

HUMORAL RESPONSE AND NEUTRALIZATION  
CAPACITY OF SHEEP SERUM INOCULATED  
WITH NATURAL AND COBALT 60-IRRADIATED  
*Crotalus durissus terrificus* VENOM  
(LAURENTI, 1768)

D. P. Netto<sup>1</sup>✉, S. B. Chiacchio<sup>2</sup>, P. L. Bicudo<sup>2</sup>, A. A. Alfieri<sup>1</sup>,  
N. Nascimento<sup>3</sup>

1 Departamento de Medicina Veterinária Preventiva, Centro de Ciências Agrárias, Universidade Estadual de Londrina, Londrina, Paraná, Brasil; 2 Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, São Paulo, Brasil; 3 Supervisão de Radiobiologia, Instituto de Pesquisas Energéticas e Nucleares IPEN/CNEM, São Paulo, Brasil.

**ABSTRACT:** The aim of this work was to investigate antigen irradiation on crotalic antivenom and the capacity of sheep as serum producers. Twelve sheep in two groups of six were inoculated with *Crotalus durissus terrificus* venom. One group was inoculated with natural venom (NV) and the other with Cobalt 60 gamma-irradiated venom (IrV). Three antigen doses were given to the animals at monthly intervals for immunization. The toxic activity of the venom was assessed by LD50 determination in mice. Blood samples were collected weekly analyses of serum neutralization capacity and potency. At the end of the experiment, the animals were challenged with a LD50 for sheep showed no signs of envenoming. These results showed that toxicity of the irradiated venom was 4.4 times less than the natural venom. The sera from the irradiated group neutralized LD50 14.6 times, and the sera from the natural group 4.4 times. Sera from the irradiated group were five times more potent. The two groups did not present clinical alterations. The results of this study show the potential for using sheep in crotalic antivenom production. The use of irradiated venom in sheep immunization induces a powerful and lasting humoral immune response shown by both the *in vitro* neutralization and potency tests and by the indirect ELISA antibody level detection technique.

**KEYWORDS:** gamma irradiated crotalic antisera, snake venom, ELISA, sheep, *Crotalus durissus terrificus*, cobalt60.

## INTRODUCTION

Snakebite envenoming is a major clinical problem for human and veterinary medicine, especially in tropical areas; it is also important that an efficient treatment is available.

Heterologous sera from animal plasma are used in the treatment of snake envenoming; these sera are usually obtained from venom-hyperimmunized horses (32). In the last hundred years, the horse has been the preferred animal for antivenom production because it is easy to manage and a large volume of plasma is obtained during bleeding and plasmapheresis. In spite of these advantages, there are important reasons to be considered in antivenom production, such as i) animal availability; ii) acquisition and maintenance costs; and iii) exacerbated local immune response which results in the formation of large abscesses at the inoculation site (28). Furthermore, many patients receiving equine antivenom show sensitivity to horse protein because of previous exposure and might develop a type 1 anaphylactic reaction (25).

Administration of immunoglobulin concentrates or F(ab)<sub>2</sub> derived from horse sera has been shown to cause a type-III hypersensitivity reaction mediated by immune-complexes; these are probably due to molecule bivalence and to circulating IgG titer present in hyperimmunized horses (7).

After accidents with venomous animals, the problem of choosing an adequate form of treatment for horse-serum sensitive patients is not uncommon. In these cases, alternative therapies are used, which are generally less efficient (25). To resolve this, immunization methodologies were developed using alternative animals such as rabbits, goats, and sheep, which have shown good results in serum production (5,26,28).

Hyperimmune plasma production depends on venom immunogenicity; this is related to the different molecular masses of each antigen (30). High toxic venoms exercise less stimulation on antibody formation than low toxicity venoms (27).

*Crotalus durissus terrificus* venom consists of highly toxic protein and non-protein compounds. This prevents inoculation at concentrations capable of providing adequate immune response for the needs of antivenom production without debilitating the animal producer (17). Detoxification of antigen or toxoid production requires that the venom loses its toxicity, but at the same time retains maximum immunogenicity (14). Different chemical and physical techniques have been proposed to detoxify snake venom, such as i) treatment with formaldehyde (14), glutaraldehyde (12), and iodine (23); ii) linking to carboximethyl-cellulose (16); iii) exposure to X and ultraviolet irradiation (9,31); iv) functional group blockage (3) by adsorption with tannin (20) and by encapsulation in liposomes, treated or non-treated with osmium tetroxide (10).

The use of electromagnetic radiation or even photon radiation projects an electron and an atom, resulting in the creation of a pair of ions, positive and negative. This phenomenon, called ionization, is the main means by which the energy of ionizing radiation is transferred to biological tissues without producing radioactivity (24). As gamma radiation with Cobalt 60 depending on dose rates does not heat the sample, this type of energy can be applied in the attenuation of biological product, which is usually susceptible to relatively high temperatures (11).

The great availability, easy handling, low acquisition, and maintenance costs led Sjoström *et al.* (28) to consider the use of ovine in antivenom production. The animals presented greater tolerance both to Freund's adjuvant and other adjuvants, with no local lesions. High IgG circulating levels were quickly obtained, but IgGT was not detected (28).

This study was carried out to assess by *in vitro* (indirect ELISA) and *in vivo* methods, the humoral immune response in sheep inoculated with natural venom and Cobalt 60-irradiated venom from *Crotalus durissus terrificus*.

## MATERIALS AND METHODS

### ANIMALS

#### **Sheep**

Twelve 1-2-year old sheep were used in this study, eight females and four males of mixed Ile de France breed, with mean weight 44 kg. Two groups of six animals were formed randomly, with two males and four females in each group. One group was inoculated with natural venom (NV) and the other with irradiated venom (IrV).

#### **Mice**

Thirty-day old Swiss albino mice (*Mus musculus*) of both sexes, weighing between 18 and 20 grams were used to assess the toxic activity (LD<sub>50</sub>) (n=56) for NV *in vitro* neutralization (n=48) and potency (n=88) of the anticrotalic serum from the NV and IrV sheep.

### VENOM

Venom was obtained from *Crotalus durissus terrificus* snake, freeze dried, divided into two aliquots of 200 mg, and kept at 4°C. One aliquot (IrV) was submitted to gamma radiation at the Institute of Energetic and Nuclear Research (IPEN, University of São Paulo, São Paulo/SP). The other aliquot (NV) was not treated at all.

#### **Venom irradiation**

The freeze-dried venom aliquot was diluted in acidified saline solution (NaCl 150mM, pH 3.0) and irradiated by a Cobalt 60 source at a dose of 5.54 x 1.102 Gy/h and a concentration of 2.000 Gy in the presence of O<sub>2</sub> and at room temperature. After irradiation, it was kept at -20°C until use.

#### **Toxic activity**

The NV toxicity was assessed by determining LD<sub>50</sub> in mice. One milligram of NV was diluted in 10 ml of buffered saline solution (PBS). Aliquots containing 7.5, 9.8, 12.7, 16.3, 20.9, 27.66, and 35.7µg of freeze-dried venom were obtained using a 1.3 as the progression factor. The Spearman-Kärber method was used to calculate the lethal dose (LD<sub>50</sub>) according to the WHO guidelines (32). Mice were divided into seven groups of eight, which were inoculated IP with 0.5 ml of each concentration. The death rate was determined after 48 hours.

#### **Venom protein concentration**

Protein concentration of IrV and NV was determined by the spectrophotometric method described by Bradford (2).

### **SHEEP IMMUNIZATION**

The two groups of sheep received three venom inoculations: the first on day zero; the second, day 30; and the third, day 60. The NV and IrV groups received 1.0 mg and 2.0 mg, respectively, diluted in 5.0 ml of PBS, pH 7.0. The first, second, and third inoculations were made with equal volume of Freund's Complete (Sigma, USA), Freund's Incomplete (Sigma, USA), and aluminium hydroxide adjuvants, respectively. The animals were inoculated SQ in the right (5 ml) and left (5 ml) subscapular region in a total of 10 ml of antigen/adjuvant emulsion. On day zero and weekly thereafter, the animals were bled by the jugular vein to obtain serum for later analysis by indirect ELISA.

All sheep in the two inoculated groups were assessed weekly for inflammatory reactions, hemorrhage alterations, abscess formation, and lymphonode increase at inoculation sites.

### **NEUTRALIZATION AND POTENCY OF SHEEP SERA**

#### ***In vitro* test**

The neutralization capacity of the two sera from NV and IrV sheep was assessed by the *in vitro* test 60 days after the first inoculation. A pool of sera was diluted in PBS (1:10) and one aliquot (250 $\mu$ l) was incubated with an equal volume of a PBS solution containing NV and IrV concentrations equivalent to 1, 5, 10, 15, and 20 LD<sub>50</sub>. After homogenization, the mixture (500 $\mu$ l) was incubated at 37°C and inoculated individually into four mice. Venom toxicity (1 LD<sub>50</sub>/500 $\mu$ l) and serum innocuity (500 $\mu$ l/1:10 dilution) were assessed by inoculation in two groups of four mice each.

Serum potency of the NV and IrV groups was assessed by incubating 250 $\mu$ l of the sera pool diluted 1:10, 1:20, 1:40, 1:80, 1:100, 1:200, 1:400, 1:800, and 1:1000 in PBS with 250 $\mu$ l of a solution containing 5 LD<sub>50</sub> of NV or IrV, also diluted in PBS. The mixture was homogenized and incubated at 37°C/1h and inoculated individually IP in groups of four mice. Toxicity of the venoms was also assessed in mice inoculated with 5 LD<sub>50</sub>/500 $\mu$ l.

Serum potency of each experimental group was calculated by the probit analysis (8), which assesses the quantity of venom ( $\mu$ g/ml) neutralized by the serum pool. Neutralization capacity of antigens (NCA) was calculated by the formula developed by Kaiser *et al.* (13).

#### ***In vivo* test**

The neutralizing capacity of antibodies induced by NV and IrV was assessed 60 days after the last inoculation. One sheep from the IrV group and one from the NV group were challenged IM with 1 mg/kg live weight of NV, as described by Araujo and Belluomini (1). The animals were observed for 48 hours post-inoculation for envenoming symptoms.

### **ENZYME IMMUNOASSAY (ELISA)**

The indirect ELISA technique was performed using microplates (Nunc-Immuno Plate-Maxisorp, USA) adsorbed with 1 $\mu$ g/well of antigens (NV and IrV) diluted in carbonate/bicarbonate 0.05M buffer, pH 9.6, and kept in a damp chamber at 4°C for 24 hours. After five washings with PBS/Tween 20, 100 $\mu$ l of each dilution on base two (1:1,000 to 1:1,024,000) of the immunized sheep sera was added to each well. After

incubation for one hour at room temperature, the plates were again washed in PBS/Tween 20. Then, 100 $\mu$ l/well of a 1:1,250 dilution of anti-sheep IgG peroxidase conjugated (Sigma/USA) was added. After incubation for one hour at room temperature, the plates were again washed and the reaction revealed by adding 100 $\mu$ l of substrate consisting of H<sub>2</sub>O<sub>2</sub> (0.03%) and dihydrochloride o-Phenylenediamine (2.5 mg/ml) diluted in citric acid buffer solution 0.1M, pH 5.0. After 20 minutes at room temperature, the reaction was interrupted by adding 30 $\mu$ l/well of 1M H<sub>2</sub>SO<sub>4</sub> solution. The reactions were read in an automatic microplate reader (Emax Precision Microplate Reader, Molecular Devices, USA) using a 490nm filter.

## STATISTICAL ANALYSES

The variables were studied in the two groups (treatments) with 15 repetitions (ELISA) each; analysis of variance in subplots was used (21). The F statistics was considered significant when  $p < 0.05$ . Significance tendency was  $0.05 > p > 0.10$ . The contrasts between pairs of means were studied by calculating the minimum significant difference for  $p = 0.05$  by the Tukey test.

To apply the cut off points to each of the serum dilutions in the results of the indirect ELISA test, optical density values equal or superior to the mean of the week plus two standard deviations were considered positive.

## RESULTS AND DISCUSSION

The LD<sub>50</sub> values were 0.09 (0.05-0.14)  $\mu$ g/g and 0.40 $\mu$ g/g mouse for NV and IrV, respectively. The LD<sub>50</sub> for IrV was 4.4 times less toxic than NV. Similar results were reported by other authors (4,18,19), who suggested that toxicity of irradiated venom would be reduced by the formation of aggregates, enabling the formation of a new complex.

The results of serum neutralization tests at 1:10 dilution indicated that IrV sheep sera neutralized 105.12 $\mu$ g/ml venom, equivalent to 14.6 times LD<sub>50</sub>. NV sheep sera at the same dilution neutralized 31.68 $\mu$ g/ml equivalent to 4.4 times LD<sub>50</sub>. This shows that serum from IrV sheep performed better than NV sheep sera. Mandal *et al.* (15) immunized rabbits with *Vipera ruselli* snake IP and observed that 0.1ml of serum from these animals neutralized five LD<sub>50</sub> in mice. Egen *et al.* (6) compared the natural *Crotalus adamanteus*, *Crotalus atrox*, *Crotalus scutulatus*, and *Agkistrodon piscivorus* antivenom produced in sheep and commercial antivenom produced in equine and found that the sheep serum was 7 to 11.7 times more effective than the equine serum.

When determining the neutralization capacity of crotalic antivenom from sheep hyper-immunized with IrV, using variable concentrations of serum and fixed concentrations of NV (5 LD<sub>50</sub>), protection was obtained in 50% of the animals tested at the 1:200 serum dilution. Sera from sheep inoculated with natural crotalic venom only gave protection up to 1:40 dilution. These results show that antibodies induced by IrV were 5 times more potent than NV. Rawat *et al.* (22) compared the ED<sub>50</sub> (effective dose 50%) of sheep sera and equine commercial sera collected in the seventh and fourteenth weeks after the first inoculation. Sheep serum neutralized 2 to 5 times the LD<sub>50</sub> of *Micrurus fulvius fulvius* venom. Commercially-produced sera were less effective with 2 LD<sub>50</sub> and totally ineffective with 5 LD<sub>50</sub>.

The animals in the two groups did not show any local alteration when clinically examined, even with double concentration of IrV. A small volume increase in the pre-scapular lymphonodes occurred in the two weeks after administration but disappeared in the following weeks. Sjostrom *et al.* (28) found that sheep tolerated venoms of different snakes emulsified in Freund's adjuvant and other adjuvants, without

developing local lesions and quickly reached high circulating IgG class antibody levels. The two sheep challenged with 1mg/kg of NV, one from each group, did not show any clinical alteration of envenoming throughout the observation period.

Indirect ELISA test was used to monitor and compare antibody titers in sheep of both groups. Both groups were immunogenic and induced specific antibody formation. Means and standard deviation of optical density detected by ELISA for the 1:1,000 and 1:16,000 dilutions of pre- and post-immune sera show no significant difference ( $p>0.05$ ) between the two groups. There was no interaction effect ( $p>0.05$ ) between the treatments, but there was similarity in behavior. However, there was a small time effect in the set of the two groups ([Tables 1](#) and [2](#) and [Figure 1](#)).

**Table 1.** Means and standard deviation of optic density (490 nm) for indirect immunoenzymatic assay (ELISA), using sheep (n=12) sera (1:1,000) inoculated at days zero, 30, and 60 with natural (I) and Cobalt60-irradiated (II) crotalic venom.

WEEKS															
GROUP	0*	1	2	3	4*	5	6	7	8*	9	10	11	12	1	
I	0.162	0.242	1.576	1.652	1.656	1.703	1.712	1.527	1.777	1.808	1.792	1.815	1.733	1.7	
	±	±	±	±	±	±	±	±	±	±	±	±	±	±	
	0.045	0.228	0.495	0.485	0.499	0.470	0.500	0.826	0.428	0.410	0.469	0.453	0.452	0.4	
II	0.147	0.303	1.654	1.655	1.589	1.669	1.847	1.888	1.774	1.751	1.807	1.782	1.776	1.7	
	±	±	±	±	±	±	±	±	±	±	±	±	±	±	
	0.036	0.239	0.425	0.483	0.509	0.476	0.448	0.427	0.431	0.404	0.414	0.424	0.404	0.3	
MEAN	0.154	0.272	1.615	1.654	1.623	1.686	1.779	1.708	1.776	1.780	1.799	1.799	1.754	1.7	
TIME	(a)	(a)	(b)	(b)	(b)	(b)	(b)	(b)	(b)	(b)	(b)	(b)	(b)	(b)	

\* Inoculations

Interaction:  $F = 0.81$ ;  $p > 0.10$

Difference between groups:  $F = 0.01$ ;  $p > 0.50$ ,  $G I = G II$ .

Difference between times:  $F = 54.42$ ;  $p < 0.05$ .

**Table 2.** Means and standard deviation of optic density (490 nm) for indirect immunoenzymatic assay (ELISA), using sheep (n=12) sera (1:16,000) inoculated at days zero, 30, and 60 with natural (I) and Cobalt60-irradiated (II) crotalic venom.

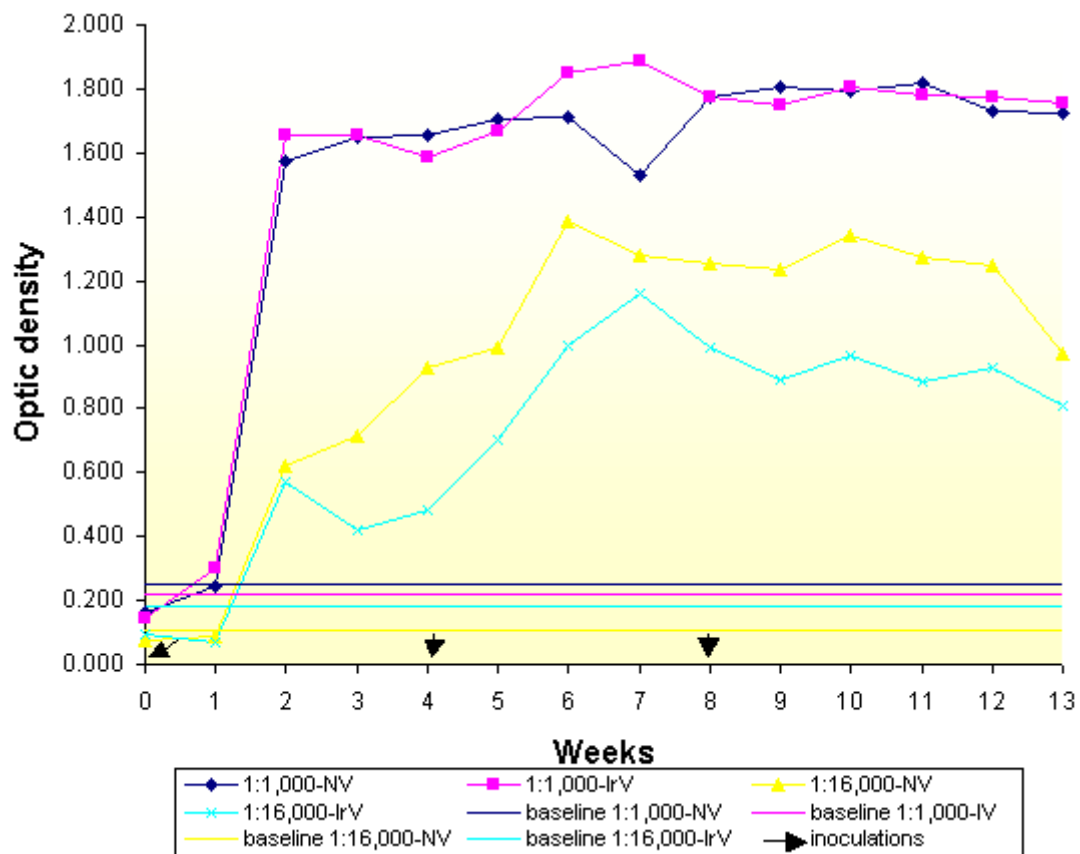
WEEKS															
GROUP	0*	1	2	3	4*	5	6	7	8*	9	10	11	12	13	
I	0.075	0.087	0.623	0.712	0.930	0.988	1.388	1.282	1.256	1.236	1.346	1.273	1.250	0.9	
	±	±	±	±	±	±	±	±	±	±	±	±	±	±	
	0.015	0.021	0.305	0.353	0.445	0.519	0.319	0.341	0.333	0.357	0.357	0.418	0.267	0.2	
II	0.091	0.070	0.571	0.419	0.481	0.705	0.997	1.161	0.988	0.890	0.965	0.882	0.931	0.8	
	±	±	±	±	±	±	±	±	±	±	±	±	±	±	
	0.045	0.018	0.289	0.235	0.308	0.302	0.515	0.427	0.495	0.435	0.505	0.464	0.365	0.3	
MEAN TIME	0.083	0.078	0.597	0.565	0.705	0.846	1.192	1.221	1.122	1.063	1.155	1.077	1.091	0.88	
	(a)	(a)	(b)	(b)	(b)	(b)	(c)	(c)	(c)	(c)	(c)	(c)	(c)	(b)	

\* Inoculations

Interaction:  $F = 1.50$ ;  $0.05 < p < 0.10$

Difference between groups:  $F = 2.40$ ;  $p > 0.10$ ,  $G1 = G11$ .

Difference between times:  $F = 35.13$ ;  $p < 0.05$ .



**Figure 1.** Mean of optic density (490nm) for indirect immunoenzymatic assay (ELISA), using sheep (n=12) sera (1:1,000 and 1:16,000) inoculated with natural (NV) and Cobalt60-irradiated (IrV) crotalic venom.

The groups were similar ( $p>0.05$ ) at 1:32,000 dilution, and therefore, there was an interaction effect ( $p<0.05$ ) between treatments. Significant differences were seen ( $p<0.05$ ) between the moments in each group and between the groups at each moment, with greater optical density values for NV in the sixth and tenth weeks (Table 3 and Figure 2). The same results were seen at 1:64,000 dilution, where the best indexes were observed in sera collected in the 6th, 7th, and 10th weeks (Figure 2).

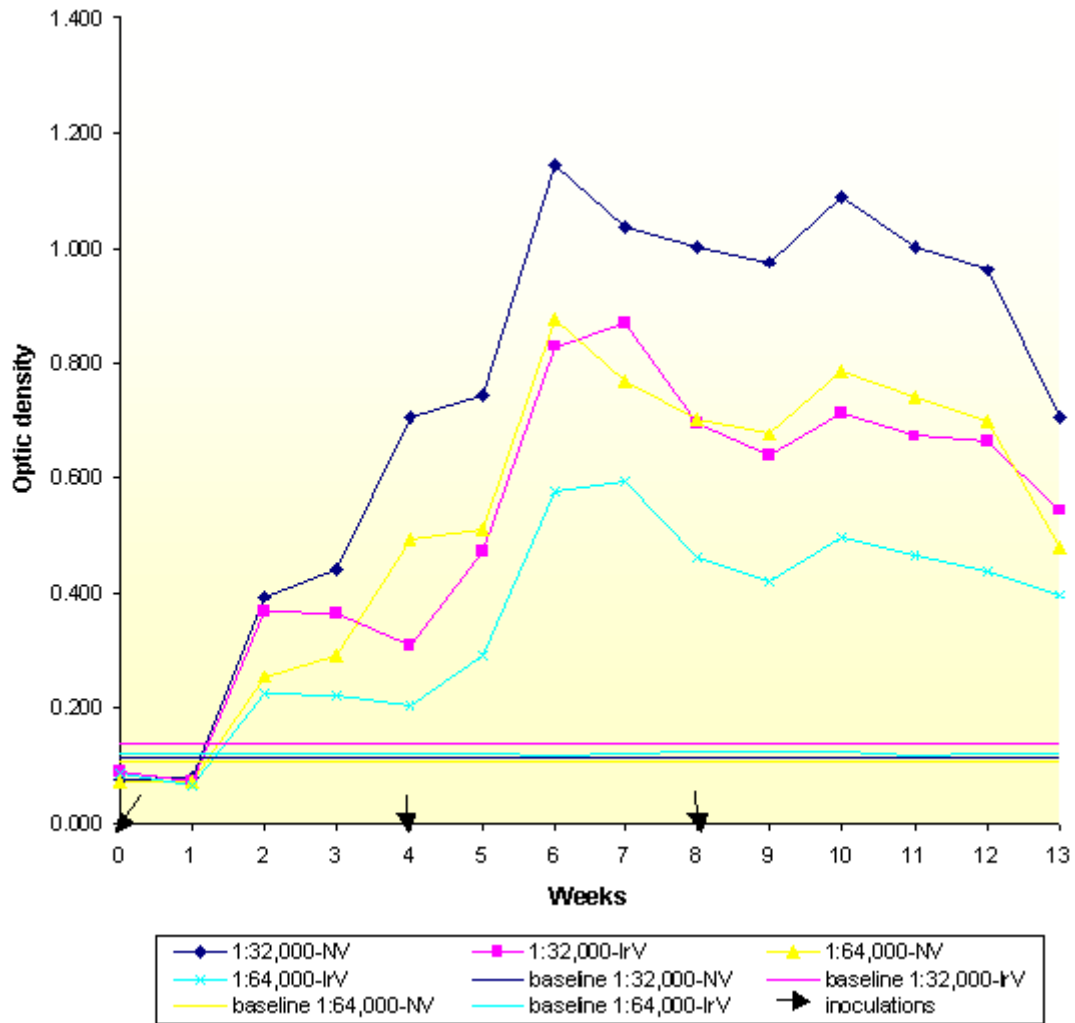
**Table 3.** Means and standard deviation of optic density (490 nm) for indirect immunoenzymatic assay (ELISA), using sheep (n=12) sera (1:32,000) inoculated at days zero, 30, and 60 with natural (I) and Cobalt60-irradiated (II) crotalic venom.

WEEKS															
GROUP	0*	1	2	3	4*	5	6	7	8*	9	10	11	12	1	
I	0.075	0.077	0.396	0.441	0.706	0.743	1.146	1.035	1.003	0.977	1.088	1.002	0.963	0.71	
	(Aa)	(Aa)	(Ab)	(Ab)	(Bb)	(Ab)	(Ac)	(Ac)	(Ac)	(Ac)	(Bc)	(Ac)	(Ac)	(Ab)	
	±	±	±	±	±	±	±	±	±	±	±	±	±	±	
	0.020	0.019	0.191	0.231	0.440	0.497	0.331	0.337	0.324	0.373	0.357	0.385	0.250	0.2	
II	0.088	0.070	0.367	0.365	0.311	0.473	0.828	0.870	0.696	0.639	0.714	0.673	0.663	0.5	
	(Aa)	(Aa)	(Aa)	(Aa)	(Aa)	(Aa)	(Ab)	(Ab)	(Ab)	(Ab)	(Ab)	(Ab)	(Ab)	(Ab)	
	±	±	±	±	±	±	±	±	±	±	±	±	±	±	
	0.026	0.015	0.192	0.196	0.192	0.241	0.469	0.424	0.369	0.402	0.443	0.417	0.295	0.2	
MEAN	0.081	0.073	0.382	0.403	0.608	0.608	0.987	0.953	0.850	0.808	0.901	0.838	0.813	0.6	
TIME															

\* Inoculations

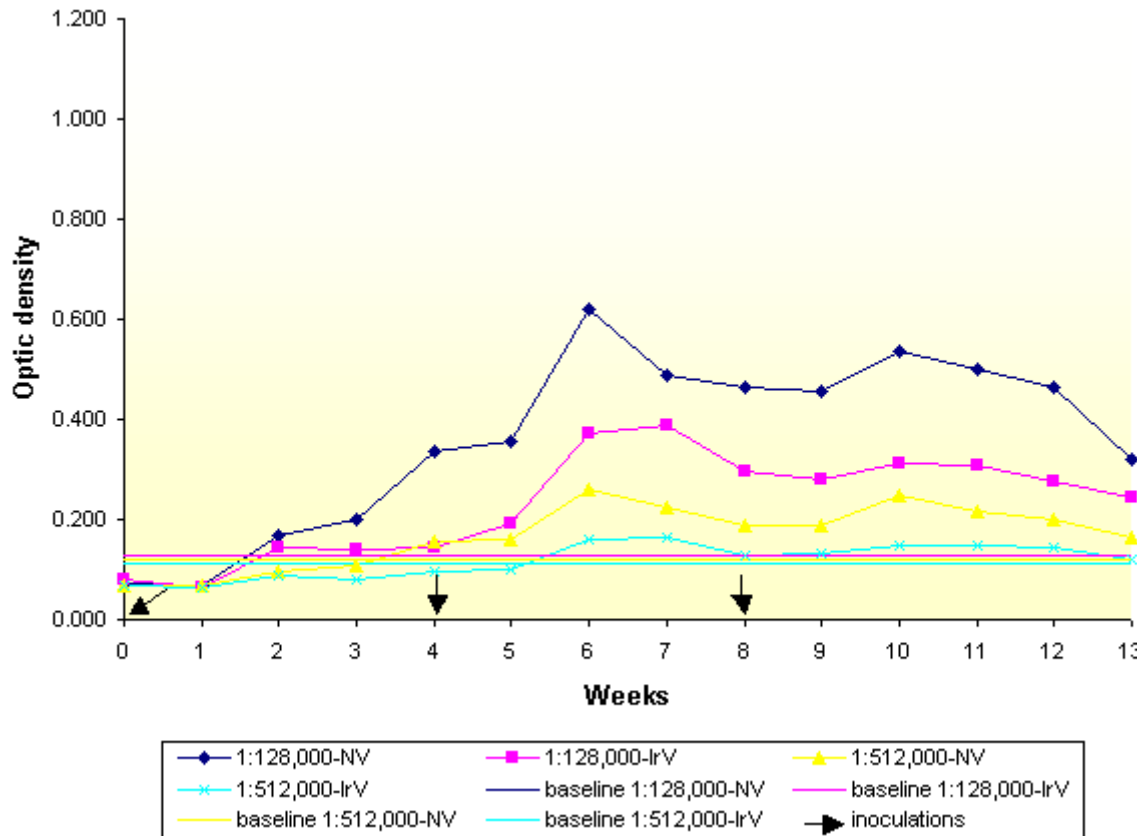
Difference between groups:  $F=1.99$ ;  $p>0.10$ ,  $G1=GII$ .

Interaction:  $F=1.90$ ;  $p<0.05$



**Figure 2.** Mean of optic density (490nm) for indirect immunoenzymatic assay (ELISA), using sheep (n=12) sera (1:32,000 and 1:64,000) inoculated with natural (NV) and Cobalt60-irradiated (IrV) crotalic venom.

When the sera were tested at dilutions 1:128,000 and 1:512,000, analysis of the results showed a statistically similar behavior to that of the initial dilution (Figure 3). Serum antibody levels showed results considered positive at 1:128,000 dilution two weeks after inoculation in both groups (Figure 3). The NV group was still positive at 1:512,000 dilution up to the 24th week (Figure 3).



**Figure 3.** Mean of optic density (490nm) for indirect immunoenzymatic assay – ELISA, using sheep (n=12) sera (1:128,000 and 1:512,000) inoculated with natural (NV) Cobalt60-irradiated (IrV) crotalic venom.

Indirect ELISA detected the presence of antibodies at levels considered positive up to 1:64,000 dilution, which persisted to the end of the experiment, that is, 24 weeks after the first IrV inoculation (Figure 2). In the NV group all serum dilutions tested remained within values considered positive up to the 24th week (Figure 2).

The results of this study were better than those found in literature (22,28,29). Sjostrom *et al.* (28) tested antivenom produced in sheep and compared it with equine commercial antivenom; positive results were obtained by ELISA at 1:25,000 dilution for sheep serum and 1:7,000 for commercial serum. Rawat *et al.* (22) also tested serum obtained in sheep and found that the antibody levels in these animals rose rapidly, and that six weeks after the first immunization, they were positive at dilutions higher than 1:100,000 and remained so even after the end of the experiment.

The results of this study show the potential of using sheep in crotalic antivenom production. The use of IrV in sheep immunization induces a potent and lasting humoral immune response, which can be shown both by neutralization and potency tests performed *in vitro* to detect antibody levels by indirect ELISA.

These results open the perspective of sheep immunization with *Crotalus durissus terrificus* venom for commercial production of antivenom in treating reptile envenoming in humans and animals.

## REFERENCES

- 1 ARAÚJO P., BELLUOMINI HE. Toxicidade de venenos ofídicos – I - Sensibilidade específica de animais domésticos e de laboratório. *Mem. Inst. Butantan*, 1960-62, 30, 133-42. [ [Links](#) ]
- 2 BRADFORD MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 1976, 72, 248-54. [ [Links](#) ]
- 3 CHICHEPORTICHE R., ROCHAT C., SAMPIERI F., LAZDUNSKI M. Structure-function relationships of neurotoxins isolated from *Naja naja* venom: physicochemical properties and identification of the active site. *Biochemistry*, 1972, 11, 1681-91. [ [Links](#) ]
- 4 CLISSA PB., NASCIMENTO N., ROGERO JR. Toxicity and immunogenicity of *Crotalus durissus terrificus* venom treated with different doses of gamma rays. *Toxicon*, 1999, 37, 1131-41. [ [Links](#) ]
- 5 DART RC., SIEFERT SA., CARROL L., CLARK RF., HALL EL., BOYER HASSEN LV., CURRY SC., KITCHENS CS., GARCIA RA. Human trial of an affinity purified antibody fragment for snake venom poisoning. *Vet. Hum. Toxicol.*, 1994, 36, 363. [ [Links](#) ]
- 6 EGEN N., RUSSEL F., CONSROE P., GERRISH K., DART R. A new ovine Fab antivenom for north American venomous snakes. *Vet. Hum. Toxicol.*, 1994, 36, 362. [ [Links](#) ]
- 7 EK N. Serum levels of the immunoglobulins IgG and IgG(T) in horses. *Acta Vet. Scand.*, 1974, 15, 609-19. [ [Links](#) ]
- 8 FINNEY DJ. *Probit analysis*. Cambridge: Cambridge University Press, 1971: 333. [ [Links](#) ]
- 9 FLOWERS HH. Effects of X-irradiation on the antigenic character of *Agkistrodon piscivorus* (cottonmouth moccasin) venom. *Toxicon*, 1966, 3, 301-4. [ [Links](#) ]
- 10 FREITAS TV., FREZARD F. Encapsulation of native for the production of antivenom and vaccination against *Crotalus durissus terrificus* venom. *Toxicon*, 1997, 35, 91-100. [ [Links](#) ]
- 11 GUIDOLIN R., CORRÊA A., CICARELLI RMB., PREVIDE E., MORAIS JF., HIGASHI HG. Esterilização de soros e vacinas por radiação gama de Cobalto. *Rev. Saúde Públ. (São Paulo)*, 1988, 22, 113-7. [ [Links](#) ]
- 12 GUIDOLIN R., DIAS DA SILVA W., HIGASHI HG., CARICATI CP., LIMA MLR., MORAIS JF., PINTO JR., MARCELINO JR. Hiperimunização de cavalos soroprodutores com venenos botrópicos e crotálico tratados por glutaraldeído. *Mem. Inst. Butantan*, 1989, 51, 85-90. [ [Links](#) ]
- 13 KAISER II., MIDDLEBROOK JL., CRUMRINE MH., STEVENSON WW. Cross-reactivity and neutralization by rabbit antisera raised against crotoxin, its subunits and two related toxins. *Toxicon*, 1986, 24, 669-78. [ [Links](#) ]
- 14 KHAN ZH., LARI FA., ALI Z. Preparation of toxoids from the venoms of Pakistan species of snakes (*Naja naja*, *Vipera russelii* and *Echis carinatus*). *Jpn. J. Med. Sci. Biol.*, 1977, 30, 19-23. [ [Links](#) ]

- 15 MANDAL M., HATI RN., HATI AK. Neutralization of pathophysiological manifestations of Russel's viper envenoming by antivenom raised against gamma-irradiated toxoid. *Toxicon*, 1993, 31, 213-6. [ [Links](#) ]
- 16 MOROZ C., GOLDBLUM N., VRIES A. Preparation of *Vipera palestinae* antineurotoxin using carboximethyl-cellulose-bound neurotoxin as antigen. *Nature*, 1963, 16, 697-8. [ [Links](#) ]
- 17 MURATA Y., ROGERO JR. Análise cromatográfica por exclusão em amostras de veneno de cascavel irradiadas com Co60. *IPEN – Inst. Pesq. Energ. Nucl.*, 1988, 154, 1-7. [ [Links](#) ]
- 18 MURATA Y., NISHIKAWA AK., NASCIMENTO N., HIGASHI HG., DIAS DA SILVA W., ROGERO JR. Gamma irradiation reduces the toxic activities of *Crotalus durissus terrificus* venom but does not affect their immunogenic activities. *Toxicon*, 1990, 28, 617-8. [ [Links](#) ]
- 19 NASCIMENTO N. *Caracterização bioquímica e imunológica dos principais produtos gerados pela irradiação de crotoxina*. São Paulo: Universidade de São Paulo, IPEN - Inst. Pesq. Energ. Nucl., 1995, 77p. [Tese- Doutorado]. [ [Links](#) ]
- 20 OKONOGI T., HATTORI Z., OGISO A., MITSUI S. Detoxification by persimmon tannin of snake venoms and bacterial toxins. *Toxicon*, 1979, 17, 524-7. [ [Links](#) ]
- 21 PIMENTEL GOMES F. *Estatística experimental*. São Paulo: Nobel, 1969: 446. [ [Links](#) ]
- 22 RAWAT S., LAING G., SMITH DC., THEAKSTON D., LANDON J. A new antivenom to treat eastern coral snake (*Micrurus fulvius fulvius*) envenoming. *Toxicon*, 1994, 32, 185-90. [ [Links](#) ]
- 23 ROCHA OA., BICALHO AFX., SILVEIRA JN., LOPES ES., HENEINE IF. A nontoxic derivative of *Bothrops jararaca* venom suitable to generate antibodies against the native venom. *Dtsch. tierärztl. Wochenschr.*, 1992, 99, 143-5. [ [Links](#) ]
- 24 ROGERO JR., NASCIMENTO N. Atenuação da toxicidade de venenos ofídicos por meio da radiação ionizante. *Biotechnol. Cien. Desenvolv.*, 1997, 1, 24-6. [ [Links](#) ]
- 25 RUSSEL FE., TIMMERMAN WF., MEADOWS PE. Clinical use of antivenin prepared from goat serum. *Toxicon*, 1970, 8, 63-5. [ [Links](#) ]
- 26 SALAFRANCA ES. Irradiated cobra (*Naja naja philippinensis*) venom. *Int. J. Appl. Radiat. Isot.*, 1973, 24, 60. [ [Links](#) ]
- 27 SCHÖTTLER WHA. Antigen-antibody relations in the present antivenin production of Brazil. *Am. J. Trop. Med.*, 1951, 31, 500-9. [ [Links](#) ]
- 28 SJOSTROM L., AL-ABDULLA IH., RAWAT S., SMITH DC., LANDON J. A comparison of ovine and equine antivenoms. *Toxicon*, 1994, 32, 427-33. [ [Links](#) ]
- 29 SMITH D., REDDI KR., LAING G., THEAKSTON RGD., LANDON J. An affinity purified ovine antivenom for the treatment of *Vipera berus* envenoming. *Toxicon*, 1992, 30, 865-71. [ [Links](#) ]
- 30 SOERENSEN B. Produção de soros antipeçonhentos e características imunológicas de cada soro. Anavenenos. In: \_\_. *Animais peçonhentos*. Rio de Janeiro: Atheneu, 1990: 91-107. [ [Links](#) ]

31 TEJASEN P., OTTOLENGHI A. The effect of ultra-violet light on the toxicity and the enzymatic and antigenic activities of snake venom. *Toxicon*, 1970, 8, 225-33. [ [Links](#) ]

32 WHO. *Progress in the characterization of venoms and standardization of antivenoms*. Geneva: World Health Organization, 1981: 23-4. [ [Links](#) ]

*Received October 18, 2001*

*Accepted November 30, 2001*



**CORRESPONDENCE TO:**

D. P. Netto - Departamento de Medicina Veterinária Preventiva, Centro de Ciências Agrárias, Universidade Estadual de Londrina, Campus Universitário, 86051-990, Londrina, Paraná, Brasil.

[netto@uel.br](mailto:netto@uel.br)