Sea cucumber (Isostichopus badionotus) body-wall preparations exert anti-inflammatory activity in vivo


A Centro de Investigación y de Estudios Avanzados del IPN – Unidad Mérida, Laboratorio de Inmunología y Biología Molecular, Antigua Carretera a Progreso Km. 6, 97310 Mérida, Yucatan, Mexico
b School of Medicine, Medical Sciences and Nutrition, University of Aberdeen, AB25 2ZD Aberdeen, UK
c Applied Science Research Foundation, A.C., Calle 26 No. 144 x 21 y 21A, Col. San Pedro Cholul, 97318 Mérida, Yucatan, Mexico
d Universidad Autónoma del Estado de Morelos, Departamento de Fisiología y Farmacología, Facultad de Medicina, Calle Leñeros s/n, Col. Los Volcanes, 62350 Cuernavaca, Morelos, Mexico

Laboratorio de Anatomía Patológica (ANAPAT), Av. Yucatán 630, Pracc. Jardines de Mérida, 97135 Mérida, Yucatan, Mexico

University of the Philippines Los Baños College, Institute of Chemistry, 4031 Laguna, Philippines

IMDEA Food Institute, CSE UAM + CSIC, Carretera de Canillo blanco 8, 28049 Madrid, Spain

Centro de Investigación y de Estudios Avanzados del IPN – Unidad Mérida, Departamento de Física Aplicada, Antigua Carretera a Progreso Km. 6, 97310 Mérida, Yucatan, Mexico

Instituto Politécnico Nacional, Unidad de Investigación, Desarrollo e Innovación Médica y Biotecnológica (UDIMERB), Prolongación de Carpio y Plan de Ayala s/n, Col. Santo Tomás, Del. Miguel Hidalgo, 11340 Ciudad de México, Mexico

ABSTRACT

Sea cucumbers contain many bioactive compounds with potential health benefits. Their widespread use in East Asia, in traditional medicines and as food supplements, are depleting many local stocks and thus increasing their harvest worldwide. In recent years this has included heavy fishing of Isostichopus badionotus from the Yucatan Peninsula. The bioactivities in sea cucumber are known to vary greatly with species and growth conditions. Despite this, little study has been done on the capacity of I. badionotus captured from Yucatan to modulate health in vivo. Sea cucumbers were harvested from the Yucatan coast and body wall prepared, freeze dried and ground. The anti-inflammatory properties were evaluated using a hen’s egg test – chorioallantoic membrane (HET-CAM) assay, a rat feeding trial, and with a mouse ear inflammation model. Additional analysis was done by histology and qRT-PCR. Extracts of lyophilized I. badionotus exerted a strong anti-inflammatory activity in each of the assays. They attenuated histological disruption caused by inflammatory agents, repressed the expression of pro-inflammatory genes including TNFα, iNOS, COX2, NFκB or IL-6, and slightly enhanced the expression of anti-inflammatory or survival genes. Our study demonstrates that sea cucumber I. badionotus from the Yucatan Peninsula exhibits potent anti-inflammatory activity in vivo.

1. Introduction

Chronic metabolic and autoimmune diseases, including diabetes, arthritis, CNS disorders and IBD are an increasing worldwide problem [1,2]. Inflammation is a causal or exacerbating factor in these disorders [3], necessitating regular use of anti-inflammatory drugs in their management. However, most of the currently available drugs have significant long-term debilitating side-effects. Therefore, new nutraceutical or pharmaceutical anti-inflammatory products are urgently needed, and in fact they are heavily investigated [4,5].

Many marine organisms express novel bioactive factors that have the potential to ameliorate disease or promote health [6]. Sea cucumbers contain factors, such as lectins, bioactive peptides, fucosylated chondroitin sulphates, and fucoidans, and have been ascribed with a

https://doi.org/10.1016/j.phanu.2018.03.002

Received 22 February 2018; Received in revised form 24 March 2018; Accepted 26 March 2018

Available online 27 March 2018

2213-4344/ © 2018 Elsevier B.V. All rights reserved.
range of therapeutic properties in vivo, including antimicrobial, antithrombotic, anticoagulant, wound healing, antioxidant, anti-hypertension, anti-tumor and anti-inflammatory [7-10]. As such, they have been employed as food supplements and in the traditional medicines for centuries in many East Asian countries.

The demand for sea cucumber products was previously met mainly through harvest of local species. However, depletion of East Asian stocks has now led to heavy fishing of edible sea cucumbers around the world [11,12]. In recent years, this has included capture of *Isostichopus badionotus* [Selenka 1867] from the Yucatan Peninsula (Mexico). This sea cucumber species is widely distributed throughout the western Atlantic Ocean, from South Carolina (USA) to Brazil, including the Caribbean Sea, but can also be found at Ascension Island in the Mid-Atlantic and in the Gulf of Guinea, Western Africa [13].

The composition and bioactivity of edible sea cucumbers varies between species and is greatly affected by regional growth conditions [9,10]. Despite the upsurge in capture of *I. badionotus* from around Yucatan, little robust study of its possible therapeutic properties has been reported. In one of the few published studies, body wall preparations were shown to be hypolipidemic when incorporated into the diet of rats [14]. In the present study the anti-inflammatory properties of *I. badionotus* from the Yucatan have been investigated.

2. Material and methods

2.1. Sea cucumber collection, handling and processing

Adult *Isostichopus badionotus* [Selenka, 1867] were collected from the sea floor off the coast of Sisal, Yucatan, Mexico under the auspices of SAGARPA permit No. DGOPA/1009/210809/08761. The organisms were individually placed in plastic bags while still at the sea bottom, then brought to the surface and kept in marine water at 22 to 24 °C, a temperature range like that of the collection site: This procedure was done to prevent proteolysis or autolysis during transportation of organisms. Immediately on arrival at the laboratory, the animals were placed in tanks with filtered marine water under controlled conditions (23–24 °C, 20 organisms/m2 stocking density).

Animals were eviscerated immediately after removal from the holding tank, leaving the body wall which was then washed extensively with cold sterile distilled water. This material was further processed by three previously described methods [14]: lyophilizing [LS, lyophilized sea cucumber]; cooking in water for 1 h at 100 °C followed by lyophilizing [CS, cooked sea cucumber]; or oven-drying at 70 °C for 12 h [OS, oven-dried sea cucumber]. After drying, each batch was milled, first with a coffee grinder (Krupps Spiver Grinder GX4100) and then with a sample mill (Cyclotec, FOSS Tecator®, Hilleroed, Denmark). Powdered samples were stored in sealed plastic bags at 4 °C until use. The LS had a very high salt content (> 60 g/100 g) and was therefore dialyzed exhaustively against water (4 °C with a dialyzing membrane (Spectra/Por®) of 12 to 14 kDa cut off. Finally, the dialyzed sample was re-dried [LWS, desalted, lyophilized sea cucumber].

2.2. Hen’s egg test – chorio-allantoic membrane assay

The Hen’s Egg Test – Chorio-Allantoic Membrane (HET-CAM) assay was performed as described elsewhere [15]. Briefly, extract anti-inflammatory activity was compared with that of the anti-inflammatory agent hydrocortisone (commonly used in this method). Substances were prepared in phosphate-buffered saline (0.02 M PBS) at 0.5 μg/μL. Three treatments were prepared: eggs treated with sodium dodecyl sulfate (SDS) as an irritant; untreated eggs (control); and eggs treated with PBS alone (control). Each treatment had three replicates. Eggs were opened to expose the CAM, and 0.3 mL of the test extract and 0.3 mL 1% SDS applied simultaneously to the CAM surface. After 20 s, the CAM was rinsed with 5 mL water and evaluated for signs of inflammation, such as irritant endpoints hyperemia (increased blood flow), hemorrhage (ruptured blood vessels), and coagulation (blood clots formation). Observations were made at 0, 0.5, 2 and 5 min using a scoring system developed previously [15]. The total score was taken as the total irritation score for each tested extract. Each extract’s inhibitory effects on irritation were calculated from this score.

2.3. Experimental animals

Male CD1 mice (30 ± 5 g) were obtained from the Center for Research and Advanced Studies of the National Polytechnic Institute (Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional – CINVESTAV-IPN) at Zacatenco, Mexico and housed in an experimental animal facility at CINVESTAV-IPN Mérida under standard conditions (12 h light/dark cycle, 23 ± 2 °C, 65% humidity) with free access to water and pelleted food. They were acclimatized for at least seven days prior to the experiment. All protocols were approved by the Institutional Animal Care and Use Committee of CINVESTAV-IPN (No. 0126-15) and complied with the applicable Mexican Official Norm (NOM-062-ZOO-1999), “Technical Specifications for the Care and Use of Laboratory Animals”, as well as all applicable federal and institutional regulations.

For rat experiments, male Wistar (Harlan strain) rats (60 days old) received either an isonitrogenous diet containing 2% cholesterol (CC) or an experimental diet which contain 2% cholesterol and 50% protein from lyophilized-washed sea cucumber (LWS) meal. Rats received the supplementation during 16 days (n = 5 per group). At the end of the supplementation, animals were euthanized after overnight fasting and small intestine tissues obtained, cleaned and immediately frozen for further analysis. Detailed animal protocol and diet are found in Olivera-Castillo et al. [14].

2.4. Mouse ear anti-inflammatory activity

A mouse ear model of cutaneous irritation was used to evaluate anti-inflammatory activity [16]. Acute inflammation was induced in anesthetized (pentobarbital 25 mg/kg animal weight) mice by topical application of 12-O-tetradecanoylphorbol-13-acetate (TPA; phorbol myristate acetate; Sigma Cat. P8139) to both surfaces of the right ear at a concentration of 2.5 μg in 10 μL acetone. 10 μL of vehicle was topically applied to each surface of the left ear. One of the extracts (125 μg/20 μL water/ear, each surface) or the anti-inflammatory dexamethasone (DXA) was applied (250 μg/10 μL water/ear, each surface) 15 min after the TPA. After six hours, the mice were re-anesthetized, and an electronic micrometer used to measure ear edema and thickness.

The mice were then euthanized by cervical dislocation, and both ears removed and weighed separately. A sterile biopsy punch (Integra Ear Biopsy Punch, Basingstoke, UK) was used to remove two representative dissections (5 mm diameter discs) from each ear, and the discs weighed. Inflammation was quantified as the difference between the average weight of the right ear discs (TPA alone or TPA + dexamethasone or TPA + extract) and that of the left ear discs (vehicle: acetone). Samples of ear discs were also taken for histological evaluation (fixed in 10% formalin) and qRT-PCR gene expression analysis (immersed in 400 μL RNAlater (Ambion) and stored at −80 °C).

2.5. Histological analysis

Formalin-fixed samples were processed using an AutotechniconDuo® System, mounted in paraffin blocks (Richard-Allan Scientific Paraffin Type 6®) and cut into sections (2 μm thickness) with a microtome (ThermoScientific Microm HM 325®). Sections were stained simultaneously with hematoxylin-eosin (H&E), examined with a conventional optical microscope and images taken with a digital camera (Evolution™ LC Color). A representative area was selected for qualitative light microscopic analysis. Histology scores were generated by two independent pathologists and averaged for analysis. Briefly, samples
were evaluated for the presence of neutrophil infiltration and edema, and each factor deemed to be either null, slight, moderate or intense (Supplementary Fig. S1). Numerical values were assigned to each intensity: null (0); slight (1); moderate (2); and intense (3). A sample’s total score was calculated by adding the values assigned each factor.

2.6. Gene expression analysis

Initial examinations for anti-inflammatory effects of lyophilized sea cucumber (LWS) was done using small intestine samples collected in a previous study in which rats received a high cholesterol diet with or without LWS [14]. After collection, the tissues were snap frozen in liquid nitrogen and stored at −70 °C until analysis. RNA was isolated from tissue using Trizol reagent (Invitrogen, USA), analyzed by 1% agarose gel electrophoresis, and quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific). Samples of RNA were reverse transcribed using Improm II Reverse transcriptase (Promega) following manufacturer instructions. Eighty-four unique genes related to inflammatory response and autoimmunity were analyzed using the RT2 Profiler PCR Array (Qiagen; Cat. PARN-077Z for rat). Quantitative real-time PCR (qRT-PCR) was performed using miScript SYBR Green Master Mix (Qiagen) following manufacturer instructions and using a 7900HT Real-Time PCR System (Applied Biosystems).

For the ear tissue samples, RNA was isolated from both healthy (non-inflamed) and treated ears at 6 h. Samples (≈10 mg) were frozen with liquid N2 and ground in a sterile mortar and pestle to produce a fine powder. Lysis buffer (Norgen, Cat. 25700) was added and grinding continued until the sample was homogenized. RNA was isolated using the Animal Tissue RNA Purification Kit (Norgen), following manufacturer instructions. After isolation, RNA was eluted in ultrapure nuclease-free distilled water (Invitrogen, Cat. 10977015) and stored at −80 °C. The quantity and quality (purity and integrity) of the RNA were evaluated with a NanoDrop 2000c and by visual analysis on 1.2% agarose gels. Genomic DNA (gDNA) was removed using the TURBO DNA-free™ Kit (Ambion, Cat. AM19907) per manufacturer instructions. Complete elimination of residual gDNA was confirmed by PCR endpoint using the RNA-treated as a template and a pair primer for the 18s rRNA gene (product of 215 bp). Synthesis of cDNA was done using the RevertAid H Minus First Strand cDNA kit (Thermo Scientific, Cat. K1632), following manufacturer instructions. Single-strand DNA (ssDNA) was diluted at a 1:20 ratio for further use in real-time PCR (qRT-PCR). Quantitative real-time PCR (qRT-PCR) assays were run using specific primer pairs (Supplementary Table S1) in a Rotor Gene Q (2-plex) real-time PCR detection system with QuantiNova SYBR Green PCR master mix (Qiagen, Cat. 208056). All reactions were performed in quadruplicate, including non-template reactions as negative controls. Thermo-cycling conditions were 5 min at 95 °C, followed by 40 cycles of 15 s at 94 °C, and 45 s at 62 °C (annealing-extension). Dissociation curves were generated to verify amplification of a single product and non-specific products (shoulders), contamination or dimers. (β-actin (ACTB) and elongation factor 2 (EEF2) were used as housekeeping genes. Results were expressed relative to the normalized transcription levels of the control sample (calibrator) (i.e. healthy ear sample). Relative expression was calculated using the 2−ddCT method, as previously described [17].

2.7. Statistical analysis

Statistical analysis was done using a Generalized Linear Modelling (GLM) procedure to estimate the effect of the factor levels (i.e. ear weight results), as well as for the variable of gene expression (fold change, RQ) for genes. Over-dispersion was detected (from residual Poisson regression model) and corrected using the Poisson GLM. The standard errors were multiplied by the square root of the dispersion parameter using a quasi-Poisson GLM model. Variance (Var[Yi] = μiφi, where μ is the mean and φ the dispersion parameter) was calculated with the GLM model. Significant difference was set at P-value < 0.05, and only differences versus vehicle + TPA shown. A customized R (www.r-project.org) function was used for all statistical analyses.

3. Results

3.1. Initial evidence of anti-inflammatory action of I. badionotus from the Yucatan Peninsula

To determine if I. badionotus from Yucatan might exhibit anti-inflammatory activity in vivo, intestine samples previously collected from rats fed a diet containing lyophilized and washed sea cucumber body wall (LWS) were run through a qRT-PCR analysis of 84 key genes related to inflammatory cytokines and receptors (Fig. 1). In the intestine from rats fed high cholesterol diet supplemented with LWS, the expression of inflammation-associated genes including interleukin 6 (IL-6); interleukin 1 beta (IL-1); integrin beta 2 (ITGB2); interleukin 1 receptor accessory protein (IL1RAP); chemokine [C-X-C motif] ligand 9 (CXCL9); and toll-like receptor 3 (TLR3), was lowered compared to that in intestine from rats fed only a high cholesterol diet. Further, dietary sea cucumber increased the expression of other genes with immunoregulatory activity, including interleukin 9 (IL9), which dampens down Th17 cell activity in autoimmune diseases [18]; chemokine [C-C motif] ligand 7 (CCL7), involved in cellular trafficking and tissue regeneration [19,20]; and chemokine [C-C motif] receptor 7 (CCR7), a cellular homeostasis regulator in mucosal tissue [21,22]. These preliminary results suggested that sea cucumber I. badionotus had anti-inflammatory and immunomodulatory effects in rodents.

3.2. Sea cucumber extracts exert in vitro anti-inflammatory effects

To determine whether I. badionotus could also ameliorate acute inflammation, its efficacy was first assessed in irritation assay in vitro. The HET-CAM assay is widely used to assess inhibition of irritation responses to a stresor, in this case sodium dodecyl sulphate (SDS) [15]. LWS, cooked and lyophilised (CS) and oven-dried (OS) sea cucumber meals all inhibited irritation induced by SDS in the assay (Fig. 2A). Irritation was reduced by more than sixty percent in all cases (Fig. 2B).
3.3. Sea cucumber extracts exert in vivo anti-inflammatory effects in mice

To further confirm this anti-inflammatory effect, a mouse model was used in which ear inflammation was induced by TPA. This model had been used previously by others to screen for natural anti-inflammatory molecules [16]. Representative discs taken at six hours from the TPA-treated left ear of mice were substantially heavier than similar discs from the non-treated (right) ear (Fig. 3A), indicative of TPA-mediated inflammation. Topical co-treatment with LWS, CS or OS preparations reversed this ear response to TPA, with LWS being most effective.

Histological analysis of the TPA-treated ears (Fig. 3B) identified an inflammation inhibition/regression phenotype associated with co-administration of LWS, OS or CS. TPA-induced inflammatory cell infiltration and edema was diminished in tissue from co-treated mouse ear. This histological analysis therefore confirmed the anti-inflammatory properties of the sea cucumber preparations in vivo.

With a second cohort of mice it was confirmed that the optimal inflammatory response to TPA alone occurred at six-hour post-treatment (Fig. 4A) and that LWS effectively ameliorated TPA’s effects on ear weight (Fig. 4B), histopathology (Fig. 4C and Supplementary Fig. S2 for higher resolution images) and histopathology score (Fig. 4C) at this timepoint.

3.4. Lyophilized sea cucumber exerts anti-inflammatory effects by targeting key genes related to inflammation

To explore the possible molecular mechanisms modulated by LWS in this assay, the expression of eight inflammation-associated genes related was analyzed in ear samples: tumor necrosis factor alpha (TNFa); interleukin 6 (IL-6); interleukin 10 (IL-10); interleukin 11 (IL-11); nuclear factor kappa B (NF-xB); inducible nitric oxide synthase (iNOS); cyclooxygenase 2 (COX2); and signal transducer and activator of transcription 3 (STAT3). Tissue expression of these genes was upregulated in response to the irritant TPA (Fig. 5). LWS limited the effects of TPA on gene expression and was more effective than dexamethasone (Fig. 5) indicative of potent anti-inflammatory activity (Fig. 5).

4. Discussion

In the present study lyophilized sea cucumber (Isostichopus badionotus) [LWS] from the Yucatan Peninsula had in vitro (HET-CAM assay) and in vivo (rat small intestine/diet trial, and mouse ear/TPA inflammation model) anti-inflammatory actions. Dietary LWS down-regulated inflammation-associated genes in rat intestine and enhanced expression of pathways important for homeostasis and gut barrier integrity, even in the absence of overt inflammation in the tissue. Topical LWS greatly reduced irritation caused by SDS in the HET-CAM assay and limited the upregulation of pro-inflammatory genes, inflammatory cell infiltration and edema induced in mouse ears by TPA.

The components of I. badionotus responsible for its anti-inflammatory activity remain to be elucidated. This species contains high amounts of bioactive factors, such as fucosylated chondroitin sulphates, fucoidans, and lectins [23], which have been linked with the anti-inflammatory activities of other sea cucumber species [6,24,25]. The anti-inflammatory activity of I. badionotus may therefore be a result of the actions of these constituents, alone or in combination, but the involvement of other bioactive factors [6,10], in the protective effects of I. badionotus cannot be excluded.

The mechanisms involved in I. badionotus’s anti-inflammatory actions are also unclear but may be linked to the Nuclear Factor kappa-B (NF-xB) molecular signaling pathway [26]. In the present study, this was modulated by I. badionotus thereby limiting expression of the inflammation-associated down-stream genes COX2 and TNF. Holothurian factors have been reported to act on this molecular signaling pathway in part by preventing cytosolic release or translocation of active NF-xB [RelA(p65)/p50] to the nucleus, thereby limiting down-
stream gene induction [31].

An intriguing finding of this study was that the basal levels of inflammation in the rat intestine were lowered by dietary *I. badionotus*. Low grade inflammation is a feature of the normal healthy intestine; there to protect the host during transient loss of epithelial integrity due to damage or imbalanced cell production and shedding or interaction with and sampling of antigens or bacteria [32]. While dietary *I. badionotus* may have had direct anti-inflammatory actions on the gut, it also upregulated pathways important in gut homeostasis and integrity. The observed reduction in intestinal inflammation could therefore be a result of enhanced gut integrity facilitated by *I. badionotus*. Further, sea cucumbers contain many antimicrobial factors [6,10,23]. These may have altered the intestinal microbiome and thereby lowered the basal levels of inflammation in the intestine [33]. Either way, dietary *I. badionotus* has the capacity to modify gut metabolism and promote homeostasis. It could therefore have potential as a food supplement to aid in gut recovery from disease.

5. Conclusion

In the present study, body wall preparations of *I. badionotus* exhibited pharmacologically promising anti-inflammatory activity in vivo. In addition, dietary *I. badionotus* has previously been shown to be hypcholesterolemic [14]. This indicates overall that *I. badionotus* from Yucatan peninsula has considerable potential as a source of nutraceuticals to combat disease and promote health. However, robust clinical trials need to be done to establish if this sea cucumber or its bioactive constituents have demonstrable and reproducible health benefits for humans.

Author contributions


Funding information

This study was supported in part by different grants and funds: CONACyT fondos mixtos Yucatán (M0023-A1) “El pepino de mar como un alimento funcional: obtención de sus principios bióactivos, caracterización biológica, efectos sobre el metabolismo y sistema inmune utilizando un modelo murino”; CONACyT basic science grant (10017) “Actividad antiinflamatoria y cicatrizante del pepino de mar (*Isostichopus badionotus*) en un modelo murino: caracterización de la actividad farmacológica y los mecanismos moleculares involucrados” to R R-C; INFRA-252665 and CB-254742 grants to MA F-H; by the Spanish Agencia Estatal de Investigación (AGL2016-78922-R) and European FEDER Funds to A.D.; and by the “For Women in Science Program” for the L’Oréal – UNESCO – Conacyt – AMC fellowship to MA F-H.

Conflict of interest

The authors declare no conflict of interest.
Fig. 4. LWS from sea cucumber (I. badionotus) exerts anti-inflammatory effects. Mouse ears exposed to vehicle (control, left ear), or inflammatory agent TPA alone, or TPA followed by lyophilized sea cucumber extract (LWS) (right ear). A) Time-course of ear inflammation model. Difference in ear weights: right-left (blank); n ≥ 3 per group. B) Difference in ear weights: right-left (blank); n ≥ 5 per group. C) Representative photomicrographs of transverse section of mouse ears sensitized with TPA or vehicle, stained with haematoxylin-eosin (magnification 10×). Images are representative of three independent experiments with similar results. D) Quantification of skin micrographs in B. Values are the mean ± SEM, n = 3 per group. * Significant difference (P < 0.05) from vehicle + TPA. (a.u.), arbitrary units.

Fig. 5. LWS from sea cucumber (I. badionotus) targets principal inflammation-related genes. Mouse ears were exposed to vehicle alone (left ear), or inflammatory agent TPA followed by lyophilized sea cucumber, or TPA followed by dexamethasone (DXA) as an anti-inflammatory control (right ear). Genes were evaluated by qRT-PCR. Values (fold change) are the mean ± SEM. * Significant difference (P < 0.05) from vehicle + TPA.
Acknowledgements

The authors thank Victor May Solis and Angel Fuentes Chim for their assistance in sample preparation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.phanu.2018.03.002.

References