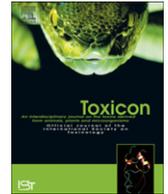




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Histopathological changes induced by extracts from the tissue covering the stingers of *Potamotrygon falkneri* freshwater stingrays

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ABSTRACT

Pain is the most conspicuous symptom observed in patients wounded by stingrays, and skin necrosis is common in accidents by freshwater stingrays. The extract from the stinger integumentary tissue of *Potamotrygon falkneri* containing toxic components (venom) was tested for its ability to induce histopathological changes in the dorsal skin of mice at different times. 3–6 h after injection, foci of necrosis in isolated basal epidermal cells were observed. Full coagulative necrosis of the skin, subcutaneous tissue and skeletal muscle was evident as soon as 24 h after venom exposure, with a clear demarcation from the normal skin. After 48 h, round collections of necrotic cells start to coalesce originating extensive skin necrotic plaques that detach from viable tissue after 72–96 h. Inflammatory infiltrate was observed after 6 h, but was always mild. Acute vascular thrombosis was rare, and hemorrhage was not present at any time. Superficial bacterial infection was present in two of the examined cases. In conclusion, the venom of *P. falkneri* is responsible for the development of an early necrosis with mild inflammatory reaction, probably due to direct action of the venom. The severe local damage is probably worsened by the mechanical trauma caused by the stinger.

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1. Introduction

Envenomations by freshwater stingrays are characterized by intense pain and pathological alterations at the injury site. These include edema, erythema and, in most cases, necrosis (Haddad et al., 2004). The damage is caused by the stinger located in the back of the stingray tail, which is used by the animal to defend itself (Charvet-Almeida et al., 2002; Garrone Neto et al., 2007). Integumentary and glandular tissues cover the stinger where the toxins are produced (Pedroso et al., 2007). The anatomical regions

most afflicted in injuries caused by stingrays are the hands and feet (Haddad et al., 2004; Brisset et al., 2006; Lim and Kumarasinghe, 2007; Garrone Neto and Haddad, 2009). Lethal injuries rarely occur except for cases where the stinger reaches vital organs (Isbister, 2001; Garrone Neto and Haddad, 2009). Specific antivenom is not available for the treatment of stingray injuries, and the therapeutic approach is based on the use of analgesic and anti-inflammatory drugs, hot water to relieve the excruciating pain and antibiotics to prevent secondary infection (Haddad et al., 2004; Clark et al., 2007; Dehghani et al., 2009; Garrone Neto and Haddad, 2010). In Brazil, the distribution of freshwater stingrays has gradually increased due to environmental alterations mainly represented by the construction of hydroelectric power plants (Barbaro et al., 2007; Garrone Neto et al., 2007; Garrone Neto and Haddad, 2010).

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The ability of the extracts obtained from the tissue covering the stingers of *Potamotrygon falkneri* to cause toxic activities such nociception, edema, myotoxicity, necrosis and lethality has already been reported (Barbaro et al., 2007). Many enzymes such as proteases and hyaluronidase were detected in the extract obtained from *Potamotrygon* freshwater stingray (Haddad et al., 2004; Barbaro et al., 2007; Magalhães et al., 2008). In addition, peptides effective in the microcirculatory environment were isolated from *Potamotrygon* gr. *orbigny* venom by Conceição et al. (2006, 2009).

The histopathological features after injection of toxins extracted from the stingray stingers are practically unknown. The aim of this study is to characterize the main histological alterations in mice skin induced by experimental envenomation using extracts from the tissue covering the stingers of *P. falkneri*.

2. Materials and methods

2.1. Animals and tissue extracts

Swiss mice (18–20 g) were provided by the Butantan Institute Animal House. Animals received food and water *ad libitum*. Specimens of *P. falkneri* (Myliobatiformes, Potamotrygonidae) were collected in the Paraná River, on the border of São Paulo and Mato Grosso do Sul States in Southwestern Brazil. Tissue extracts were obtained from the integumentary tissue covering the stinger as previously described (Haddad et al., 2004). The protein content of tissue extract pools (23 stingers) was determined by bicinchoninic acid method (Smith et al., 1985), using bovine serum albumin as a standard. The procedures involving animals were conducted in conformity with national laws and policies controlled by the Butantan Institute Animal Investigation Ethical Committee (protocol n 333/2006).

2.2. Local reaction and dermonecrotic activity induced by *P. falkneri* tissue extracts

Local reaction (edema/erythema and paleness/ecchymosis areas) and necrosis were determined by i.d. injection of 400 µg of *P. falkneri* tissue extracts (this dose is able to induce an intense inflammatory reaction and necrosis as described by Barbaro et al., 2007), dissolved in 0.1 ml of PBS, into the mouse dorsum skin (3 animals for each time period). Animals were sacrificed by CO₂ inhalation and the inner dorsum skin was examined. Areas of local reaction and necrosis were inspected 3, 6, 24, 48, 72 and 96 h after injection and reported as the mean of the three measurements (mm²) for each parameter studied. Animals injected only with PBS were used as control.

2.3. Histology

Skin squares of about 1 cm² of the injected area were removed and fixed in 4% paraformaldehyde in PBS 0.1 M, pH 7.2 for 24 h. The samples were dehydrated in ethanol and embedded in paraffin. Sections of 4 µm were obtained in a Microm HM340E microtome, stained with hematoxylin-eosin and examined under a light microscope. Photomicrographs were obtained with a Zeiss Axioskop 2 plus

microscope equipped with a digital camera (AxioCam) and the software Axiovision (Zeiss).

3. Results

3.1. Macroscopic local reaction

The *P. falkneri* tissue extract evoked a local reaction. Areas of intense inflammatory reaction at the injection site were characterized by edema, erythema, paleness and necrosis (Table 1 and Fig. 1). The control animal injected with PBS did not show any inflammatory reaction.

3.2. Histological assessment of skin damage

Three hours after injection, nuclear contraction and hyperchromasia was observed in a few basal epidermal cells and hair follicles, with initial detachment of the epidermis from the dermis, which showed evidence of mild edema, but no inflammatory infiltrate or hemorrhage (Fig. 2A). Skeletal muscle cells showed mild hypereosinophilia and focal cytoplasmic degeneration; acute thrombosis was seen in only one blood vessel in deep dermis (Fig. 2B).

After 6 h of injection, multiple foci of epidermal detachment from the superficial dermis were detected (Fig. 2C). Besides edema, a very mild inflammatory infiltrate was observed, composed of neutrophils and macrophages, particularly at the subcutaneous tissue. There was acute thrombosis of few blood vessels in deep dermis and foci of coagulative necrosis of skeletal muscle cells (Fig. 2D). No hemorrhage was verified.

After 24 h of injection, coagulative necrosis of the full skin was evident, with a clear-cut demarcation from the viable skin. The necrotic changes affected the epidermis, dermis, subcutaneous tissue and skeletal muscle (Fig. 2E). Foci of epidermal erosion and mild acute inflammatory infiltrate as well as round collections of cellular debris in the upper dermis and epidermis were present (Fig. 2F). No hemorrhage was verified and very few blood vessels showed thrombosis. Superficial epidermal bacterial infection was present in one of the samples.

After 48 h of injection, coagulative necrosis of skin, subcutaneous and skeletal muscle tissue was evident (Fig. 3A). The epidermis and the dermis showed mild acute inflammatory infiltrate and collections of cellular debris,

Table 1
Macroscopic measurements of local reaction and necrosis induced by *P. falkneri* stinger tissue extracts.

Time (hours)	Necrosis (mm ²)	Local reaction (mm ²)
3	–	234.3 ± 5.0
6	–	165.0 ± 15.5
24	2.3 ± 4.0	148.7 ± 37.1
48	3.3 ± 2.9	132.7 ± 56.1
72	8.7 ± 9.6	70.0 ± 26.2
96	19.3 ± 10.1	114.3 ± 46.5

Mice were injected i.d. with 400 µg of *P. falkneri* tissue extracts diluted in 0.1 ml of PBS. Local reaction (edema, erythema and paleness) and necrosis were evaluated after 3, 6, 24, 48, 72 and 96 h. Data are expressed as mean ± SD.

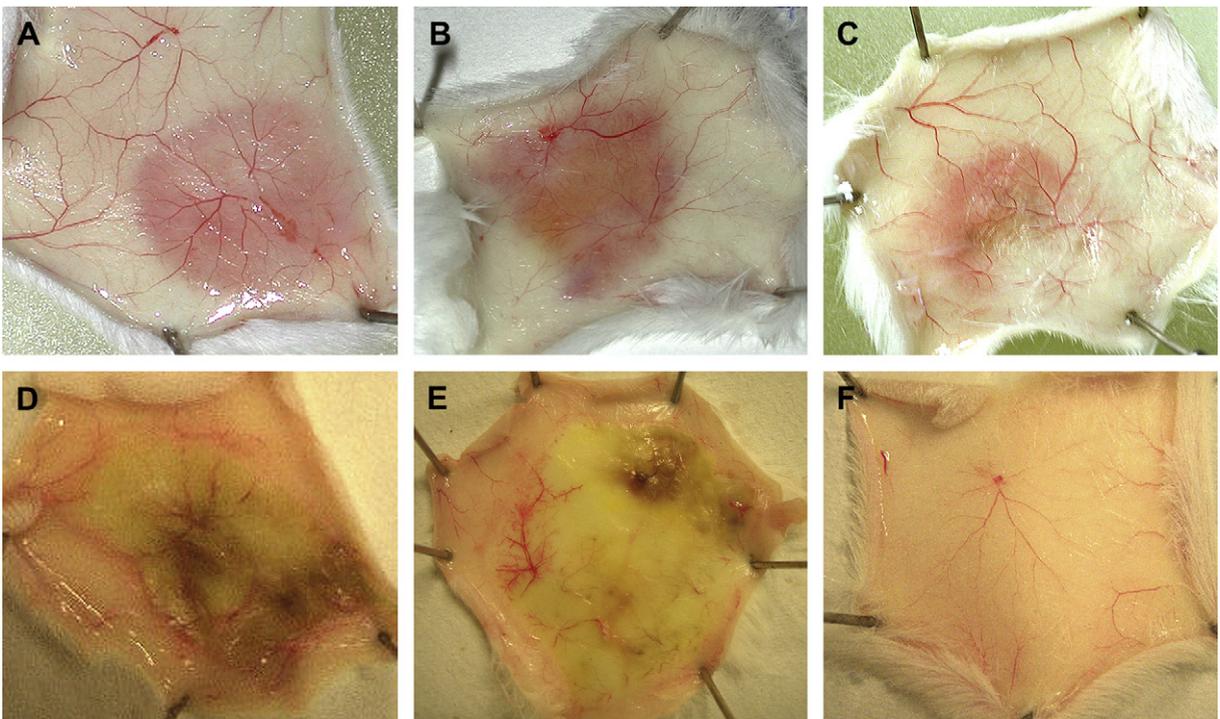


Fig. 1. Macroscopic aspect of mice skin (inner side) after injection of *P. falkneri* stinger tissue extract, at different times. Local reaction and necrosis were evaluated after 3, 6, 24, 72 and 96 h (A–E, respectively). Control animal (PBS) (F).

characterizing micro-abscesses (Fig. 3B). Few blood vessels in the dermis and subcutaneous tissue presented thrombosis. No hemorrhage was verified.

After 72 h of injection, the necrotic tissue presented cellular debris in the form of numerous round collections or diffuse infiltration, constituting a necrotic plaque focally detached from the deep tissue (Fig. 3C). Regenerative hyperplasia of epidermal cells appeared at the lesion borders (Fig. 3D). A mild inflammatory infiltrate was observed around viable blood vessels in the deep subcutaneous tissue.

After 96 h of injection, the regenerative hyperplasia of epidermal cells at the necrotic skin border was more evident (Fig. 4A). The coagulative necrosis of the tissue was clear, affecting the skeletal muscle and presenting cellular debris infiltration. In one of the samples the epidermis was missing in some areas and superficial bacterial infection appeared (Fig. 4B). No hemorrhage or blood vessel thrombosis was detected.

In animals of the control group no evidence of necrosis was noted although mild edema and mononuclear cell infiltration of dermis and subcutaneous tissue were focally present. Moreover, control animals did not show any histological abnormalities in most of the skin, and subcutaneous and skeletal muscle tissue (Fig. 4C,D).

4. Discussion

There are few reports in literature on the toxic effects of freshwater stingray venom. Under our experimental conditions, we verified that the tissue extract of *P. falkneri*

could induce necrosis and an inflammatory reaction at the site of injection. These data are in agreement with reports of accidents in humans (Haddad, 2000; Haddad et al., 2004; Garrone Neto and Haddad, 2010). They are also in agreement with an experimental model (Barbaro et al., 2007) demonstrating that necrosis and local inflammation are much more prominent in injuries caused by freshwater stingrays when compared with those caused by marine species. Our histological study demonstrated that necrosis occurs very soon after the exposure; foci of epidermal necrosis with initial detachment from the dermis were detected 3–6 h after extract injection. Moreover, at these times, signs of initial necrosis of skeletal muscle were observed. Necrosis was clearly established 24 h after extract injection, affecting all the soft tissues (skin, subcutaneous and skeletal muscle tissue), presenting a clear-cut demarcation with the viable neighboring skin and showing a coagulative appearance. This appearance indicates the occurrence of protein denaturation, which is compatible with the action of proteases. Furthermore, our study showed no evidence of significant vascular thrombosis or hemorrhage at any time, which reinforces the hypothesis that the venom induces tissue necrosis probably by the direct action of toxins/enzymes (Barbaro et al., 2007).

Envenomations caused by some species of snakes (Gutiérrez et al., 2005; Moura-da-Silva et al., 2007), spiders (Ospedal et al., 2002; Hogan et al., 2004) and fish (Lima et al., 2003; Pareja-Santos et al., 2009) are also characterized by severe local tissue damage. The venom of these animals has enzymes involved in the pathogenesis of local myonecrosis, skin damage with intense inflammatory

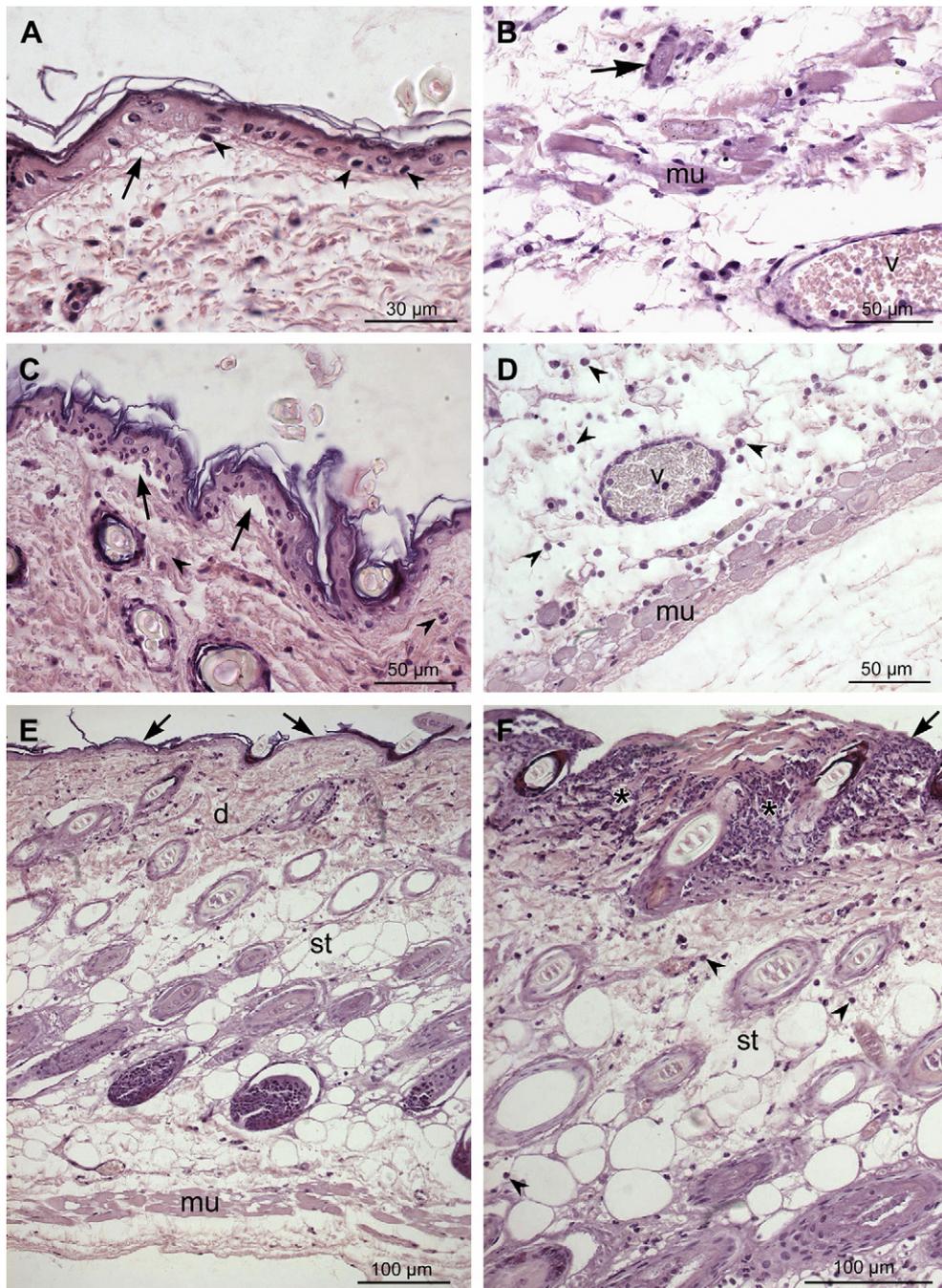


Fig. 2. Effect of *P. falkneri* stinger tissue extract in mouse skin. A and B: 3 h after injection. Epidermis presents initial detachment from the dermis (arrow, Fig. 2A). Basal epidermal cells show nuclear contraction and hyperchromasia (arrowheads). Muscle cells (mu) present mild hyper eosinophilia and focal cytoplasmic degeneration. Most of the blood vessels are patent (v), but one single blood vessel (arrow, Fig. 2B) shows acute thrombosis. C and D: 6 h after injection. Multiple foci of epidermal detachment from dermis are seen (arrows). There is edema and scarce inflammatory cells (arrowheads) in the subcutaneous tissue plus foci of coagulative necrosis of muscle cells (mu). A patent blood vessel (v) can be seen. E and F: 24 h after injection. Coagulative necrosis affects epidermis (arrows), dermis (d), subcutaneous tissue (st) and skeletal muscle (mu). There are foci of epidermal erosion (arrows) and round collections of cellular debris in the upper dermis and epidermis (asterisks). Scarce inflammatory cells (arrowheads) are seen in the subcutaneous tissue.

reaction. [Barbaro et al. \(2007\)](#) showed that *P. falkneri* tissue extract contains enzymes capable of degrading distinct proteins such as casein, gelatin and fibrinogen. These data suggest that such proteases could contribute to

degradation of proteins and extracellular matrix components, favouring the establishment of local injury. Additionally, the detection of hyaluronidase activity in *Potamotrygon* tissue extract seems to constitute strong evidence that in this

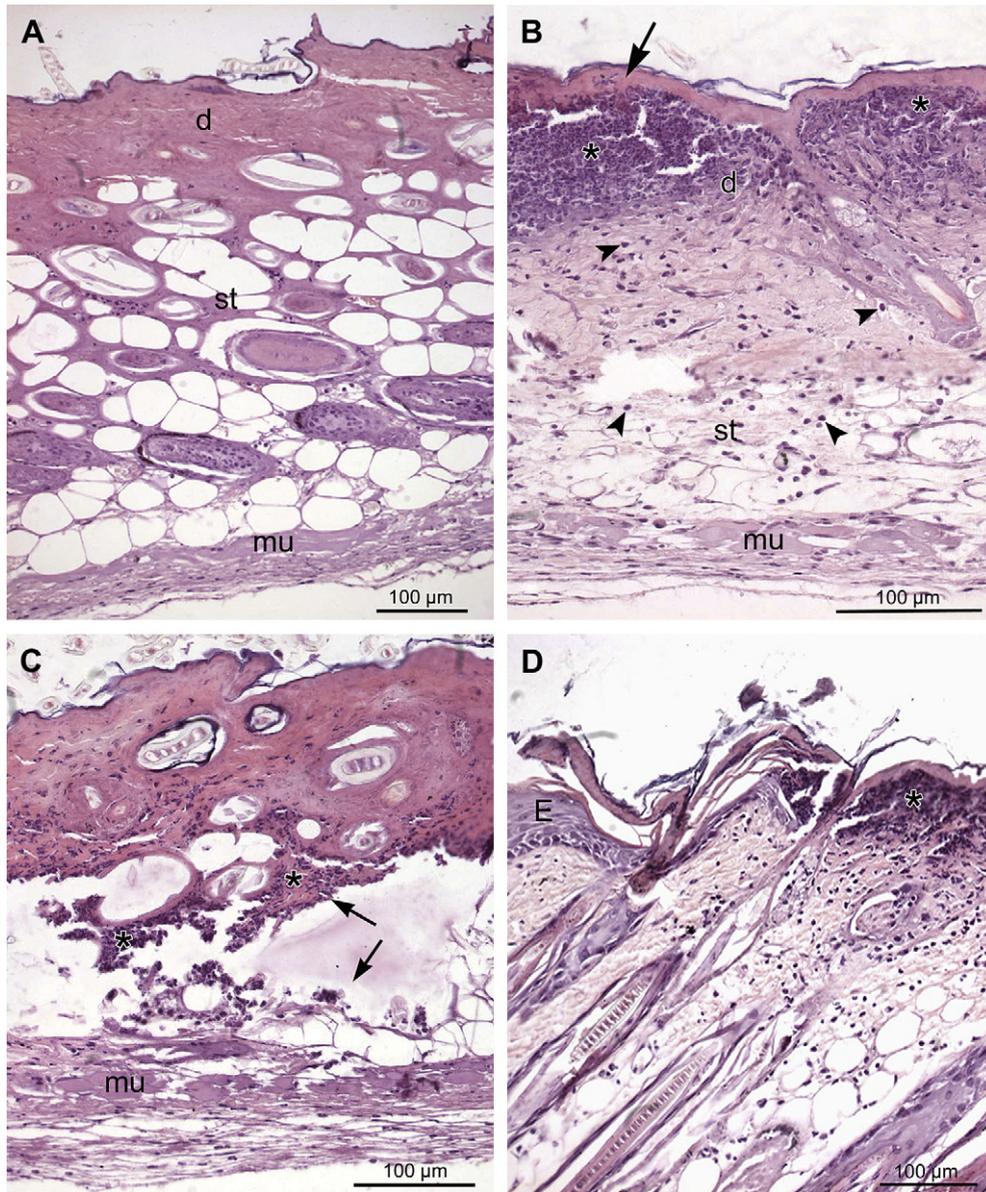


Fig. 3. Effect of *P. falkneri* stinger tissue extract in mouse skin. A and B: 48 h after injection. Coagulative necrosis of epidermis (arrow), dermis (d), subcutaneous (st) and skeletal muscle tissue (mu) is evident. There are micro-abscesses characterized by collections of acute inflammatory cells and cellular debris affecting the epidermis and superficial dermis (asterisks). Inflammatory cells are present in the dermis and subcutaneous tissue (arrowheads). C and D: 72 h after injection. There is cleavage of the necrotic tissues at the level of sub cutis (arrows), with collections of cellular debris (asterisks). Regenerative hyperplasia of epidermal cells (E) begins at the border of the lesions.

genus there is an amplification of the local damage caused by toxins as well as of the injury caused by the stinger (Haddad et al., 2004; Barbaro et al., 2007; Magalhães et al., 2008). Other species of *Potamotrygon* genus (*Potamotrygon* cf. *scobina* and *P. gr. orbigny*) can also cause necrosis as reported by Magalhães et al. (2006). The authors also observed that the mucus, which covers the animal, could augment this necrotic activity.

Secondary infection is usually found in patients injured by marine (Clark et al., 2007; Dehghani et al., 2009) or freshwater (Haddad et al., 2004) stingrays. In our experiments, two

samples showed bacterial infection, one 24 h and the other 96 h after venom injection indicating that the site of injury becomes a breeding ground for bacterial contamination. Studies are being conducted to determine which bacterial strains are more commonly associated with this type of envenoming.

In conclusion, the toxins found in the tissue covering the stingers of *P. falkneri* were able to cause severe local damage, characterized mainly by early necrosis. The association of the action of these toxins with the mechanical trauma caused by the stinger can explain the local necrosis

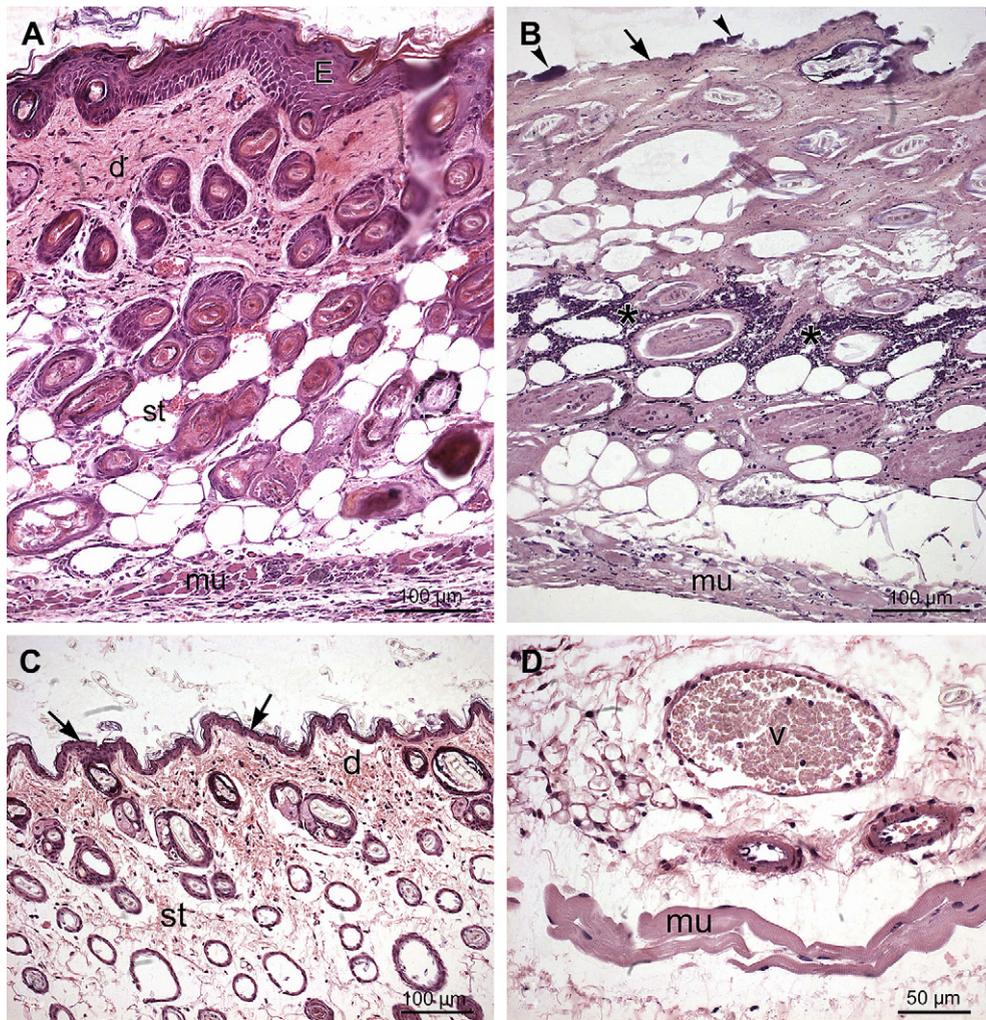


Fig. 4. Effect of *P. falkneri* stinger tissue extract in mouse skin. A and B: 96 h after injection. There is an evident regenerative hyperplasia of epidermal cells (E) bordering the necrotic skin, which shows intense coagulative necrosis that reaches the skeletal muscle (mu, Fig. 4B). In some areas the epidermis was totally lost (arrow) and superficial bacterial infection was present in one sample (arrowheads) Note the presence of cellular debris in the necrotic tissue (asterisks). C and D: Control group. No evidence of necrosis. The skin did not show abnormalities, although mild edema and mononuclear cell infiltration of dermis (d) and subcutaneous tissue (st) were focally present. v—blood vessel.

and the severe sequelae observed in humans injured by freshwater stingrays.

Conflict of interest

The authors declare that there are no conflicts of interest.

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References

- Barbaro, K.C., Lira, M.S., Malta, M.B., Soares, S.L., Garrone Neto, D., Cardoso, J.L., Santoro, M.L., Haddad Jr., V., 2007. Comparative study on extracts from the tissue covering the stingers of freshwater (*Potamotrygon falkneri*) and marine (*Dasyatis guttata*) stingrays. *Toxicon* 50, 676–687.
- Brisset, I.B., Schaper, A., Pommier, P., Haro, L., 2006. Envenomation by amazonian freshwater stingray *Potamotrygon motoro*: 2 cases reported in Europe. *Toxicon* 47, 32–34.
- Charvet-Almeida, P., Araújo, M.L.G., Rosa, R.S., Rincón, G., 2002. Neotropical freshwater stingrays: diversity and conservation status. *IUCN Shark News* 14, 47–51.

- Clark, R.F., Girard, R., Rao, D., Ly, B.T., Davis, D.P., 2007. Stingray envenomation: a retrospective review of clinical presentation and treatment in 119 cases. *J. Emerg. Med.* 33, 33–37.
- Conceição, K., Konno, K., Melo, R.L., Marques, E.E., Hiruma-Lima, C.A., Lima, C., Richardson, M., Pimenta, D.C., Lopes-Ferreira, M., 2006. Orpotrin: a novel vasoconstrictor peptide from this venom of the Brazilian stingray *Potamotrygon* gr. *orbignyi*. *Peptides* 27, 3039–3046.
- Conceição, K., Santos, J.M., Bruni, F.M., Klitzke, C.F., Marques, E.E., Borges, M.H., Melo, R.L., Fernandez, J.H., Lopes-Ferreira, M., 2009. Characterization of a new bioactive peptide from *Potamotrygon* gr. *orbignyi* freshwater stingray venom. *Peptides* 30, 2191–2199.
- Dehghani, H., Sajjadi, M.M., Rajaian, H., Sajedianfard, J., Parto, P., 2009. Study of patient's injuries by stingrays, lethal activity determination and cardiac effects induced by *Himantura gerrardi* venom. *Toxicon* 54, 881–886.
- Garrone Neto, D., Haddad Jr., V., Vilela, M.J.A., Uieda, V.S., 2007. Registro de ocorrência de duas espécies de potamotrigonídeos na região do Alto do Rio Paraná e algumas considerações sobre biologia. *Biota Neotropica* 7 (1). <http://www.biotaneotropica.org.br/v7n1/pt/abstract?shortcommunication+bn00707012007> 1676–0603 Jan/Abr 2007.
- Garrone Neto, D., Haddad Jr., V., 2009. Acidentes Por Raias. In: Cardoso, J.L. C., França, F.O.S., Wen, F.H., Málague, C.M., Haddad Jr., V. (Eds.), *Animais Peçonhentos No Brasil: Biologia, Clínica E Terapêutica Dos Acidentes*, second ed. Sarvier, São Paulo, pp. 295–305.
- Garrone Neto, D., Haddad Jr., V., 2010. Arraias em rios da região Sudeste do Brasil: locais de ocorrência e impactos sobre a população. *Rev. Soc. Bras. Med. Trop.* 43, 82–88.
- Gutiérrez, J.M., Rucavado, A., Escalante, T., Diaz, C., 2005. Hemorrhage induced by snake venom metalloproteinases: biochemical and biophysical mechanisms involved in microvessel damage. *Toxicon* 45, 997–1011.
- Haddad Jr., V., 2000. Atlas de animais aquáticos perigosos do Brasil: guia médico de identificação e tratamento. Editora Rocca, São Paulo. pp. 123–128.
- Haddad Jr., V., Garrone Neto, D., de Paula, N.J.B., Marques, F.P.L., Barbaro, K.C., 2004. Freshwater stingrays: study of epidemiologic, clinic and therapeutic aspects based on 84 envenomings in humans and some enzymatic activities of the venom. *Toxicon* 43, 287–294.
- Hogan, C.J., Barbaro, K.C., Winkel, K., 2004. Loxoscelism: old obstacles, new directions. *Ann. Emerg. Med.* 44, 608–624.
- Isbister, G., 2001. Venomous fish stings in tropical northern Austrália. *Am. J. Emerg. Med.* 19, 561–565.
- Lim, Y.L., Kumarasinghe, S.P.W., 2007. Cutaneous injuries from marine animals. *Singapore Med. J.* 48, 25–28.
- Lima, C., Clissa, P.B., Piran-Soares, A.A., Tanjoni, I., Moura-da-Silva, A.M., Lopes-Ferreira, M., 2003. Characterisation of local inflammatory response induced by *Thalassophryne nattereri* fish venom in a mouse model of tissue injury. *Toxicon* 42, 499–507.
- Magalhães, K.W., Lima, C., Piran-Soares, A.A., Marques, E.E., Hiruma-Lima, C.A., Lopes-Ferreira, M., 2006. Biological and biochemical properties of the Brazilian *Potamotrygon* stingrays: *Potamotrygon* cf. *scobina* and *Potamotrygon* gr. *orbignyi*. *Toxicon* 47, 575–583.
- Magalhães, M.R., da Silva, N.J., Ulhoa Jr., C.J., 2008. A hyaluronidase from *Potamotrygon motoro* (freshwater stingrays) venom: isolation and characterization. *Toxicon* 51, 1060–1067.
- Moura-da-Silva, A.M., Butera, D., Tanjoni, I., 2007. Importance of snake venom metalloproteinases in cell biology: effects on platelets, inflammatory and endothelial cells. *Curr. Pharm. Des.* 13, 2893–2905.
- Ospedal, K.Z., Appel, M.H., Fillus Neto, J., Mangili, O.C., Veiga, S.S., Gremski, W., 2002. Histopathological findings in rabbits after experimental acute exposure to the *Loxosceles intermedia* (brown spider) venom. *Int. J. Exp. Pathol.* 83, 287–294.
- Pareja-Santos, A., Saraiva, T.C., Costa, E.P., Santos, M.F., Zorn, T.T., Souza, V. M., Lopes-Ferreira, M., Lima, C., 2009. Delayed local inflammatory response induced by *Thalassophryne nattereri* venom is related to extracellular matrix degradation. *Int. J. Exp. Pathol.* 90, 34–43.
- Pedroso, C.M., Jared, C., Charvet-Almeida, P., Almeida, M.P., Garrone Neto, D., Lira, M.S., Haddad Jr., V., Barbaro, K.C., Antoniazzi, M.M., 2007. Morphological characterization of the venom secretory epidermal cells in the stinger of marine and freshwater stingrays. *Toxicon* 50, 688–697.
- Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150, 76–85.