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^{60}Co gamma irradiation prevents *Bothrops jararacussu* venom neurotoxicity and myotoxicity in isolated mouse neuromuscular junction

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Abstract

The ability of gamma radiation from ^{60}Co (2000 Gy) to attenuate the toxic effects of *Bothrops jararacussu* venom was investigated on mouse neuromuscular preparations in vitro. A comparative study between the effects of native and irradiated venoms was performed on both phrenic-diaphragm (PD) and extensor digitorum longus (EDL) preparations by means of myographic, biochemical and morphological techniques. Native venom (10 and 20 μg/ml) induced a concentration-dependent paralysis of both directly and indirectly evoked contractions on PD preparations. At 20 μg/ml, it also caused a pronounced myotoxic effect on the EDL muscle preparation that was characterized by an increase of creatine kinase release and by several morphological changes of this preparation. By contrast, irradiated venom, even at concentrations as high as 40 μg/ml, induced neither paralyzing nor myotoxic effects. It was concluded that ^{60}Co gamma radiation is able to abolish both the paralyzing and the myotoxic effects of *B. jararacussu* venom on the mouse neuromuscular junction. These findings support the hypothesis that gamma radiation could be an important tool to improve antisera production by reducing toxicity while preserving immunogenicity. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Gamma radiation; *Bothrops jararacussu* venom; Neuromuscular junction

1. Introduction

Serum therapy is the most effective treatment for snakebites. Ophidic antisera are produced by immunization of horses with crude venoms. However, snake venoms are, in general, weak immunogens inducing low humoral and cellular immune responses (Magalhães et al., 1986). In addition, the high toxicity of snake venoms reduces the useful life of immunized horses, limiting the antisera productivity. Our group has been working on snake venom irradiation, intending to decrease venom toxicity while preserving its immunogenicity, in order to improve antisera production (Murata et al., 1990; Nascimento et al., 1996a,b; Gallacci et al., 1998, 2000). Proteins irradiated, either in dry state or in solution, undergo chemical and physicochemical changes that can alter their primary, secondary and tertiary structures (Skalka and Antoni, 1970). These structural changes result in a decrease or loss of the enzymatic and toxic activities of the proteins (Gallacci et al., 1998, 2000). However, irradiated proteins retain epitopes that induce efficient protective responses when employed as immunogens (Murata et al., 1990; Nascimento et al., 1996a,b; Clissa et al., 1999). These findings point toward venom irradiation as a promising tool for venom detoxification.

Snakes of the genus *Bothrops* are responsible for 90% of the ophidic accidents occurring in Brazil (Jorge and Ribeiro,

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1990). Venoms from these snakes are complex mixtures of toxins, enzymes and active peptides that induce multiple systemic alterations and pronounced local changes such as pain, edema, hemorrhage and myonecrosis (Vital Brazil, 1972; Rosenfeld, 1971; Mebs et al., 1983). The myotoxic effect of these venoms is particularly important, not only because it may lead to permanent tissue loss disabling the victim (Gutiérrez and Lomonte, 1995), but also because it may induce severe cutaneous lesions on the animals chronically exposed to the venom during the immunization process, reducing antisera productivity. Myonecrosis may be due to the vascular degeneration and ischemia caused by venom metalloproteinases, or it may result from a direct action of myotoxins upon the plasma membrane of muscle cells (Gutiérrez and Lomonte, 1995; Ownby et al., 1999). Such myotoxins are widely distributed among different venoms of *Bothrops* species (Moura-Da-Silva et al., 1990; Lomonte et al., 1990).

Bothrops jararacussu venom is one of the most myotoxic venoms from this genus since about 30% of its total proteins are myotoxins (Rodrigues-Simioni et al., 1983; Homsi-Brandeburgo et al., 1988). In addition, this venom also induces the blockade of both directly and indirectly evoked contractions in frog nerve–muscle preparations (Rodrigues-Simioni et al., 1983). Previous work showed that gamma radiation reduces the acute toxicity while preserving the antigenic and immunogenic properties of *B. jararacussu* venom (Spencer, 1996; Spencer et al., 1997). Nevertheless, in order to propose the use of irradiated venoms as new immunogens, an extensive investigation of their biological characteristics is necessary. Thus, the influence of ^{60}Co gamma radiation (2000 Gy) upon the toxic activities of the *B. jararacussu* venom at the neuromuscular junction was investigated in this work. The effects of both native and irradiated venoms were investigated in mouse neuromuscular preparations by means of myographic, biochemical and morphological techniques.

2. Material and methods

2.1. Venom and irradiation

Whole air-dried venom *B. jararacussu* was purchased from Instituto Butantan (São Paulo, Brazil). Venom samples of 2 mg/ml in NaCl (0.15 M) were irradiated in a ^{60}Co gamma source (GAMMACELL 220, Atomic Energy Commission of Canada, Ltd). A dose of 2000 Gy was applied to the samples at the rate of 450 Gy/h at room temperature.

2.2. Animals

Adult male mice weighing 25–30 g were maintained under a 12 h light–dark cycle (lights on at 07:00 h) in a temperature controlled environment (22 ± 2 °C) for at least 10 days prior to the experiments. Food and water were freely available. Animal procedures were in accordance with the guidelines for animal care prepared by the Committee on Care and Use of Laboratory Animal Resources, National Research Council, USA.

2.3. Myographic study

Animals were killed by exsanguination after previous ether anesthesia. The phrenic-diaphragm (PD) preparation was removed and mounted vertically in a conventional isolated organ-bath chamber containing 20 ml of physiological solution of the following composition (mmol/l): NaCl, 135; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; $NaHCO_3$, 15; Na_2HPO_4, 1; glucose, 11. This solution was gassed with O_2 (95%) + CO_2 (5%) and kept at 35 ± 1 °C. The preparation was attached to an isometric force transducer (Gould, GM2/GM3) for recording the twitch tension. The transducer output was displayed on a polygraph recorder (Beckman, R-511A). The resting tension was 2 g. Indirect contractions were evoked by supramaximal strength pulses (0.5 Hz; 0.5 ms), delivered from an electronic stimulator (Narco Bio-System) and applied on the phrenic nerve by means of a suction electrode. Direct contractions were evoked by supramaximal pulses (0.2 Hz, 5 ms) through a bipolar electrode positioned on opposite sides of the muscle. Experiments of direct contractions were performed in the presence of pancuronium bromide ($2 \times 10 - 6$ M). The preparations were allowed to stabilize for 45 min before the addition of a single concentration of *B. jararacussu* venom, native or irradiated.

2.4. Creatine kinase (CK) release

Since cutting of diaphragm muscle during its removal from the animal can lead to artificially high basal levels of CK release, the myotoxic activity was assayed on the extensor digitorum longus (EDL) muscle that can be removed without cutting the muscle. The EDL muscle was isolated and transferred to a chamber containing 5 ml of physiological solution with the composition stated earlier and maintained at 35 ± 1 °C. The preparations were washed three times at 15 min intervals to allow the tissue to stabilize before the addition of *B. jararacussu* venom, native or irradiated. Control experiments were performed in the absence of the venoms. Samples of the bath solution (0.2 ml) were collected before as well as 40, 80 and 120 min after the addition of the venoms. CK levels were immediately determined using a commercially available kit (CK-NAC-CELM, Brazil).

2.5. Morphology

After the collecting samples for CK assay, EDL muscles were frozen in liquid nitrogen. Transverse sections (8 μm thick) were cut out at −20 °C in a cryostat and stained with haematoxylin and eosin (HE) prior to examination by light microscopy (Mc Manus and Mowry, 1960).

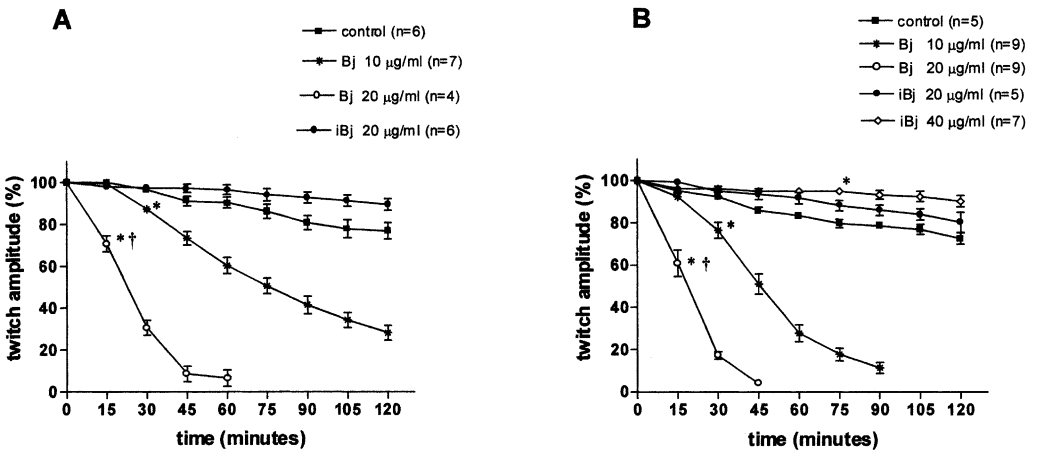

Fig. 1. Effects of *B. jararacussu* venom (Bj) and irradiated Bj venom (iBj) on directly (A) and indirectly (B) evoked twitches in phrenic-diaphragm preparations of mice. The ordinate represents the % amplitude of twitches relative to the initial amplitude. The abscissa indicates the time (min) after addition of Bj or iBj to the organ bath. Vertical bars represent the SEM. * indicates the point from which there are significant differences relative to control.; † indicates the point from which there are significant differences relative to the respective iBj concentration. ($P < 0.05$).

Samples of muscle were also fixed in Karnovsky's fixative (2.5% glutaraldehyde, 2% paraformaldehyde, 0.1 M phosphate buffer, pH 7.4) for 4 h, and washed in 1% osmium tetroxide. The tissue was dehydrated in ascending concentrations of acetone and embedded in Epon resin. Thick (1.5 μm) sections were stained with uranyl acetate and lead citrate and examined by electron microscope.

2.6. Statistical analysis

Results are expressed as mean ± SE. Data were analyzed by Student's *t*-test (for comparison of two samples) and analysis of variance complemented by the Tukey–Kramer test (for comparison of more than two samples). Values of $P < 0.05$ were considered significant.

3. Results

3.1. Myographic studies

Native *B. jararacussu* venom (10 and 20 μg/ml) induced a concentration-dependent decrease of both directly and indirectly evoked twitches (Fig. 1(A) and (B)). However, the mean times for 50% paralysis were significantly higher ($P < 0.05$) for directly than for indirectly evoked contractions, at venom concentrations of 10 μg/ml (78.87 ± 16.1 min, $n = 7$ vs. 45.95 ± 8.1 min $n = 9$), and 20 μg/ml (25.05 ± 2.7 min, $n = 4$ vs. 18.03 ± 4.7 $n = 9$). The paralyzing effects of native *B. jararacussu* venom on both direct and indirect contractions could not be reversed by washing the preparation for at least 30 min with venom-free physiological solution.

In contrast to native venom, irradiated *B. jararacussu* venom did not induce the blockade of direct or indirect contractions at concentrations as high as 20 or 40 μg/ml (Fig. 1(A) and (B)). Furthermore, some degree of facilitation of both directly and indirectly evoked contractions compared to control preparations was observed in the presence of irradiated venom. This effect was significant for indirect contractions.

3.2. CK release

Native *B. jararacussu* venom (20 μg/ml) induced a significant increase of CK release from the EDL muscle compared to the control preparation (Fig. 2). This effect was significant from 20 min of contact with the preparation. On the other hand, irradiated venom, even at a higher concentration (40 μg/ml) did not induce significant increase of CK release within 120 min of contact with the preparation.

3.3. Morphology

Figs. 3(A) and 4(A) and (B) show the light and ultrastructural appearance of control EDL muscles. The light microscopic examinations of EDL muscles exposed to native *B. jararacussu* venom (20 μg/ml) for 120 min revealed a large number of muscle fibers drastically affected by the formation of dense clumps of hypercontracted myofibrils alternating with areas of cytoplasm apparently devoid of myofibrils (Fig. 3(B)). The ultrastructural analysis showed disorganization or loss of myofilaments and mitochondrial swelling with absence or vesiculation of cristae (Fig. 4(C) and (D)). On the other hand, the histological analysis of EDL muscle submitted to irradiated venom revealed a large number of fibers with normal aspect and few muscle cells presenting edema in the connective tissue (Fig. 3(C)). The ultrastructural study showed few muscle fibers with focal areas of

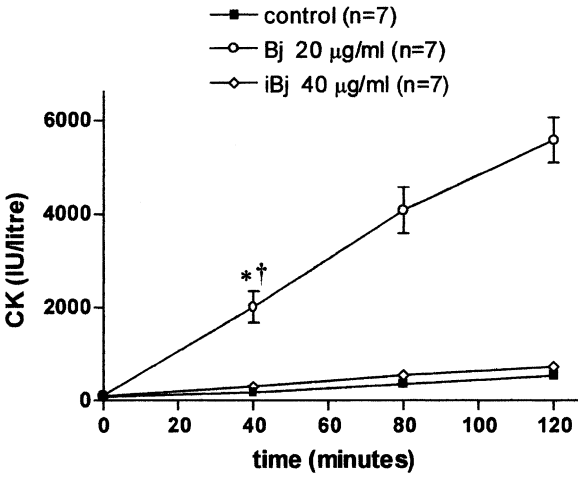

Fig. 2. Creatine kinase (CK) release from mouse EDL muscle preparation in control situation and after 120 min of incubation with *B. jararacussu* (Bj) venom or irradiated Bj (iBj). The ordinate represents the CK concentration (IU/l) in the bath medium and the abcissa indicates the time (min). Vertical bars represent SEM. * indicates the point from which there are significant differences relative to control. † indicates the point from which there are significant differences relative to the respective iBj concentration. ($P < 0.05$).

degeneration and disorganization of myofibrils (Fig. 4(E) and (F)).

4. Discussion

Although *B. jararacussu* venom is a complex mixture of macromolecules, all the effects of this venom observed in the present work have been mainly attributed to the action of its myotoxins, bothropstoxins I and II (Homsi-Brandeburgo et al., 1988; Gutierrez et al., 1991). It has been suggested that *Bothrops* myotoxins increase the muscle-cell membrane permeability, leading to the collapse of ionic gradients (Rodrigues-Simioni et al., 1983; Gutierrez et al., 1991). The muscle-cell depolarization, consequent to the membrane leakiness, has been indicated as the most probable cause of the muscle paralysis induced by either *B. jararacussu* venom or bothropstoxin-I (Rodrigues-Simioni et al., 1983; Heluany et al., 1992). This hypothesis is supported by the observation that *B. jararacussu* venom has no effect on nerve endings in frog nerve–muscle preparations (Rodrigues-Simioni et al., 1983) and by the fact that bothropstoxin-I does not affect endplate nicotinic receptors in chicken preparations (Heluany et al., 1992). In this work, we observed that indirectly evoked contractions of mouse PD preparation were more vulnerable to the paralyzing effect of native *B. jararacussu* venom than the directly evoked one. Since the endplate region represents a very limited fraction of the muscle fiber, it seems conceivable that the process of neuromuscular transmission might be more susceptible to

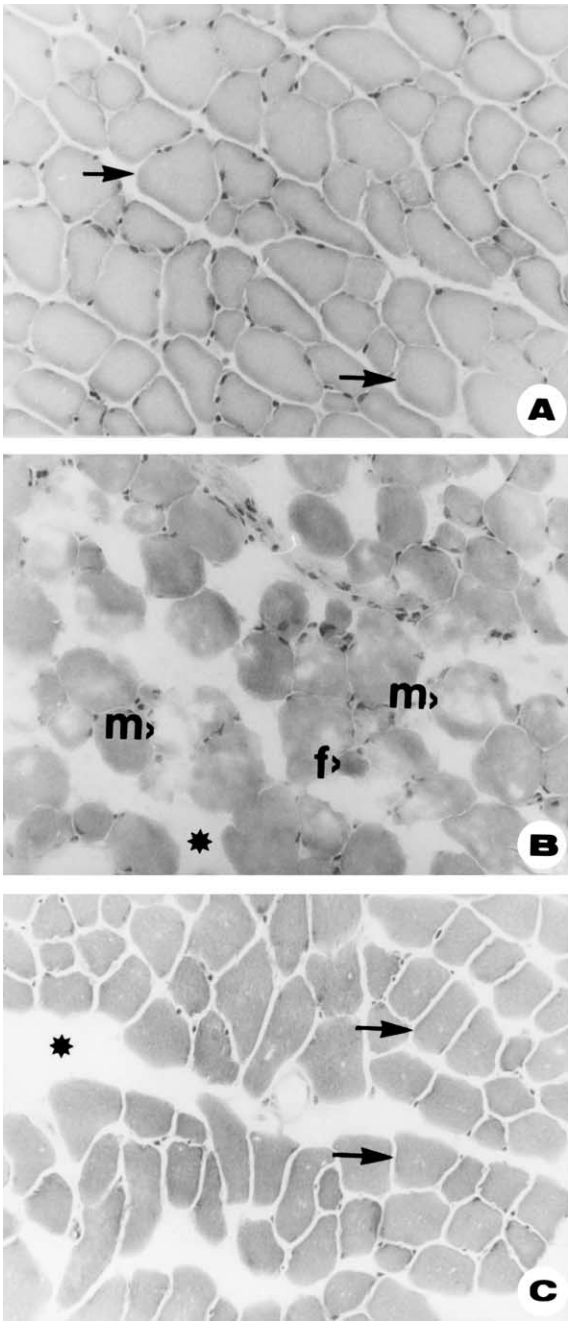

Fig. 3. Light micrographs of mice EDL muscles submitted to haematoxylin and eosin (HE) stain. (A): control muscle with normal fiber morphology (arrows), X 62.5. (B): muscle exposed to *B. jararacussu* venom (20 μg/ml). Note a large number of round fibers with loss of myofibrils (m); hypercontracted fibers (f) and oedema (*), X 62.5. (C): muscle exposed to irradiated *B. jararacussu* venom (40 μg/ml) presenting most fibers with normal aspect (arrows) and some areas with oedema (*), X 62.5.

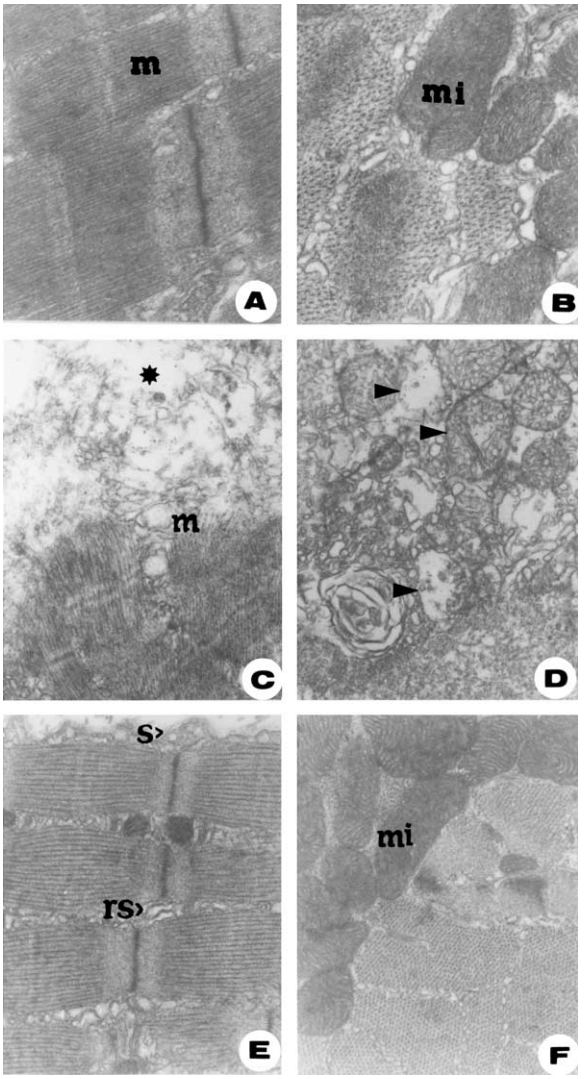

Fig. 4. Electron micrographs of mice EDL muscles. (A) and (B): control muscle with normal myofibrils (m) presenting well-defined sarcomeres; mitochondrias (mi). (A) X 37.800 and (B) X 41.000. (C) and (D): muscles exposed to *B. jararacussu* venom (20 μg/ml). Fibers with disorganization of myofibrils (m) and cytoplasmatic area devoid of myofilaments (∗); several mitochondrial swelling, with absence or vesiculation of cristae (arrow head). (C) X 27.000 and (D) X 18.850. (E) and (F): muscles exposed to irradiated *B. jararacussu* venom (40 μg/ml). Normal aspect of myofibrils, sarcoplasmic reticulum (rs); mithocondria (mi) and sarcolema (s). (E) X 29.400 and (F) X 25.200.

the venom-induced depolarization than the contractile process.

The myotoxic activity of *B. jararacussu* venom has also been credited for a change in the normal ionic environment within the sarcoplasm induced by the venom myotoxins (Rodrigues-Simioni et al., 1983; Gutierrez et al., 1991). A prominent calcium influx following the membrane destabilization would be responsible for a variety of cell degenerative mechanisms such as hypercontraction of myofilaments, mitochondrial alterations and activation of calcium-dependent proteases and phospholipases (Rodrigues-Simioni et al., 1983; Gutierrez et al., 1991; Gutiérrez and Lomonte, 1995).

Irradiated *B. jararacussu* venom did not show paralyzing or myotoxic activities in mouse neuromuscular preparations. The detoxification of snake venoms in water solution by ionizing radiation results from the interaction of the venom components, proteins and peptides, with the free radicals formed by water radiolysis, mainly hydroxyl radicals and hydrated electrons (Butler et al., 1984). These radicals act by removing hydrogen, breaking disulfide bonds, promoting deamination, as well as inducing the formation of intramolecular and intermolecular covalent bonds (Alexander and Hamilton, 1962; Butler et al., 1984; Halliwell and Guitteride, 1989; Souza-Filho et al., 1992). Previous work (Spencer, 1996) has shown that irradiated *B. jararacussu* venom had its chromatographic and electrophoretic profiles drastically changed, suggesting that new molecular species were formed. These neoformed molecules could be soluble aggregates of integral components of the venom and/or covalent complexes of protein fragments resulting from the action of free radicals generated during the irradiation process. The results of this work indicate that the structural changes induced by gamma radiation must also affect the bothropstoxins present in *B. jararacusssu* venom.

Previous work has shown that irradiated *B. jararacussu* venom preserves its immunogenic properties (Spencer, 1996; Spencer et al., 1997). The findings of the present work show that gamma radiation abolishes both the paralyzing and myotoxic activities of *B. jararacussu* venom. Taking together, these data indicate that the irradiated venom could be a better immunogen, able to induce a protective immune response without the deleterious effect of the native venom. As a whole, the present data support the idea that gamma radiation is a venom-detoxification method that could help to solve the chronic problems of antiophidic sera production.

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