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LABORATORY EVALUATION OF YOUNG OVINES INOCULATED WITH NATURAL OR ^{60}Co-IRRADIATED *Crotalus durissus terrificus* VENOM DURING HYPERIMMUNIZATION PROCESS

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ABSTRACT: Laboratory profile of young ovines was studied in order to evaluate and compare their antiserum production from natural and Cobalt-60 irradiated *Crotalus durissus terrificus* (*C.d.t.*) venoms. The parameters analyzed included complete blood count, and urea, creatinine, aspartate aminotransferase, total proteins, albumin and globulin serum measurements. Three groups of six animals each were used. Group 1 (G1) received natural *C.d.t.* venom; Group 2 (G2) received irradiated *C.d.t.* venom; and Group 3 (G3) was used as control and did not receive venom, only adjuvants, using seven venom inoculations. During the experimental period, animals were fortnightly weighed. According to clinical and weight evaluation, sheep in post-weaning phase showed no changes in their physiological profiles but had excellent weight gain. The parameters analyzed were not statistically different (p<5%) among the groups tested. The hyperimmunization process was successfully accomplished with the production of specific antibodies against *Crotalus durissus terrificus* venom. Results bring a new possibility of utilizing ovines in the commercial production of anticrotalic serum, which may be used to treat human and animal envenomation. Its production cost may be reduced by subsequent use of hyperimmunized sheep for human consumption.

KEY WORDS: *Crotalus durissus terrificus*, hyperimmunization, ovines, antivenom, irradiation.

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INTRODUCTION

Accidents by the species *Crotalus durissus terrificus* account for 14% of the ophidic accidents in Brazil with a high mortality rate (12, 28).

Venom from rattlesnakes is extremely toxic although poorly immunogenic (14, 15), which is partially due to the presence of immunosuppressant components (11, 15, 17, 38). Moreover, damage caused to animals after inoculation of crude venom contributes to low antivenom production (31).

Problems observed in patients allergic to equine serum have led to the development of alternative immunization techniques using other animals, which, besides the low cost, have presented excellent results in heterologous serum production (18, 19, 32-37).

Sjostrom *et al.* (37) produced antivenoms in sheep and compared them with commercial equine serum. Sheep showed tolerance to adjuvants, no local alterations, and fast increase of highly specific antibody titer.

Many researchers have been seeking alternatives to prepare toxoid through venom biological detoxification, which would keep its immunogenicity and minimize the damages to serum producer animals (5, 13, 26, 39).

Gamma radiation has been efficient in attenuating ophidic venoms and in decreasing toxicity without altering immunogenicity, and the addition of substances to the venom was not necessary (1, 2, 8, 10, 16, 20, 21, 32).

Crotalus durissus terrificus venom causes hematological alterations in erythrocytes, leukocytes, platelets, coagulation factors (6) and fibrinogen (3, 40) when inoculated into humans and animals (6).

The aim of the present paper was to evaluate and compare the laboratory profile of young ovines inoculated with natural and ^{60}Co-irradiated *Crotalus durissus terrificus* venom during the hyperimmunization process. The animals were subjected to hematological exams, biochemical measurements and parasitological tests. These laboratory parameters allowed the evaluation of helminthic infestation, hydration status, and immunological, hepatic and renal functions.

MATERIALS AND METHODS

Crude air-dried venom from a large number of South American rattlesnakes, *Crotalus durissus terrificus (C.d.t.)*, was provided from The Center for the Study of Venoms

and Venomous Animals, CEVAP, UNESP, Botucatu, Brazil. Swiss mice (18-22 g) were obtained from the animal facility at the same Institute.

We used 18 male Santa Inês and Ile de France sheep of 60-70 days old, which were kept in the Laboratory for Studies of Reproductive Biotechnology, School of Veterinary Medicine and Animal Husbandry, UNESP, Botucatu, Brazil.

Venom irradiation

*Crotalus durissus terificus w*hole 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 (9). Samples were irradiated at 5.25 KGy/h with 2000 Gy 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 (30). These experiments were performed at the Institute of Nuclear and Energetic Research, IPEN/CNEM/SP.

Sheep immunization

We used three groups of six animals each. Group 1 (G1) received natural *C.d.t.* venom; Group 2 (G2) received irradiated *C.d.t.* venom; and Group 3 (G3) was used as control and did not receive venom, only adjuvants.

Inoculation occurred at six different moments (M): At day one, animals received 500 µg venom diluted with 1 ml saline solution (PBS) homogenized in 1 ml of Freund's Complete Adjuvant (FCA), by intradermal route; at days 14 and 28, they received 1 mg venom diluted with 1 ml PBS homogenized in 1 ml of aluminium hydroxide ($AlOH_3$), by subcutaneous route; at day 42, they received 1.5 mg venom diluted with 2 ml PBS, by subcutaneous route; and at days 56 and 70, animals received 2 mg venom diluted with 2 ml PBS, by subcutaneous route. At day 84, animals were bled and did not receive treatment.

Each animal was injected with 2 ml venom into four different regions of the neck.

Laboratory Evaluation

Complete blood count, biochemical and parasitological exams were performed every 14 days during the experimental period, resulting in seven different moments (M1, M2, M3, M4, M5, M6, M7).

During this period, animals were also weighed.

Total red blood cells and leukocytes count was performed in an automatic cell counter. Hemoglobin was determined by the colorimetric method based on cyanmethemoglobin formation, and globular volume was assessed by the microhematocrit method.

Differential leukocytes count was performed in 100 cells in smears stained according to Rosenfeld method (23).

Plasma total protein concentration was measured by refractometry, and fibrinogen was assessed by the method of heat precipitation (56ºC), as indicated by Kaneko & Harvey (25), and subsequent refractometry.

Samples for cell blood count and plasma total protein and fibrinogen were obtained in EDTA-containing tubes.

Serum samples for the biochemical tests were obtained by centrifugation of total clotted blood. They were stored in 1.5-ml aliquots in polyethylene tubes at -20ºC until use. Biochemical tests were analyzed by spectrophotometry:

All reagents were pro-analysis grade.

- *Urea:* colorimetric enzymatic method.
- *Creatinine:* colorimetric method with kinetic alkaline picrate reaction.
- *Total serum protein:* colorimetric method with biuret reaction.
- *Albumin:* colorimetric method with bromocresol green reaction.
- *Globulin:* difference between total serum protein and albumin concentrations.
- *Aspartate aminotransferase (AST):* optimized kinetic UV method.

Animals received a dose of 1 ml/40 kg weight of vermifuge Levamisol (Ripercol L-150F®), which was repeated after 15 days. The number of eggs per feces [epf] (22) was counted three times: at days 1, 15 and 30, respectively; the first count was before treatment for control.

Statistical analysis

Groups and moments (days) mean values were compared by analysis of repeated measures of the groups mean profiles (or a similar nonparametric procedure) according to Johnson and Wichern (24). Significance level was set at 5% in the F test.

RESULTS AND DISCUSSION

Red blood cells, hemoglobin counts and globular volume showed values within the reference range in the three groups tested with no statistical difference among them. We also noticed a slight increase throughout the experiment.

These results demonstrate that crotalic venom used in the hyperimmunization process did not interfere in sheep red blood cells.

Measurement of plasma protein and fibrinogen showed values within the reference range for the three groups tested with no statistical difference among them.

Platelet count values were not within the reference range for the three groups tested but no statistical difference among them was observed. We noticed a significant increase at day 84 (M7) in the three studied groups.

Transitory thrombocytosis may occur due to the epinefrin action during stress, causing splenocontraction and releasing high number of platelets into circulation. Cytokines (IL1, IL3, IL6 and IL11), when produced in inflammatory processes and in reactions that stimulate the immune system, activate megakaryocytes colony factor and can also cause thrombocytosis (27).

As electronic cell count device can count the small ovine erythrocytes as platelets, there may occur technical interference (23), but it would affect all moments of the experiment.

White blood cells count showed that the mean number of leukocytes and neutrophils was within the reference range or limits in the three groups tested, except at day 14 (M2) for the control group, in which an animal had 22,300 leukocytes (/µl) and 13,200 neutrophils (/µl), elevating the group mean values because of an abscess caused by the first inoculation with Freund's Complete Adjuvant. As treatment, we used 5 mg/kg Enrofloxacine (Baytril®) once a day for 5 days. Thereafter, values were within the reference range again. No statistical difference was observed among the groups studied.

Total count of eosinophils and monocytes showed that the mean values found were within the reference range in all the groups without any statistical difference among them.

Serum urea concentration was within the reference range values in the three groups tested, except for Groups 2 and 3 at M1. There was not statistical difference among groups. These animals might have presented values slightly higher than normal due

to prerenal causes such as: increased protein ingestion, dehydration, gastrointestinal hemorrhage, heart disease, septic or traumatic shock (7, 27).

Serum creatinine measurement showed values below the reference range in all the groups tested at every moment studied, but no statistical difference was observed among them.

Serum albumin concentration was within the reference range values for the three groups tested, except at days 28 (M3) and 42 (M4), when all groups had values slightly below the ones considered normal for the species. No statistical difference among groups was observed.

According to serum globulin measurement, the values found were within the reference range for all the groups tested at days 01 (M1), 14 (M2), and 70 (M6). There was not statistical difference among groups.

Albumin:globulin relationship might be altered when there is high production of globulins (immunoglobulins). The liver then decreases albumin production to keep the normal relationship. In inflammatory processes, the liver diminishes the albumin yield to produce acute-phase proteins [inflammatory proteins] (29).

Aspartate aminotransferase (AST) measurement showed values within the reference range in the three groups tested. No statistical difference was observed among groups.

The laboratory parameters for the species had been based on Jain NC (23).

All the animals had excellent weight gain throughout the experimental period. The three groups studied did not show any statistical difference.

According to these observations, we can suggest the production of antiserum from young and young-adult sheep, since the hyperimmunization process caused no effects on the animals growth and weight gain; instead, they produced antibodies against *Crotalus durissus terrificus* venom.

Through parasitological monitoring, we could observe a decrease of endoparasites when counting the number of eggs per gram of feces (epg) at three moments throughout the experiment. The antihelmintic scheme adopted showed a tendency of a decrease of infestation by parasites (4).

Animals from different rural properties showed high parasite infestation rates, which was resolved by the correct use of vermifuge. Animals receiving appropriate parasitological control tend to show better weight gain.

The immunized animals presented an excellent immune response, producing an antiserum of high neutralizing capacity. These results will be shown in a future publication.

Analyzing the results all together, we can conclude that:

In the post-weaning phase, sheep presented no alteration in their physiological profiles and had excellent weight gain, indicating that neither natural nor irradiated venom caused debility or nutritional deficiency.

Since development of the sheep tested was normal, hyperimmunization process was successfully accomplished with the production of specific antibodies against *Crotalus durissus terrificus* venom.

Utilization of post-weaning ovines as serum producer animals may be an excellent alternative for sheep raisers. According to these results, they could use these animals as food after the hyperimmunization process, and their blood would be another lucrative byproduct available to them.

The present experiment, with confined young animals, will be extended to field animals in order to evaluate the experimental model efficiency. This model will also be tested with venoms from other snakes that commonly cause accidents in Brazil.

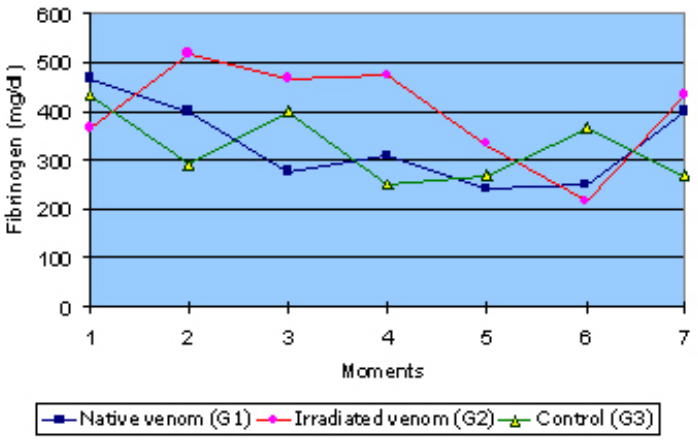

Figure 1: Mean values of fibrinogen dosage (mg/dl) in ovine groups inoculated with natural (G1) and ^{60}Co-irradiated (G2) *C.d.t.* venom, and control group (G3), at different moments.

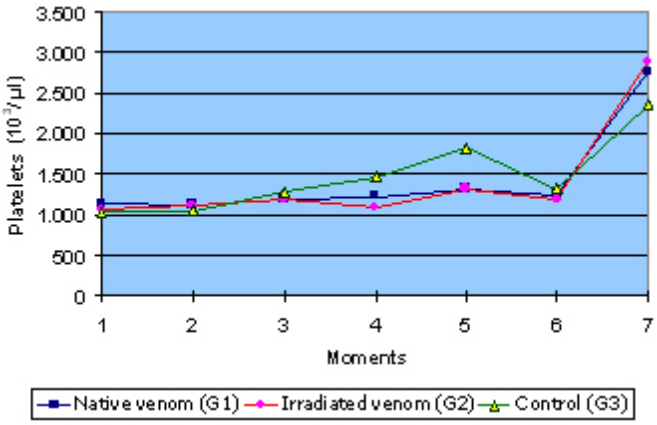

Figure 2: Mean values of total platelets count (103/µl) in ovine groups inoculated with natural (G1) and ^{60}Co-irradiated (G2) *C.d.t.* venom, and control group (G3), at different moments.

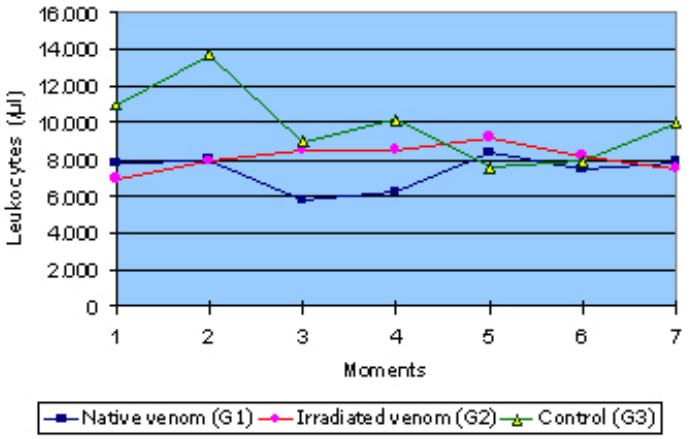

Figure 3: Mean values of total leukocytes count (/µl) in ovine groups inoculated with natural (G1) and ^{60}Co-irradiated (G2) *C.d.t.* venom, and control group (G3), at different moments.

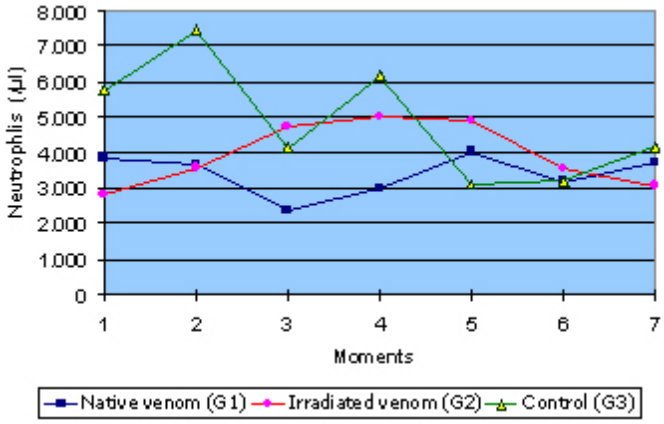

Figure 4: Mean values of total segmented neutrophils count (/µl) in ovine groups inoculated with natural (G1) and ^{60}Co-irradiated (G2) *C.d.t.* venom, and control group (G3), at different moments.

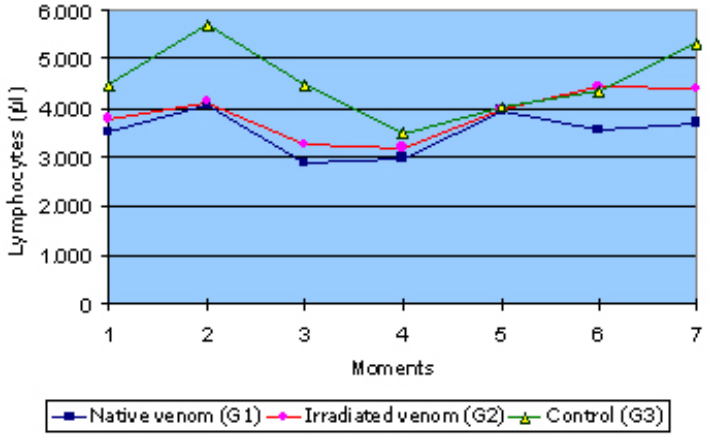

Figure 5: Mean values of total lymphocytes count (/µl) in ovine groups inoculated with natural (G1) and ^{60}Co-irradiated (G2) *C.d.t.* venom, and control group (G3), at different moments.

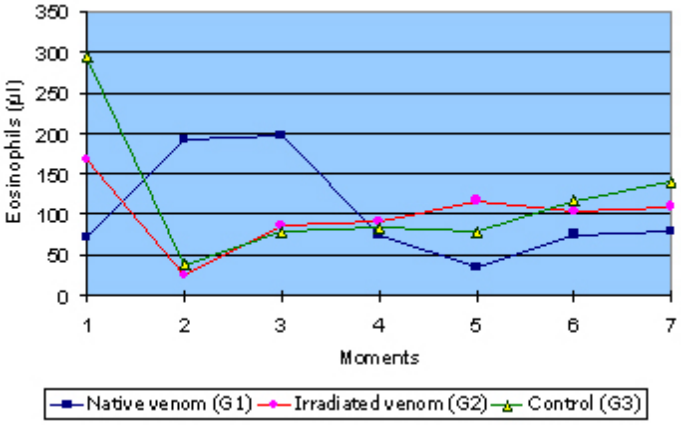

Figure 6: Mean values of total eosinophils count (/µl) in ovine groups inoculated with natural (G1) and ^{60}Co-irradiated (G2) *C.d.t.* venom, and control group (G3), at different moments.

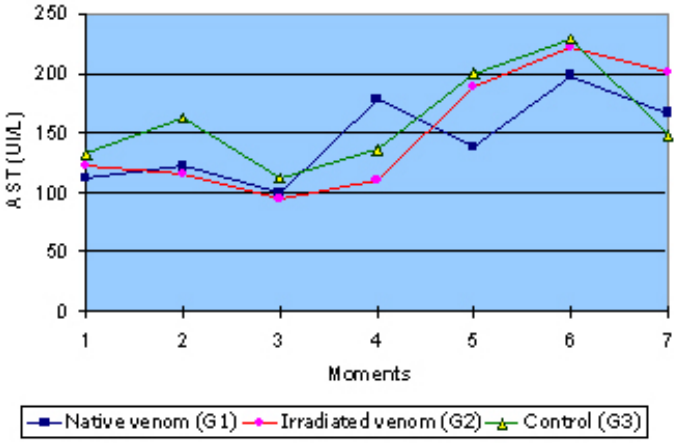

Figure 7: Mean values of aspartate aminotransferase (AST) dosage (UI/l) in ovine groups inoculated with natural (G1) and ^{60}Co-irradiated (G2) *C.d.t.* venom, and control group (G3), at different moments.

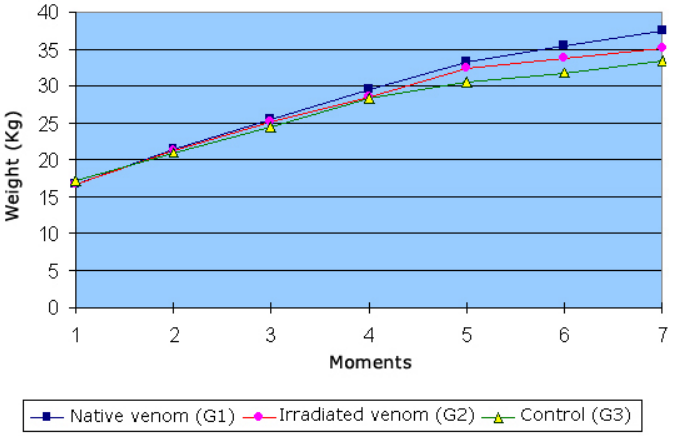

Figure 8: Mean values of weight in ovine groups inoculated with natural (G1) and ^{60}Co-irradiated (G2) *C.d.t.* venom, and control group (G3), at different moments.

Moments: 1= day 1; 2 = day 14; 3 = day 28; 4 = day 42; 5 = day 56; 6 = day 70; 7 = day 84

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