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PREVIOUS 60-CO RADIATION FROM PARATRYGON AIEREBA MUCUS INDUCES THE PRODUCTION OF HIGHLY RESPONSIVE ANTIBODIES AND A BETTER IMMUNE RESPONSE IN MICE

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ABSTRACT

Wounds from stinging freshwater stingrays are painful, difficult to heal and cause extensive necrosis and systemic phenomena. The treatment is symptomatic, of low efficiency and there is no therapy, which causes more suffering to the injured. This study aimed to evaluate the immune response induced by the native or irradiated by 60-Co gamma from *Paratrygon aiereba* mucus. IPEN's Committee on Ethics in the Use of Animals (n.º126/2013) and lanes captured under license from the Chico Mendes Institute for Biodiversity Conservation (n.º6781-1/2014) approved this research. For the assays, sera from Swiss mice previously immunized against native or irradiated mucus were used. The proliferation of splenic B cells

in response to mucus was evaluated by the In Vitro Induced Antibody Production method and serum and splenic cytokines were also quantified. Our data demonstrate that the irradiated mucus of *P. aiereba* induces greater production of antibodies and more immunological memory in the mice. Spleen cells from animals immunized against irradiated mucus produced IFN-γ, TNF-α and IL-10, and serum TNF-α (immunized group against irradiated mucus) and IL-6 and IL-17 (immunized group against native mucus). The results corroborate the use of ionizing radiation, with production of highly responsive antibodies and better immune response, besides proving that *Paratrygon aiereba* mucus is capable of stimulating cellular and humoral adaptive immune response, contributing to the continuity of associated investigations.

1. INTRODUCTION

Freshwater stingrays are venomous animals, frequently associated with accidents in northern Brazil where the shallow waters and the human use of the rivers favour close proximity between the fish and potential victims [1]. Stingray stings induce painful lesions, edema, necrosis [2], [3], [4] and the mucus that covers the body of these fishes may increase the severity of the wounds [5].

Few studies have been dedicated to the mucus of specimens of *Potamotrygonidae* family stingrays. Some works were performed using the venom (extract of sting) [6], [7]. However there is little information about the mucus of *Paratrygon aiereba*, one of the freshwater stingray found Amazônica and Tocantins-Araguaia basin [8], [9].

Serotherapy is specific and effective treatment for venomous animals bites or stings. This treatment consists on the administration of antivenom obtained from immunized animals [10]. In the case of accidents by stingrays the treatment is symptomatic, of low efficiency and there is no therapy, which causes more suffering to the injured. In this sense, to evaluate the immune response induced by *Paratrygon aiereba* mucus is more important for to subsidiary futures studies serotherapycs. Also using the ionizing radiation that has shown advantageous in the attenuation of animal toxins resulting in better immunogens for the production of sera [11].

2. MATERIAL AND METHODS

The Ethics Committee on Animal Use of IPEN (protocol n. °126/2013), approved this research. The capture of the stingrays was accomplished by means of environmental license (n. º 6781-1 /2014), granted by the Chico Mendes Institute of Biodiversity Conservation. All the captured stingrays were deposited in the Fish Collection of the Laboratory of Systematic Ichthyology of the Federal University of Tocantins (UFT), Campus of Porto Nacional, state of Tocantins.

2.1 Obtention and processing of the venom and mucus samples

The extraction of the mucus of the stingrays was performed by scraping of the epithelium of the dorsum and the samples were stored at -20ºC until used [5]. All the samples were dissolved in 150mM NaCl, centrifuged at 1190xg and the resulting supernatant was filtered through a 0,22μm membrane. The protein dosage was performed by the colorimetric method of Bradford (1976) [12].

2.2 Irradiation of the samples

Samples of *Paratrygon aiereba* mucus (800µg/mL) were irradiated with 2 kGy of gamma rays from a 60-Co Gamacell 220 (Atomic Energy Canada Ltd) with a dose rate of 1,031kGy/h, at room temperature and in the presence of atmospheric oxygen.

2.3 Immunization of animals

Groups of Swiss mice (n=20) were immunized with four doses fortnightly of native or irradiated mucus, in the concentration of 1.5µg/mL (0.2mL/ animal, diluted in saline 0.9%). The first dose of immunization was performed in the presence of Freund's incomplete adjuvant and the other doses with native or irradiated mucus in saline solution. The obtaining serum from the mice was performed by bleeding orbital plexus fifteen days after each immunization. Control of mice received one dose of incomplete adjuvant followed by 0.9% saline inoculum.

2.4 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA plates were coated overnight at 4°C with 5 µg/mL of the *Paratrygon aiereba* mucus in carbonate buffer (Na_2CO_3-$NaHCO_3$ 0,1M, pH 9,5). The plates were washed with PBST (PBS containing 0.05 % Tween-20) and blocked with 0.3 % milk (Molico®) in PBST for 1h at 37°C. After blocking, the sample serum diluted in PBST was added for 1h at 37°C. Next, the plates were washed and appropriately diluted anti-mouse IgG peroxidase-conjugated antibodies were added (Sigma Aldrich®). After further washes, the bound conjugate was revealed with 3,3',5,5'-tetramethylbenzidine (TMB - Sigma Aldrich®) for 30min. The reactions were stopped by adding sulfuric acid (H_2SO_4) 5%. The absorbance at 492nm was determined using multi-mode microplate reader (Labsystems Multiskan MS®, Midland, ON, CA) [13].

2.5 In vitro Induced Antibody Production (IVIAP)

Sterile 96-well culture plates were coated for 4h at 4°C with 100µL of mucus (5µg/mL) de *Paratrygon aiereba* in carbonate buffer (Na_2CO_3-$NaHCO_3$ 0,1M pH 9,5). Plates were washed with PBST, blocked with 2% bovine serum albumin (BSA) sterile in PBS for 2h at 37°C. After, plates were washed. Spleen cells were obtained from mice immunized with irradiated mucus of *Paratrygon aiereba* (n=3), mice immunized with native mucus of *Paratrygon aiereb*a (n=3) and control mice not immunized (n=3). The spleens were dissociated with needles in RPMI 1640 culture medium with amphotericin B, 5 mg/mL; penicillin, 500 UI/mL; streptomycin, 200 mg/mL; and β-mercaptoethanol (complete culture medium). Separation of the cells to obtain the lymphocytes was performed with Ficoll-Paque™ Premium 1084 (GE Healthcare®), following the manufacturer's guidelines. After separation of the cells, they were suspended in 1 ml of RPMI 1640 culture medium (Gibco®), counted in Neubauer's chamber and adjusted to a concentration of $2X10^6$ cells/ml. Cells were distributed in the culture plates pre-coated. Then, 100µl of culture medium containing 10% fetal bovine serum and 10µg/mL of *Paratrygon* mucus was added to each well. Cells were incubated in 5% CO_2 at 37°/C for 48h. After, the supernatant was recovered and stored for next test and the plates were washed with PBST, and anti-mouse peroxidase-conjugated IgG was added in a volume of 100µL/well (1:10.000), plates were then incubated for 1 h at 37°C. After further washings, TMB were added for 30 min. Reactions were stopped with sulphuric acid 5%, and absorbance 492 nm was determined using an automatic microplate reader. All steps were conducted in laminar flow hood with sterile handling [14].

2.6 Detection of cytokines by cytometry

The supernatants obtained from splenic cell cultures from the IVIAP assays were used and sera from mice immunized with native and irradiated mucus and control mice. The Cytometric Bead Array® (CBA) Mouse Th1, Th2 and Th17 (BD Biosciences®) commercial cytometric kit was used according to the manufacturer's instructions. The analyzes were conducted on the flow cytometer (BD Biosciences® LSRFortessa), the data collected by BD FACSDIVA® software (BD Biosciences®) and analyzed using FCAP Array V 3.0® software (BD Biosciences®).

2.7 Statistical analysis

Quantitative data were analyzed statistically through analysis of variance and Bonferroni type post-test, where comparisons with $p < 0.05$ were considered significant, using the GraphPad Prism 5.0 statistical package (GraphPad software, Inc San Diego , California, USA).

3. RESULTS

The *in vitro* production of IgG antibodies, performed on spleen cells cultured by mucus stimulated, was higher in response to irradiated mucus ($p < 0.05$) by comparing the responses of groups previously immunized against native mucus and not immunized (Tab. 01).

Table 1: Detection of IgG antibodies by spleen cells of Swiss mice immunized with native or irradiated *Paratrygon aiereba* mucus by IVIAP

Group	Absorbance (450 nm)
C	0,0097
INM	0,0145
IIM	0,0474*

C - Control (not immunized); INM - Immunized native mucus; IIM - immunized irradiated mucus. Asterisks indicate statistical difference in relation to the control (serum of unimmunized mice): * $p < 0.05$.

Cytokines produced by splenic lymphocytes in response to mucus stimulation were analyzed by comparing the results found in groups previously immunized against native or irradiated mucus and non-immunized group. We observed significant production of IL-10 ($p < 0.01$), IFN-γ and TNF-α ($p < 0.05$) in splenic cultures of mice immunized against irradiated mucus.

Table 2: Expression of cytokines (pg/ml) by splenic cells in response at the mucus re-stimulation

Cytokine	C	INM	IIM
IL-10	139,1	360,4	654,5**
IFN-Y	1,2	128,6	395,8*
TNF-α	193,8	1146,2	1401,5*

C - Control (not immunized); INM - Immunized native mucus; IIM - immunized irradiated mucus. Asterisks indicate statistical difference in relation to the control group: * $p<0.05$ and ** $p<0.01$.

In the serum of mice immunized against irradiated mucus, higher TNF-α production was detected ($p<0.05$), while native mucus was found IL-17A and IL-6 ($p<0.05$) (Tab. 03).

Table 3: Serum cytokine (pg/ mL) expression against native or irradiated mucus

Cytokine	C	INM	IMI
TNF-α	0,0	11,6	34,7*
IL-6	0,0	24,0*	0,0
IL-17A	0,0	14,6*	0,0

C - Control (not immunized); INM - Immunized native mucus; IIM - immunized irradiated mucus. Asterisks indicate statistical difference in relation to the control group: * $p<0.05$.

4. DISCUSSION

The humoral immune response triggered by the irradiated mucus of *Paratrygon aiereba* was evidenced by the proliferation of splenocytes *in vitro* (IVIAP). The spleen cells of mice (immunized with native or irradiated and non-immunized mucus) had their growth restimulated against mucus, corroborating their ability to induce adaptive immune response, an important component for humoral immunity [15].

We can highlight the ability of irradiated mucus antibodies to recognize and respond to mucus in their native form, and the splenocytes of the animals immunized against the irradiated mucus are more responsive than the other groups. These data are in agreement with works that use the irradiation of toxins or venoms of snakes and also of parasites, since the gamma radiation is able to improve the immunogenic properties of these compounds [16], [17].

It is known that animal poisons, including fish, are composed of a variety of biologically active proteins and peptides [18], [19], [20], [21]. The nature of the antigen, as in the case of this work, a native or irradiated protein, may play an important role in directing the triggered immune response. In recent years, studies have shown that after the irradiation process, the protein undergoes changes in the tertiary and secondary structure, a fact that is directly related to the changes in its biological functions. In some cases the proteins, after being irradiated in

solution, have insoluble aggregates visible to the naked eye. Part of the sample may also reveal intermediate forms of conformation that present as soluble aggregates [22], [23], [24], [25], [26].

The mechanism by which cytokines induce selective differentiation of $CD4^+$ T cells into Th1 and Th2 is not fully known. But it is known that they can act when the T cell is activated for the first time by an APC or during the proliferative phase [18]. The nature of the antigen, as in the case of this work, a native or irradiated protein, may play an important role in directing the triggered immune response. According to the results found, it is possible to notice that the animals presented a Th1 and Th2 profile.

The results obtained by IVIAP corroborate the results of splenic cytokine dosing in response to irradiated mucus. In this assay the group immunized against irradiated mucus was more responsive to mucus than the other groups, which meets the result of the dosage cytokines produced by splenic cells in the group of animals immunized against irradiated mucus was more responsive , producing more cytokines (TNF-α, IFN-γ and IL-10) than the other groups.

In this work the serum cytokine levels were: irradiated mucus induced higher expression of TNF-α, which is produced by Th1 cells; and native mucus induced more IL-6 and IL-17α, which induce Th17 response [27].

A similar result was reported demonstrated the capacity of the total venom of marine fish *Thalassophryne natteri* to stimulate balanced immune responses Th1/Th2, attributing to isolated toxins the induction of Th1 or Th2. The toxin Nattectin induces Th1 response, characterized by the production of IFN-γ and IgG2a [28] and another toxin, Natterina, induces Th2-type response, characterized by the production of IgG1[29]. Higher levels of TNF-α produced by spleen cells, followed by IL-10 and IFN-γ was verified in the group immunized with irradiated mucus. TNF-α and IFN-γ are produced by Th1 cells, whereas IL-10 is produced by Th2 cells [30].

The research of cytokines and chemokines induced by the venom of freshwater rays has been reported in the literature. Monteiro dos Santos et al. (2011) verified the induction of IL-1, IL-6 and KC by the mucus and venom of *Potamotrygon henlei* and MCP-1 only by the venom [5]. And in 2014, Kimura et al. verified the induction by *Potamotrygon motor* venom of IL-6, KC and MCP-1 [6]. In both studies, the assays were carried out from the venom inoculation in the paw of mice and the cytokines/chemokines detected by ELISA. Although different methods were employed, serum IL-6 expression in this work, a proinflammatory cytokine, was also verified in the group immunized against native mucus.

Venom has also been described as inducing the production of cytokines and chemokines, such as the venom of the fish *Thalassophryne nattereri* and *T. maculosa*, which induced the production of TNF-α, IL-1β and IL-6 [30]. The venom of the *Crotalus durissus* terrificus (TNF-α, IFN-γ, IL-4 and IL-10) [31], the *Loxoceles* gaucho spider (IL-6, MCP-1 and KC) [7]. Alves (2009) found that Bothropstoxin-1 (BTHX-1) one of the toxins of the irradiated *Bothrops jararacussu* snake induces the expression of IFN-γ and IL-2 in isogenic mice, that is, Th1 response pattern [32]. Cells from the spleen of the group immunized against mucus irradiated from *Paratrygon aiereba* also produced IFN-γ.

5. CONCLUSIONS

The results presented here corroborate with studies developed by our group that ionizing radiation is able to maintain the antigenic properties capable of inducing humoral and cellular adaptive immune response, allowing the continuity of associated research.

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