

**IMMUNIZATION WITH NATIVE AND COBALT 60-IRRADIATED *Crotalus durissus*  
*terrificus* VENOM IN SWISS MICE: ASSESSMENT OF THE NEUTRALIZING  
POTENCY OF ANTISERA**

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**ABSTRACT:** ELISA was used to evaluate, follow, and compare the humoral immune response of Swiss mice during hyperimmunization with natural and Cobalt 60-irradiated ( $^{60}\text{Co}$ ) *Crotalus durissus terrificus* venom. Potency and neutralization were evaluated by *in vitro* challenges. After hyperimmunization, immunity was observed by "in vivo" challenge and the side effects were assessed. The animals immunization with one LD<sub>50</sub> of the venom was on days one, 15, 21, 30, and 45, when blood samples were collected; the challenges occurred on the 60<sup>th</sup> day. Results showed that ELISA was efficient in evaluating, following, and comparing mouse immune response during hyperimmunization. Serum titers produced with natural venom were similar to those produced with irradiated venom. Immunogenic capacity was maintained after  $^{60}\text{Co}$  irradiation. Serum produced from *Crotalus durissus terrificus* irradiated venom showed higher potency and neutralization capacity than that from natural venom. All antibodies were able to neutralize five LD<sub>50</sub> from these venoms. Clinical alterations were minimum during hyperimmunization with irradiated venom.

**KEY WORDS:** hyperimmunization, irradiation, *Crotalus durissus terrificus*, ELISA, serum neutralization, detoxification.

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## INTRODUCTION

The treatment of accidents caused by snakebites uses heterologous serum from animal plasma, generally equine (11, 27, 39) and ovine (18, 26, 33), hiperimmunized with snake venoms (46).

*Crotalus durissus terrificus* (*C.d.t.*) venom is poorly immunogenic despite being clearly toxic (12). One of the reasons for obtaining an adequate humoral immune response against this venom is the presence of immunosuppressant components (9, 12, 18, 41). In addition, the injury suffered by the animals after the injection of crude venom contributes to low productivity of antivenin (34).

Crotoxin, one of the *C.d.t.* venom components, represents the main obstacle in the production of crotalic antivenom, since its high toxicity causes severe symptoms in serum-producer animals. Systemic reactions, which can also lead the animal to death, are very frequently observed (8, 22).

As sera production depends directly on the immunogenic capacity, this problem needs to be solved. Thus, the researchers have been searching for alternatives preparing toxoids by venom biological detoxification, but keeping its immunogenicity (11, 46).

In regard to the techniques used in the toxoids preparation for antivenin preparation, innumerous works, in which chemical and/or physical agents were used for this purpose, are found. Among the chemical agents are: carboximetyl cellulose (28); photooxidation in the presence of methylene blue (25); chelating agents (20); formalin (14); iodine (16); glutaraldehyde (22); and others. The principal physical agents are: x-ray radiation (19), ultraviolet radiation (44), heat (14), and gamma radiation (23). This last one, according to previous works (1, 6, 8, 12, 13, 15, 17, 21, 29, 31, 33, 34, 36, 40, 42, 43), has been proved to be an excellent tool of detoxification, since it is able to decrease the toxicity without altering the immunogenicity. In addition, this radiation does not add any new substance to the venom.

Thus, alternatives to improve the heterologous sera productivity preparing less damaging toxoids and preserving their immunogenic capacity (11, 46) have been searched. Therefore, the aims of this work were to compare the humoral immune response, to determine and compare the neutralizing potency and capacity of the serum obtained from mice inoculated with *Crotalus durissus terrificus* venom in its native and cobalto 60-irradiated form, in order to use these results in the serum production.

## **MATERIAL AND METHODS**

Crude air-dried venom from a large number of South American rattlesnakes, *Crotalus durissus terrificus*, was provided from The Center for the Study of Venoms and Venomous Animals – CEVAP – UNESP. Mice were obtained from the colony housed available at the same institute.

### ***Venom irradiation***

Whole *C.d.t.* venom was dissolved in saline solution (0.15 M NaCl adjusted to pH 3.0 with concentrated HCl), and its protein concentration adjusted to 2 mg/ml as determined by the Bradford method (7). Samples were irradiated at a dose of 5,25 KGy/h with 2000 Gy using gamma rays derived from a <sup>60</sup>Co source, Gammacell 220 (Atomic Energy Agency of Canada Ltd), in the presence of O<sub>2</sub> at room temperature (31). These experiments were performed at the Institute of Nuclear and Energetic Research – IPEN/CNEM/SP.

### ***Production of antibodies***

Three groups of eight Swiss male mice, weighing 18-22 g, were immunized. Each inoculum received a LD<sub>50</sub> of native (Group I) and irradiated (Group II) *Crotalus durissus terrificus* venom, corresponding to 0.148 µg/g mice. (31).

The inocula were performed on the first day, using a complete Freund's adjuvant intradermally; on the 15<sup>th</sup> day, using aluminum hydroxide subcutaneously; and on the 21<sup>st</sup>, 30<sup>th</sup>, and 45<sup>th</sup> days, using PBS intraperitoneally.

The control group received the inocula on the same days, but just with the adjuvants. All the animals were inoculated with 200 µl of a solution containing 100 µl of the excipient and 100 µl of the venom diluted in saline solution. The control group received 200 µl of a solution containing 100 µl of the excipient and 100 µl of saline solution.

Before receiving each inoculum, all the animals were bled by the retro-orbital plexus, a sample of 100 µl blood was collected and the serum was separated. From each group of animals, a serum pool was prepared, frozen, and kept at -20°C.

### ***Enzyme-linked immunoassays (ELISA)***

This assay was performed in order to detect the antibodies produced against native and irradiated *C.d.t.* venom in the animal sera. They were accomplished on the 15<sup>th</sup>,

21<sup>st</sup>, 30<sup>th</sup>, 45<sup>th</sup>, and 60<sup>th</sup> days, performed as described by Nascimento *et al.*, (31). On the 60<sup>th</sup> day, the titration of the sera from the two groups studied was performed based on the dilution of 1:200 to 1:204800.

### ***Evaluation of the potency involving in vitro preincubation of serum and venom***

To determine the antivenom potency at the 60<sup>th</sup> day after the first inoculation, an aliquot of 100 µl of the pool of each serum was incubated with 100 µl of a solution containing variable quantities of native venom. For this purpose, different dilutions in PBS equivalent to 1, 3, 5, 10, and 15 LD<sub>50</sub> were prepared. The incubation was performed in Eppendorf tubes kept in an incubator at 37°C for 30 minutes (26). Then, 200 µl of each solution was intraperitoneally inoculated into mice.

For control, eight mice were inoculated: four received 200 µl of a solution containing five LD<sub>50</sub> of the native venom diluted in PBS to confirm its toxicity, the remaining animals received only 100 µl of an aliquot of serum diluted in 100 µl of PBS, in order to evaluate its innocuousness. After 48 hours, the mortality rate was recorded.

### ***Protective ability of the antivenom***

To determine the antivenom neutralization capacity at the 60<sup>th</sup> day after the first inoculation, a constant amount of *C.d.t.* venom (74 µg/ml) was mixed with different dilution of Swiss mice antivenom raised against the non-irradiated (native venom) or irradiated venom. Following incubation at 37°C for 30 minutes, the mixtures were injected i.p. into mice at a dose of 10 µl/g body weight (26). The toxin neutralizing capacity (µg of toxin/ml of antivenom) was calculated as described by Kaiser *et al.*, (24).

For control, eight mice were inoculated: four received 200 µl of a solution containing five LD<sub>50</sub> of the native venom diluted in PBS to confirm its toxicity, the remaining animals received only 100 µl of an aliquot of serum diluted in 100 µl PBS in order to evaluate its innocuousness. After 48 hours, the mortality rate was recorded.

### ***Evaluation of the “in vivo” neutralizing capacity***

In order to verify the “in vitro” neutralizing capacity of the antibodies produced by the animals, at the end of the immunization process, four mice of each group studied were weighed and individually challenged with 5 LD<sub>50</sub> of the native venom diluted in

PBS. Each animal received, intraperitoneally, 200  $\mu$ l of this solution. After 48 hours, the mortality rate was recorded.

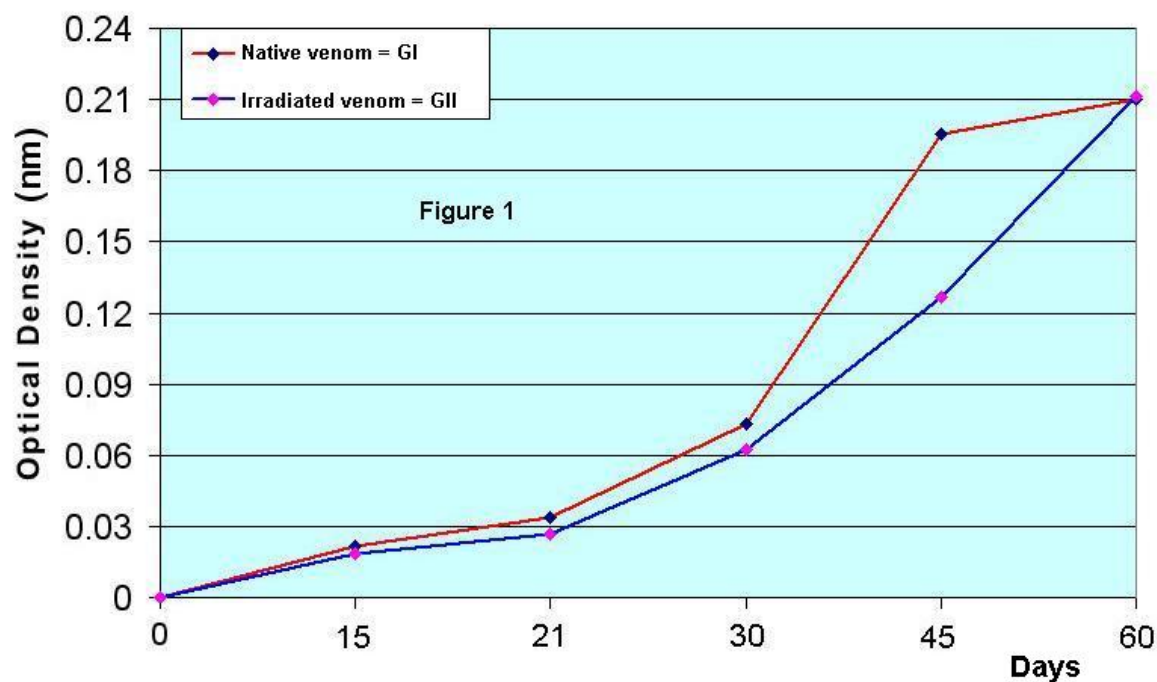
### Statistical analysis

The significance of differences between the means of two experimental groups was determined by the Snedecor's *F*-test. Values of  $p < 0.05$  were considered statistically significant (15).

## RESULTS

Results of the ELISA method for the serum produced from native and irradiated *Crotalus durissus terrificus* venom

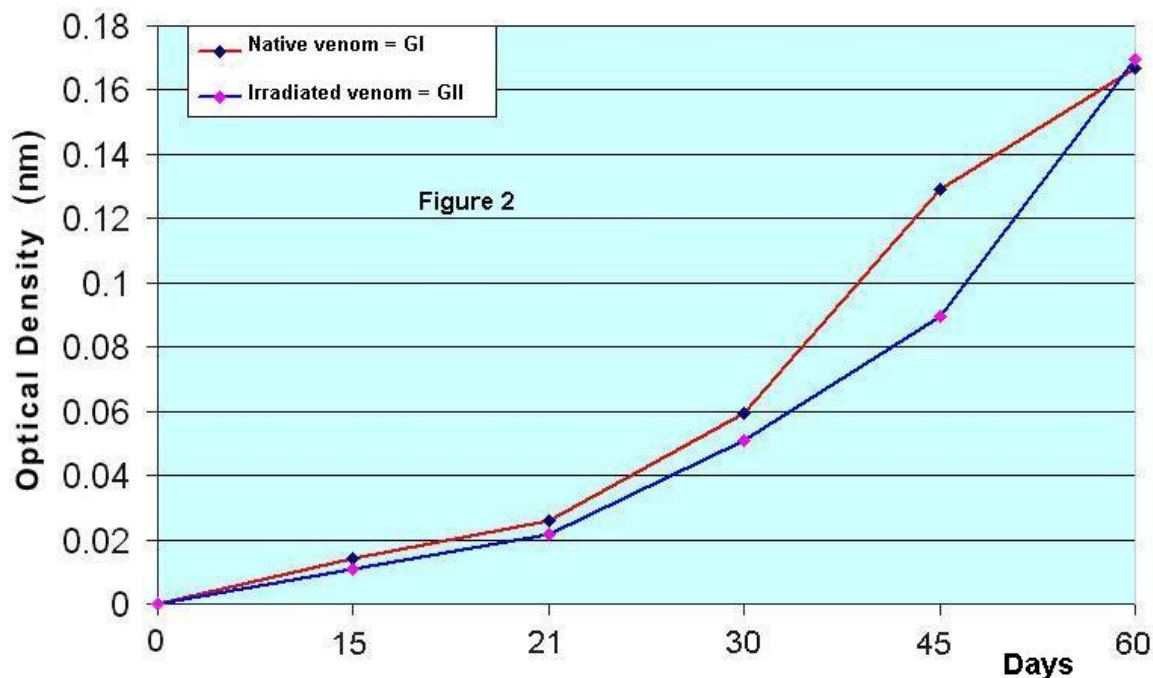
### Enzyme-linked immunoassays (ELISA)



$G I = G II$ ;  $F_{Lines} = 1.89 < F_{Critical} = 7.71$  ( $\alpha = 5\%$ ).

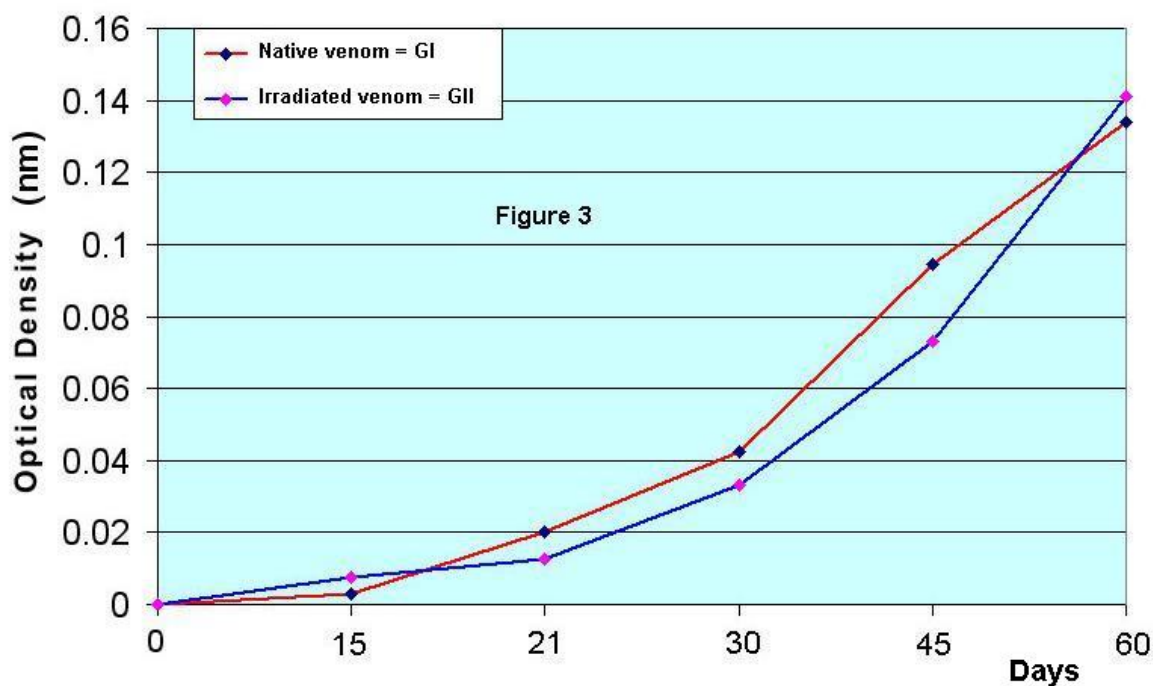
$D_0 \neq D_{15} \neq D_{21} \neq D_{30} \neq D_{45} \neq D_{60}$ ;  $F_{Columns} = 34.16 > F_{Critical} = 6.39$  ( $\alpha = 5\%$ ).

Figure 1: Distribution of the optical densities (nm) mean values in the ELISA test, in different moments, using serum extracted from animals inoculated with native (GI) and cobalt 60-irradiated (GII) venom, diluted in 1:200.



$G I = G II$ ;  $F_{Lines} = 2.04 < F_{Critical} = 7.71$  ( $\alpha = 5\%$ ).

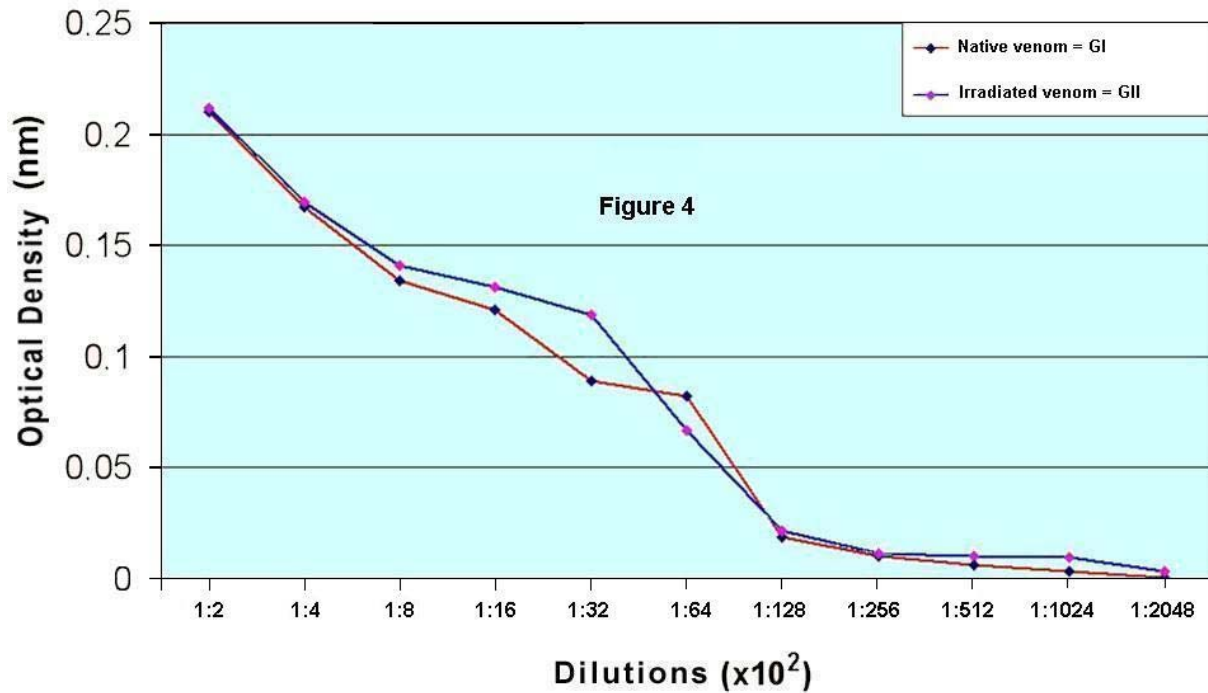
$D_0 \neq D_{15} \neq D_{21} \neq D_{30} \neq D_{45} \neq D_{60}$ ;  $F_{Columns} = 60.81 > F_{Critical} = 6.39$  ( $\alpha = 5\%$ ).



$G I = G II$ ;  $F_{Lines} = 1.11 < F_{Critical} = 7.71$  ( $\alpha = 5\%$ ).

$D_0 \neq D_{15} \neq D_{21} \neq D_{30} \neq D_{45} \neq D_{60}$ ;  $F_{Columns} = 89.71 > F_{Critical} = 6.39$  ( $\alpha = 5\%$ ).

Figures 2 and 3: Distribution of the optical densities (nm) mean values in the ELISA test, in different moments, using serum extracted from animals inoculated with native (GI) and cobalt 60-irradiated (GII) venom, diluted in 1:400 and 1:800 respectively.



$G I = G II$ ;  $F_{Lines} = 2.36 < F_{Critical} = 4.96$  ( $\alpha = 5\%$ ).

$D_{1:2} \neq D_{1:4} \neq D_{1:8} \neq D_{1:16} \neq D_{1:32} \neq D_{1:64} \neq D_{1:128} \neq D_{1:256} \neq D_{1:512} \neq D_{1:1024} \neq D_{1:2048}$ ,  
 $F_{Columns} = 202.86 > F_{Critical} = 2.98$  ( $\alpha = 5\%$ ), ( $D \times 10^2$ )

Figure 4: Distribution of the optical densities mean values in the ELISA test, at the 60<sup>th</sup> day, in different dilutions (1:200 to 1:204800), using serum extracted from animals inoculated with native (GI) and cobalt 60-irradiated (GII) venom.

### Neutralizing capacity and potency

Table 1: Determination of the potency of *Crotalus durissus terrificus* antivenom prepared with native venom (GI) or irradiated venom (GII)

Venom in LD <sub>50</sub> quantity	Mortality (%)	
	GI	GII
1	0	0
3	0	0
5	50	25
10	100	75
15	100	100
-----	0	0
1	100	100

Table 2: Serum dilution for determination of the neutralizing capacity of *Crotalus durissus terrificus* antivenom prepared with native venom (GI) or irradiated venom (GII).

Serum Dilution	Mortality (%)	
	GI	GII
Pure	50	25
1:5	100	50
1:10	100	75
1:20	100	75
1:40	100	100
1:80	100	100



We observed that 1.0 ml of the antivenom prepared with *C.d.t.* irradiated venom could neutralize 296 µg of native venom, whereas 1.0 ml of the antivenom prepared with native venom could neutralize only 59.2 µg.

### **“In vivo” neutralizing capacity**

From the groups studied, none of the animals died after 48 hours observation.

## **DISCUSSION**

The most efficient treatment known until the present moment for accidents caused by venomous snakes is the specific heterologous serum (4, 45). The production of antivenom has been performed throughout the history in large animals, particularly Equidae in Brazil (11, 22, 39, 45).

The production of specific serum against *Crotalus durissus terrificus* venom is a difficult process because, despite being toxic, it has an inhibitory effect in the antibodies production (9, 18, 37, 41).

The hiperimmunization with crude venoms can cause important side effects, including death (2). According the same authors, few studies on the importance of the deleterious effects caused by venoms during the hiperimmunization process were performed.

In order to overcome these difficulties, several methods of detoxification have been studied in order to minimize the alterations caused by the venom (14, 16, 19, 20, 22, 23, 25, 28, 44). In addition, the researchers look for alternatives of antibodies production with high quality and the highest seric levels.

In this context, gamma radiation has been showed to be an excellent tool for snake venom attenuation. Some authors (12, 30, 31, 32) verified that, after the irradiation, the *C.d.t.* venom and its fractions antigenic and immunogenic properties were preserved, and its toxicity was reduced.

Murata *et al.* (29), using *C.d.t.* crude venom verified that the irradiation of 2000 Gy is the ideal dose for snake venoms detoxification, since it keeps many immunological properties. Nascimento *et al.* (30, 31, 32) found similar results using purified crotoxin. In the present study, the efficiency of the irradiation technique of *Crotalus durissus terrificus* venom with <sup>60</sup>Co in the production of antivenomous serum in mice was investigated.

The ELISA technique was used in the detection and following of the titer of antibodies during the hiperimmunization process. This technique has been showed to be the method with the best specificity, sensitivity, fastness, simplicity, and low costs (3, 5, 30, 35, 38, 45).

Clissa *et al.*, 1999, evaluated the immunogenic activity of native and irradiated *C.d.t.* venom in rabbits by the ELISA assay. Nascimento *et al.* (31) evaluated the ionizing radiation influence on the *C.d.t.* venom crotoxin. According to these authors the antigenic and immunogenic properties were preserved after the irradiation with cobalt 60.

De Paula *et al.* (17), studying the production of antivenoms from irradiated *C.d.t.* crotoxin and crude venom in rabbits, verified that the sera produced were able to recognize the native toxins.

The results obtained showed that the optical density values from the ELISA test to the several dilutions of native and irradiated *C.d.t.* venom did not show statistical differences between the two groups studied.

It was also verified that the antibodies could be detected 15 days after the first inoculum. On the last day of the experiment, the 60<sup>th</sup> day, the titration of the antibody levels in the dilutions from 1:200 to 1:204800 was performed. It was observed that there was no statistical difference between the groups evaluated.

The titles of antibodies produced permit the conclusion that both the group of mice hiperimmunized with native venom and the group of mice hiperimmunized with irradiated venom were immunogenic, with production of antibodies able to recognize native *C.d.t.* venom. These results confirm the data obtained by other authors (12, 32).

Tests of neutralization potency and capacity were performed after the animals of the group studied produced high titers of antibodies.

The “in vitro” neutralization capacity of the serum produced from the irradiated *C.d.t.* venom was higher than that of the serum produced from native venom.

The serum produced from native *C.d.t.* venom neutralized 0.592 mg/ml, equivalent to 200 LD<sub>50</sub> of the native venom, while the serum produced from the irradiated venom neutralized 5.92 mg/ml, equivalent to 2000 LD<sub>50</sub> of the native venom.

The results observed in the determination of the sera pool potency showed that, despite the titers of antibodies were similar according to the ELISA assay, the serum produced from the irradiated venom showed to be efficient.

According to Cardi *et al.* (8), the gamma irradiation proved to be the most successful method for the crotoxin detoxification. These authors (8) suggest that the reduction of toxicity is due to a precocious endocytosis of crotoxin by phagocytic cells, improving the antigen processing. This occurs because the irradiation promotes molecule oxidation facilitating its phagocytosis due to the presence of scavenger receptors in the macrophages surface.

It is known that the antigens, as they enter the organism, suffer an oxidation process by the defensive cells to facilitate the phagocytosis process (36). In irradiated samples, the macrophagus find these molecules oxidated, and therefore, they eliminate this step of the process. A better processing, associated to a faster antigen presentation, makes the immunologic system produce more complete antibodies against a higher number of antigen epitopes (36).

The results observed in this study agree with those already obtained by other authors (12, 30, 31, 33, 34, 36).

None of the animals from the groups studied died during the “in vivo” tests when challenged with 5 LD<sub>50</sub> of the native venom after 48 hours observation.

With regard to the side effects caused by venoms, Carvalho *et al.* (10), cite the venom and adjuvants toxicity, mainly the Freund’s Complete Adjuvant, as the major problem in the production of commercial antivenoms. They cause inflammation and lesions in the inoculum site, contributing to the reduction of the longevity of animals that produce immunoglobulines.

In this experiment, all the mice were daily observed in order to detect clinical alterations and lesions in the inoculation sites. None of the animals died, and no local or systemic alterations were observed.

Therefore, these results demonstrate that the gamma radiation can be used as an important tool for the anticrotalic serum production in the process of animal immunization; since the neutralization capacity and potency of the serum produced from the irradiated venom were higher despite the titers of antibodies found by the immunization process were similar.

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