



Freshwater stingrays: study of epidemiologic, clinic and therapeutic aspects based on 84 envenomings in humans and some enzymatic activities of the venom

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Abstract

Freshwater stingrays are very common in the Paraná, Paraguay, Araguaia, and Tocantins Rivers and tributaries in Brazil. This study presents the clinical aspects of 84 patients injured by freshwater stingrays. Intense pain was the most conspicuous symptom. Skin necrosis was observed in a high percentage of the victims, mostly fishermen and bathers. The initial therapeutic procedures, like immersion of the affected member in hot water were effective in the initial phases of the envenoming, especially in the control of the acute pain; however, they did not prevent skin necrosis. By SDS–PAGE, the freshwater stingray (*Potamotrygon falkneri*) venom extract presented a major band of approximately 12 kDa. Several other components distributed between 15 and 130 kDa were detected in the venom extract. Many components with molecular mass above 80 and 100 kDa have gelatinolytic and caseinolytic activities, respectively. Hyaluronidase activity was detected only in a component around 84 kDa in *P. falkneri* venom extract. Our results demonstrated that the presence of these enzymes could explain partially the local clinical pictures presented by patients wounded by freshwater stingray.

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1. Introduction

The Neotropical freshwater stingrays of the family Potamotrygonidae are the only elasmobranchs totally restricted to fluvial systems in South America (Thorson

et al., 1978). These stingrays belong to a larger group of predominantly marine elasmobranchs (Nishida, 1990) and are considered to have been derived from a marine ancestor during the marine incursions in the Miocene, approximately 20 million year ago (Lovejoy, 1997; Lovejoy et al., 1998; Marques, 2000). To date, there are about 20 species of potamotrygonids, which are commonly found resting on sandy or muddy bottoms in shallow waters of the major river basins of South America such as Atrato and Magdalena in Colombia, Orinoco and Maracaibo in Venezuela, and Amazon, Paraná and Paraguay in Brazil and Argentina (Rosa, 1985). These elasmobranchs are conspicuous

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creatures of the South American fauna not only because they are the only elasmobranchs found in continental waters of the Neotropical region, reaching up to 1 m in diameter and more than 30 kg of weight, but also because they are known as dangerous animals by members of local communities who are terrified by being stung by them.

Like some marine rays, the freshwater stingrays possess one or more stings at the base of their tails, which possess serrated edges and a very sharp tip. Its position on the tail certainly is responsible for the effectiveness of the defensive response when a person steps on or handles these elasmobranchs. In these cases, a powerfully struck blow of the tail toward the stimulus is likely to result in the penetration of the sting into the body of the victim. The sting is covered by an epithelium that possesses great quantities of glandular cells producing venom (Halstead, 1970), which, when compressed during penetration liberate their content into the tissues of the victim. As a complicating factor, the sting might break and provoke the retention of dentine fragments in the wound. Bacterial infections, especially that are caused by *Pseudomonas* spp. and *Staphylococcus* spp., are also commonly associated with these injuries (Haddad, 2000). In most cases, accidents involving freshwater stingrays cause very severe injuries, a fact reported by various authors (Vellard, 1932; Couto de Magalhães, 1934; Fróes, 1935; Mello-Leitão, 1948; Fonseca, 1952; Lisboa, 1967).

In several areas of Brazil those accidents are greatly feared, as they are almost always associated to cases of temporary or permanent physical incapacity. Although proven reports of deaths provoked by freshwater stingrays do not exist, it is possible that these happen due to the serious processes of skin necrosis and high indexes of bacterial infections that are present. The victims are fishermen, who are daily handling these animals frequently captured by their fishing gear. However, bathers are also exposed to the danger of being injured by stingrays, especially during the dry season when sand banks along the rivers are exposed and provide recreational areas for members of the communities nearby. However, these shallow water habitats are also sought by freshwater stingrays to feed and rest during the day.

Detailed documentation of these accidents is rare and difficult, for they happen at distant and isolated places most of the time. In addition, most of the patients seek for help only after a long time since the accident took place. These are important points, because they contribute to the lack of knowledge on the subject.

Additional problems in the injuries caused by freshwater stingrays are the aquarists, responsible for massive importation of the fishes in Europe, Japan and the USA. The loss of information about the risks of how to maintain freshwater stingrays in an aquarium can precipitate injuries in the hands of the aquarists, sometimes with severe consequences (Schiera et al., 2002).

Little is known about the toxic activities of the venom of marine and freshwater stingrays. The lack of data is mainly due to the difficulty to extract venom, once it is very difficult and dangerous to capture the animals, the quantity of the venom is very low and it is termolabile (Russell and Van Harreveld, 1954; Russeffl et al., 1957, 1958). The integument covering the sting must be intact to allow expression of the venom. Neurotoxicity (Vellard, 1931, 1932), cardiotoxicity (Fleury, 1950) and circulatory disturbances (Russell and Van Harreveld, 1954; Russell et al., 1957; Rodrigues, 1963, 1972) probably by direct effect on the myocardium were described for some authors.

In this study, we discuss epidemiological, clinical and therapeutic aspects of the envenoming and some enzymatic activities of the venom of freshwater stingrays based on the observation and treatment of 84 injuries involving inhabitants and tourists of two great river basins of Brazil.

2. Patients, material and methods

2.1. Patients

We carried out a prospective study during the period of January 2000–March 2003, in which 84 cases of injuries caused by freshwater stingrays were documented in the tributaries and marginal ponds of the Paraguay, Paraná, Tocantins, and Araguaia Rivers, in the cities of Corumbá (Paraguay River, Mato Grosso do Sul State), Presidente Epitácio, Três Lagoas and Foz do Iguaçu (Paraná River, São Paulo and Paraná States), Araguacema and Araguaina (Araguaia River, Tocantins State) (Fig. 1). The patients were observed in local hospitals and Centers of Health, with the help of local Health staff and fishermen associations in variable periods of Brazilian winter. The criteria for observation were the presence of the victim in a hospital or Health Center and the presence of the author. The patients gave written or oral permission for the photographs and the ethics committees of the local Health centers were consulted and they approved the observations, because they do not know any method capable to relieve the manifestations of the envenoming.

To systematically document the history of the injuries, the injury was photographed and the victims were asked to answer a semi-structured questionnaire considering the following variables: sex, age, date, hour and place of occurrence, activity performed at the moment of injury, time elapsed between the accident and the arrival at the unit of health care that assisted the case, whether medicines or other methods used in popular medicine were employed, and signs and symptoms reported by the victim. With the data at hand, we sought for a profile of the epidemic, clinical, and therapeutic aspects of the injuries.

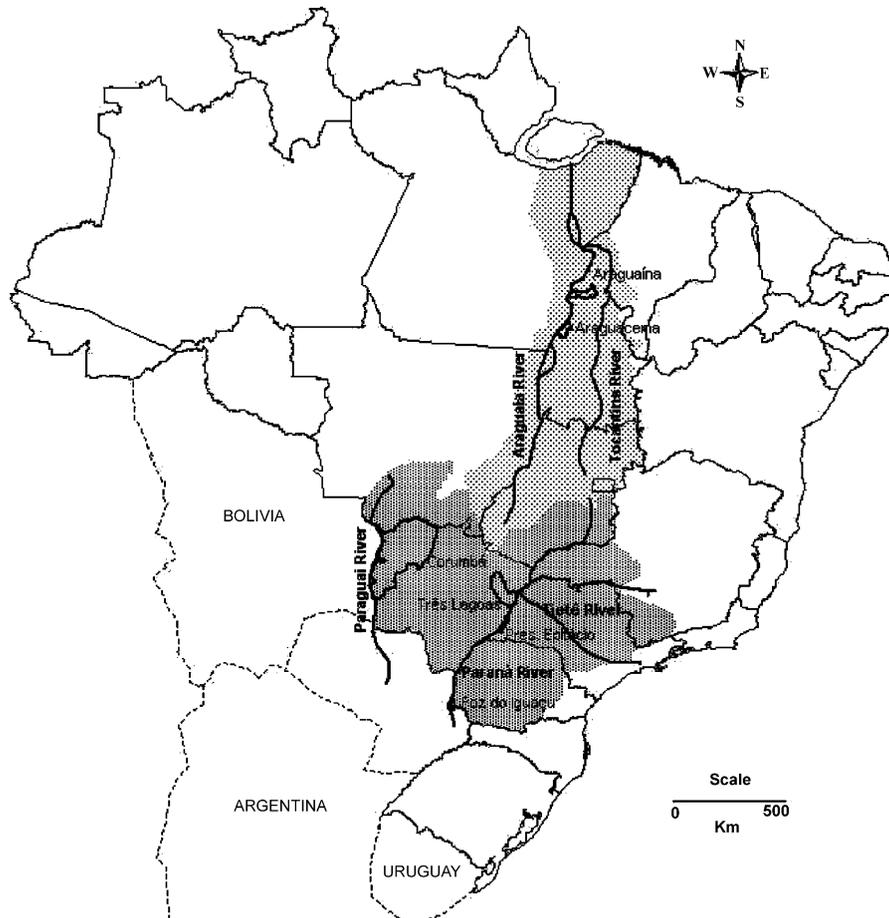


Fig. 1. Tocantins/Araguaia and Paraguay/Paraná River systems.

2.2. Venom

Specimens of *Potamotrygon falkneri* were collected on Paraná River in the Presidente Epitácio region (SP, Brazil). The epithelium (that cover the sting) obtained from 10 animals were scratched and dissolved in PBS pH 7.4. The venom extract sample was centrifuged at 5000 g for 10 min. The protein content of the supernatant was determined using bicinchoninic acid solution according to Smith et al. (1985) using bovine serum albumin (Sigma Chemicals, St Louis, MO, USA) as protein standard.

2.3. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

The proteins (10 µg) of *P. falkneri* venom extract were analyzed by SDS–PAGE (12% acrylamide resolution gels) under non-reducing conditions using the method of Laemmli (1970). Prior to electrophoresis, the samples were mixed with buffer and the proteins revealed by the silver stain method (Blum et al., 1987). Pre-stained

molecular weight (Sigma Chemicals, St Louis, MO, USA) were used as molecular weight markers.

2.4. Protease assays

Casein, and gelatine were used as substrates and the method was adapted from Heussen and Dowdle (1980). Briefly, either 2 mg/ml casein (Merck, Darmstadt, Germany), or 2 mg/ml gelatine (Type B from bovine skin, Sigma Chemicals, St Louis, MO, USA) was incorporated as substrate in the 10% resolving gel with a 4% stacking gel containing no substrate. Samples of *P. falkneri* venom extract (10 µg) were loaded in non-reducing sample buffer and the gels were run at 15 mA/gel, at 4 °C. After electrophoresis the SDS was removed by washing the gel twice for 20 min in 2.5% Triton X-100 before incubation in 20 mM Tris, 0.4 mM calcium chloride pH 7.4 at 37 °C for 16 h and staining with 0.125% Coomassie blue. Clear areas in the gel indicate regions of enzyme activity. Pre-stained molecular weight (BioRad, Hercules, CA, USA) were used as molecular weight markers.

2.5. Hyaluronidase assay

Hyaluronidase activity was assayed by hyaluronic acid substrate SDS–PAGE. The method was modified from [Miura et al. \(1995\)](#). Briefly, 170 µg/ml hyaluronic acid (Rooster comb, Sigma Chemicals, St Louis, MO) was incorporated as the substrate in the 10% resolving gel with a 4% stacking gel contained no substrate. Samples of *P. falkneri* venom extract (10 µg) were loaded in non-reducing sample buffer and the gels were run at 15 mA/gel, at 4 °C. After electrophoresis, the SDS was removed by washing the gel device for 20 min in 2.4% Triton X-100 before incubation in 20 mM Tris, 0.5 mM calcium chloride pH 7.4 at 37 °C for 16 h. Residual proteins (which may interfere with gel staining) were removed by adding 0.2 g/ml *Streptomyces griseus* protease (Sigma Chemicals, St Louis, MO, USA) and incubating at 37 °C for a further 2.5 h. The gel was then soaked in destain (25% methanol, 10% acetic acid) for 20 min, stained with Alcian blue (0.5% in destain) for 1 h and destained. Clear areas in the gel indicate regions of enzyme activity. Pre-stained molecular weight (BioRad, Hercules, CA, USA) were used as molecular weight markers.

3. Results

3.1. Clinical, epidemiological and therapeutic aspects

3.1.1. Paraná and Paraguay rivers and tributaries

Fifty-nine injuries had occurred in Paraná and Paraguay Rivers. Eighteen occurred in the area of the Paraguay River, Southern Pantanal (Corumbá, Mato Grosso do Sul State), and 41 in the Paraná River, in a boundary between States of São Paulo and Mato Grosso do Sul (Três Lagoas and Presidente Epitácio) and near the frontier of Brazil, Paraguay and Argentina, in the lake formed by the Itaipu hydroelectric power station (Foz do Iguaçu). The stings occurred during a period of 39 months (January 2000–March 2003) and the criteria of the patients included were cited in Section 2.

In Paraguay and Paraná Rivers, the 59 injuries were caused by *Potamotrygon motoro* ([Fig. 2](#)) and *P. falkneri*, the latter being the most common potamotrygonid stingray found in the area. Specimens of the two species were collected and identified by an author (VHJ). The injuries occurred mainly in men (about 90%), in Brazilian winter (period of drought), during daylight (nearly 85%). Most of the wounds took place when the victims walked through shallow waters on sand banks either because they were picking baits for recreational fisheries, or rendering small services in the Corumbá harbor or moorings at the municipal districts of Presidente Epitácio, Três Lagoas and Foz do Iguaçu (51 cases recorded). Children and adults swimming in areas of shallow waters in the Paraguay River were injured on four occasions. Three fishermen were injured

during the course of their work, approximately 5% of the total of the cases reported for this area. The small number of accidents upon fishermen probably is a result of the fear they have for these fishes. As a result, they seem to be extremely careful when they capture stingrays. Finally, one of the patients got injured in circumstances not known. Injuries to the lower limbs comprised nearly 90% of the accidents (53 cases), the remaining injuries affected other areas such as hands and arms.

All victims reported great pain immediately after being stung, which is caused by the venom and the serrated and bony stingers on the tail ([Fig. 3](#)). After the appearance of edema and local erythema, we observed cutaneous necrosis of variable gravity in almost all the patients. The pattern of the resulting ulcer can be diagnosed by the presence of necrosis, which starts from the center of the wound and followed by concentric formations of new necrotic areas—reminiscent of the concentric circles formed by a stone-shot in the water, or like the layers of an onion ([Fig. 4](#)). In about 25% of the patients this pattern is not observed, which makes it difficult to diagnose the cause of the injury. The ulcers heal after months of evolution, leaving big scars



[Fig. 2](#). Above: *Potamotrygon motoro* is the most common species of freshwater stingray in all Brazilian rivers. Below: *Potamotrygon orbignyi*, the predominant species stingray of Araguaia/Tocantins Rivers systems.



Fig. 3. Above: Close-up of stingers of *Potamotrygon motoro*. Note the serrated edges and the sharp tip of the bony stinger. Below: early injury in the dorsum of the foot of a male patient. Note the necrotic area demarcated within approximately one day of evolution.

during the processes of cicatrization. The average time of cicatrization was three months. In addition, fever, cold sweating, nausea, vomiting and agitation were among the systemic effects manifested in the patients. Some patients applied urine, ashes, alcohol and other substances on the injured point, without relief of the pain. In about one third of the patients we utilized the immersion of the affected member in hot water and the intensity of the pain decreased, but the necrosis was similar to that observed in patients without treatment.

3.1.2. Araguaia and Tocantins rivers

In the Tocantins State, Northern Brazil, the 25 injuries caused by freshwater stingrays concentrate near to areas formed by the Tocantins and Araguaia rivers and their respective tributaries. Nineteen were from Araguaia River and six from Tocantins River. The accidents happened predominantly during the afternoons (between 13 and 18 h) of July and August. The prevalence of accidents during this time of the day is probably related to activities such as fishing, washing clothes, recreation, or hygiene (baths, cleaning of domestic utensils).



Fig. 4. Chronic ulcers caused by stings of freshwater stingrays. The pattern of the lesion is like a 'cut onion' or 'stone-shot in the water'. The wound possesses a central necrosis and surrounding subsequent extension.

The injuries occurred in any age group for both sexes (there was a peak between 20 and 30 years old), with prevalence in men (80%). A striking observation was that many of the accidents occurred to non-local people, but mainly to tourists, who during this time of the year come to these areas to enjoy the beaches formed by sand banks exposed along the river due to low water levels, ignoring the fact that stingrays also prefer these areas. Most of the accidents were caused by *Potamotrygon orbignyi* (Fig. 2). The species of *P. orbignyi* was identified by an author (Fernando Portella de Luna Marques) and about ten specimens were collected in the area of study.

The main symptom associated to the injuries was the immediate pain of great intensity after the sting, and reported by those who experienced it as a burning pain ('deep pain'). The pain lasted, in general, for approximately 2 h at least. Patients reported local paresthesia, edema, erythema and hemorrhagic and necrotic areas. In some cases, we observed the development of abscess, cellulitis and tissue loss. Thus, the injury usually resulted in a considerable scar after cicatrization. Injuries in great vessels

Table 1

Signs and symptoms manifested by 84 patients injured by freshwater stingrays in the Paraguay, Paraná and Tocantins/Araguaia Rivers and tributaries

Signs and symptoms	N (%)
Intense pain	84 (100)
Erythema	84 (100)
Edema	84 (100)
Skin necrosis	76 (90.4)
Ulcers	76 (90.4)
Systemic manifestations	66 (80.9)

can provoke massive bleeding. Other reported symptoms were dizziness, myalgia, migraine, fever, and vomiting.

In this group, seven patients were treated with hot water with good results to relieve the pain, but again the necrosis was similar to those without initial treatment.

A summary of the clinical pattern reported by the 84 patients is found in Table 1

3.2. Enzymatic activities

3.2.1. Analysis of the venom by SDS–PAGE

At least 18 components were detected by SDS–PAGE (Fig. 5A). The major band of *P. falkneri* venom extract was located around 12 kDa. Other bands strongly stained were detected between 70 and 25 kDa. Several other weakly stained bands (by silver nitrate) between 15 and 130 kDa, were also observed.

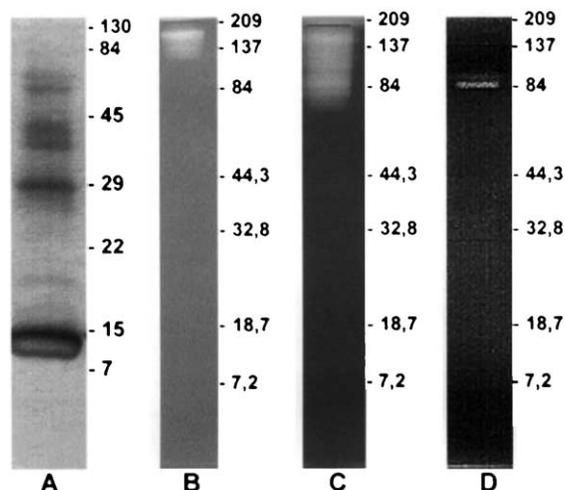


Fig. 5. (A) Proteins (10 μ g) of *Potamotrygon falkneri* venom extract were separated by SDS–PAGE (12% acrylamide resolution gels) and stained by silver nitrate method. (B) Casein, (C) Gelatine or (D) Hyaluronic acid substrate SDS–PAGE (10%) analysis of *P. falkneri* venom extract (10 μ g). The position of pre-stained molecular mass standards (kDa) is shown to the right.

3.2.2. Caseinolytic, gelatinolytic and hyaluronidase activities

The technique of substrate SDS–PAGE was used to detect enzymatic activities present in the *P. falkneri* venom extract. It was possible to identify four very evident components with caseinolytic activity between 200 and 100 kDa (Fig. 5B). The venom extract also had large array of gelatinolytic proteases. Seven components with molecular mass between 200 and 80 were detected (Fig. 5C). Hyaluronidase activity was detected in only a single band located around 84 kDa by hyaluronic acid substrate SDS–PAGE (Fig. 5D).

4. Discussion

The main effect of the envenoming by freshwater stingrays in this study was the excruciating pain in all the patients, together with local edema and erythema. The systemic symptoms were frequent (nearly 80%) in the acute phase. In later phases, we observed a great percentage of skin necrosis (about 90%), and chronic ulcers (about 90%). An important fact is that the pattern of the necrosis and of the pain caused by the freshwater stingrays is similar in all of the species studied.

The injuries caused by freshwater stingrays are treated by an enormous variety of home-made medicines, associated in these communities, with many myths and superstitions (Castex, 1963a,b; Castex and Loza, 1964; Castex et al., 1964 a,b; Castex, 1965). Among those, member of riverside communities believe that the use of urine at the place of the injury or to scrub the eyes of the stingray on the attacked point, or still, the placement of the stung area in a female genitalia, helps to control the pain. However, the only indication now accepted in the acute phase of the injury is the immersion of the affected part in hot water. There are various theories about the effect of the hot water: high temperatures would inactivate the proteins discharged by the glandular cells on the sting thus relieving the pain, or local vasodilatation resulting from immersion in this very warm water dissipates the venom of the site of the sting or the vasodilatation provoked by the hot water could bloke the pain and ischemia associated with the vasoconstriction caused by the venom (Haddad, 2000).

Other important procedures are surgical exploration and intense cleaning of wounds especially the lacerated ones. Prophylaxis of tetanus, the use of systemic antibiotics in all the lacerated wounds should also be included. Anesthetic infiltration in the place and systemic analgesics, including opiate, should be applied to control the pain if that was not reached in the first 2 h after the accident by using hot water.

The complications of the later phases of injuries are treated with the same procedures applied to cicatrization of chronic ulcers. It is important to rest, intensive washing with water and soap, potassium permanganate compress and topical antibiotics.

In any way, the difficulty of serial observations of the patients with several late infectious processes were not observed, therefore the average of observations in each patient did not pass at once, and this was examined in different phases of the envenoming, what also hindered the application of initial measures of the treatment, as the immersion in hot water.

There is no definitive and practicable therapy for the envenoming. However, the control of the initial pain can be obtained with the procedures described above. The inactivation of the venom is the only ideal procedure to minimize the consequences of the sting. The production of antivenin serum is unrealistic in view of the low number of accidents reported and the high cost of the development and production of the antivenin.

In general, the procedures described above are applicable in all injuries caused by stingrays. When compared to marine stingrays, the injuries caused by freshwater stingrays seem to be graver. However, the intense pain and the introduction of bacterial infections are common in both cases. Nonetheless, the proportion of cutaneous necrosis in twelve accidents caused by marine stingrays was of 25%, whereas in approximately 90% of the injuries caused by potamotrygonids resulted in the development of necrosis (Haddad Jr., 2000).

The result of biochemical venom characterization indicates that at least 18 components were detected by SDS–PAGE on *P. falkneri* venom extract. The major band was located around 12 kDa. Some of the components revealed by SDS–PAGE, probably, belong to the epithelium that recover the glandular tissue constituted by dispersed cells over the sting (Halstead, 1970) that is removed with the glandular tissue during the scratching.

In the injuries caused by freshwater stingrays, besides the intense local pain observed the presence of edema, erythema and necrosis are common. These events suggest extracellular matrix disturbances. Many authors describe the degradation of the extracellular matrix, for enzymes present in animal venom. Metalloproteinases present in venom of different species of snakes are able to degrade molecules of extracellular matrix such as fibronectin, entactin, collagen type IV. Besides, they are involved in some hemorrhagic effects observed in the envenoming (Bjarnanson and Fox, 1995). Sphingomyelinase, protease, among others, contribute to the development of the cutaneous necrosis observed in patients bitten by *Loxosceles* spiders (Futrell, 1992; Feitosa et al., 1998). Hyaluronidase is an enzyme frequently found in animal venoms (Tan and Ponnunduraj, 1992). It is an important spreading factor due to its ability to hydrolize connective tissue helping the action of the gelatinases (Birkedal-Hansen et al., 1993). In our study we verified that *P. falkneri* venom extract has proteolytic, gelatinolytic and hyaluronidase activities. Recently, Magalhães (2001) detected 5'-nucleotidase, phospholipase, acid phosphatase, hyaluronidase and proteases in *P. motoro* venom obtained from animals of

Crixás–Açú River (Goiás, Brazil). However, phosphodiesterase and alkaline phosphatase activities were not verified. The enzymes that we detected in our experiments act directly on extracellular matrix components contributing for their degradation and collaborating to the establishment of the dermonecrotic lesion. The hyaluronidase activity associated with the proteolytic activity could contribute to the increased local damage already caused by the sting.

Our observations point out to the conclusion that the envenoming caused by freshwater stingrays is very severe and should be considered as one of the most important injuries caused by aquatic animals in South America. This is the first time that a large number of accidents of this sort are addressed in the literature. Thus, we hope to contribute to a better understanding on therapeutics of these harmful injuries.

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