Brief Communication

Pro-inflammatory effects of the aqueous extract of *Echinometra lucunter* sea urchin spines

Juliana Mozer Sciani[1,2], Bianca Cestari Zychar[3], Luis Roberto de Camargo Gonçalves[3], Thiago de Oliveira Nogueira[3], Renata Giorgi[3] and Daniel Carvalho Pimenta[1,2]

[1]Laboratório de Bioquímica e Biofísica, Instituto Butantan, 05503-900, São Paulo; [2]Centro de Biologia Marinha, Universidade de São Paulo, 11600-000, São Sebastião; [3]Laboratório de Fisiopatologia, Instituto Butantan, 05503-900, São Paulo, SP, Brazil
Corresponding author: Daniel Carvalho Pimenta, Avenida Vital Brazil, 1500, São Paulo – SP, Brazil. Email: dcpimenta@butantan.gov.br

Abstract

The sea urchin, *Echinometra lucunter*, can be found along the Western Central Atlantic shores. In Brazil, it is responsible by circa 50% of the accidents caused by marine animals. The symptoms usually surpass trauma and may be pathologically varied and last differently, ranging from spontaneous healing in a few days, to painful consequences lasting for weeks. In this work, we have mimicked the sea urchin accident by administering an aqueous extract of the spine into mice and rats and evaluated the pathophysiological developments. Our data clearly indicate that the sea urchin accident is indeed a pro-inflammatory event, triggered by toxins present in the spine that can cause edema and alteration in the leukocyte–endothelial interaction. Moreover, the spine extract was shown to exhibit a hyperalgesic effect. The extract is rich in proteins, as observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, but also contains other molecules that can be analyzed by reversed phase high-performance liquid chromatography. Altogether, these effects corroborate that an *E. lucunter* encounter is an accident and not an incident, as frequently reported by the victims.

Keywords: *Echinometra lucunter*, toxins, inflammation, hyperalgesia

Experimental Biology and Medicine 2011; **236**: 277–280. DOI: 10.1258/ebm.2010.010257

Introduction

Echinometra lucunter (Echinodermata: Echinoidea) (Linnaeus, 1758) is an edible widespread sea urchin species, commonly found in the Western Central Atlantic. In Brazil, it is the most abundant sea urchin species.[1] The outer bodies of sea urchins are covered by appendages (calcified spines, in the case of *E. lucunter*) that are mainly involved in the passive and/or active defense of the animal.[2] This characteristic, associated to its human beloved habitat, makes this urchin responsible by circa 50% of the accidents caused by marine animals in Brazil.[3]

Symptoms from sea urchin accidents usually surpass trauma and may be pathologically varied. Spine penetration causes intense and immediate pain, bleeding, erythema, edema and local myalgia.[4–6] Without treatment, these acute symptoms usually spontaneously heal in a few days, or weeks in the worst cases. However, injuries may complicate due to secondary infections or the development of a chronic inflammatory response, normally associated to the presence of spine fragments and a resultant granuloma formation.[7–9]

Taking into account the clinical reports of often painful Brazilian sea urchin accidents, we have assessed the pro-inflammatory activities of the aqueous spine extract of *E. lucunter*. In order to achieve this, initial biochemical characterization was performed and the main symptoms observed in the victims were mimicked by *in vitro* and *in vivo* assays.

Material and methods

Sea urchins and aqueous extract collection

Specimens of *E. lucunter* were collected in São Paulo, Brazil (23°49′53′S; 45°31′18′W), under license number 13852-1 from the Brazilian Environmental Agency (IBAMA – Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis). Animals were collected without distinction of sex, age or size. A hundred and fifty spines were removed from five animals, washed very quickly with distilled water and immediately immerged in ammonium acetate (100 mmol/L, pH 7.5) for 24 h, at 4°C. The solution was centrifuged and the supernatant was lyophilized and diluted in

ISSN: 1535-3702

sterile saline solution or in compatible buffers, depending on the subsequent assay.

Biochemical characterization

In order to assess the protein contents of the *E. lucunter* spine extract, a 12% polyacrylamide gel electrophoresis, containing sodium dodecyl sulfate (SDS-PAGE), was performed, in reducing conditions, according to Laemmli.[10] The aqueous extract also was analyzed by reversed phase high-performance liquid chromatography (RP-HPLC) using a binary HPLC system (20A Prominence, Shimadzu Co, Kyoto, Japan), in a C18 column (ACE C18, 5 μm, 100 Å, 50 mm $\times$ 4.6 mm) with trifluoroacetic acid (TFA)/H_2O (1:1000) and TFA/acetonitrile (ACN)/H_2O (1:900:100) solvents (A and B, respectively). The column was eluted at a constant flow rate of 1.2 mL/min with a 0–100% gradient of solvent B over 10 min.

Biological assay

Male Swiss mice (20–25 g) and male Wistar rats (180 g) used in experiments were treated and maintained under ethical conditions at animal housing facilities of Instituto Butantan (protocol 438/07).

Leukocyte responses within mouse cremaster venules were assessed by intravital microscopy. Aqueous extract (10 μg/100 μL) or sterile saline (control) were injected in mice ($n = 5$), into the subcutaneous tissue of the scrotal bag. After 2, 4, 24 or 48 h, animals were anesthetized with injection (subcutaneously) of ketamine (100 mg/kg) and xylazine (10 mg/kg) and the cremaster muscle was exteriorized for microscopic examination *in situ* as previously described by Baez.[11]

Paw edema was induced by intraplantar injection of 10 and 20 μg of aqueous extract (diluted in 30 μL of sterile saline), into the footpad of mice ($n = 5$). The contralateral paw received the same volume of saline (control paw). Paw edema was evaluated by a plethysmometer (Letica, Barcelona, Spain) at 15, 30 min, 1, 2, 3 and 4 h after injection. The results were expressed as the difference (%) of volume between paws injected with extract and saline.

Rats ($n = 8$) were evaluated by the paw pressure test before and at different times (1, 2 and 4 h) after intraplantar injection of the spine aqueous extract (10 and 20 μg) or sterile saline (control). The pain threshold was measured using an Ugo Basile® pressure apparatus, essentially as described elsewhere.[12]

The hemorrhagic activity of aqueous extract was verified, according to the modified method described by Kondo *et al.*[13] Briefly, 10 and 20 μg of the spine aqueous extract were injected (intradermic route) into the abdominal skin of mice ($n = 5$). Three hours after injection, animals were euthanized, and the skin was removed in the area of injection to determine hemorrhagic spots in the internal face of the skin.

The results are presented as mean $\pm$ standard error of mean (SEM). Statistical evaluation of data was carried out by Student's *t*-test or repeated-measures two-way analysis of variance followed by Bonferroni post-test (GraphPad Prism 5, GraphPad Software Inc, LaJolla, CA, USA). Differences of results were considered statistically significant when $P < 0.05$.

Results

Biochemical characterization

The aqueous extract of *E. lucunter* spines presented a number of proteins, ranging from ~15 to ~150 kDa (Figure 1a). One must take into account that it is a buffered aqueous extract and not a cell lysate; therefore, the presence of such numerous proteins is noteworthy. RP-HPLC analysis (Figure 1b) shows that the vast majority of the extract eluted at the void volume, which reaches up to 1 AU, but there are several molecules eluting along the gradient, which are very likely not to be proteins. Except for peaks eluting at ~6 to ~8′ that present some 254–280 nm

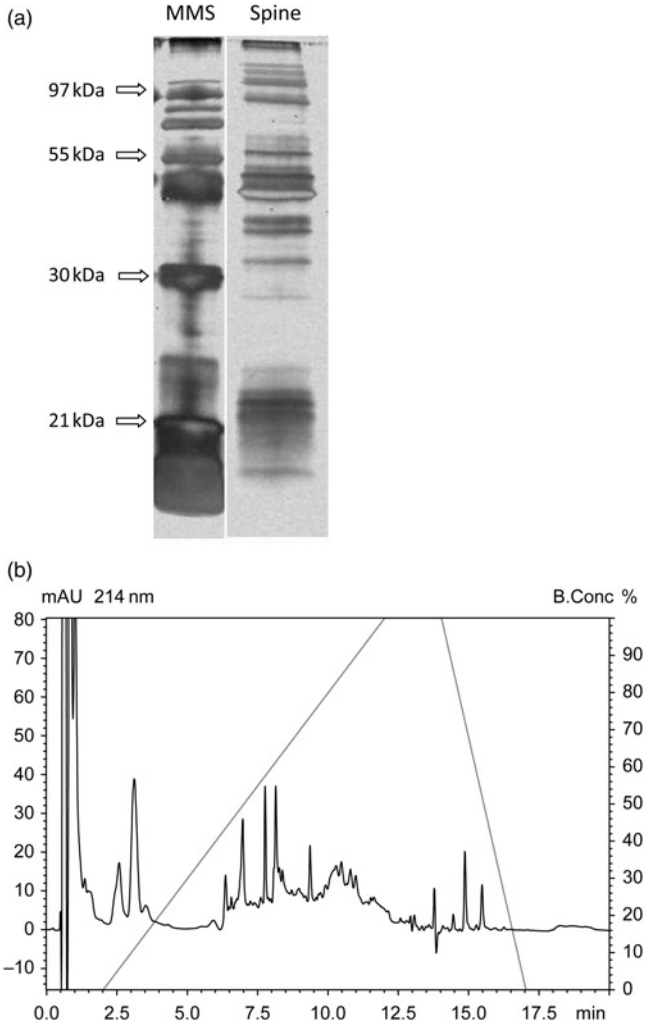

Figure 1 Biochemical characterization of the aqueous extract of *Echinometra lucunter* spines. (a) Silver-stained 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 50 μg extract. MMS, Molecular mass standards (Broad range; Amersham Biosciences, Waukesha, WI, USA). Some of the bands of the marker are indicated by arrows. (b) C18 RP-HPLC profile of aqueous extract from *E. lucunter* spine, monitored at 214 nm

absorbance, all other peaks presented maximum absorbance at 214–222 nm (data not shown, photodiode array detection).

Biological assay

Significant cell recruitment could be observed two hours after the 10 μg spine aqueous extract administration, as assessed by the increased rolling cells count that also significantly adhered and migrated (Figure 2). At four hours, rolling had diminished, while adhesion and migrated cells were still increasing. Adhesion and migration reached a maximum after 24 h, while rolling decreased, once all adhered cells migrated. After 48 h, rolling cells have returned to the basal state, indicating the end of inflammation; nevertheless, few adhered and migrated cells could still be observed.

Intraplantar administration of the aqueous extract (10 and 20 μg) was able to induce a dose-dependent paw edema (Figure 3). Although not intense (28%, in comparison to saline administration), this event was both rapidly triggered and dose dependent ($P < 0.05$).

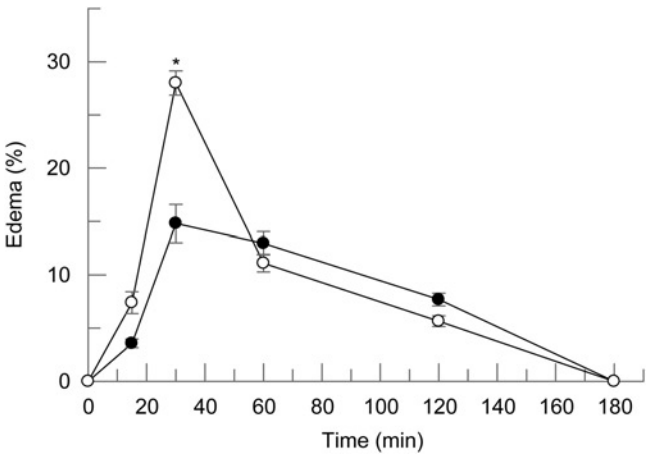

Figure 3 Edematogenic effect caused by aqueous extract from *Echinometra lucunter* spine on mice ($n = 5$). Data are present as mean $\pm$ SEM of the difference (%) in volumes of paw inject with extract and saline. Black circles correspond to 10 μg and white circles correspond to 20 μg. *Indicates $P < 0.05$

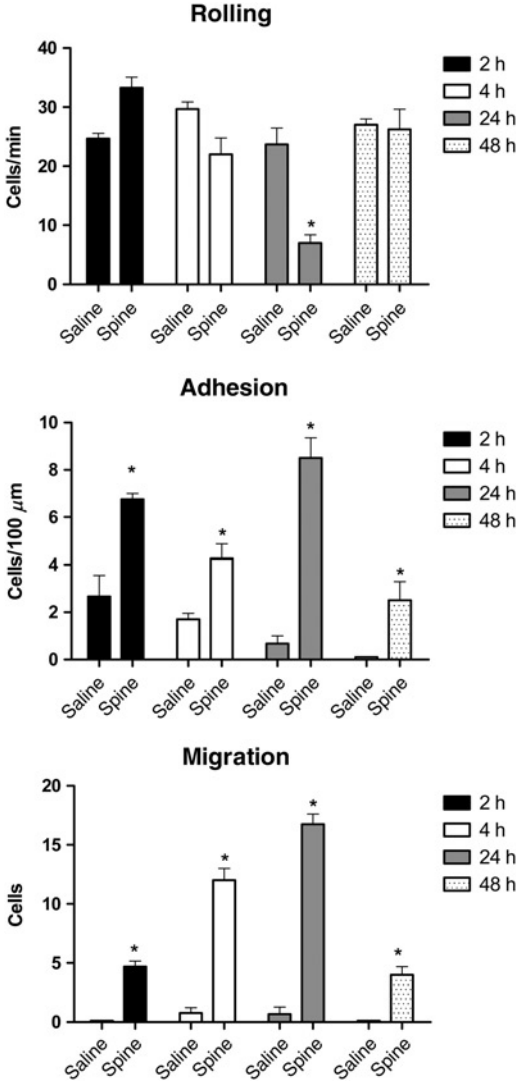

Figure 2 Alterations on leukocyte–endothelium interactions induced by aqueous extract from *Echinometra lucunter* spine (10 μg). The counting of rolling, adhered and migrated cells are represented in 2, 4, 24 and 48 h after injection. Data are present as mean $\pm$ SEM ($n = 5$). *Indicates $P < 0.05$ compared with the respective control group

The pain threshold significantly diminished one hour after the aqueous extract injection for both groups (10 and 20 μg), in comparison to the control group, as depicted in Figure 4. After two hours, the animals that received 20 μg of extract still presented a diminished pain threshold. Only four hours after the aqueous extract administration did the pain threshold value return to the initial level for the 20 μg group.

No hemorrhagic effect could be observed for 10 and 20 μg of the aqueous extract, at the tested conditions (data not shown).

Discussion

The protein nature of the *E. lucunter* aqueous extract indicates that the spine is a rich environment in which active and seemingly constitutive secretory pathways take place. It is noteworthy that these proteins are present in the

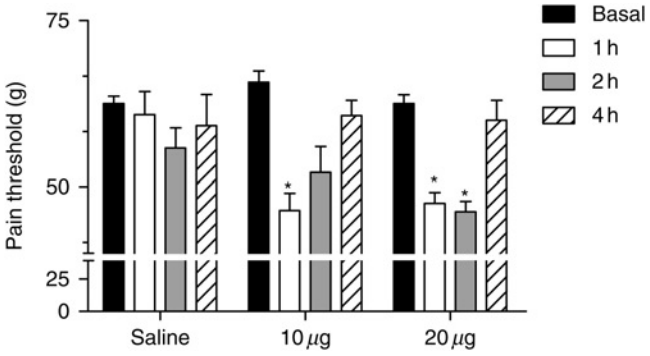

Figure 4 Effects of aqueous extract from *Echinometra lucunter* spine (10 and 20 μg) on mechanical hyperalgesia of rats. Pain threshold in response to mechanical stimulation was measured before (basal measurement) and after treatment using the paw pressure test. Data are present as mean $\pm$ SEM. *Indicates $P < 0.05$

aqueous extract and are not originated from a cell lysate. Furthermore, the RP-HPLC analysis indicates that there is also a number of non-protein components present in the spine aqueous extract.

Our experimental model successfully reproduced the main pharmacological symptoms usually described by the sea urchin victims, as reported by the coastal medical facilities and the scientific literature.[3,7] According to our results, the main pathological effect observed was inflammation, by alterations in the leukocyte–endothelial interactions, edema as well as hyperalgesia. It is noteworthy to mention that the spines' extraction buffer was inoculated in both bacterium and fungus-specific growth media and no colony formation could be observed, corroborating the fact that all pharmacological effects reported here originated from the urchin itself.

In this work, we demonstrated for the first time, that the aqueous extract of *E. lucunter* spines is rich in toxins, e.g. molecules that may take part in the protection of the animal, as an additional defense mechanism to the spine puncturing itself. Identification of the toxins secreted into the spine, responsible for the effects observed in this work is currently ongoing. Altogether, these results may lead to the development of proper treatment for the sea urchin victims, one that could cause faster relief and better recovery, in addition to the recommended precocious removal of the spines.

Author contributions: All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript; JMS and DCP were responsible for collection of sea urchins and obtainment of aqueous extract of spines, conducted biochemical experiments and wrote the manuscript; BCZ and LRCG conducted experiments of leukocyte–endothelium interaction and hemorrhagic studies; TON and RG performed paw pressure test and paw edema experiments.

ACKNOWLEDGEMENTS

Supported by funds provided by FAPESP (2007/02476–1 [DCP], 2007/08478–6 [JMS]) and CNPq 470873/2007–8 (DCP). DCP is also a CNPq fellow researcher 302405/2008–9 and member of the INCTTOX PROGRAM – CNPq/FAPESP.

REFERENCES

1 Hiratsuka Y, Uehara T. Feeding rates and absorption efficiencies of four species of sea urchins (genus Echinometra) fed a prepared diet. *Comp Biochem Physiol Part A* 2007;**148**:223–9

2 Dubois P, Ameye L. Regeneration of spines and pedicellariae in echinoderms: a review. *Microsc Res Tech* 2001;**55**:427–37

3 Haddad V Jr, Lupi O, Lonza JP, Tyring SK. Tropical dermatology: marine and aquatic dermatology. *J Am Acad Dermatol* 2009;**61**:733–50

4 Cracchiolo A, Goldberg L. Local and systemic reactions to puncture injuries by the sea urchin spine and the date palm thorn. *Arthritis Rheum* 1977;**20**:1206–12

5 Smith ML. Skin problems from marine echinoderms. *Dermatol Ther* 2002;**15**:30–3

6 Nassab R, Rayatt S, Peart F. The management of hand injuries caused by sea urchin spines. *J Hand Surg Br* 2005;**30**:432–3

7 Rossetto AL, Mora JM, Haddad V Jr. Sea urchin granuloma. *Rev Inst Med Trop S Paulo* 2006;**48**:303–6

8 Rocha G, Fraga S. Sea urchin granuloma of the skin. *Arch Derm* 1962;**85**:406–8

9 Kabigting FD, Kempiak SJ, Alexandrescu DT, Yu BD. Sea urchin granuloma secondary to *Strongylocentrotus purpuratus* and *Strongylocentrotus franciscanus*. *Dermatol Online J* 2009;**15**:9

10 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;**227**:680–5

11 Baez S. An open cremaster muscle preparation for the study of blood vessels by *in vivo* microscopy. *Microvasc Res* 1973;**5**:384–94

12 Randall O, Selitto JJ. A method for measurement of analgesic activity on inflamed tissue. *Arch Int Pharmacodyn Ther* 1957;**111**:409–19

13 Kondo H, Kondo S, Ikezawa K, Murata R. Studies on the quantitative method for determination of hemorrhagic activity of Habu snake venom. *Jpn J Med Sci Biol* 1960;**13**:43–52

(Received August 25, 2010, Accepted December 15, 2010)