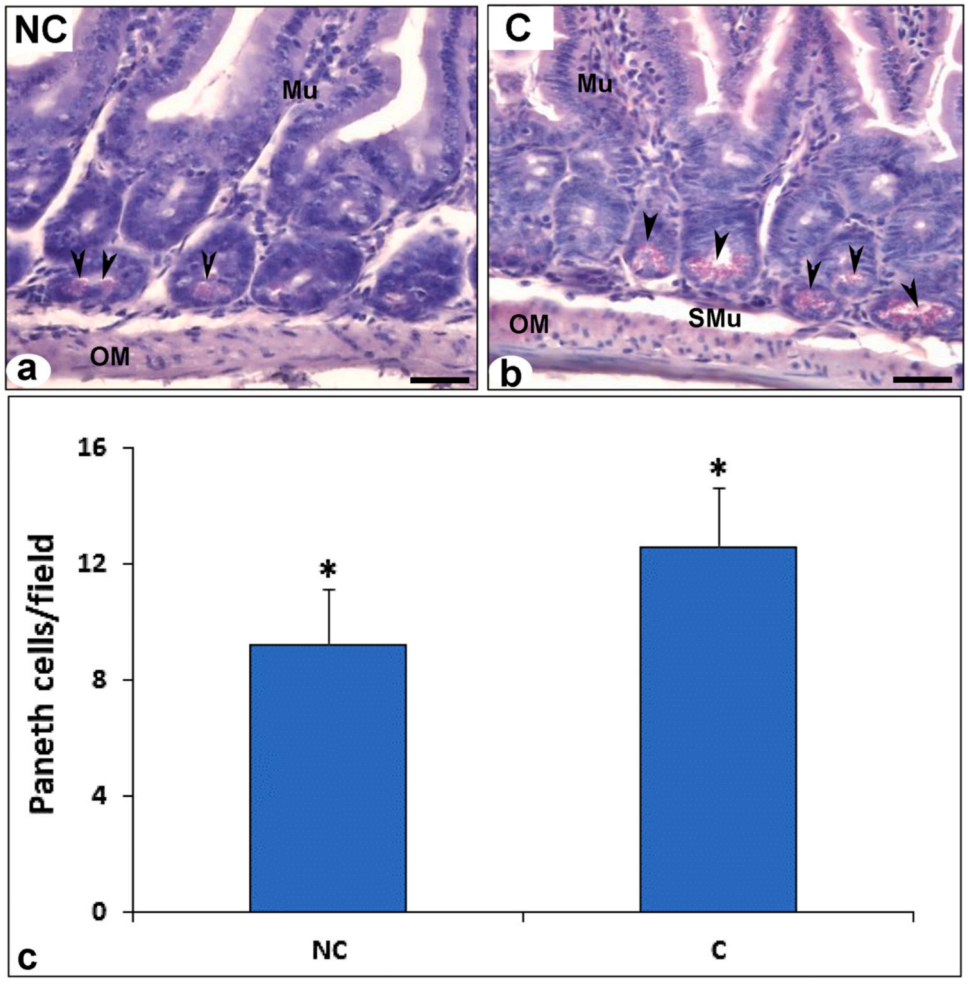

Fig. 2. (**a,b**) Representative cross-sectional images of the small intestine from animals fed uncontaminated fish (NC) and contaminated fish (C) showing the presence of Paneth cells (arrowheads) in Lieberkühn crypts. In (**c**) a graph is shown with the number of Paneth cells per field at 400x magnification in each of the experimental groups. The bars represent the means ± SD (n = 5). The identical symbol (*) on different bars indicates significant differences between them ($*p < 0.05$). Mu: Mucosa; OM: Own muscle; SMu: Submucosal layer. Scale bars: (**a,b**) 30 µm; Staining: H-Er.

2.5. Statistical analysis

Statistical analysis was performed with SPSS.25 software. We compared the distinct treatments by means of a one-way analysis of variance (ANOVA) with a Tukey multiple comparison post-test. Significance was set a $p < 0.05$. Results are expressed as means ± SD.

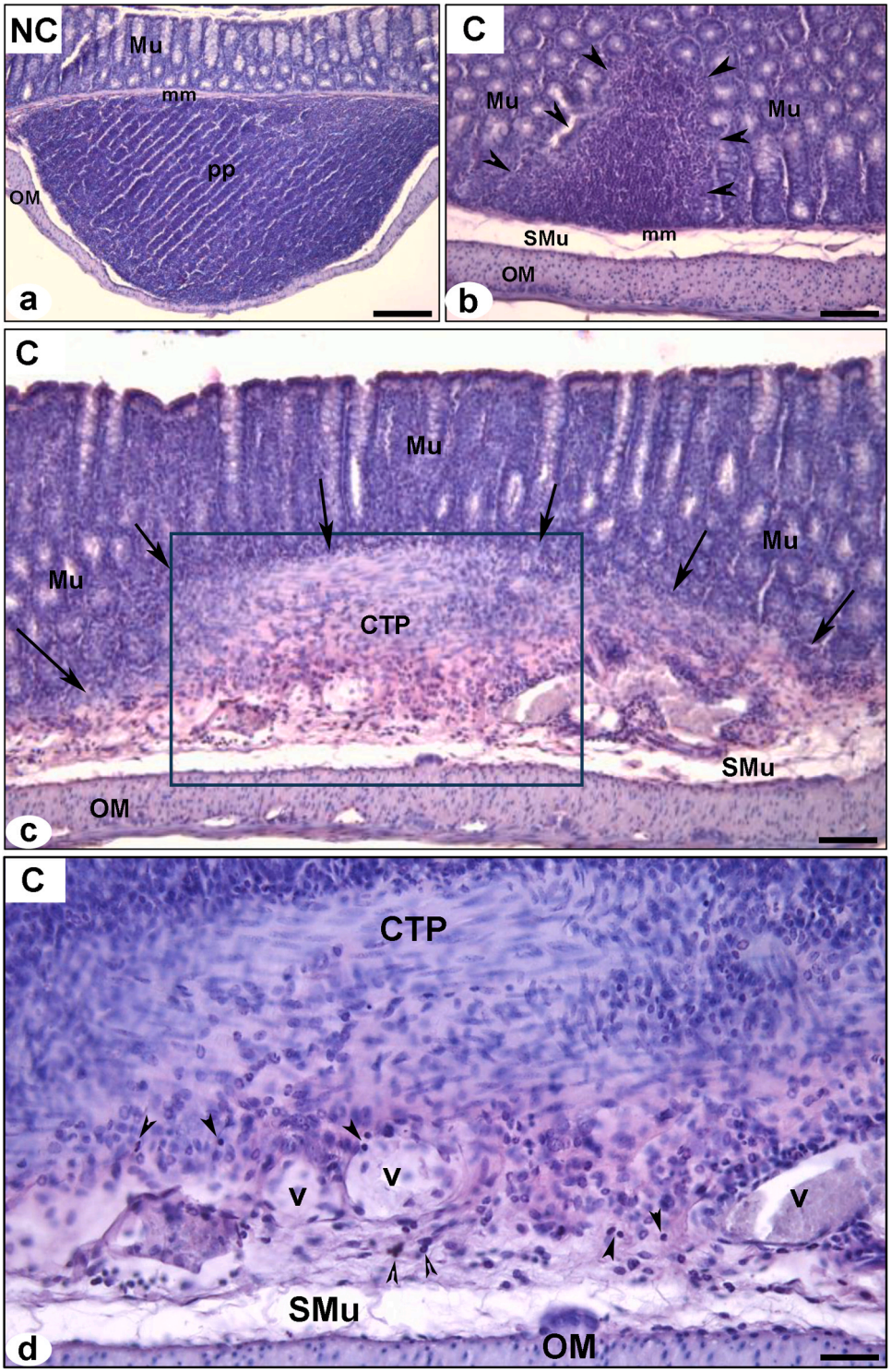

Fig. 3. (**a**,**b**) Representative images of the small intestine in an animal from group NC showing the usual location of a Peyer's patch (lymphoid follicle) in the submucosal region (SMu) (**a**), and from an animal from group C showing the presence of a lymphocytic proliferation in the mucosal layer (arrowheads) (**b**). (**c**) Representative image of the small intestine of an animal from group C showing the presence of a connective tissue proliferation (CTP) with abundant cellularity in the intestinal mucosal layer (arrows). (**d**) Magnification image of the boxed area showing the presence of large blood vessels (v) and pyknotic nuclei (arrowheads) in the connective tissue proliferation (CTP). CTP: Connective tissue proliferation; mm: Muscularis mucosae; Mu: Mucosa; OM: Own muscle; pp: Peyer patches; SMu: Submucosal layer; v- blood vessel. Scale bars: (**a**) 130 μm; (**b**) 100 μm; (**c**) 120 μm; (**d**) 60 μm. Staining: H-Er.

3. Results

3.1. Experimental design

A batch of mice was used to analyze mouse tissue modifications using food-fish contained with CTXs. Toxic and non-toxic fish muscle was administered by controlled oral administration to 20 g Swiss albino male mice at 1/10 × DL_{50} every week over a prolonged period at sub-lethal doses of ten weeks. Thus, samples of muscle of toxic amberjack (1.10 μg/kg equiv. CTX1B) were lyophilized, powdered, and sieved to obtain fish flour. Mice were fed weekly (0.50 ng equiv. CTX1B/dose) over a period of ten weeks. Weight control during the experiment revealed an 8–10 % decrease in weight in the group of animals fed with toxic muscle compared to the control group, however, no other external nor behavioral signs were observed. After this period, the individuals were sacrificed, and necropsied. Samples of liver, kidney, heart, spleen, intestine, brain, lung, and stomach from each animal were extracted and carefully analyzed to detect the histological alterations induced by CTXs.

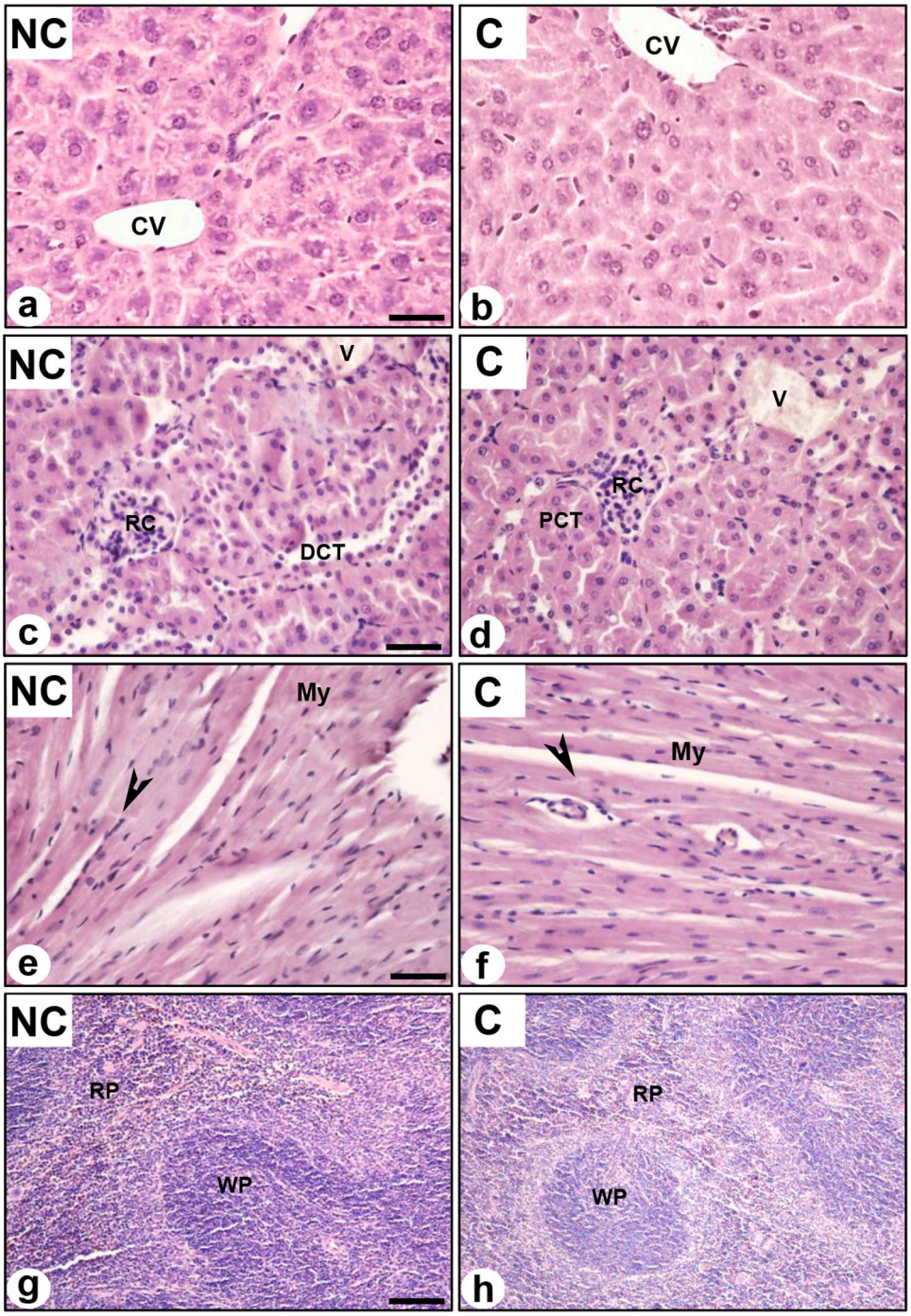

Fig. 4. Representative semi-panoramic images showing the tissue architecture of the liver (**a**,**b**), kidney (**c**,**d**), heart (**e**,**f**) and spleen (**g**,**h**) in the two experimental groups NC and C. CV: Centrilobular vein; RC: Renal corpuscle, DCT: Distal convoluted tubule, PCT: Proximal convoluted tubule, V: Blood vessel; My: Myocardium; RP: Red pulp, WP: White pulp. The arrowheads in images (**e**) and (**f**) indicate the intercalary disc. Scale bars: (**a**-**f**): 40 μm, (**g**,**h**): 100 μm. Staining: H-Er.

3.2. Histological studies

Histological examination of organs in animals fed with contaminated fish (C), showed changes in the mucosal layer of the small intestine. On one hand, changes in cell composition in the epithelial layer were detected in Paneth cells, which presented a significant increase of 27 % compared to animals fed with not contaminated fish (NC) (Fig. 2a-c).

On the other hand, lymphocytic proliferation in the connective tissue of the lamina propria was observed in different regions of the small intestine in animals fed with contaminated fish. Unlike Peyer's patches, located in the submucosal layer, these lymphocytic proliferations were observed within the mucosal layer (Fig. 3a,b). Likewise, within the mucosal layer and along the intestinal wall, foci of connective tissue proliferation compatible with fibrous lesions were observed in animals fed contaminated fish. Inside these proliferative regions, abundant vascularization and pyknotic nuclei compatible with apoptotic cells were observed (Fig. 3c,d).

The rest of the organs examined presented a conserved tissue architecture without changes at cellular level in the two experimental groups. The liver showed a normal structure with hepatocytes arranged in cords bordering the sinusoid capillaries. The vascular spaces of portal and hepatic systems present normal size and morphology as well as the

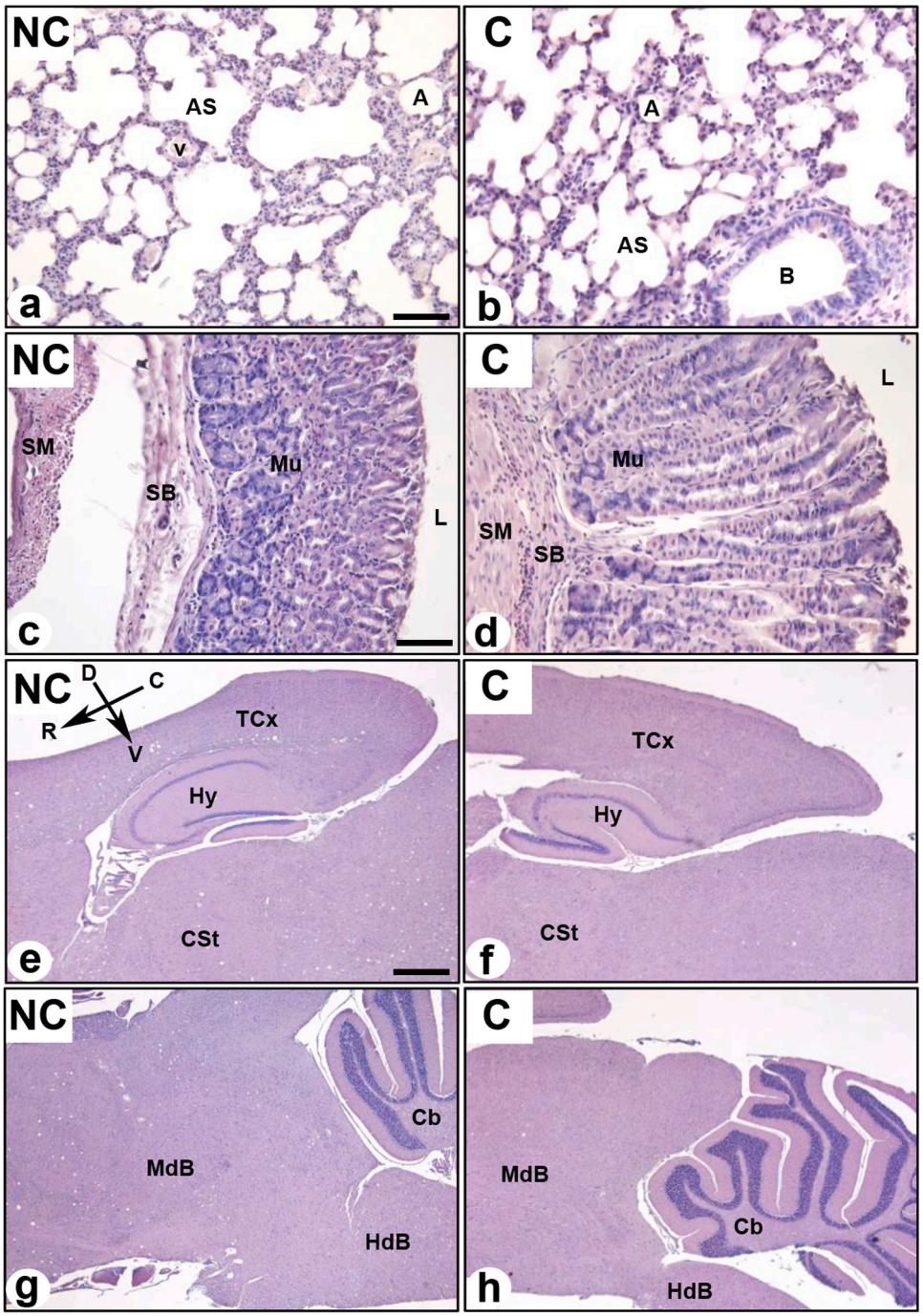

Fig. 5. Representative semi-panoramic images showing the tissue architecture of the lung (**a**,**b**), stomach (**c**,**d**) and brain (**e**-**h**) in the two experimental groups NC and C. AS: alveolar sac, A: pulmonary alveolus, B: bronchiole; SM: Smooth muscle, SB: Submucosal layer, Mu: Mucosal layer, L: Gastric lumen; TCx: Telencephalic cortex, Hy: Hippocampus, CSt: Striatum, MdB: Midbrain, Cb: Cerebellum, HdB: Hindbrain. The arrows in image (**e**) indicate the rostro-caudal and dorso-ventral axes. Scale bars: (**a**-**f**): 100 μm, (**g**,**h**): 500 μm. Staining: H-Er.

bile ducts (Fig. 4a,b). The kidneys showed a preserved tissue architecture with normal distribution of elements in cortex and medulla. Cortico-medullary interface with normal vascular elements was clearly seen. Medullary rays, calyxes and pelvis, of normal size and structure were also found (Fig. 4c,d). The heart presented a normal histological structure without significant changes. The myocardium showed cardiomyocytes with preserved morphology, showing nuclei in a central position and intercalary discs with normal distribution (Fig. 4e,f). The spleen showed a normal tissue architecture without significant changes in cell composition and distribution, observing the differentiation between white and red pulp (Fig. 4g,h).

The lung showed normal tissue architecture without significant changes. No signs of fibrosis were detected. Vascular and air spaces of normal size and morphology and normal epithelial characteristics were observed. Alveolar and pulmonary sacs presented normal structure without significant changes nor thickening of the walls (Fig. 5a,b). The stomach presented a normal histological structure, clearly differentiating the mucosal, submucosal, and muscular layers without alterations or significant changes (Fig. 5c,d). The brain presented a normal histological structure in rostral and caudal areas, without significant changes in the distribution of gray and white matter. All cortical and subcortical structures were observed, clearly differentiating the olfactory bulbs, telencephalic cortex, hippocampi, striatum, as well as the different thalamic and hypothalamic nuclei in the region of midbrain. In the hindbrain, the different centers were clearly observed, as well as the cerebellum with a preserved structure in the cortex and region of the deep nuclei. Ventricles of preserved size and morphology were also observed in which the choroid plexus of normal structure could be identified (Fig. 5e-h).

4. Discussion

The analysis of tissues derived from the oral treatment results, in the small intestine, in a higher presence of Paneth cells, which increased by 27 % compared to control animals. There was proliferation of lymphoid tissue and connective tissue in the lamina propria, the latter generating fibrous masses with apoptotic nuclei, and an 8–10 % decrease in weight in animals fed with toxic muscle compared to the control group. No other external nor behavioral signs were observed.

Paneth cells are a type of cell found in the small intestine. These highly specialized cells were described by Gustav Schwalbe and Josef Paneth in the late 19th century as columnar epithelial cells possessing prominent eosinophilic granules in their cytoplasm (Paneth, 1888). Paneth cells are in the small intestinal crypts of Lieberkühn and play an integral role in maintaining intestinal homeostasis and modulating the physiology of the small intestine and its associated microbial flora (Wallaeys et al., 2023). Paneth cells produce and secrete antimicrobial peptides and proteins that regulate the epithelial environment and help defend the host against invading microorganisms. Paneth cells are also involved in the production of digestive enzymes and mucin, a protein that helps protect the lining of the small intestine from damage. Paneth cells secrete to both modulate the microbiome and mediate the inflammatory response. IgA is one such component which may be produced by plasma cells in the lamina propria before accumulating and associating in Paneth cell granules. This in turn plays a significant role in secondary regulation of the host microvasculature, the normal injury and repair mechanisms of the intestinal epithelial layer, and the levels of intestinal inflammation (Lueschow, McElroy, 2020). Autophagy is also an important process for Paneth cells. Because Paneth cells tend to live longer than most other cells of the gut and have many aggregated proteins that could be recycled by other neighboring cells, as damage and stressors occur to the cells, autophagy becomes activated. The position of the Paneth cell in the intestinal crypts also allows them to interact directly with intestinal stem cells, influencing the intestinal stem cells niche as has recently been revealed (Cray et al., 2021).

CTXs are toxins produced by certain species of marine algae that can accumulate in the flesh of some fish and cause a condition known as ciguatera, which is characterized by gastrointestinal, cardiovascular, and neurological symptoms. To the best of our knowledge, until now, no relationship has been documented between Paneth cells and ciguatoxins despite their involvement in digestive health and intestinal homeostasis. Variations in the number of Paneth cells, both reductions or increases, have been described in humans and animal models associated with different pathological conditions such as infections, ischemic processes, or inflammation (Tanaka et al., 2001; Gassler, 2017). An increment of Paneth cells can lead to a condition known as Paneth cell metaplasia (PCM), which is characterized by an abnormal accumulation of these cells in the intestinal crypts, or Paneth cell hyperplasia (PCH), characterized by an increased number of Paneth cells in their usual location. Both conditions are indicators of chronic epithelial disease cell damage (Jenkins et al., 1997) and trigger a disruption of the intestinal epithelial barrier and an increase in inflammation, which can cause a variety of digestive and immunological problems such as Crohn's disease. Some of the most common symptoms include abdominal pain and bloating, malabsorption of nutrients, diarrhea, and weight loss (Tanaka et al., 2001; Simmonds et al., 2014). Other potential problems associated with an increased number Paneth cells include a high risk of infection due to an imbalance of bacterial colonies in the intestine, as well as the increased risk to develop certain types of cancer, such as colorectal cancer (López-Arribillaga et al., 2021). In this work, the presence of PCH in the small intestine of animals, fed with contaminated fish, suggests a response to injury of the intestinal barrier caused by CTXs, which could be mediated by a direct or indirect action of CTXs on Paneth cells.

Alterations of lymphoid tissue in the small intestine can lead to a variety of conditions and complications. These problems include gastrointestinal infections that can lead to reduced production of immunoglobulins, and an increased risk of infection in the gastrointestinal tract, and malnutrition due to decreased absorption of nutrients. Altered lymphoid tissue in the small intestine can also contribute to the development of inflammatory bowel disease, as well as the risk of developing autoimmune disorders and food allergies (Eberl and Lochner, 2009; Zheng, Zhu, 2022). In this study, the increase in lymphocyte proliferation observed in the lamina propria also suggests a response to the injury caused by CTXs on the intestinal barrier. In this sense and considering the increase observed in Paneth cells, there could be a correlation between both facts. As described above, some components, such as IgA, are produced by plasma cells (stimulated B lymphocytes) of the lamina propria before being accumulated and associated in Paneth cells. Therefore, the increase in both cell populations seems logical as a joint response by the intestinal mucosa to the presence of CTXs.

Fibrous lesions in the small intestine are a common complication of inflammatory bowel disease and may be a sign of a more serious underlying medical condition such as Crohn's disease or celiac disease. Treatment for these conditions may involve dietary changes and medications to reduce inflammation and pain. In some cases, surgery may be necessary to remove the lesions. If left untreated, these conditions can lead to serious complications, including malnutrition and bowel obstruction (Wu et al., 2023). Our results in the experimental conditions, in which the animals were fed with contaminated fish for 10 weeks, the proliferation of lymphocytes and the presence of proliferative fibrous lesions in the mucosal layer of the small intestine, are clear signs of local inflammatory response, suggestive of direct and sustained damage to the intestinal epithelial barrier by the action of CTXs. The presence of pyknotic nuclei compatible with apoptotic cells is equally indicative of direct tissue damage. On the other hand, the presence of fibrous lesions along the small intestine represents an increase in the thickness of the mucosal layer, compatible with an inflammatory process, that could interfere with a normal intestinal absorption (Rieder et al., 2012), thus justifying the 8–10 % weight loss observed in animals fed with CTX-contaminated fish.

The analysis of the rest of the organs of the digestive apparatus as well as organs of other apparatus and systems did not reveal significant

histological alterations or changes at cellular level in any of them with the treatment regimen used. In contrast, there are studies in which using different treatment regimens, specifically higher concentrations of CTXs for shorter periods of time have described alterations in organs such as the heart (Terao et al., 1991; 1992).

5. Conclusions

CP syndrome affects humans as a consequence of the ingestion of CTX-contaminated fish through the trophic chain. In general, there is abundant information about the digestive, cardiovascular and neurologic effects of ciguateric syndrome in terms of symptomatology; nonetheless, data on the damage that toxins may produce on the different organs of affected individuals are scarce. In this work, we have focused on analyzing the damage caused in organs of mice. The effects were analyzed using Swiss albino mice treated by oral administration of low concentration of CTX-enriched food-fish. Thus, the experiment was performed by feeding mice with CTX-contaminated fish muscle at concentrations 10-times below the median lethal dose (LD_{50}). All animals were necropsied, and their organs examined. Alterations were found mainly in the small intestine. The analysis of tissues from the feeding experiment resulted in an increase of Paneth cells, as well as presence of lymphoid tissue proliferation and fibrous lesions in the mucosal layer and a decrease in weight animals treated with contaminated fish. These findings are suggestive signs of a response to direct and sustained damage to the epithelial barrier of the small intestine by the action of CTXs. Thus, the administration of CTX-contaminated fish at low doses, in which no ciguateric syndrome symptoms nor behavioral changes were observed in the individuals, causes alterations in gastrointestinal tissues and decreasing weight. These facts reinforce the need and relevance of accurate detection methods of CTXs in the seafood trophic chain.

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CRediT authorship contribution statement

Fernando Real: Writing – original draft, Investigation. **Ana R. Diaz-Marrero:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Conceptualization. **Natalia García-Álvarez:** Investigation. **Víctor Hernández-López:** Writing – original draft, Investigation. **Ricardo Reyes:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **José J. Fernández:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization.

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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Institutional Review Board Statement

All animal procedures were performed in accordance guidelines and regulations approved by the Ethics Committee of University of La Laguna (ULL). This study was completed in strict accordance with the authorization and protocol reference number ULL-CEIBA2020–0397

References

Bottein, M.Y., Wang, Z., Ramsdell, J.S., 2011. Toxicokinetics of the ciguatoxin P-CTX-1 in rats after intraperitoneal or oral administration. Toxicol *284* (*1-3*), 1–6. https://doi.org/10.1016/j.tox.2011.02.005.

Caillaud, A., Eixarch, H., de la Iglesia, P., Rodriguez, M., Dominguez, L., Andree, K.B., Diogène, J., 2012. Towards the standardisation of the neuroblastoma (neuro-2a) cell-based assay for ciguatoxin-like toxicity detection in fish: application to fish caught in the Canary Islands. Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. *29* (*6*), 1000–1010. https://doi.org/10.1080/19440049.2012.660707.

Eur-lex.europa.eu:Regulation (EU) 2017/625 (https://eur-lex.europa.eu/eli/reg/2017/625/oj); Regulation (EU) 2019/627 (https://eur-lex.europa.eu/eli/reg_impl/2019/627/oj)..

Chinain, M., Gatti, C.M.I., Darius, H.T., Quod, J.P., Tester, P.A., 2021. Ciguatera poisonings: A global review of occurrences and trends. Harmful Algae *102*, 101873. https://doi.org/10.1016/j.hal.2020.101873.

Costa, P.R., Churro, C., Rodrigues, S.M., Frazão, B., Barbosa, M., Godinho, L., Soliño, L., Timóteo, V., Gouveia, N., 2023. A 15-Year Retrospective Review of Ciguatera in the Madeira Islands (North-East Atlantic, Portugal). Toxins *15*, 630. https://doi.org/10.3390/toxins15110630.

Cray, P., Sheahan, B.J., Dekaney, C.M., 2021. Secretory Sorcery: Paneth Cell Control of Intestinal Repair and Homeostasis. *Cell Mol. Gastroenterol. Hepatol. 12* (*4*), 1239–1250. https://doi.org/10.1016/j.jcmgh.2021.06.006.

Eberl, G., Lochner, M., 2009. The development of intestinal lymphoid tissues at the interface of self and microbiota. Mucosal Immunol. *2* (*6*), 478–485. https://doi.org/10.1038/mi.2009.114.

FAO and WHO. 2020. Report of the Expert Meeting on Ciguatera Poisoning. Rome, 19–23 November 2018. *Food Safety and Quality* No. 9. Rome. https://doi.org/10.4060/ca8817en.

Gassler, N., 2017. Paneth cells in intestinal physiology and pathophysiology. World J. Gastrointest. Pathophysiol. *8*, 150–160. https://doi.org/10.4291/wjgp.v8.i4.150.

Jenkins, D., Balsitis, M., Gallivan, S., Dixon, M.F., Gilmour, H.M., Shepherd, N.A., Theodossi, A., Williams, G.T., 1997. Guidelines for the initial biopsy diagnosis of suspected chronic idiopathic inflammatory bowel disease. The British Society of Gastroenterology Initiative. J. Clin. Pathol. *50* (*2*), 93–105. https://doi.org/10.1136/jcp.50.2.93.

Lewis, R.J. Detection of toxins associated with ciguatera fish poisoning. In Manual on Harmful Marine Microalgae; UNESCO: Paris, France, 2003; pp. 267–277.

Lewis, R.J., Sellin, M., Poli, M.A., Norton, R.S., MacLeod, J.K., Sheil, M.M., 1991. Purification and characterization of ciguatoxins from moray eel (Lycodontis javanicus, Muraenidae). Toxicon *29* (*9*), 1115–1127. https://doi.org/10.1016/0041-0101(91)90209-a.

López-Arribillaga, E., Yan, B., Lobo-Jarne, T., Guillén, Y., Menéndez, S., Andreu, M., Bigas, A., Iglesias, M., Espinosa, L., 2021. Accumulation of Paneth Cells in Early Colorectal Adenomas Is Associated with Beta-Catenin Signaling and Poor Patient Prognosis. Cells *10* (*11*), 2928. https://doi.org/10.3390/cells10112928.

Lueschow, S.R., McElroy, S.J., 2020. The Paneth Cell: The Curator and Defender of the Immature Small Intestine. *Front. Immnunol. 11*, 587. https://doi.org/10.3389/fimmu.2020.00587.

Paneth, J., 1888. Ueber die secernirenden Zellen des Dünndarm-Epithels. Arch. Mikrosk. Anat. *31*, 113–191.

Ramos-Sosa, M.J., García-Álvarez, N., Sanchez-Henao, A., Padilla, D., Sergent, F.S., Gago-Martínez, A., Diogène, J., Caballero, M.J., Fernández, A., Real, F., 2023. Ciguatoxin-like toxicity distribution in flesh of amberjack (*Seriola* spp.) and dusky grouper (*Epinephelus marginatus*). Environ. Res. *228*, 115869 https://doi.org/10.1016/j.envres.2023.115869.

Raposo-García, S., Boente-Juncal, A., Rodriguez-Vieytes, M., Camiña, M., Costas, C., Cao, A., Louzao, M.C., Cifuentes, M., Vale, C., Botana, L.M., 2022. In vivo subchronic effects of ciguatoxin-related compounds, reevaluation of their toxicity. *Arch. Toxicol. 96* (*9*), 2621–2638. https://doi.org/10.1007/s00204-022-03315-0.

Rieder, F., Kessler, S., Sans, M., Fiocchi, C., 2012. Animal models of intestinal fibrosis: new tools for the understanding of pathogenesis and therapy of human disease. Am. J. Physiol. Gastrointest. Liver Physiol. *303* (*7*), G786–G801. https://doi.org/10.1152/ajpgi.00059.2012.

Sanchez-Henao, A., García-Álvarez, N., Padilla, D., Ramos-Sosa, M., Silva Sergent, F., Fernández, A., Estévez, P., Gago-Martínez, A., Diogène, J., Real, F., 2021.

Accumulation of C-CTX1 in Muscle Tissue of Goldfish (*Carassius auratus*) by Dietary Experience. Animals *11* (*1*), 242. https://doi.org/10.3390/ani11010242.

Satake, M., Murata, M., Yasumoto, T., 1993. The structure of CTX3C, a ciguatoxin congener isolated from cultured *Gambierdiscus toxicus*. Tetrahedron Lett. *34* (*12*), 1975–1978. https://doi.org/10.1016/S0040-4039(00)91978-6.

Satake, M., Ishibashi, Y., Legrand, A.M., Yasumoto, T., 1996. Isolation and structure of ciguatoxin-4A, a new ciguatoxin precursor, from cultures of dinoflagellate *Gambierdiscus toxicus* and parrotfish *Scarus gibbus*. *Biosci. Biotech. Biochem. 60* (*12*), 2103–2105. https://doi.org/10.1271/bbb.60.2103.

Simmonds, N., Furman, M., Karanika, E., Phillips, A., Bates, A.W., 2014. Paneth cell metaplasia in newly diagnosed inflammatory bowel disease in children. BMC Gastroenterol. *14*, 93. https://doi.org/10.1186/1471-230X-14-93.

Soliño, L., Costa, P.R., 2018. Differential toxin profiles of ciguatoxins in marine organisms: Chemistry, fate and global distribution. Toxicon *150*, 124–143. https://doi.org/10.1016/j.toxicon.2018.05.005.

Soliño, L., Costa, P.R., 2020. Global impact of ciguatoxins and ciguatera fish poisoning on fish, fisheries and consumers. Environ. Res. *182*, 109111 https://doi.org/10.1016/j.envres.2020.109111.

Spielmeyer, A., Loeffler, C.R., Bodi, D., 2021. Extraction and LC-MS/MS Analysis of Ciguatoxins: A Semi-Targeted Approach Designed for Fish of Unknown Origin. Toxins *13*, 630. https://doi.org/10.3390/toxins13090630.

Tanaka, M., Saito, H., Kusumi, T., Fukuda, S., Shimoyama, T., Sasaki, Y., Suto, K., Munakata, A., Kudo, H., 2001. Spatial distribution and histogenesis of colorectal Paneth cell metaplasia in idiopathic inflammatory bowel disease. J. Gastroenterol. Hepatol. 16, 1353–1359. https://doi.org/10.1046/j.1440-1746.2001.02629.x.

Terao, K., Ito, E., Oarada, M., Ishibashi, Y., Legrand, A.M., Yasumoto, T., 1991. Light and electron microscopic studies of pathologic changes induced in mice by ciguatoxin poisoning. Toxicon *29* (*6*), 633–643. https://doi.org/10.1016/0041-0101(91)90056-w.

Terao, K., Ito, E., Yasumoto, T., 1992. Light and electron microscopic studies of the murine heart after repeated administrations of ciguatoxin or ciguatoxin-4C. Nat. Toxins *1* (*1*), 19–26. https://doi.org/10.1002/nt.2620010106.

UBC ACC TECH 09a Oral Dosing (Gavage) in Adult Mice SOP (Last date revised: February 2021; version No. 2; https://animalcare.ubc.ca/sites/default/files/documents/TECH%2009a%20-%20Oral%20Dosing%20%28Gavage%29%20in%20Adult%20Mice%20April%202021%20Final.pdf; (Last viewed: 04/07/2024)).

Wallaeys, C., Garcia-Gonzalez, N., Libert, C., 2023. Paneth cells as the cornerstones of intestinal and organismal health: a primer. EMBO Mol. Med. *15* (*2*), e16427 https://doi.org/10.15252/emmm.202216427.

Wu, X., Lin, X., Tan, J., Liu, Z., He, J., Hu, F., Wang, Y., Chen, M., Liu, F., Mao, R., 2023. Cellular and molecular mechanisms of intestinal fibrosis. Gut Liver *17*, 360–374. https://doi.org/10.5009/gnl220045.

Yasumoto, T., Murata, M., 1993. Marine toxins. Chem. Rev. *93* (*5*), 1897–1909. https://doi.org/10.1021/cr00021a011.

Zheng, M., Zhu, J., 2022. Innate lymphoid cells and intestinal inflammatory disorders. *Int. J. Mol. Sci. 23* (*3*), 1856. https://doi.org/10.3390/ijms23031856.