Immune response and neutralization capacity of antibodies produced in young sheep immunized with *Crotalus durissus terrificus* native or Cobalt-60 irradiated venom

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Abstract

The ELISA technique was used to evaluate and compare young ovine humoral immune response during crotalic antiserum production. Animals were clinically evaluated throughout this process, and the neutralizing capacity of antisera raised against natural (NV) and Cobalt-60 irradiated (IrV) venoms of C*rotalus durissus terrificus* (*C.d.t.*) was verified by means of *in vitro* challenges. Three groups of six animals each were used: G1 received NV; G2 was inoculated with IrV; and G3 was used as control. Animals received six immunizations during 84 days at 14-day intervals. ELISA of antibody profile showed significant difference ($p<5\%$) between experimental groups (G1<G2). These results justify the use of gamma radiation to detoxify *Crotalus durissus terrificus* venom like alternative to antiserum production. The neutralizing capacity of antiserum raised against IrV was fivefold higher than that of antiserum raised against NV. Results showed a new possibility of using ovines to produce commercial crotalic antiserum, which may be employed in the treatment of human and animal envenomation. Production cost might be reduced by the subsequent utilization of hyperimmunized ovines as food.

Key words:
Crotalus durissus terrificus.
Hyperimmunization.
Ovines.
Crotalic antiserum.
Irradiation.

Introduction

The venom of *Crotalus durissus terrificus* snakes is highly toxic; however it is poorly immunogenic [1] partially due to the presence of immunosupressor components [2,3,4]. Also, damage caused to animals after crude venom inoculation contributes to low antivenom production.[5]

Researchers have been seeking alternatives to prepare toxoids through venom biological detoxification, which would keep its immunogenicity and minimize damages caused to antiserum producer animals.[6,7]

Gamma radiation has been efficient in attenuating ophidic venoms and capable of decreasing their toxicity without altering immunogenicity, without addition of any substance to the venom. [8,9,10,11]

During the last century, horse has been the animal of choice for antivenom production because it is easy to manage and produces high quantities of antisera.[12,13] However, the high costs to obtain and maintain horses stimulate research on alternative hyperimmunization schemes.[14]

Thus, the aims of the present paper were to compare the humoral immune response, to determine and compare the

potency and the neutralizing capacity of antisera raised in young ovines against natural and Cobalt-60 irradiated *Crotalus durissus terrificus* venoms.

Materials and Method

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, Brazil. Swiss mice (18-22g) were obtained from the colony housed at the same Institute.

Eighteen male sheep, 60-75 days old, Santa Inês and Ile de France breeds, were kept at the Laboratory for Studies on Biotechnology and Reproduction, School of Veterinary Sciences and Animal Husbandry, UNESP, Botucatu, Brazil.

Study approved by the Committee of Ethics in Animal Experimentation in the Botucatu Medical School - UNESP, Brazil (Protocol 609/07)

Whole *C.d.t.* venom was dissolved in saline solution (0.15M NaCl adjusted to pH 3.0 by using concentrated HCl), and its protein concentration adjusted to 2 mg/ml as per the Bradford[15] method. Samples were irradiated at 5.25KGy/h with 2000Gy using gamma rays derived from a ^{60}Co source, Gammacell 220 (Atomic Energy Agency of Canada Ltd), in the presence of O_2 at room temperature. [16] These experiments were performed at the Institute of Nuclear and Energetic Research – IPEN/CNEM /SP, Brazil.

Antibody production

Three groups of six sheep each were used. G1 received natural *C.d.t.* venom; G2 received irradiated *C.d.t.* venom; and G3 was used as control and did not receive venom, only adjuvants. This methodology was standardized previously for our group.

Inoculations were carried out at six different moments (M), as follows: At day 1 (M1), animals received intradermally 500μg venom diluted in 1mL saline solution (PBS) homogenized with 1mL Freund's Complete Adjuvant (FCA); at days 14 (M2) and 28 (M3), they received subcutaneously 1mg venom diluted in 1mL PBS homogenized with 1mL aluminum hydroxide ($AlOH_3$); at day 42 (M4), they received subcutaneously 1.5mg venom diluted in 2mL PBS; at days 56 (M5) and 70 (M6), animals were subcutaneously inoculated with 2mg venom diluted in 2mL PBS. At day 84 (M7), animals were bled.

Each animal received 2mL of the solution (0.5mL into four different points of the lateral region of the neck). Antisepsis with iodated alcohol and hair removal were performed at the inoculation site.

Before venom inoculation, 20mL blood was collected from each animal for ELISA.

Enzyme-linked immunosorbent assay (ELISA): this test was carried out as described by Nascimento et al.[16], from M1 to M7 in order to detect antibodies produced against natural and irradiated *C.d.t.* venoms. On the 84th day (M7), sera titration was performed based on the dilution of 1:100 to 1:51200 for the two groups studied.

To determine the antivenom neutralizing capacity on the 84th day (M7) after the first inoculation, the same quantity of *C.d.t.* venom (5 LD_{50}, 0.74μg/ml) was mixed with different dilutions of antivenoms of sheep obtained from the non-irradiated and irradiated venoms. After incubation at 37°C for 30 minutes, mixtures were intraperitoneally injected into mice at the dose of 10μl/g body weight[17]. The toxin neutralizing capacity (μg toxin/ml antivenom) was calculated as by Kaiser et al. [18], using the following equation:

Neutralization capacity of toxin (NCT) = $(D - DL_{50}) \times 1 \times 10^5 X (1/V_{50})$,

Where D = total dose of the toxin (mg/g); DL_{50} in mg/g; V_{50} = Volume of antivenom that reduces the lethality of 1 ml of solution of the toxin injection to 50%. The lethality of 50% will mean that the antiserum was able to reduce the effective dose for 1 DL_{50}.

For control, four mice received 200μl of a solution containing 5 LD_{50} of natural venom diluted in PBS to confirm its toxicity,

and another four mice received only 100µl of an aliquot of serum diluted in 100µl PBS to evaluate its innocuousness. After 48 hours, mortality rate was recorded.

To determine the antivenom potency on the 84th day (M7) after the first inoculation, a 100µl aliquot of each serum pool was incubated with 100µl of a solution containing variable quantities of natural venom (different PBS dilutions equivalent to 1, 3, 5, 10, and 15 LD_{50}). Incubation was carried out in Eppendorf tubes kept at 37°C for 30 minutes.[17] Then, 200µl of each solution was intraperitonially inoculated into mice.

Eight animals were used as control: four received 200µl of a solution containing 5 LD_{50} of natural venom diluted in PBS and the remaining animals received 100µl of an aliquot of serum diluted in 100µl PBS. After 48 hours, mortality rate was recorded.

Animals were observed throughout the hyperimmunization process. Alterations on the inoculation site, skin and/or subcutaneously such as pain, hyperthermia, edema, hemorrhage, fistula, abscess, and necrosis as well as the number of dead animals were descriptively reported.

Responses by the three groups at seven different moments were evaluated by means of repeated measures analysis (or similar non-parametric proceeding) of mean profiles, according to Johnson and Wichern[19]. Test results obtained by F statistics were significant at 5% level, in moments M1 to M7.

Results and Discussion

Enzyme-linked immunosurbent assay (ELISA)

ELISA results of antisera raised against natural and irradiated *Crotalus durissus terrificus* venoms.

In the present study, analysis of optical density values obtained by ELISA for several dilutions showed that the sera pool from ovines hyperimmunized with irradiated venom had titers higher than those of sera pool from animals hyperimmunized with natural venom (Figure 1).

Irradiated venom produced high

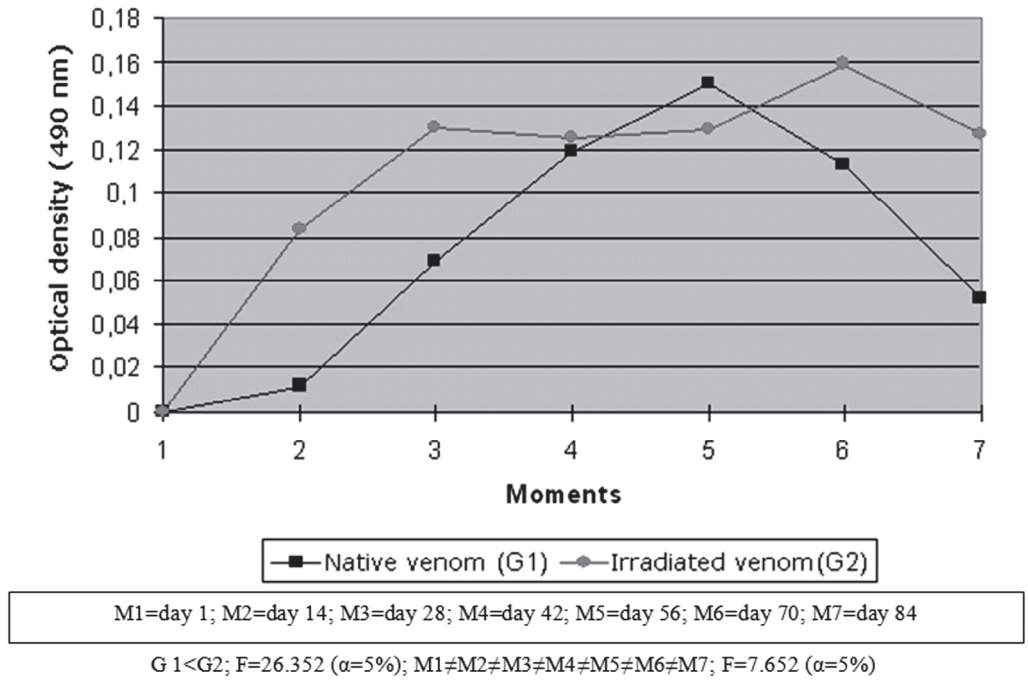
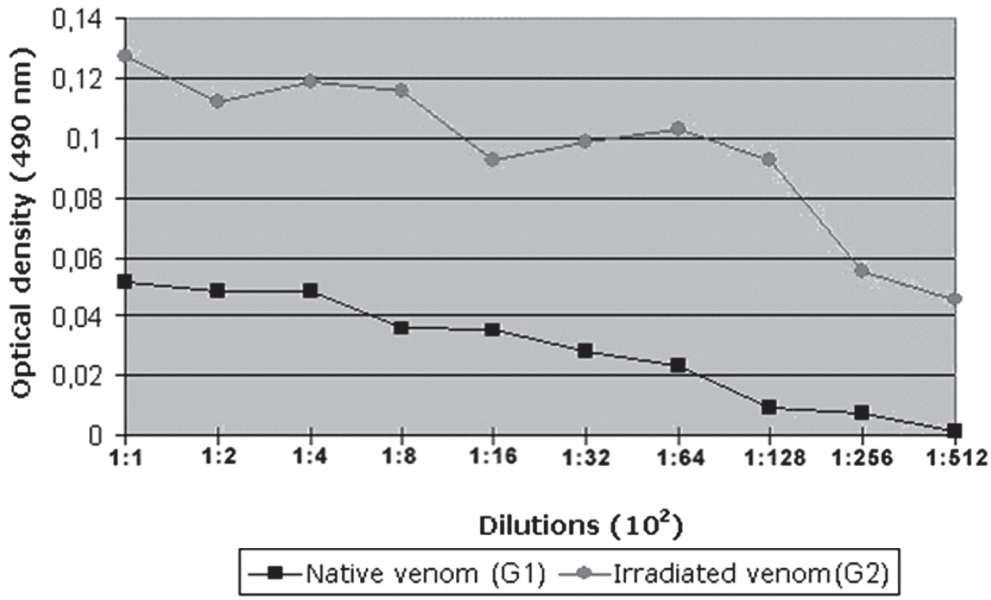


Figure 1 - Optical density mean values (nm) of antisera raised in animals inoculated with natural (G1) and Cobalt-60 irradiated (G2) venoms (diluted 1:100) at different moments



G1<G2; F=164.46 (α=5%); $D_{1:100} \neq D_{1:200} \neq D_{1:400} \neq D_{1:800} \neq D_{1:1600} \neq D_{1:3200} \neq D_{1:6400} \neq D_{1:12800} \neq D_{1:25600} \neq D_{1:51200}$; F=6.54 (α=5%).

Figure 2 - Optical density mean values (nm) of antisera raised in animals inoculated with natural (G1) and Cobalt-60 irradiated (G2) venoms at different dilutions 10^2 (1:100 to 1:51200) on the 84th day (M7)

antibody levels since the 14th day after the first inoculation. On the last day (day 84), titration of antibody levels (from 1:100 to 1:51200) showed statistical difference between groups (Figure 2), with higher titers in the group inoculated with irradiated venom.

Therefore, the Cobalt-60 irradiated *C.d.t.* venom was immunogenic, could produce higher levels of antibodies capable of recognizing natural *C.d.t.* venom, and achieved production peak faster. These results confirm data obtained by several authors.[8,20,21,22]

Venom neutralizing capacity and potency

One ml of antivenom raised against irradiated *C.d.t.* venom could neutralize 296μg venom, whereas 1.0ml of antivenom raised against natural venom could neutralize only 59.2μg.

In both tests mentioned above, mortality was 100% when venom alone was administered and 0% when serum alone was administered.

Potency and neutralizing capacity tests were carried out after animals presented high antibody titers.

Antibodies produced by both experimental groups were effective in neutralizing natural *C.d.t.* venom *in vitro* (Table 1). Sera pool from animals inoculated with Cobalt-60 irradiated *C.d.t.* venom had higher potency and neutralizing capacity than

Table 1 - Antiserum dilution for determination of the neutralizing capacity of *Crotalus durissus terrificus* antivenom raised in animals inoculated with natural (G1) and irradiated (G2) venoms

Antiserum dilution	Mortality (%)	
	G1	G2
Pure	0	0
1:5	87.5	0
1:10	100	87.5
1:20	100	100
1:40	100	100
1:80	100	100

that of animals inoculated with natural venom (Table 2).

Table 2 - Determination of the potency of *Crotalus durissus terrificus* antivenom raised in animals inoculated with natural (G1) and irradiated (G2) venoms

Venom (LD_{50})	Mortality (%)	
	G1	G2
1	0	0
3	0	0
5	75	0
10	87.5	37.5
15	100	87.5

Potency G1 = 59.2µg/ml; G2 = 296µg/ml

The present results corroborate studies [22,23,24] which assert that ionizing radiation can attenuate ophidic venom toxicity without reducing its antigenic capacity.

Clinical evaluation

Animals were daily observed throughout the hyperimmunization process and only one animal died due to intoxication by copper present in the food. On the seventh day after the first inoculation, all animals showed alterations at the inoculation site such as hair fall, volume increased in 3-6cm diameter, ulcerated areas with purulent exudates (Figure 3), rigidity, increased temperature, abscess, and enlarged supraclavicular lymph nodes.

Lesions reduced after daily local cleaning; however, rigid fibrotic abscesses remained until the end of the experiment.

Parenteral administration of heterologous antivenom has been the main treatment for snake envenomation since the pioneer works by Calmette, Phisalix and Bertrand [25,26]. Antivenom production has been carried out in big animals, particularly equine

Figure 3 - Suppurative abscess and ulcerated areas in animals from the three groups on the 7th day after the first inoculation

in Brazil [27,28].

Production of *Crotalus durissus terrificus* antiserum is a difficult process since it is highly toxic, inhibits antibody production [29] and may cause severe lesions to antiserum producer animals, leading to death. Accidents caused by *C.d.t.* in Brazil account for more than 2000 cases per year with a high mortality rate [30].

Consroe et al. [31] stated that antivenom raised in animals such as ovines are less immunogenic than equine antibodies. This fact leads to research on novel hyperimmunization schemes to obtain high titers of specific antibodies in those animals.

The present work used natural and Cobalt-60 irradiated venoms in order to investigate and compare their efficiency in the production of crotalic antiserum raised in young ovines as well as to evaluate the antibody production and the antiserum potency and neutralizing capacity.

Several researchers have successfully used ovines for antivenom production.[20,32] Affection is not an obstacle to the use of these animals, which are free from infectious diseases responsible for great damages to producers.[7]

The main advantage of using sheep is their excellent, fast humoral immune response. High titers of specific antibodies are produced in 100% animals, remaining high for a long period when immunization is continued.[32]

On the other hand, hyperimmunized horses produce high levels of an immunoglobulin called IgG_T [33], which has high protective capacity [although it is more immunogenic than IgG][20,34] and causes

anaphylactic reactions in patients presensitized to equine proteins[13]. This immunoglobulin was not detected in ovines.[7]

Clark et al.[28] reported the efficacy of using equine antivenom to treat neurotoxicity caused by North-American rattlesnake venom.

Netto et al.[8] and Ferreira Junior et al.[22] showed an excellent possibility of using ovines in the production of *Crotalus durissus terrificus* antivenom.

Irradiation causes chemical and physicochemical changes in the proteins secondary and tertiary structures but keeps their immunogenic properties. This detoxification may be an effective method to reduce the venom toxic effects in immunized animals and improve antigens for toxoid and vaccine preparation.[9,24]

According to Cardi, Nascimento e Andrade[35], gamma irradiation is the most successful method to detoxify crotoxin, an important toxin found in crotalic venom.

Clinical evaluation demonstrated that, approximately seven days after the first inoculation with Freund's Complete Adjuvant, all animals showed significant local alterations such as necrosis, fistulas and abscesses. As the control group received only adjuvant and saline solution, lesions could be associated with the adjuvant and not with the natural or the irradiated crotalic venom.

Carvalho et al.[36] cited the venom and Freund's Adjuvant toxicity as the major problem in the production of commercial antivenom, since they cause inflammations and lesions at the inoculation site reducing the antiserum producer animals longevity.

Some moderate local alterations such as edema, abscess, fistula and fibrosis can be observed during hyperimmunization with snake venoms, mainly those of *Bothrops* snakes.[37] However, according to Ferreira Junior et al.[22], problems can be eliminated when Cobalt-60 irradiated venom is used.

Conclusions

Since the tested ovines development was normal, hyperimmunization process was successfully performed, and specific antibodies against *Crotalus durissus terrificus* venom were produced.

The results of the present study justify the use of gamma radiation to detoxify *Crotalus durissus terrificus* venom in order to improve antiserum production.

The titles of antivenom produced with irradiated venom, measured by ELISA, were higher than the titles of antivenom obtained from the native venom of *Crotalus durissus terrificus*. This was also observed with neutralization capacity.

The present experiment with confined young animals will be extended to field animals in order to evaluate the efficiency of this experimental model, which should also be tested with venoms from other snakes that commonly cause accidents in Brazil. In the future we intend to challenge the serum produced for this method front to the commercial anti-venoms.

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Resposta imune e capacidade de neutralização de anticorpos produzidos em ovinos jovens imunizados com veneno de *Crotalus durissus terrificus* nativo e irradiado com Cobalto 60

Resumo

A técnica de Elisa foi utilizada para avaliar e comparar a resposta imune humoral de ovinos jovens para a produção de soro anticrotálico. Durante o processo de soroprodução, foi realizada a avaliação clínica dos animais. A capacidade de neutralização do soro produzido a

Palavras-chave:
Crotalus durissus terrificus.
Hipermunização.
Ovinos.
Soro anticrotálico.
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partir de veneno de serpente C*rotalus durissus terrificus*, nativo (VN) e irradiado (VIr) com Cobalto–60 foi verificada por meio de desafios *in vitro*. Um grupo de seis animais recebeu veneno nativo, o segundo grupo recebeu veneno irradiado e o terceiro grupo foi o controle. Os animais receberam seis imunizações durante 84 dias com intervalo de 14 dias. Houve diferença significativa ($p<5\%$) no teste de ELISA do perfil de anticorpos produzidos pelos grupos experimentais (VN<VIr). O grupo imunizado com veneno irradiado apresentou perfil de anticorpos maior que o grupo imunizado com veneno nativo. A capacidade de neutralização do soro produzido a partir do VIr foi cinco vezes maior quando comparado ao soro produzido com VN. Estes resultados justificam o uso da radiação gama na destoxicação do veneno de *Crotalus durissus terrificus* como alternativa na produção de antiveneno.

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