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HISTOPATHOLOGICAL EVALUATION IN EXPERIMENTAL ENVENOMATION OF DOGS WITH *Crotalus durissus terrificus* VENOM

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ABSTRACT: The present work evaluated histopathological aspects in experimental envenomation of dogs with *Crotalus durissus terrificus* venom. Twenty-eight mixed breed adult dogs were divided into three groups of seven animals each: Group I – only venom; Group II – venom + 50ml antiophidic serum + fluid therapy; Group III – venom + 50ml antiophidic serum + fluid therapy + urine alkalization. Lyophilized venom of *Crotalus durissus terrificus* was reconstituted in saline solution and inoculated subcutaneously at the dose of 1mg/kg body weight. Three animals of each group were subjected to euthanasia, and their muscular tissue, brain, spleen, kidneys, heart, lungs, stomach, small and large intestines, and popliteal lymph node fragments were collected for histopathological evaluation. There was myonecrosis in the inoculated limb, renal tubular degeneration, lymphoid hyperplasia of spleen, and unspecific reactive hepatitis. These results show the antigenicity and action of the venom on the immune system.

KEY WORDS: histopathology, *Crotalus durissus terrificus*, dogs, treatment, intoxication.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Ophidic accidents represent a serious public health problem in tropical countries because of the high frequency in which they occur and the high morbidity and mortality they cause (27).

Crotalus venom is considered of great importance for both medicine and veterinary medicine due to the severe clinical signs it causes, and it may be fatal in many cases mainly when the treatment with specific serum is not used early (5, 13, 24). *Crotalus durissus terrificus* venom has as main actions: neurotoxic (36, 37), myotoxic (7, 22), coagulant (2) and hemolytic activities *in vitro* (29). The existence of hemolytic action *in vivo* is not clear (2, 25). The fractions that constitute the venom are frequently the subject of scientific research due to their high number of pharmacologically and biochemically active proteins (35). However, few papers have reported the histopathological alterations in animals and humans envenomed with crotalic venom.

The patient who suffered an accident with *Crotalus durissus terrificus* venom shows discreet alterations at the bite site; however, severe systemic signs are observed in most cases (8). In addition to specific serum therapy, complementary measures are necessary such as the use of sodium bicarbonate to alkalinize the urine, since acid urine potentiate the intratubular myoglobin precipitation (10).

MATERIALS AND METHODS

This experiment was approved by the Ethics Committee of São Paulo State University, UNESP, Botucatu, São Paulo State, Brazil.

Animals

Twenty-one young adult dogs, male and female, were used. They had no defined breed, were clinically healthy (17) and weighed from 4 to 7kg.

Experimental Groups

Three experimental groups (Groups I, II, III) were constituted of seven dogs each. Group I was inoculated with *Crotalus durissus terrificus* venom; Group II received the venom and was treated six hours after inoculation with 50ml (50mg) of anti-bothropic-crotalic serum (Vencofarma®), intravenously, associated with fluid therapy containing sodium chlorate (0.9% NaCl, dose 50ml/kg); Group III was inoculated with venom and treated with 50ml (50mg) of anti-bothropic-crotalic serum intravenously and fluid

therapy (0.9% NaCl, dose 50ml/kg) containing 8.4% sodium bicarbonate (dose 4mEq/kg).

Venom

Crotalus durissus terrificus venom was provided by the Center for the Study of Venoms and Venomous Animals, CEVAP-UNESP, Botucatu, São Paulo State, Brazil.

It was obtained by compressing the venom glands and was further lyophilized and stored at -20ºC, being dissolved in sterile saline solution at the moment of administration in order to obtain the concentration of 40mg/ml. The dose of 1mg/kg was administered to the middle third of the lateral face of the thigh, after local trichotomy and antisepsis.

Three animals from each group were euthanized one week after the administration of crotalic venom. For such procedure, acepromazine (0.2% Acepran-Univet®) was used as preanesthetic agent and pentobarbital sodium (Hypnol 3%-Fontoveter®) was used as anesthetic agent, followed by the administration of 19.1% potassium chlorate solution (Aster®). Then, dogs were sent to the Pathological Anatomy Service at University of Western São Paulo [Universidade do Oeste Paulista – UNOESTE], Presidente Prudente, São Paulo State, Brazil, where they were subjected to complete necropsy according to the routine techniques of the cited Service.

During examination, samples of spleen, brain, heart, stomach, liver, popliteal lymph node, small and large intestines, lungs, kidneys as well as skeletal muscles from the right and left femoral biceps and left and right semitendinosus muscles were collected.

The collected samples were fixed in 10% buffered formalin solution for 48h. They were sectioned and histologically processed for inclusion in paraffin, and 5μ sections were made in rotary microtome. Sections were stained with Hematoxylin-Eosin and modified Masson's Trichrome and examined by optical microscopy (Nikon® Microscope, 400X and 1,000X magnification).

RESULTS

Femoral Biceps and Semitendinosus Muscles

All the evaluated animals presented discreet edema at the site of inoculation of *Crotalus durissus terrificus* venom. The muscles presented well-delimited focal areas

of whitish color due to degeneration and necrosis (Figure 1). Femoral biceps and semitendinosus muscles were macro and microscopically affected (inoculation site). In the adjacent muscles and in the contralateral hind limb, no alteration was found and the muscles were entire in shape, color and texture. The muscle alterations caused by the crotalic venom were characterized by a focal (sparse) or diffuse (severe) necrotic myositis (Figure 2).

As for the myoregenerative activity, among envenomed animals, discreet regeneration was noticed in two and remarkable regeneration in one animal from Group I. All animals from Group II presented discreet regeneration of the muscular fibers. In animals from Group III, a remarkable regeneration was observed (Figures 3 and 4).

Kidneys

All necropsied animals presented tubular degeneration (Figure 5). Tubular necrosis was also found in two animals from Groups I and III and in one animal from Group II (Figures 6 and 7).

Spleen

Reactive lymphoid hyperplasia was present in all animals (Figure 8). Two animals from Group I presented sites of thrombocytosis and in three animals from Group II, there were sites of extramedullary hematopoiesis, evidenced by the presence of megakaryocytes (Figure 9). Hemosiderosis was also seen in two animals from Group III (Figure 10).

Liver

All animals from Groups I and II presented severe sinusoidal congestion and three animals from Group III presented mild sinusoidal congestion after crotalic envenomation. Hemosiderosis in Küpffer cells was noticed in one animal from Group III and multifocal centrilobular necrosis, in two animals from Groups I and II (Figure 11). Two animals from Group III and one animal from Group II presented mild portal mononuclear inflammatory infiltrate. Küpffer cell hyperplasia was seen in one animal from Group III (Figure 12).

Stomach

Moderate gastric mucosal hyperemia was noticed in three animals from Group I and in two animals from Group II which were inoculated with crotalic venom, characterizing acute gastritis (Figure 13).

Popliteal lymph node

The popliteal lymph node from the limb inoculated with crotalic venom showed reactive hyperplasia.

Lungs, brain, heart and large intestine

For Groups I, II and III, which were inoculated with crotalic venom, these organs were also subjected to macro and microscopic analysis; however, no significant alteration was found.

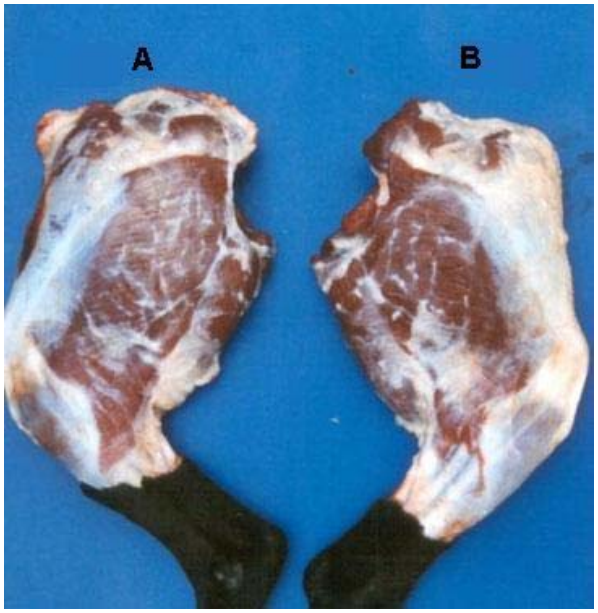

Figure 1. A: Macroscopic aspect of the muscles of the thigh from a dog experimentally envenomed with crotalic venom, showing focal areas of whitish color. B: Muscles of the contralateral hind limb of the animal in A, showing normal color.

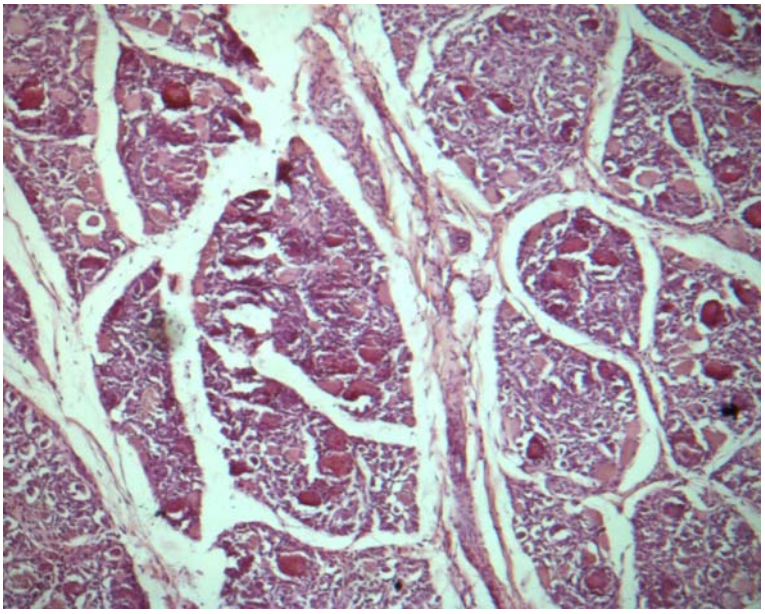

Figure 2. Necrotic muscular fibers. There are some areas of edema dividing the fibers (HE, 100X).

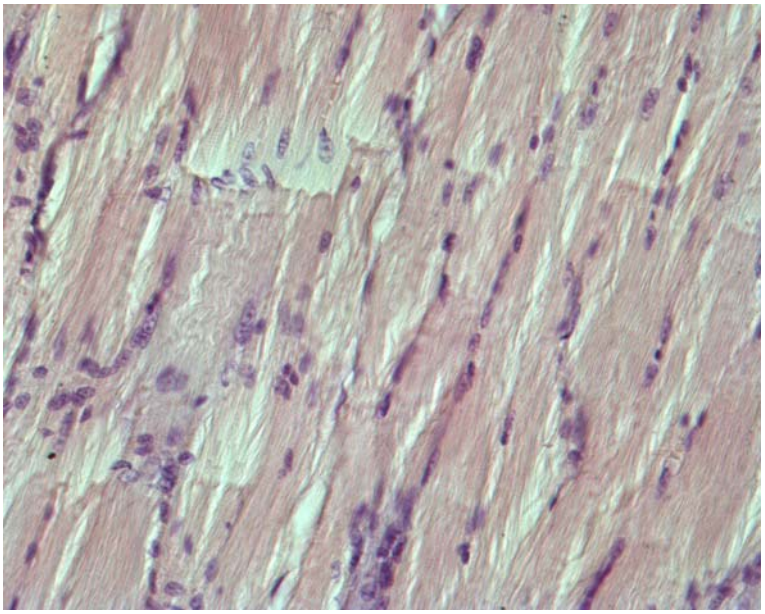

Figure 3. Muscular fibers under remarkable regeneration, with presence of satellite cells, in one animal from Group III (HE, 400X).

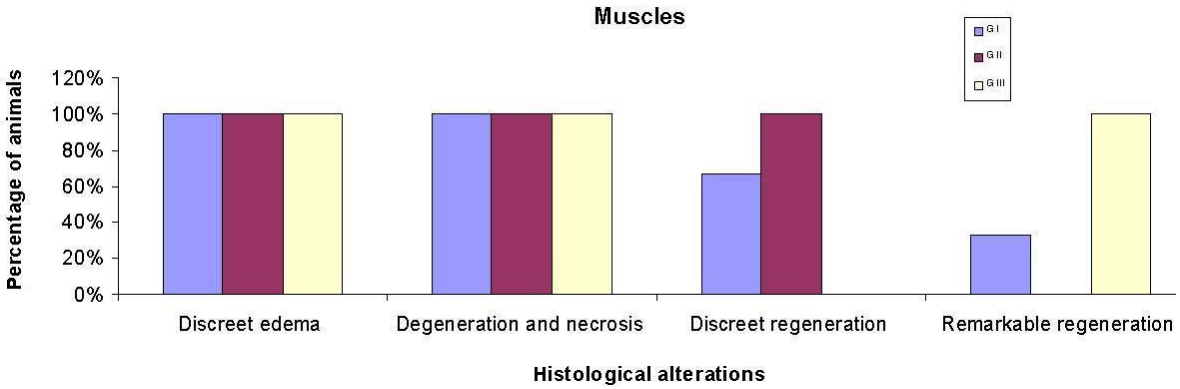

Figure 4. Percentage of animals that developed muscular lesions.

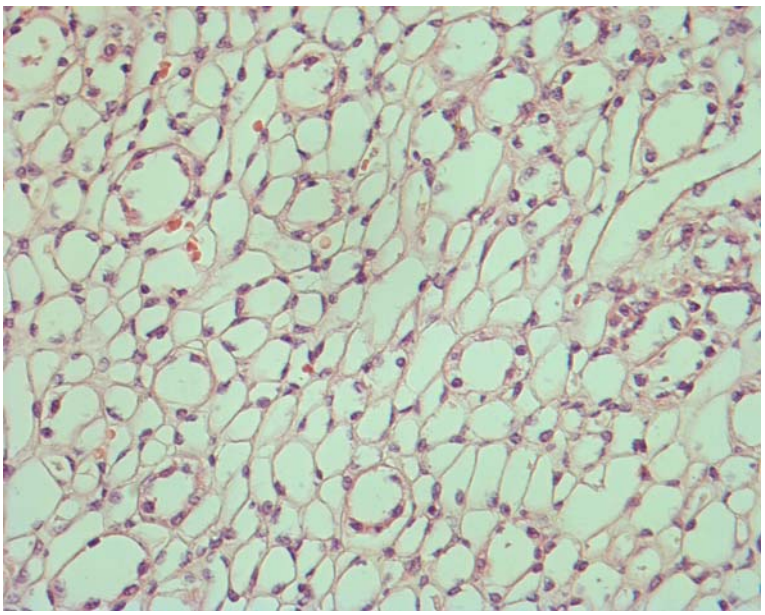

Figure 5. Microscopic aspect of the kidney from a dog inoculated with crotalic venom, showing tubular degeneration (HE, 400X).

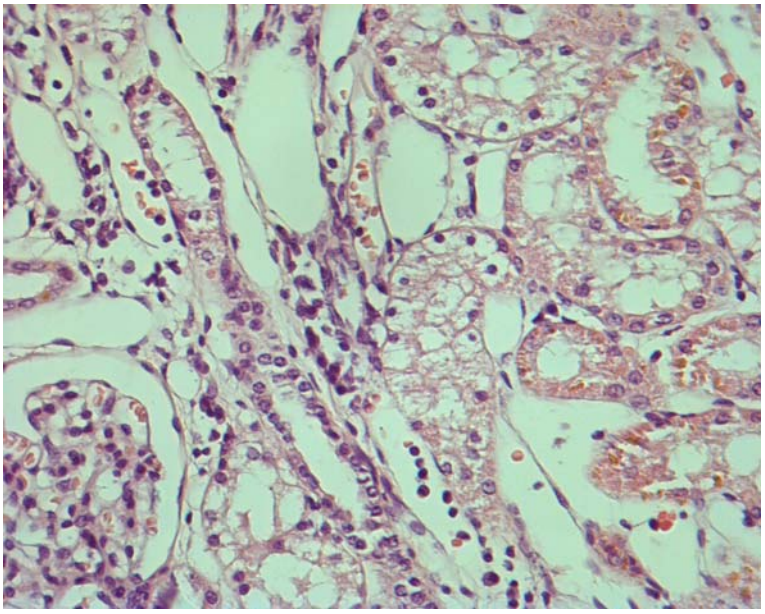

Figure 6. Renal tubular necrosis, with loss of epithelial integrity and presence of inflammatory infiltrate (HE, 400X).

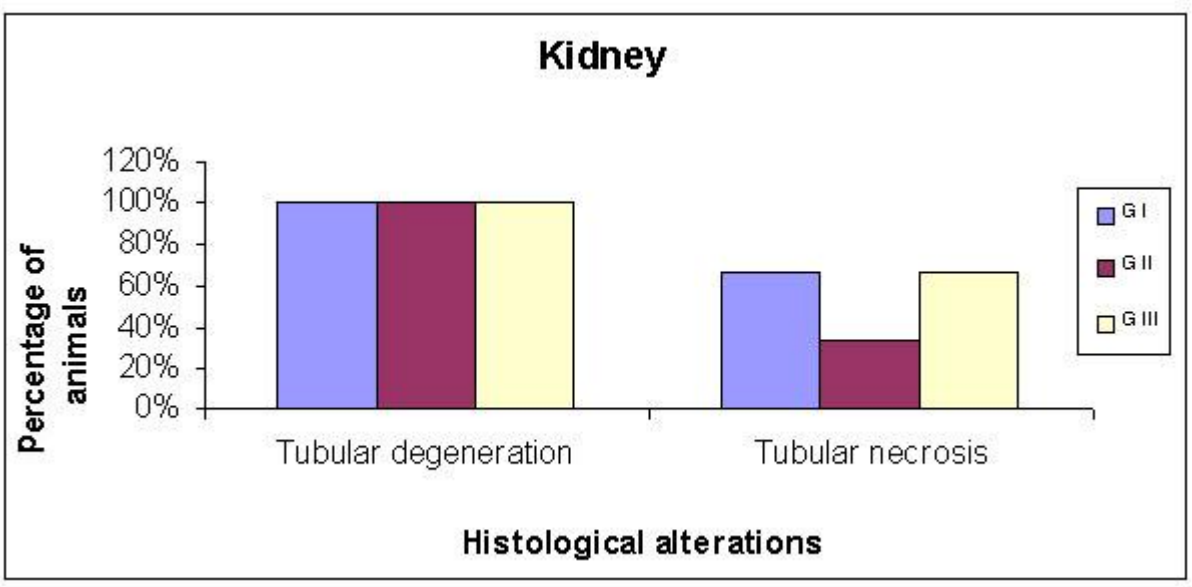

Figure 7. Percentage of animals that developed renal lesions.

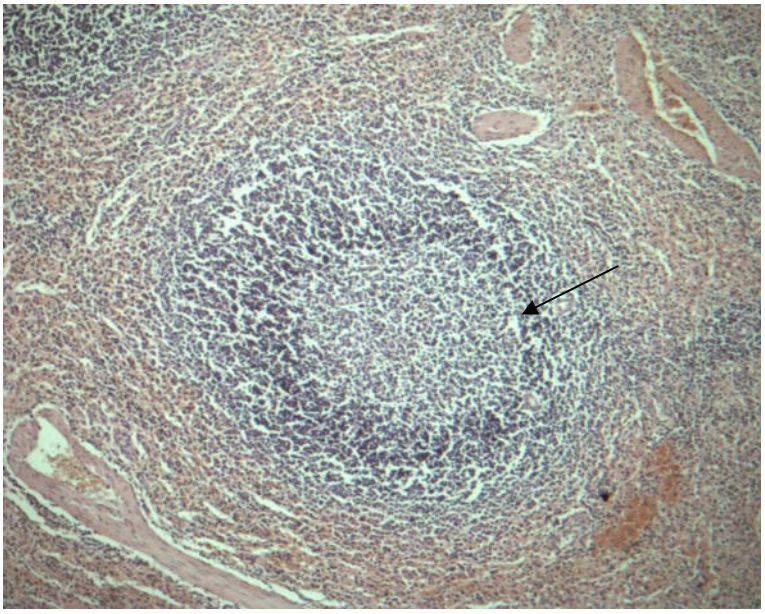

Figure 8. Microscopic aspect of the spleen from a dog inoculated with crotalic venom, showing hyperplasia of the lymphoid tissue in the white pulp, evidenced by the expanded germinative center of the lymphoid nodule (arrow) (HE, 100X).

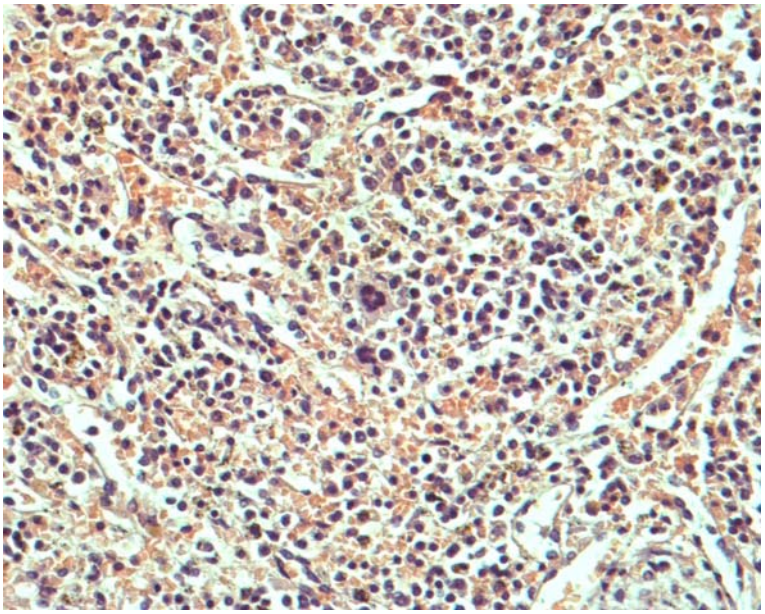

Figure 9. Presence of a megakaryocyte (center of the figure) in the spleen, showing extramedular hematopoiesis (HE, 400X).

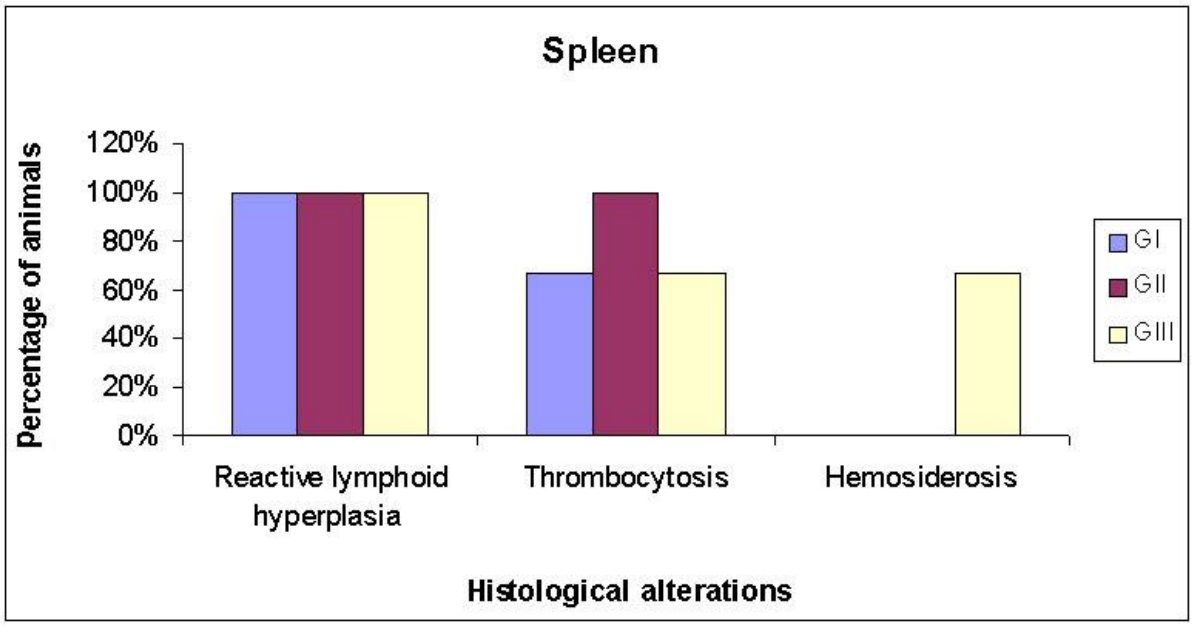

Figure 10. Percentage of animals that developed splenic lesions.

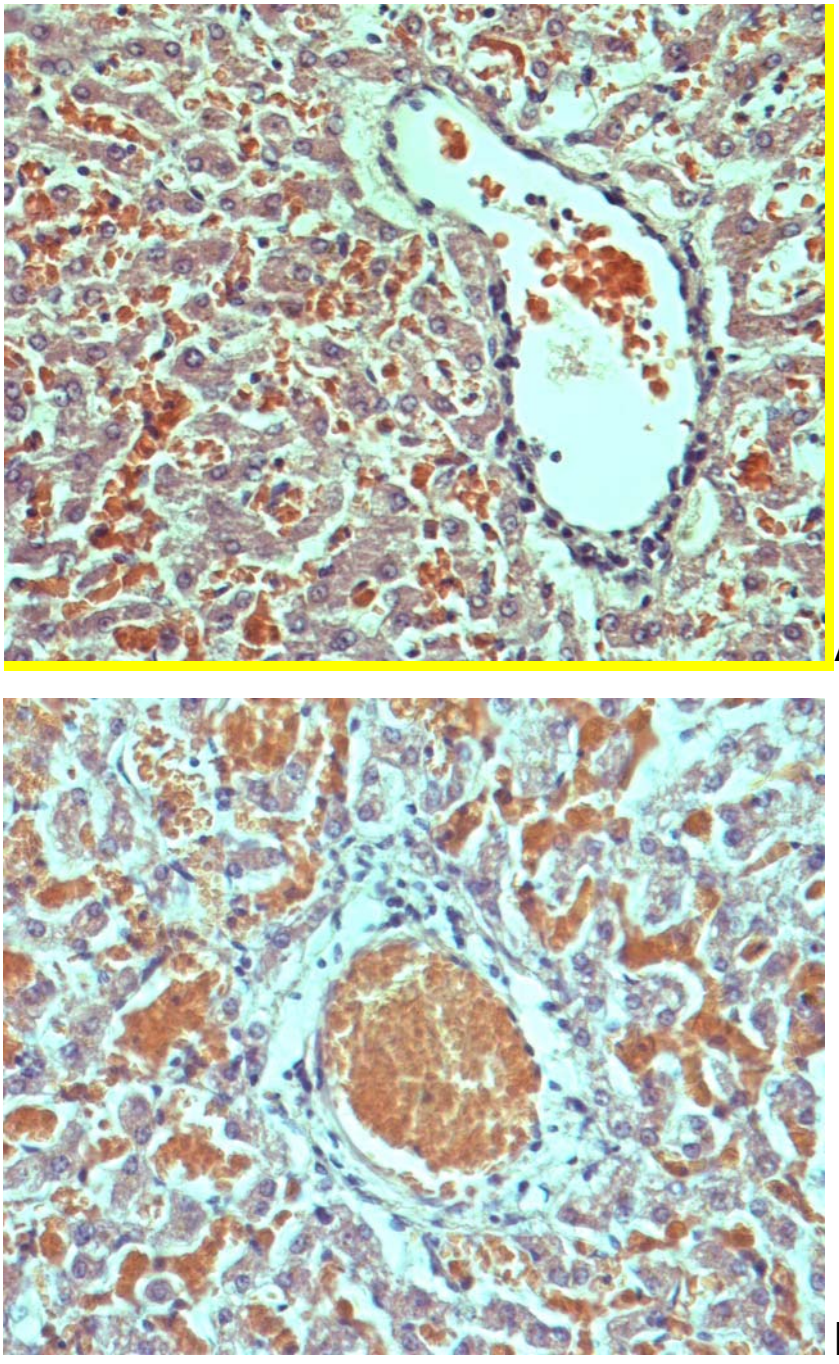

Figure 11. Microscopic aspect of the liver, showing congestion of hepatic sinusoids and centrilobular necrosis in dogs inoculated with crotalic venom. A: (HE, 400X), B: (HE, 1000X).

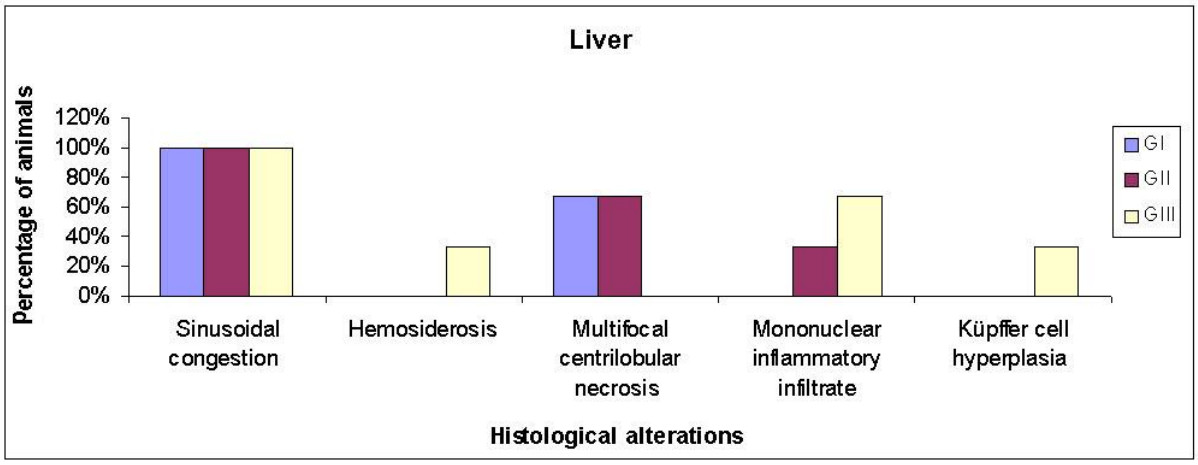

Figure 12. Percentage of animals that developed hepatic lesions.

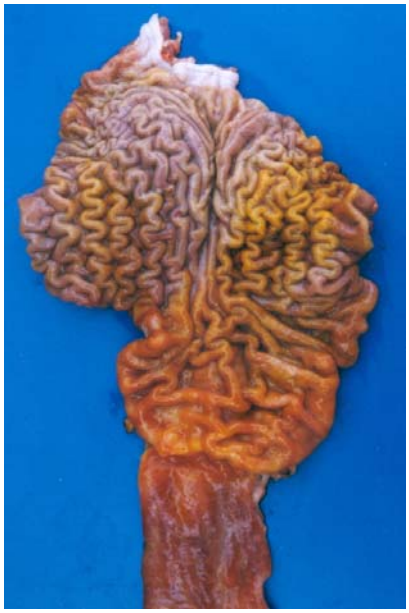

Figure 13. Macroscopic aspect of the stomach from a dog inoculated with crotalic venom, showing acute catarrhal gastritis.

DISCUSSION

The macro and microscopic alterations found in the muscles of animals inoculated with crotalic venom were consistent with rhabdomyolysis and justify the occurrence of myoglobinuria reported by some authors in patients envenomed by *Crotalus* snakes (14, 22).

The effects of crotoxin, crotapotin and phospholipase A_2 on the skeletal muscles have been studied (16, 18). Salvini *et al*. (31) noticed muscular alterations after intramuscular administration of crotoxin to mice, with presence of hypercontractility of

muscular fibers and edema three hours after such administration and myonecrosis up to three days after the administration, as well as fibers at different stages of regeneration. Myotoxicity and myonecrosis, which may be observed in the first hours after crotalic accident, are related to the types of components present in the venom. Some days after envenomation, partial or complete regeneration of the muscular fibers is usually noticed (26).

Azevedo-Marques *et al*. (7) observed myonecrosis at the bite site and at some sites far from it in human patients. Such findings are similar to those found by Koscinczuk *et al*. (21), who described the case of two dogs bitten by a *Crotalus durissus terrificus* snake and that presented edema and necrosis at the affected limb as well as occasional myonecrosis in the muscular fibers of the contralateral limb. These findings differ from those of the present study in which muscular alterations were not found in sites other than that of venom inoculation.

Acute renal failure has been cited by several authors as one of the main complications and most frequent cause of death in humans and animals envenomed with crotalic venom (3, 6, 7, 14, 15, 22, 23, 28, 32). Amaral *et al*. (3) reported nephron involvement, with glomerulonephritis, acute tubular necrosis and renal cortical necrosis in humans who were victims of crotalic accident. Such lesions were attributed to the direct nephrotoxic action of the venom, arterial hypotension, shock and myoglobinuria secondary to the rhabdomyolysis. Focal glomerulonephritis has also been seen in bovines (12). In these studies, inflammation becomes evident, which did not occur in the dogs from our experiment.

Amorin *et al*. (4) researched renal alterations in 15 dogs experimentally envenomed with crotalic venom. They noticed vacuolar degeneration in the ascending limb of the loop of Henle and distal convoluted tubule. The lesions were more remarkable at the transition zone between the renal cortex and the medulla. Inflammatory sites with presence of neutrophils were seen in a small number of dogs and, in most of them, signs of glomerulonephritis were absent. These findings are similar to those of our experiment in which no inflammatory activity was noticed and only tubular degeneration was observed.

In our study, the noticed renal alterations did not have a high degree of severity. The time spent from the venom inoculation until euthanasia (144h) may have favored a stabilization of the renal lesion and partial recomposition of the affected areas.

Another possibility is that the used venom dose was not high enough to cause severe renal lesions (24).

The significant alterations seen in the spleen of animals inoculated with crotalic venom were reactive hyperplasia of lymphoid tissue and extramedullary hematopoiesis. There are no reports of such alterations in the spleen of animals inoculated with any kind of ophidic venom. This organ removes both antigenic particles and old blood cells from the circulation (34). However, part of the circulating antigens from the crotalic venom was captured during the passage through the spleen, causing hyperplasia of the lymphoid tissue.

In the liver, a mild portal mononuclear inflammatory activity with little or no lobular inflammatory activity predominated in the animals that received crotalic venom in our study. There was Küpffer cell hyperplasia in one case and variable degrees of sinusoidal congestion. These findings characterize an unspecific reactive hepatitis (20). The hepatotoxicity of *Crotalus durissus terrificus* venom was initially demonstrated in humans. The alterations are due to two mechanisms: lesion in the mitochondria and effect of cytokines, mainly interleukin-6 (IL-6) and interleukin-8 (IL8) on hepatocytes (9, 11).

The gastritis noticed in animals from Groups I and II justify the emesis shown by some animals after envenomation. The animals from Group III, which received sodium bicarbonate during fluid therapy, did not show histopathological alterations compatible with gastritis, probably due to the anti-acid property of the bicarbonate (33).

CONCLUSIONS

The alterations noticed in our study demonstrate the nephrotoxicity and myotoxicity previously reported by other authors (1, 4, 14, 19, 23, 30). It was proven that crotalic venom causes renal tubular degeneration and acute gastritis in dogs. The administration of sodium bicarbonate intravenously was efficient as a complementary therapy, avoiding the development of acute gastritis and emesis. However, it did not prevent the occurrence of renal lesions. The present study brings new data about the antigenicity of crotalic venom, since it was able to cause important alterations in the spleen and liver. Also, it showed the role of the spleen in combating the venom toxicity, which was demonstrated by the histological findings of hyperplasia of the lymphoid tissue.

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