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EVALUATION OF MIOTOXIC ACTIVITY OF BOTHROPSTOXIN-1 IRRADIATED WITH ^{60}Co GAMMA RAYS

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ABSTRACT

Ionizing radiation has been successfully employed to modify the immunological properties of biomolecules and has been proven to be a powerful tool to attenuate snake venoms toxicity without affecting their immunogenic properties. Snake venoms and their isolated toxins showed structural modifications after gamma radiation process, in aqueous solution, mainly by water radiolysis sub products. Free radical scavengers, such as $NaNO_3$ and t-butanol, present selective effects on specific radical from water radiolysis. The $NaNO_3$ has affinity by aqueous electron, while the t-butanol has affinity by hydroxyl radical. At the present work, we have investigated the miotoxic activity of bothropstoxin-1 (BTHX-1), a K49 phospholipase, present in *Bothrops jararacussu* crude venom, before and after irradiation process, with or without scavenger substances presence. BTHX-1 was irradiated with 2kGy of ^{60}Co gamma rays, in aqueous solution and in the presence of oxygen. BALB/c mice were inoculated with either native or irradiated toxin, with or without scavenger substances. After 3 hours, blood samples were collected and the miotoxic activity was evaluated by LDH (lactate dehydrogenase) release. The muscular tissue damage was directly related to the LDH amounts released. Irradiated bothropstoxin-1, with or without $NaNO_3$ substance, caused less damage than their native counterpart. But irradiated toxin, in the presence of t-butanol, was so miotoxic as the native BTHX-1. These results indicate that irradiation of toxic proteins can promote significant modifications on their structures, but still retaining many of the original biological properties of their native counterparts. Additionally, some scavengers substances can change these gamma radiation effects.

1. INTRODUCTION

Gamma radiation consists on electromagnetic waves resulting from nuclear transitions and can interact with biomolecules in two ways: directly, when the radiation hits the molecule, or indirectly when free radicals are generated and react with the target molecule. With proteins, ionizing radiation promotes changes in their enzymatic, pharmacological and immunological properties; the two latter being more radioresistant [1, 2].

Snake crude venoms and also their isolated toxins present structural modifications if submitted to gamma radiation effects, in aqueous solution [3]. This occurs due to reactions

with the products of water radiolysis. Some scavenger substances, such as $NaNO_3$ and t-butanol, remove selectively the water radiolysis products [4].

Bothrops venoms are complex mixtures of components with a wide range of biological activities. Among these substances, miotoxins have been investigated by several groups. Bothropstoxin-1 (BTHX-1) is a phospholipase A2-like basic miotoxin from *Bothrops jararacussu* [5]. The BTHX-1 is capable of blocking the neuro-muscular transmission [6].

The obtention of modified antigens with lower toxicity and preserved or improved biological properties would be useful. Numerous researches [3, 7, 8] have shown the power of radiation to modify proteins, improving its biological properties. In this way, studies involving a better characterization of response generated against an irradiated protein would be very important. Therefore, in the present work we investigated the miotoxic behavior of BTHX-1, a K49 phospholipase, present in *Bothrops jararacussu* crude venom, before and after irradiation, in the presence of selective scavengers substances.

2. MATERIALS AND METHODS

2.1 Reagents

All reagents were commercially obtained and were of analytical grade. Bothropstoxin-1 was purified from *Bothrops jararacussu* crude venom (Butantan Institute).

2.2 Animals

BALB/c isogenic mice were obtained from the animal housing facility of IPEN/CNEN/SP and maintained in sterilized isolators and absorbent media, with food and water *ad libitum*. The manipulation of these animals before or during the experiments was according to the "Principles of Laboratory Animal Care" (NIH publ. N^o 86-23, revised in 1985) and to the "Principles of Ethics in Animal Experimentation" (SBCAL/COBEA).

2.3 Ion-exchange chromatography

Bothropstoxin-1 was purified using a single-step purification method. *Bothrops jararacussu* venom was dissolved in 1 ml buffer (25mM sodium phosphate, pH 7.8). After centrifugation, the supernatant was injected into a Resource-S cation exchange column connected to an FPLC system (Pharmacia®) and eluted with a linear salt gradient [5]. The samples resulting from the chromatography were collected, frozen and dried for later use.

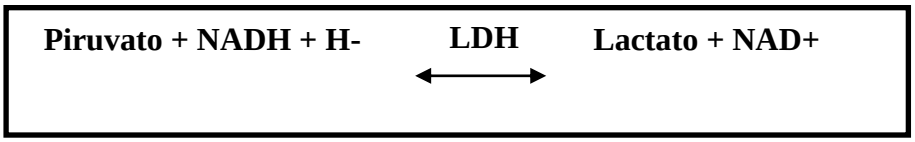
2.4 Protein irradiation

Bothropstoxin-1 was dissolved in 0,15 M NaCl to a final concentration of 2mg/mL. This solution was irradiated with a dose of 2 kGy using gamma rays derived from a ^{60}Co source (Gamma Cell, Atomic Agency of Canada Ltd) at room temperature and in the presence of atmospheric O_2, with a 2,88 kGy/h dose rate.

2.5 Muscular damage evaluation – LDH (lactate dehydrogenase)

The enzyme LDH catalyzes the conversion of pyruvate to lactate, while NADH is oxidized to NAD+. The catalytic activity is determined by the rate of disappearance of NADH, measured at 340 nm. It is determined by the decrease in absorbance at 340 nm, which is proportional to

the activity of LDH in the sample. The activity of LDH is determined according to the following reaction [9]:



(1)

For this assay using the LDH kit Liquiform of Labtest, Balb/c mice were inoculated, intraperitoneally, with either native or irradiated BTHX-1, in the presence or not of scavengers substances, diluted in saline. After a period of 3 hours, the blood was collected, using retro-orbital plexus, to get the plasma.
To verify the presence of LDH in the plasma, it has been employed a Reagent Work.

Reagent Work = Reagent 1 + Reagent 2

Reagent 1: NADH 360 µmoL/L and sodium azide 15 mmoL/L

Reagent 2: Sodium Pyruvate 6 mmoL/L and sodium azide 15 mmoL/L

Into predetermined wells, were added 2 µL of plasma and 100 µL of Reagent Work. A Spectra Max190 equipment was used to make the readings after 1 minute (A1) and 2 minute (A2) at 340 nm. The activity was determined using this formula:

$$A = \frac{A_1 - A_2}{2} \times 8095 \quad (2)$$

3. RESULTS AND DISCUSSION

The assessment of muscle damage by release of LDH, showed that a minor injury was caused when the irradiated BTHX-1 was used without scavengers and in the presence of sodium nitrate ($NaNO_3$) (Figures 1 and 2). Unlike what was observed for the toxin in its native form. The same results were observed for [10] while worked with irradiated BTHX-1, without scavengers. The native BTHX-1 was more miotoxic than the irradiated form that has induced a lower release of LDH.
The LDH catalyzes the conversion of pyruvate to lactate in the presence of NADH. The decrease in absorbance at 340 nm due to oxidation of NADH is proportional to the activity of LDH in the sample (Tables 1, 2 and 3). Therefore, the damage caused in the tissue is directly related to the amounts of LDH released [9].

Table 1: Data obtained from the readings of the release of LDH assay at 340 nm after 1 and 2 minutes application of working reagent in blood plasma of BALB/c mice, inoculated with BTHX-1 native or irradiated. Activity = [(Abs.1 - Abs.2) / 2] x 8095.

	SAMPLES (BTHX-1)	Abs. 1 (after 1 min.)	Abs. 2 (after 2 min.)	ACTIVITY
NATIVE	1	0,6146	0,5902	**98,759**
	2	0,6078	0,5957	**48,97475**
	3	0,6272	0,6160	**45,332**
	4	0,5979	0,5915	**25,904**
IRRADIATED	1	0,6111	0,5959	**61,522**
	2	0,5899	0,5758	**57,06975**
	3	0,6292	0,6164	**51,808**
	4	0,6114	0,5991	**49,78425**

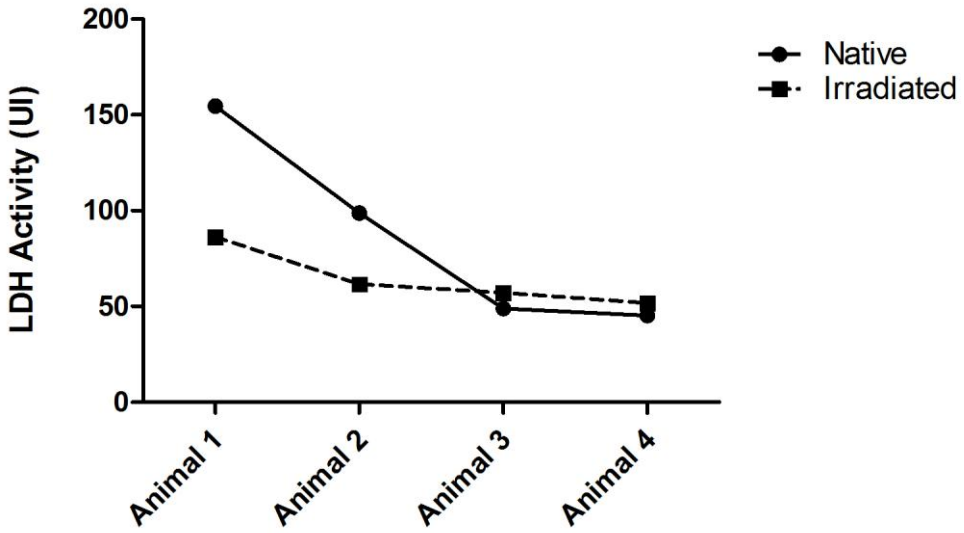


Figure 1 - LDH release assay to evaluate the muscle damage caused by BTHX-1, native or irradiated, inoculated into BALB / c mice.

Table 2: Data obtained from the readings of the release of LDH assay at 340 nm after 1 and 2 minutes application of working reagent in blood plasma of BALB/c mice, inoculated with BTHX-1 native or irradiated, in presence of sodium nitrate ($NaNO_3$). Activity = [(Abs.1 - Abs.2) / 2] x 8095.

	SAMPLES (BTHX-1)	Abs. 1 (after 1 min.)	Abs. 2 (after 2 min.)	ACTIVITY
NATIVE	1	0,5757	0,5473	**114,949**
	2	0,6352	0,6152	**80,95**
	3	0,6266	0,613	**55,046**
	4	0,6468	0,6347	**48,97475**
IRRADIATED	1	0,6012	0,5755	**104,0208**
	2	0,5457	0,5275	**73,6645**
	3	0,646	0,6334	**50,9985**
	4	0,5615	0,5554	**24,68975**

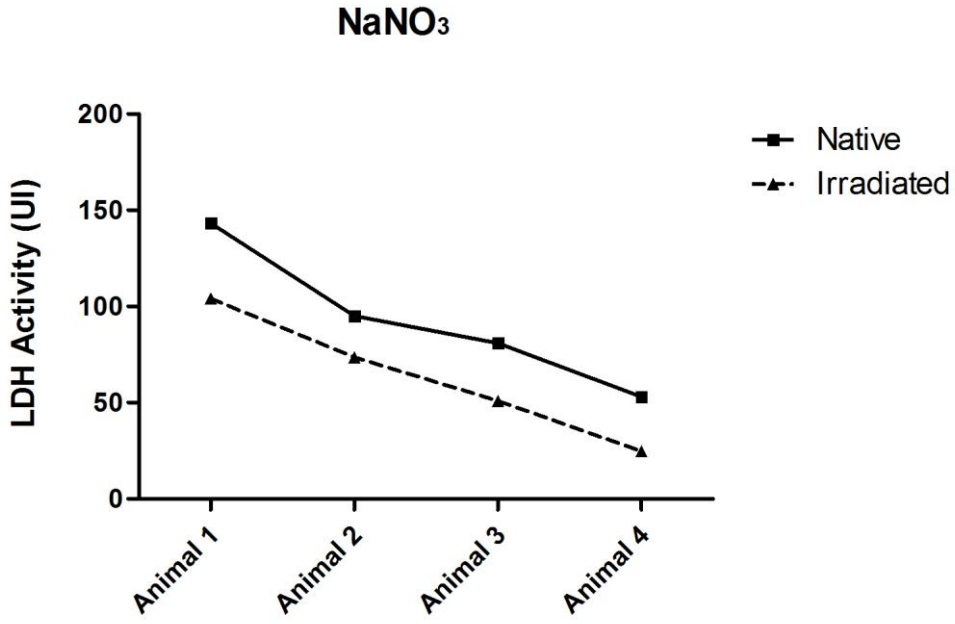


Figure 2 - LDH release assay to evaluate the muscle damage caused by BTHX-1, native or irradiated, in presence of sodium nitrate ($NaNO_3$), inoculated into BALB / c mice.

Table 3: Data obtained from the readings of the release of LDH assay at 340 nm after 1 and 2 minutes application of working reagent in blood plasma of BALB/c mice, inoculated with BTHX-1 native or irradiated, in presence of t-butanol. Activity= [(Abs.1 - Abs.2) / 2] x 8095.

	SAMPLES (BTHX-1)	Abs. 1 (after 1 min.)	Abs. 2 (after 2 min.)	ACTIVITY
NATIVE	1	0,6359	0,6189	**68,8075**
	2	0,7284	0,7146	**55,8555**
	3	0,7272	0,7138	**54,2365**
	4	0,5838	0,5757	**32,78475**
IRRADIATED	1	0,5757	0,5473	**114,949**
	2	0,6352	0,6152	**80,95**
	3	0,6266	0,613	**55,046**
	4	0,6468	0,6347	**48,97475**

In Figure 3, we can observe that the BTHX-1 irradiated in the presence of t-butanol promoted great release of LDH and, consequently, worst muscle damage when compared with native toxin, as well as for irradiated toxin without scavengers (Figure 1) and irradiated toxin in the presence of $NaNO_3$ (Figure 2). This data indicated a possible modulatory role of the hydroxyl radical (OH •) in the toxicity of the protein. This is probably due the OH• be one of the most reactive species among the products from radiolysis of water [11].

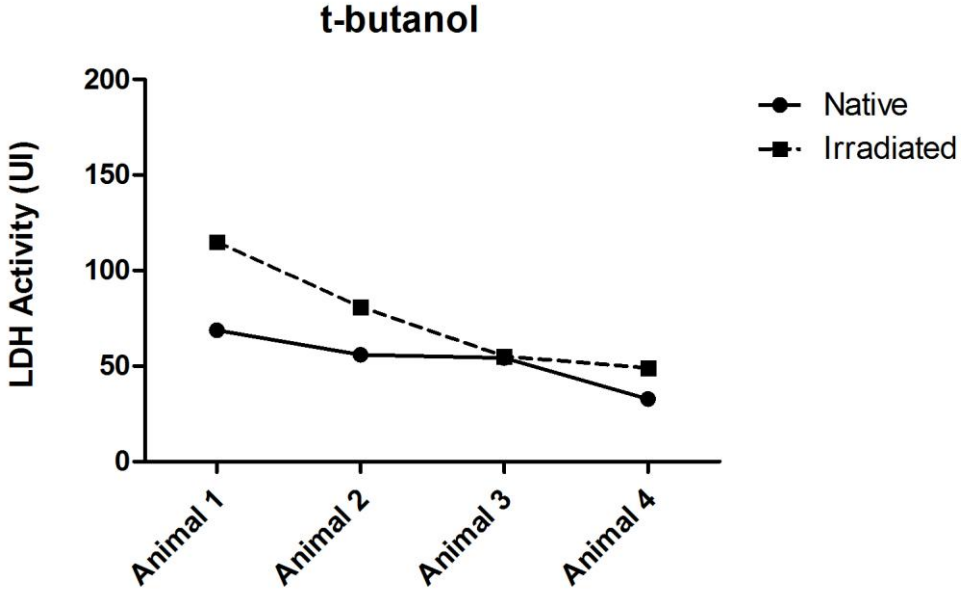


Figure 3 - LDH release assay to evaluate the muscle damage caused by BTHX-1, native or irradiated, in presence of t-butanol, inoculated into BALB / c mice.

If OH• radical is removed by t-butanol, the changes in the structure of the protein decrease, so there is a consequent decrease in the toxicity attenuation. Therefore, the muscle damage caused by the irradiated protein, in the presence of t-butanol, is quite similar to that promoted by the native form protein. When investigating the action of chitosan scavenger, [12] observed that structural changes in human albumin, caused by free radicals of oxygen were reduced. The presented results indicate that the irradiation of BTHX-1 promoted the attenuation of its toxicity, but many of the biological properties of the native form of the toxin were kept. It was also showed that the scavenger substances have in important role during irradiation process. These factors indicated that ionizing radiation has proved to be an important tool in the attenuation of animal toxins, such as BTHX-1.

4. CONCLUSIONS

The evaluation of muscle damage by LDH release indicated that a minor injury was caused when the irradiated BTHX-1 was used, without scavengers and in the presence of ($NaNO_3$). The BTHX-1 irradiated in the presence of t-butanol promoted greater release of LDH and, consequently, main muscular damage if compared with native toxin, as well as for irradiated toxin without scavengers and irradiated toxin in the presence of $NaNO_3$. These factors indicate a possible modulator role of the hydroxyl radical (OH •) in the toxicity of the protein.

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