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HUMORAL IMMUNE RESPONSE AGAINST NATIVE OR ^{60}CO IRRADIATED VENOM AND MUCUS FROM STINGRAY *PARATRYGON AIEREBA*

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ABSTRACT

Poisonings and traumas caused by poisonous freshwater fish such as rays are considered a major public health problem and draw attention because of accidents involving these animals cause serious local symptoms and are disabling, keeping the victim away from work. The therapy of these cases is based only on the symptoms of patients, which implies in its low efficiency, causing suffering for the victims. This study aims to evaluate and compare the humoral immune response in animals inoculated with native or ^{60}Co irradiated *Paratrygon aiereba* venom and mucus. Ionizing radiation has proven to be an excellent tool to decrease the toxicity of venoms and isolated toxins. The mucus and venom samples of *P. aiereba* were irradiated using gamma rays from a ^{60}Co source. Animals models were immunized with the native or irradiated mucus or venom. The assays were conducted to assess the production of antibodies by the immunized animals using enzyme immunoassay and western blotting. Preliminary results show the production of antibodies by the immunized animals. The resulting sera were also checked for antigenic cross- reactivity between venom and mucus, demonstrating the potential of mucus as an antigen for serum production for the specific treatment for accidents by stingrays. However, it is essential to carry out further tests in order to verify the neutralization of the toxin by antibodies formed by animals.

1. INTRODUCTION

Freshwater stingrays are venomous fishes found resting on the sandy and muddy bottom of shallow waters of rivers from the major hydrographic basins of South America. These fishes are strictly freshwater animals and belong to the *Potamotrygonidae* family [1].

Paratrygon aiereba or apple ray is a large animal, reaching up to 1m of disk radius and is among the larger members of the *Potamotrygonidae* family. Its tail is whip-shaped, long and thin in the juveniles, and is frequently incomplete in adults. The sting is small when compared to other freshwater rays and located close to the base of the tail (Figure 1). *P. aiereba* is widely distributed in the amazon, including the Tocantins and Araguaia river

basins. Phylogenetic studies indicate that this genus is the most basal of the *Potamotrygonidae* family [2, 3, 4].

Figure 1: *Paratrygon aiereba* (Photo: Gabriela Ortega Coelho Thomazi).

The stings of these animals are rigid structures covered by epithelium containing great quantities of glandular cells that produce venom whose composition and mechanisms of action are not yet fully elucidated. Furthermore, the dorsum of these animals is frequently covered by mucus which was shown to also present toxic activity [5, 6, 7, 8, 9].

As the sting is covered by a mucus containing anti-microbial peptides, proteolytic enzymes and other immunogenic substances, this mucus is believed to increase the severity of the wounds caused by freshwater stingrays [6].

Pain appears immediately after the sting, initially at the site of the injury, later spreading to the whole limb. It is described as an intense pain, disproportional to the size of the lesion [10, 11]. Fever, cold, sweating, nauseas, vomiting agitation and tachycardia are some systemic effects described, as well as secondary infections [12, 13].

A better knowledge of the immunological behavior of the venom and the mucus will pave the way to a possible specific treatment that might be helpful in the therapy which is currently only based on symptomatology, resulting in low efficiency, causing pain to the patients, besides corroborating to the study of fish venoms that contain a wide diversity of toxins yet to be discovered [9, 14].

Ionizing radiation can affect the molecular structure and activity of biological molecules. Several studies report the attenuation of animal toxins using gamma radiation such as crotamine, A toxin from the rattlesnake *Crotalus durissus terrificus*, which has its toxicity decreased by almost two folds, indicating the potential of gamma radiation as a detoxifying agent as an alternative for antivenom production [15, 16].

In the present work, we evaluated and compared the humoral immune response of animals injected with either venom or mucus from the stingray *Paratrygon aiereba* in their native or ^{60}Co irradiated forms. Ionizing radiation has shown to be an excellent tool to decrease the toxicity of venoms and isolated toxins, resulting in better immunogens for the production of

antivenoms, the only efficient treatment for snakebites, besides contributing for the welfare on antivenom-producing animals. In the cases of stingrays, as there is no specific treatment, the present study might lead to the development of a novel, specific, antivenom therapy.

2. MATERIALS AND METHODS

2.1 Venom and mucus from Paratrygon aiereba

2.1.1. Collection

Paratrygon aiereba stingrays (n=17) were collected in the Ribeirão do Carmo creek, an affluent of the Tocantins river, in the city of Porto Nacional – TO, coordinates: 10°42.271"S 48°30.337"W, with the help of a local fisherman. The capture of the animals was done in October 2014, with a license (n. º 6781-1/2014) granted by the Chico Mendes Institute for the conservation of biodiversity (ICMBio). All the captured animals were donated to the fish collection of the Laboratory of Systematic Ichthyology of the Federal University of Tocantins (UFT) Campus of the city of Porto Nacional – TO.

2.1.2 Obtention and processing of the venom and mucus samples.

The extraction of the venom and mucus of the stingrays was performed in the UFT Laboratory of Systematic Ichthyology, by scraping of the epithelium of the dorsum (mucus) and the sting and the samples were stored at -20ºC until used [9]. A pool of all the venom or all the mucus collected was made. All the samples were then dissolved in 150mM NaCl, centrifuged at 1190xg and the resulting supernatant was filtered through a 0,22µm membrane.

2.2 Laboratory animals

Female Swiss mice (n=34) and female New Zealand rabbits (n=5) from the IPEN animal housing facility were used for the immunization assays, after approval by the committee for Ethics in the Use of Animals (CEUA) IPEN/SP n.º 126/13.

2.3 Irradiation of the samples

Samples of *Paratrygon aiereba* mucus (800µg/mL) and venom (400µg/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.

2.4 Antibodies production

Female Swiss mice (n=28) were immunized with native *Paratrygon aiereba* mucus or venom at a concentration of 1.5µg/mL using the intra-peritoneal route. 06 mice were used as negative controls. For the irradiated mucus or venom, 4 female New Zealand rabbits were immunized and one animal served as a control. Five intradermal immunizations were done using a 15 days interval. The blood was collected and the serum was separated and frozen at -20°C.

2.5 Enzyme linked immunosorbent assay – ELISA

Microtitration plates were coated for 12h at 4°C with 100µL/well of a 5 µg/mL solution of native *Paratrygon aiereba* mucus or venom dissolved in pH 9.5 sodium carbonate/bicarbonate buffer. The plates were then blocked with 3% skim milk in Tris Buffered Saline (TBS). After the blocking step, 100µL of the sera were applied to the wells, starting from a 1:100 dilution and using a 2 fold dilution factor and incubated for one hour.

All dilutions were made in quadruplicate. After four TBS washings, the appropriate peroxidase labelled antibody (anti-mouse or anti-rabbit IgG), diluted 1:5000, was applied to the wells. After a new round of washes with TBS, the reaction was developed by incubation with 0.05% hydrogen peroxyde in the presence of 0.02% orthophenyl-diamine. The absorbance of the wells was then measured at 490 nm in a microplate reader.

2.6 Western blotting

The native or irradiated mucus and venom proteins were separated by electrophoresis as above and transfered to a nitrocelulose membrane using a semi-dry apparatus, according to the manufacturer´s instructions. After a 1 hour blocking step with skim milk in TBS, the membranes were incubated overnight with a 1:100 dilution of the appropriated serum. After four washing cycles with phosphate buffered saline pH 7.4 containing 0.05% tween 20. The bound antibodies were then detected using peroxidase labelled anti-mouse IgG (native venom and mucus) or anti-rabbit IgG (irradiated samples) antibodies, in the presence of 0.05% hydrogen peroxide, using TMB (3,3′,5,5′-Tetramethylbenzidine®) as a chromogen.

3. RESULTS AND DISCUSSION

3.1 Enzyme linked immunosorbent assay – ELISA

In the humoral immune response, properly stimulated B lymphocytes secrete antibodies that recognize and neutralize antigens such as toxins [18]. Thus this assay was performed to detect and quantify antibodies raised against *Paratrygon aiereba* mucus or venom.

The detection of antibodies against a given toxin or venom is of extreme relevance for the development of antivenoms used in the treatment of envenoming. For an antivenom therapy to be efficient, it is important for the serum to be specific for the animal involved. Furthermore, serum therapy is considered the only efficient treatment to revert the pathological effects caused by many venomous animals [19].

According to our results, we conclude that the animals immunized with either mucus or venom, irradiated or not, were able to induce antibodies, indicating that both secretions are immunogenic. We also verified that the antibodies raised against mucus or venom are cross-reactive, meaning that mucus and venom share many common epitopes. This finding is of major relevance, once the mucus is present in much higher quantities on the stingray, it is much easier to collect, without having to kill or mutilate the fish. To our knowledge, this is the first description of the immunological behaviour of the mucus and the venom of *Paratrygon aiereba* [4].

It is noteworthy that the production of antibodies by animals immunized with irradiated mucus or venom was detected. Previous works showed that ionizing radiation promote physico-chemical in proteic molecules from snake venoms, without affecting in their ability to induce a protective immune response, frequently even improving the immunological properties of the antigen [20]. The gamma ray irradiation process may attenuate the proteolytic activity of toxins, this activity being the main responsible for the lesions occurring in antivenom producing animals, as in anti-snake venom producing horses. Thus, the use of irradiated venom and or mucus might yield an effective anti-stingray venom serum, using an attenuated antigen, able to induce protective antibodies, causing less harm to the antivenom-producing animals, which is in agreement with animal welfare issues, reducing their suffering [21].

3.2 Western Blotting

In order to verify specific antibodies in the sera of immunized animals to proteins present in the mucus and venom *Paratrygon aiereba* stingray was performed Western Blotting.

The results show that there was a recognition of specific anti-mucus antibodies for both test performed with anti-mucus serum and with anti-venom serum, demonstrating again the antigenic cross reactivity between both.

In relation to the tests with the serum of immunized animals with the irradiated samples was verified that the irradiated anti-mucus serum recognized bands with molecular mass from 75kDa, with emphasis the fact that also recognized 50kDa bands of native and irradiated poison from. But the irradiated anti-venom serum recognized the same bands of the mucus, corroborating the antigenic cross-reactivity.

4. CONCLUSIONS

Preliminary results show the possibility of a specific treatment for poisoning rays, since the production of antibodies by the immunized animals was detected. It antigenic cross-reactivity

between them also observed, suggesting that mucus can replace the venom in the tests, which can be considered an advantage since the mucus is more easily obtainable.

Accidents by these animals are considered a public health problem because among venomous freshwater fish of medical importance, the stingrays draw attention because of accidents with these animals be disabling, for keeping the victim away from work, bring important consequences at the point of sting. In short, are injury with high morbidity and treatment is based solely on symptoms, causing more suffering to the injured.

However, it is essential to carry out further tests to verify the toxin neutralization by antibodies formed by the animals in order to obtain a specific treatment for these injuries, as the serum therapy.

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