

Effect of gamma irradiation on the behavioral properties of crotoxin

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Abstract

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Crotoxin has been detoxified with gamma radiation in order to improve crotalic antiserum production. Nevertheless, present knowledge of the biological characteristics of irradiated crotoxin is insufficient to propose it as an immunizing agent. Crotoxin is known to increase the emotional state of rats and to decrease their exploratory behavior (Moreira EG, Nascimento N, Rosa GJM, Rogero JR and Vassilieff VS (1996) *Brazilian Journal of Medical and Biological Research*, 29: 629-632). Therefore, we decided 1) to evaluate the effects of crotoxin in the social interaction test, which has been widely used for the evaluation of anxiogenic drugs, and 2) to determine if irradiated crotoxin induces behavioral alterations similar to those of crotoxin in the social interaction, open-field and hole-board tests. Male Wistar rats (180-220 g) were used. Crotoxin (100, 250, and 500 µg/kg) was injected intraperitoneally 2 h before the social interaction test. Similarly, irradiated crotoxin (2000 Gy gamma radiation from a ⁶⁰Co source) was administered at the doses of 100, 250, and 500 µg/kg for the hole-board test, and at the doses of 1000 and 2500 µg/kg for the open-field and social interaction tests. ANOVA complemented with the Dunnett test was used for statistical analysis (P<0.05). Crotoxin decreased the social interaction time (s) at the doses of 100, 250 and 500 µg/kg (means ± SEM) from 51.6 ± 4.4 to 32.6 ± 3.7, 28.0 ± 3.6 and 31.6 ± 4.4, respectively. Irradiated crotoxin did not induce behavioral alterations. These results indicate that 1) crotoxin may be an anxiogenic compound, and 2) in contrast to crotoxin, irradiated crotoxin was unable to induce behavioral alterations, which makes it a promising compound for the production of crotalic antiserum.

Key words

- Crotoxin
- Gamma-irradiated crotoxin
- Anxiety
- Social interaction
- Hole-board test
- Open-field test

Snake bites represent a serious health problem in many countries. The rattlesnake *Crotalus durissus terrificus* is found throughout the Brazilian territory, and is responsible for about 12% of all snake bites (1). Crotalic venom is composed of several toxins such as crotoxin, crotamine, gyroxin and convulxin (2). Considerable information on the bio-

logical effects of crotoxin, a neurotoxin, is available since this is the prevalent and most toxic component of crotalic venom (3,4).

Serotherapy is the treatment of choice for snake bites, and the availability of horse antisera is dependent on venom immunogenicity. Since the useful life of immunized horses is impaired by chronic venom toxic-

ity, it became necessary to develop techniques that would improve antiserum production. For this purpose, biochemical, immunological and radiobiological studies have been carried out with crotalic venom and crotoxin submitted to gamma radiation (5-7). These studies have shown that irradiated crotoxin is about 2-fold less toxic than crotoxin, but its immunogenicity is preserved (8). Although ionizing radiation appears to be promising as a venom detoxification method, production of anticrotalic serum from irradiated crotoxin requires extensive knowledge of its biological characteristics. On the basis of these considerations, we investigated behavioral alterations induced by irradiated crotoxin.

We have recently demonstrated in rats that crotoxin increases the emotional state and reduces the exploratory behavior evaluated in open-field and hole-board tests. Moreover, an anxiolytic dose of diazepam, a benzodiazepine receptor agonist, reversed these behavioral alterations of crotoxin, suggesting that this compound may present an anxiogenic effect (9).

Therefore, we decided 1) to evaluate the effects of crotoxin in the social interaction test, which has been widely used for the evaluation of anxiogenic drugs (10-12), and 2) to determine if irradiated crotoxin induces behavioral alterations similar to those of crotoxin in the social interaction, open-field and hole-board tests.

Male Wistar rats weighing 180-220 g were housed at room temperature ($22 \pm 2^\circ\text{C}$) under a 12-h light-dark cycle with food and water *ad libitum*. Behavioral evaluation was conducted in a dark sound-proof room with dim red lights. To minimize possible influences of circadian changes during the tests, different treatments were alternated. Before introducing each animal, the apparatus was washed with a 5% (v/v) ethanol water solution to avoid possible bias due to odor trails left by previous animals.

Crotoxin was injected intraperitoneally

at the doses of 100, 250, and 500 $\mu\text{g}/\text{kg}$ 2 h before the animals were submitted to the social interaction test. Similarly, irradiated crotoxin was administered at the doses of 100, 250, and 500 $\mu\text{g}/\text{kg}$ for the hole-board test and at the doses of 1000 and 2500 $\mu\text{g}/\text{kg}$ for the social interaction and open-field tests. Higher doses were tested because irradiated crotoxin is 2-fold less toxic than crotoxin (8).

Crotoxin was purified from *Crotalus durissus terrificus* crude venom by gel filtration on Sephadex G-75 (Pharmacia) followed by isoelectric pH precipitation. The Bradford method was used for protein determination and purity was assessed by SDS-PAGE (6). Gamma radiation with ^{60}Co was assessed with a GAMMACELL 220 source (produced by the Atomic Energy Commission of Canada, Ltd.) (8). A dose of 2000 Gy was applied at the rate of 400 Gy/h, using 2 mg/ml of crotoxin in 0.15 M NaCl adjusted to pH 3.0 with 0.1 M HCl.

The open-field apparatus was an arena similar to that described by Broadhurst (13). For the social interaction test, all observations were carried out in the open-field arena. The hole-board was an open-field arena with four equally spaced holes (3 cm in diameter) in the floor, as described by File and Wardill (14).

In the open-field test, we recorded ambulation (number of floor units entered) and rearing (number of times that the animal stood on its hind legs) for 3 min and the duration, in seconds, of grooming (time used for the animal to groom) and freezing (time that the animal remained completely immobile, often in a crouching posture, with eyes wide open and irregular respiration). In the hole-board, head-dip count and head-dipping duration (in seconds) were recorded for 5 min, and a head-dip was scored if both eyes disappeared into the hole (14).

Social interaction, which is sensitive to anxiogenic drugs (15), consisted of familiarizing each pair (cagemates) of rats with the

arena for a period of 8 min on two consecutive days. On the third day, each rat was randomly assigned to an unfamiliar partner according to weight, in groups of 12 animals (six pairs) which subsequently received the appropriate drug. These rats were then returned to their home cage with their original

cagemate until testing. After an appropriate pretreatment time, each pair of unfamiliar rats was placed in the test arena and observed for social interaction behavior and overall activity for 5 min. Social interaction time (in seconds) per pair of rats was measured as the time spent sniffing the partner,

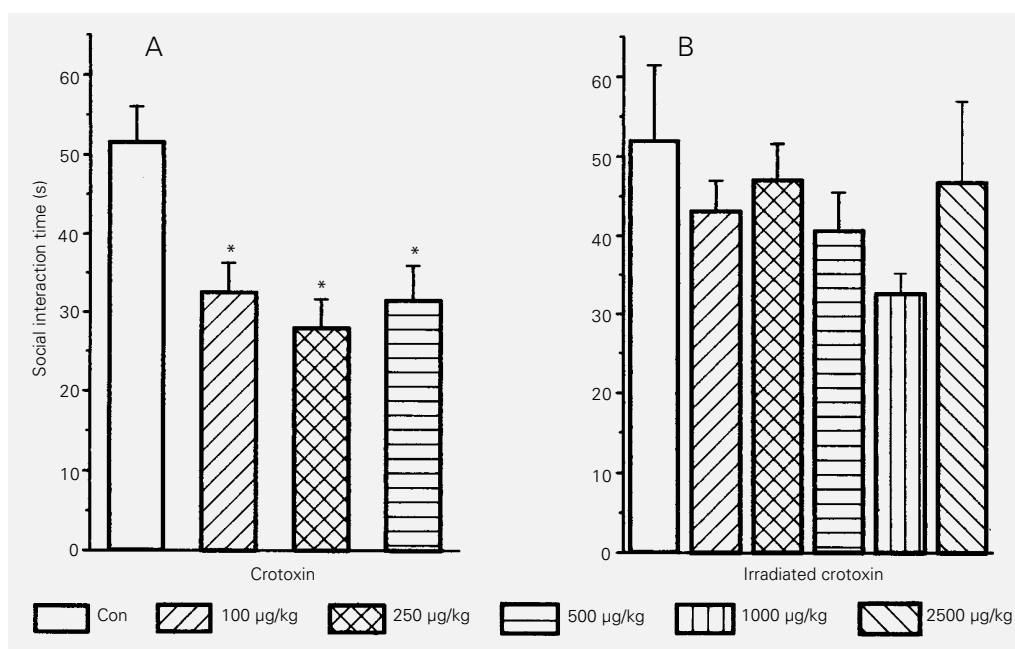


Figure 1 - Effects of crotoxin and irradiated crotoxin on rats submitted to the social interaction test. Rats were injected intraperitoneally with crotoxin (100, 250, and 500 µg/kg) (A) or irradiated crotoxin (100, 250, 500, 1000, and 2500 µg/kg) (B) or saline 2 h before the social interaction test. Data are reported as the mean ± SEM for 6 pairs of animals per group. *P < 0.01 compared to control (dose zero) (Dunnett test).

Table 1 - Behavioral effects induced by irradiated crotoxin on rats submitted to the open-field and hole-board tests.

Rats were injected intraperitoneally with irradiated crotoxin or saline 2 h before the hole-board (100, 250, and 500 µg/kg) and open-field (100, 250, 500, 1000, and 2500 µg/kg) tests. Data are reported as the mean ± SEM for 8-10 animals per group. Dose zero corresponds to control. No significant effects were demonstrated with ANOVA.

Test	Dose (µg/kg)					
	0	100	250	500	1000	2500
Open-field						
Ambulation (count)	69.75 ± 2.19	60.50 ± 3.57	69.00 ± 4.03	60.80 ± 3.85	65.57 ± 4.10	60.14 ± 2.75
Rearing (count)	24.08 ± 1.90	17.00 ± 1.35	23.50 ± 1.75	22.00 ± 2.80	23.14 ± 1.98	20.29 ± 1.51
Grooming (s)	12.23 ± 2.10	10.20 ± 1.49	11.56 ± 1.76	13.22 ± 1.68	13.71 ± 3.35	11.00 ± 1.73
Freezing (s)	1.60 ± 0.48	2.56 ± 0.73	1.56 ± 0.67	3.00 ± 0.88	2.00 ± 1.16	3.14 ± 0.86
Hole-board						
Head-dip (count)	8.50 ± 1.10	8.75 ± 0.96	6.00 ± 0.76	7.57 ± 0.97		
Head-dipping (s)	9.25 ± 1.33	12.63 ± 1.82	9.00 ± 1.41	9.57 ± 1.09		

climbing over and crawling under the partner, mutual grooming, genital investigation, and following and walking around the partner (16). Aggressive behavior was not considered to be a social interaction behavior.

Data were analyzed by ANOVA for one-way classification (17) and post-hoc tests were then performed using the Dunnett test, with the level of significance set at $P < 0.05$.

Figure 1 shows the effects of crotoxin (panel A) and irradiated crotoxin (panel B) on rats submitted to the social interaction test. ANOVA demonstrated that crotoxin reduced the duration of social interaction time ($F_{(3,23)} = 6.97$, $P < 0.01$), indicating an anxiogenic response. Post-hoc comparisons (Dunnett) revealed that crotoxin significantly modified the social interaction time at the doses of 100, 250 and 500 $\mu\text{g}/\text{kg}$. Furthermore, the social interaction time was not significantly modified by irradiated crotoxin ($P > 0.05$).

The data in Table 1 show that irradiated crotoxin was unable to modify the animal behavior evaluated in the open-field and hole-board tests (ANOVA, $P > 0.05$).

Crotoxin displayed an anxiogenic profile in the social interaction test, i.e., it reduced the time spent in active social interaction. Since anxiety is seen as a component of the emotional state (18), the crotoxin-induced anxiogenic effect is consistent with the increased emotional state reported previously by Moreira et al. (9). Furthermore, the crotoxin-anxiogenic profile is similar to that of β -carboline in the social interaction test (15), supporting our previous suggestion that crotoxin might be an inverse benzodiazepine receptor agonist (9).

Since crotoxin is a neurotoxin, one might

conclude that the behavioral alterations induced by this compound could be attributed, at least in part, to neuromuscular junction blockade. However, the crotoxin-induced behavioral alterations are inconsistent with a blockade of the neuromuscular transmission, since we have recently reported that crotoxin increased grooming in the open-field test and, although it decreased ambulation and rearing, these behaviors were reversed by an anxiolytic dose of diazepam (9).

The present data show that gamma radiation abolishes crotoxin-induced behavioral alterations. Ionizing radiation can change the biological and antigenic properties of a protein through alterations in the molecular structure. It has been suggested that the dose of 2000 Gy of gamma radiation cleaves approximately six disulfide bridges per molecule of crotoxin (6). The integrity of all disulfide bonds of crotoxin, a potent toxin of *Naja naja atra* venom, is essential for its toxicity since after reduction and reoxidation of the bridges the reformation of the bonds is necessary for the recovery of the full lethality of this toxin (19). On the basis of these considerations, we suggest that the rupture of the disulfide bonds of crotoxin by gamma radiation may be responsible for the loss of the behavioral effects of this toxin. Moreover, it should be taken into account that gamma radiation also induces aggregation and precipitation of crotoxin (6) which may impair the biodistribution of irradiated crotoxin, contributing to the loss of the behavioral effects. Since irradiated crotoxin does not induce behavioral alterations in the animal and does not interfere with immunogenicity, we conclude that it can be useful for the production of anticrotalic serum.

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