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Paratrygon aiereba irradiated anti-mucus serum reduce edematogenic activity induced in experimental *model*

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ABSTRACT

Accidents by freshwater stingrays are common in northern Brazil, there is no specific therapy for high morbidity and local tissue destruction. The irradiation of venoms and toxins by ionizing radiation has been used to produce appropriate immunogens for the production of antisera. We planned to study the efficacy of stinging mucus irradiation in the production of antisera, with serum neutralization assays of edematogenic activity and quantification of cytokines performed in animal models of immunization with native and irradiated mucus of *Paratrygon aiereba*, a large freshwater stingray. Antiserum potency and its cross-reactivity with mucus from other freshwater stingrays were detected by ELISA. Immunization models demonstrated the ability to stimulate a strong humoral response with elevated levels of serum IgG detectable by ELISA, and both native and irradiated mucus were immunogenic and capable of recognizing mucus proteins from other freshwater neotropical stingrays. Mucus *P. aiereba* causes cellular and humoral adaptive immune responses in cells of immunized mice producing antibodies and cytokines such as TNF-α, IL-6 and IL-17. Rabbit antisera immunized with mucus from *P. aiereba* irradiated at 2 kGy showed a significant reduction of mucus-induced edematogenic activity in mice. Our data suggest that the use of antisera against freshwater stingray mucus show the possibility of specific therapy for these accidents.

1. Introduction

The irradiation of venoms and toxins by gamma rays has been used successfully for the production of suitable immunogens for serum therapy (Ferreira Jr. et al., 2009) that is the only specific and effective treatment for venomous animals accidents (Chippaux and Goyffon, 1998).

Accidents by freshwater stingrays are commons in northern Brazil and there is no specific treatment for these cases. Despite their high incidence, the accidents by freshwater or marine stingrays are often under-reported due to a failure in the reporting system for venomous animals, although the country has large river basins and an extensive coastline (Reckziegel et al., 2015).

The neotropical freshwater stingrays that present endemic geographic distribution in the main fluvial systems of South America (Moro et al., 2010; Carvalho and Lovejoy, 2011), are elasmobranchs adapted to live in freshwater environments. They belong to the family Potamotrygonidae which comprises four genera, *Potamotrygon*,

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Plesiotrygon, *Heliotrygon* and *Paratrygon* (Araújo et al., 2004).

The genus *Paratrygon* is considered monospecific however studies indicate this group may comprise a species complex (Frederico et al., 2012). Their serrated sting is small relative to the other neotropical freshwater stingrays and located near the base of the tail and the oval shaped disc. It has a geographical distribution in the Orinoco, Amazon and Tocantins-Araguaia basins (Carvalho et al., 2003; Carvalho and Lovejoy, 2011).

The stingrays of the Potamotrygonidae family have one to four stings located on the tail and positioned to function defensively acting only on stimulus and constitute the only physical defensive weapon (Garrone Neto and Haddad Jr., 2010).

The entire length of the body of the stingrays is covered by mucus, including the sting, and consequently the mucus is introduced into the lesion. Previous studies show that the dorsal mucus extract is as immunogenic as the stinger extract and has antigenic cross-reactivity (Thomazi, 2016; Lameiras et al., 2017). In addition, it has already been shown that samples of the sting, back and tail of neotropical freshwater stingrays have similar biological activity (Monteiros-Dos-Santos et al., 2011; Lameiras et al., 2014).s

Accidents by freshwater stingray are always very painful. The description of the symptoms is characterized by intense pain disproportional to the size of the lesion (Halstead, 1970). Other manifestations are characterized by intense edema and erythema, associated with necrosis, vasculitis and ulcerations with healing delay (Silva Jr. et al., 2015) and secondary infections (Torrez et al., 2015).

The therapeutic in these cases is based on the extent and character of the lesion, on the clinical symptoms manifested by the victims and there is no medical protocol for caring for the injured (Garrone Neto and Haddad, 2009).

In this study, we evaluate the immunogenic properties of 2 kGy ^{60}Co irradiated *Paratrygon aiereba* mucus and the ability of a serum produced with this antigen to neutralize the edematogenic activity of the mucus, the serum neutralization of edema activity of, which would contribute to a more specific treatment of injuries caused by accidents with neotropical freshwater stingrays.

2. Materials and methods

2.1. Animals

Male Swiss mice (n = 40) weighing between 20 and 22 g and male New Zealand rabbits (n = 6) weighing between 3 and 4 kg, provided by Institute of Energy and Nuclear Research of the University of São Paulo, Brazil (IPEN) were used. Animals were housed under conditions of controlled temperature and artificial light (12-h light/12-h dark, lights on at 7:00 a.m.). Animals were transferred to a different (temperature-consistent) room and were acclimated for 10 days before beginning the experiments. The maintenance and use of the animals followed the recommendations of the National Council on the Control of Animal Experimentation (CONCEA). All animals were kept in plastic cages with sterile pinus bedding, receiving commercial Nuvital® ration and water *ad libitum*. The animals were euthanized in a CO_2 chamber following the standards of manipulation and care of laboratory animals. All studies were performed after the approval of the Committee on Care and Use of Animal Resources of the Institute of Energy and Nuclear Research of the University of São Paulo, Brazil.

2.2. Stingrays and processing of samples

Specimens of *Paratrygon aiereba* (n = 10), *Potamotrygon orbignyi* (n = 3) and *Potamotrygon rex* (n = 3), were collected in Ribeirão do Carmo, a tributary of the Tocantins river in the municipality of Porto Nacional, and in the reservoir of the Luís Eduardo Magalhães Hydroelectric Power Plant in the municipalities of Palmas and Porto Nacional, state of Tocantins, northern region of Brazil.

Mucus was obtained by scraping of the epithelium covering the animals' backs. The samples were diluted in 0.9% saline solution, centrifuged at 1190×*g* and the supernatant was filtered through a 0.22 μm filter (Millipore). The protein content was determined by the Bradford colorimetric method (Bradford, 1976). All samples were maintained at −70 °C until use.

A mucus pool of each species was made. The mucus pool of *P. aiereba* was divided into two aliquots: one aliquot was maintained in its native form and the other was subjected to the gamma ray irradiation process. Both aliquots were used in the immunization of the experimental models, and the mucus in its native form was used in the other assays described below. The mucus from *P. rex* and *P. orbgnyi* were used only to check the immunological cross-reactivity of these samples with the antibodies raised against the *P. aiereba* mucus.

2.3. Irradiation of mucus

The mucus of *P. aiereba* (800 μg/mL) obtained as described above was subjected to irradiation with a dose of 2 kGy with 90% shielding, by homogeneous exposure to γ-rays from a ^{60}Co source (Gamacell®, Atomic Energy of Canada) with a dose rate of 1.031 kGy/h, at room temperature in the presence of atmospheric oxygen (Nascimento et al., 1996). Control samples remained on the outside of the source throughout the irradiation time for evaluation of environmental conditions.

2.4. Immunization

Groups of Swiss mice (n = 20) and New Zealand rabbits (n = 04) were immunized *subcutaneously* with 04 bi-weekly doses of native (non-irradiated) or irradiated *P. aiereba* mucus. The first dose was administered in the presence of incomplete Freund's adjuvant. The groups of mice were immunized with a concentration of 1.5 μg of mucus (200μL/animal, diluted in 0.9% saline), and the rabbits immunized with a concentration of 200 μg of mucus (1mL/animal, diluted in saline 0.9%). Control groups, not immunized (mice n = 05; rabbit n = 02), were inoculated with incomplete Freund's adjuvant diluted in 0.9% saline. Serum samples for the immunological assays were obtained 15 days after the last immunization, the blood samples from the mice were obtained via the orbital plexus and from the rabbits by puncture of the marginal or central vein of the ear. The serum produced by rabbits in response to native and irradiated mucus were used in serum neutralization and ELISA assays, whereas the serum of mice was used for cytokines assays, *in vitro* induced antibody production and ELISA.

2.5. Enzyme linked immunosorbent assay (ELISA)

Microtitration plates were coated overnight at 4 °C with 5 μg of protein/mL of mucus (*P. aiereba*, *P. orbignyi* or *P. rex*) in 0.05 M carbonate buffer, pH 9.0. Plates were washed with phosphate buffered saline plus Tween 0.05%(PBST) for 5 min and blocked with 0.3% skim milk in PBST for 1 h at 37 °C. After blocking, serum of mice, rabbit and non-immunized animals diluted in PBST was added (1/200 at 1/102,400). Next, the plates were washed and incubated with diluted (1/5000) anti-IgG peroxidase-conjugated antibodies (Sigma®-Aldrich Co., St. Louis, MO, USA). After further washes, bound IgG was developed using 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma®-Aldrich Co., St. Louis, MO, USA), the reactions were stopped by adding 5% sulfuric acid. Absorbance at 492 nm was determined using multi-mode microplate reader (LabSystems Multiskan MS®, Midland, ON, CA) (Theakston et al., 1977).

2.6. Neutralization of edematogenic activity

The ability of the antibodies raised against native or ^{60}Co irradiated by *P. aiereba* mucus to neutralize the edema induced by mucus was assessed *in vitro* (Lopes-Ferreira et al., 2000).

Mucus of *P. aiereba* (50 μg/mL) was incubated for 30 min at 37 °C with rabbit anti-native or irradiated mucus sera, which were diluted 1/1000. After incubation, the samples were shaken and injected (30 μL) into the plantar pad of the hind paw of groups of Swiss mice (n = 5/group). The neutralization of edematogenic activity was measured at 1 h, 2 h, 4 h and 24 h after inoculation. In all animals the initial individual volume of each paw was verified before the injection of the samples (time zero). Volumes were measured using a plestismometer (Ugo Basile TM, IT) and the results expressed as volume variation in relation to the baseline volume in μL per period (Kimura et al., 2014).

2.7. In vitro induced antibody production (IVIAP)

All steps were performed in sterile laminar flow. Sterile 96-well flat-bottom plates were coated overnight at 4 °C with sterile 5 μg of protein/mL of mucus (native) and incubated for 48 h in a 5% CO_2 chamber at 37 °C. After, spleen cells (2×10^6 cell/well) were obtained from mice immunized with irradiated *P. aiereba* mucus (n = 3), mice immunized with native *P. aiereba* mucus of (n = 3) and naïve control mice (n = 3) 15 days after the last dose, were dissociated in sterile conditions in a laminar flow in RPMI 1640 culture *medium as described elsewhere* (Zorgi et al., 2016). The mononuclear cells were separated using Ficoll-Paque™ Premium 1840 (GE Healthcare®) according to the manufacturer's instructions. After, the cells were seeded on plates previously coated with 5 μg of protein/mL of mucus (native) for 48 h in a 5% CO_2 chamber at 37 °C.

Briefly, after 48 h culture, the supernatant was removed and the wells washed according to the previously recorded ELISA methodology (Caterino-de-Araujo, 1992). Anti-mouse IgG conjugated to peroxidase was added and incubated for 1 h at 37 °C. Wells were washed, 100 μL TMB were applied and the reaction was stopped with 5% H_2SO_4 after 30 min. The reactivity index of the samples was determined as the ratio of the absorbance from each sample by the mean absorbance of the negative control samples, used for intra-test quality control for conjugate variation (IR = Abs of positive samples/mean of Abs. of negative samples).

2.8. Production of cytokines by peripheral blood cells or spleen cells of mice immunized with native or irradiated mucus

The detection of cytokines was realized in supernatant of IVIAP assays or serum. The cytokines analysis was performed according manufacturer instructions using Cytometric Bead Array® (CBA) Th1, Th2 and Th17 (BD Biosciences®). The analysis was conducted in a flow cytometer (BD Biosciences® LSR Fortessa). The data were collected by FACSDIVA® (BD Biosciences®) software and analyzed by FCAP Array V 3.0 (BD Biosciences®).

2.9. Ethics aspects

This research was approved by the Committee on Ethics in the Use of Animals do Institute of Nuclear Energy Research of the University of São Paulo, Brazil, protocol n. ° 126/13.

The capture of the stingrays accomplished by means of environmental license n. ° 6781–1/2014, granted by the Chico Mendes Institute of Biodiversity Conservation. All the captured animals were deposited in the Fish Collection of the Laboratory of Systematic Ichthyology of the Federal University of Tocantins, Campus of Porto Nacional, state of Tocantins, Brazil.

3. Results

3.1. IgG titers of the anti-mucus of P. aiereba

The antibody titer in rabbits immunized against native (n = 2) or irradiated (n = 2) *P. aiereba* mucus demonstrated high levels of specific IgG antibodies by ELISA (Table 1). Rabbits immunized with four doses of native mucus show a high production of antibodies from the first dose, while those immunized with irradiated mucus showed a progressive increase of titers, with a greater significant difference when compared between the immunized and control groups ($p < 0{,}001$, $p < 0{,}01$, $p < 0.05$). As observed in rabbits, both native and irradiated mucus induced high levels of specific antibodies in mice (n = 20) after immunization ($p < 0{,}001$, $p < 0.05$).

3.2. Cross-reactivity of the antisera

The mice and rabbit serum produced against the native or irradiated mucus of *P. aiereba*, reacted to the mucus antigens of the others freshwater stingray species: *P. orbignyi* and *P. rex* with significant difference when compared between control groups not immunized or immunized with *P. aiereba* mucus (Fig. 1).

3.3. Neutralization of edematogenic activity

One of the most important objectives of this study was to evaluate the ability of seroneutralization of edema induced by mucus de *P. aiereba* (Fig. 2). Irradiated anti-mucus serum (rabbit), previously incubated with *P. aiereba* mucus diluted 1:1,000, was able to reduce the formation of edema in the paw of Swiss mice ($p < 0.01$), a fact not observed with native anti mucus serum in the same conditions.

3.4. IVIAP

We evaluated the production of specific IgG anti-mucus from spleen cells in culture as described in methods. Mucus specific IgG production was evaluated in groups of Swiss mice immunized with native (n = 3) and irradiated (n = 3) mucus. After the four doses of immunization, the two groups showed significant levels of anti-mucus IgG in spleen cells (Fig. 3). Animals immunized with irradiated mucus had higher levels of IgG anti-mucus than animals immunized by native mucus.

3.5. Production of cytokines

We evaluated the production of IL-17, IL-6, TNF alpha, IFN gamma, IL-10, IL-4 and IL-2 by CBA. The production of these cytokines was determined in peripheral blood of mice immunized with native and irradiated mucus and also in the supernants of spleen cells exposed to mucus. The results of cytokines produced by both groups of immunized animals (irradiated mucus and native mucus) were compared with the results of cytokines produced by not immunized animals (control) (Fig. 4).

We can observe the significant production of cytokines IL-17 A and IL-6 ($p < 0.05$ both) by peripheral blood cells in those immunized with native mucus (Fig. 4A and B) and significant production of TNF-α ($p <$

Table 1
IgG titers of the P. aiereba anti-mucus sera as compared by threshold titer by non-parametric tests.

Animal (n)	Serum collected 15 days after	Native mucus		2 kGy irradiated mucus		p native vs irradiated
		Titer	p vs control	Titer	p vs control	
Rabbit (4)	1st dose	51,200	<0.01	6400	<0.001	<0.05
	2nd dose	51,200	<0.05	12,800	<0.001	NS
	3rd dose	25,600	<0.05	25,600	<0.001	NS
	4th dose	102,400	<0.05	51,200	<0.001	NS
Mice (20)	1st dose	0	0	0	0	NS
	2nd dose	25,600	<0.001	12,800	<0.001	NS
	3rd dose	51,200	<0.05	51,200	<0.05	NS
	4th dose	51,200	<0.001	51,200	<0.05	NS

NS – not significant.

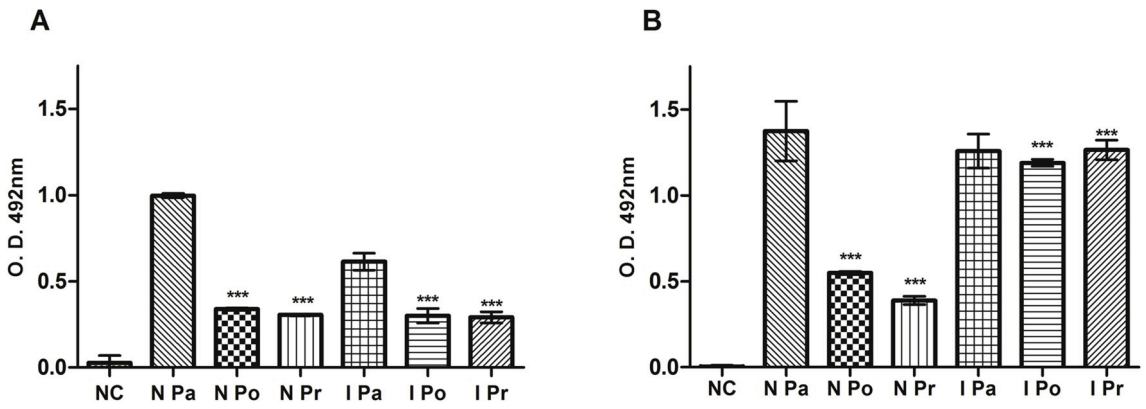

Fig. 1. Antigenic cross-reactivity against the mucus of different species of freshwater stingrays by ELISA (1:12,800). A) Serum from immunized mice (n = 20); B) Serum from immunized rabbits (n = 4). NC negative control; N Pa - native anti-mucus serum P. aiereba against mucus of *P. aiereba* (positive control of native); N Po - native anti-mucus serum *P. aiereba* against mucus of *P. orbignyi*; N Pr - anti-native *P. aiereba* mucus serum against mucus of *P. rex*; I Pa - irradiated anti-mucus serum *P. aiereba* against mucus of *P. aiereba* (positive control of irradiated); I Po - irradiated anti-mucus serum *P. aiereba* against mucus of *P. orbignyi*; I Pr - irradiated anti-mucus serum *P. aiereba* against mucus of *P. rex*. *Bars represent the mean and standard error of mean,* the asterisks indicate significant difference in relation to the negative and positive controls: ***p < 0.001.

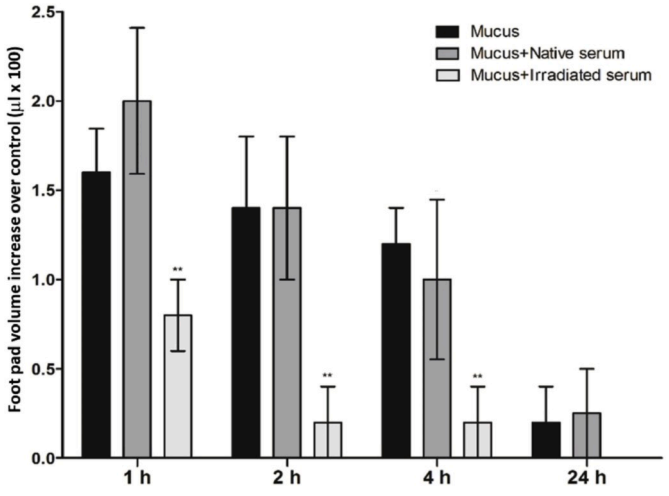

Fig. 2. In vitro serum neutralization of the edematogenic activity induced by the mucus of *P. aiereba* (50 µg) in the paw of Swiss mice (n = 5/group). Mucus of *P. aiereba* was incubated with anti-native or irradiated mucus serum produced in rabbits (1/1000). Each bar represents the mean of the readings in the plethysmometer and the standard error of the mean. Asterisks indicate statistical difference in relation to the mucus (50 µg) of P. aiereba: **p < 0.01.

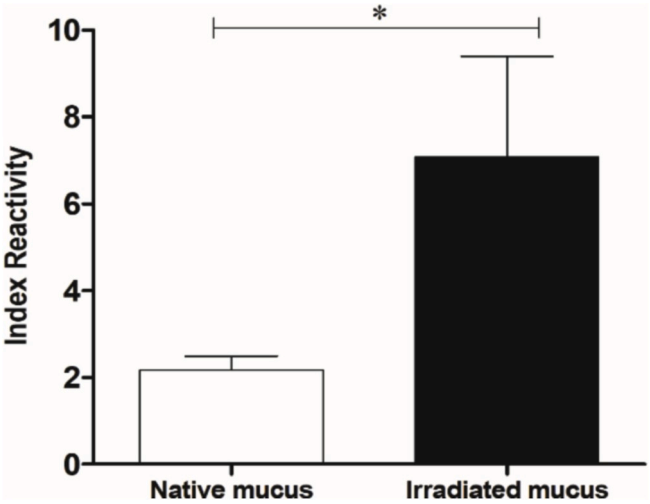

Fig. 3. Detection of specific IgG produced by spleen cells from Swiss mice immunized with native (n = 3) or irradiated (n = 3) mucus stimulated by mucus of *P. aiereba*. Bars represent the standard error of mean; the asterisk indicate statistical difference between the groups (p < 0.05).

0.05) in those immunized with irradiated mucus (Fig. 4C).

Spleen cells challenged with mucus presented a quite different response in mice immunized with irradiate mucus, producing more IFN-γ (p < 0.05) and IL-10 (p < 0.01) (Fig. 4D and E) and also TNF-α (p < 0.05) (Fig. 4F), while the spleen cells from mice immunized with native mucus shows a much less intense production of those cytokines.

4. Discussion

Accidents caused by stingrays of the Potamotrygonidae family are characterized by severe tissue damage and the local edema is one of the most common manifestations (Haddad et al., 2013). Tissue laceration caused by the sting induces extravasation of fluid into the interstitial space forming the edema (Silva Jr. et al., 2015). Likewise the participation of proteolytic enzymes present in the mucus causes cellular damage (Magalhães et al., 2006), resulting in leakage of fluid into the interstice, in degradation of components of extracellular matrix, working as diffusion factors and acting directly in the degradation of proteins (Magalhães et al., 2008), contributing to tissue injury and changes in local microcirculation (Kimura et al., 2014; Dos Santos et al., 2017). The increase in extracellular volume starts about 30 min after the inoculation and it is maintained at a high level for several hours, both in experimental models (Barbaro et al., 2007; Monteiro-Dos-Santos et al., 2011) and in accidents with human (Silva et al., 2015).

The results show that the 2 kGy irradiated mucus is able to stimulate the production of specific antibodies which recognize and partially neutralize the edematogenic components. These data suggest that mucus should have antigenic agents responsible for edema. Native anti-serum (non-irradiated) mucus was not able to inhibit the edematogenic activity of mucus. The same was observed in studies using venom of the fish *Thalassophryne nattereri* (Piran-Soares et al., 2007) and *Scatophagus argus* (Muhuri et al., 2005). Most toxins have low immunogenicity but are extremely active (Rangel-Santos and Mota, 2000; Laustsen et al., 2015, 2017). *By* its random action, radiation can induce chemical changes in protein structures (Oliveira et al., 2015), maintaining or improving their immunological properties (Nascimento et al., 1996; Abib and Laraba-Djebari, 2003; Ferreira Jr. et al., 2009; De La Rosa et al., 2018). In aqueous solution, proteins become more immunogenic, being better

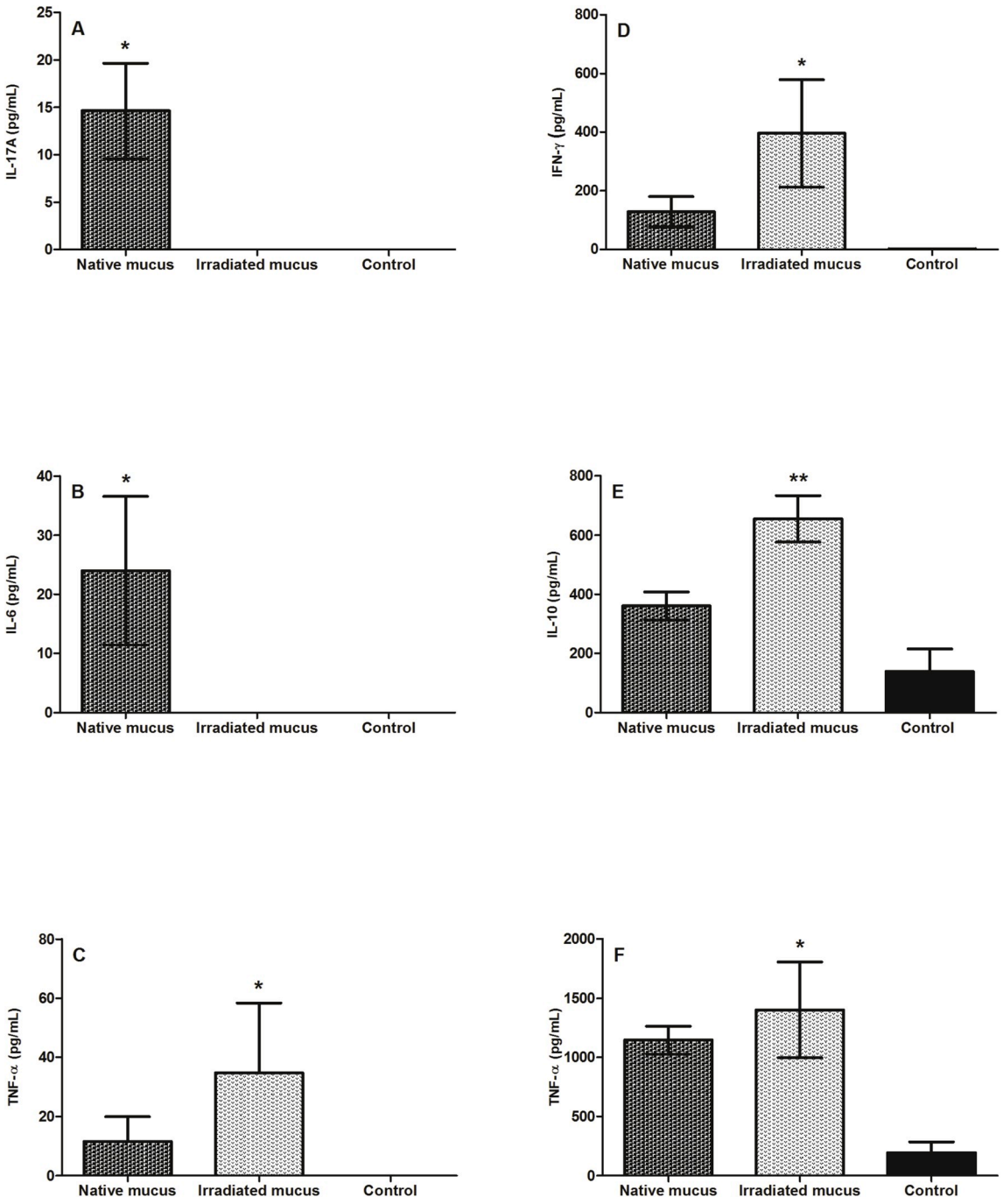

Fig. 4. Expression of cytokines (pg/mL) in response to mucus of *P. aiereba*. A) IL-17 A; B) IL-6; C) αTNF at blood. D) IFN-γ; E) IL-10; F) TNF-α at spleen cells. Native mucus (group immunized, n = 3), irradiated mucus (group immunized, n = 3), control (group not immunized, n = 3). Bars represent the mean and standard error of mean, the asterisks indicate statistical difference in relation to the control group: *$p < 0.05$, **$p < 0.01$.

recognized and processed by macrophages (Cardi et al., 1998), *improving antigen presentation by antigen-presenting cells in the previous stages of the adaptive immune response.*

In this work we observed from ELISA results that sera from animals immunized with native or irradiated mucus had similar IgG titers, but the serum neutralization test showed that even with similar IgG titers, antibody quality is different since only serum irradiated anti-mucus was able to reduce the edematogenic activity of *P. aiereba* mucus. Irradiated mucus induced a slow initial antibody response compared to the native mucus immunization, however, in the latter immunizations both showed a similar quantitative response. Our data suggest that native mucus appears to induce a nonspecific acute immune response, probably activating TH17 innate lymphoid cells (Isailovic et al., 2015), while irradiated mucus induces a regular adaptive immune response that promotes affinity selection of antibodies, but this response needs more steps in the immune response for selection of memory B cell production (Biram et al., 2019), as observed by the higher levels of memory B cells in the spleen of mice immunized with irradiated mucus by IVIAP.

Irradiated mucus only induces the production of TNF-α by peripheral blood cells, probably due to the residual toxicity. Besides that, in the native mucus was observed the production of cytokines IL-6 and IL-17. Studies using mucus and venom of *Potamotrygon henlei* and venom of *P. motoro* demonstrated stimulation for IL-1, IL-6 and KC cytokines and chemokine (Monteiro-Dos-Santos et al., 2011; Kimura et al., 2014; Dos

Santos et al., 2017). IL-6 participates in cell signaling increasing vascular permeability, contributing to the formation of edema (Alsaffar et al., 2018). The relationship between edema and IL-6 participation could be related to our results, since in sera from animals immunized against irradiated mucus no IL-6 was detected and it partially reduced mucus-induced edema. The native mucus immunization induced IL-17 producing cells. This interleukin stimulates the production of TNF-α and IL-6 also found in this profile, inducing neutrophilic inflammation, which is part of the acute phase immune response (Oliveira et al., 2011), but also interferes in the adaptive immune response being produced by innate lymphoid cells (Isailovic et al., 2015), that justify the early antibody production found with this immunization. Cytokines found after irradiated mucus immunization show activation of an adaptive immune response in spleen cells. This response was characterized by the detection of memory cells by IVIAP and by the production of effector cytokines secreted by CD4 cells. This would have the ability to promote a better selection of high affinity antibodies capable of neutralizing some of the mucus toxins, resulting in qualitative production of high affinity antibodies, a fact not observed with native mucus immunization.

IVIAP and immunoenzymatic assays showed that native and irradiated *P. aiereba* anti-mucus serum were able to react and recognize epitopes present in the mucus of *P. aiereba.* In addition, our results show cross-reactivity with other species of stingrays (*P. orbignyi* and *P. rex*) that are commonly found in Amazonia, Araguaia, Tocantins, and Orinoco River Basin. The possibility of using the same antiserum for other species may facilitate treatment in the event of an accident since identification of the species at the time of the accident is not possible in most cases. Trials demonstrating cross-reactivity between different species of stingrays have been described in the literature (Barbaro et al., 2007; Lameiras et al., 2017).

Despite the difficulty of identifying the animal, most of the papers cited the genus *Potamotrygon* as the most related to these accidents, however, due to the fact that *P. aiereba* has a large geographical distribution in several hydrographic basins (Amazonas, Tocantins, Araguaia, Orinoco), accidents with this species cannot be ruled out. Finally, the composition and mechanism of action of the toxins present in Potomatrygonidae stingrays has not been fully studied, especially on the mucus that covers the entire body of these fish and contributes to the effects caused by stings of these animals. Evidence of mucus immunogenicity and heterologous serum action is a key step in continuing the search for a treatment that reduces the effects of the accidents with neotropical freshwater stingray.

5. Conclusions

The irradiated anti-mucus serum of the *Paratrygon aiereba* is able of decrease edematogenic activity induced in mice and the *P. aiereba* mucus causes cellular and humoral adaptive immune response in immunized animals.

Ethical statement

I declare that the manuscript "***Paratrygon aiereba* irradiated anti-mucus serum reduce edematogenic activity induced in experimental models"** submitted to the Toxicon was approved by the Committee on Ethics in the Use of Animals do Institute of Nuclear Energy Research of the University of São Paulo, Brazil, protocol n. ° 126/13. The capture of the stingrays accomplished by means of environmental license n. ° 6781–1/2014, granted by the Chico Mendes Institute of Biodiversity Conservation. All the captured animals were deposited in the Fish Collection of the Laboratory of Systematic Ichthyology of the Federal University of Tocantins, Campus of Porto Nacional, state of Tocantins, Brazil.

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