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YOUNG OVINE DEATH DURING HYPERIMMUNIZATION: CROTALIC ENVENOMATION OR COPPER TOXICOSIS?

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ABSTRACT: The unfavorable evolution of a young ovine during hyperimmunization process with *Crotalus durissus terrificus* venom was investigated in order to differentiate its origin between ophidic envenomation and copper toxicosis. Clinical, laboratory, necroscopic and histological exams as well as evaluation and measurement of heavy metals (copper) in the kidneys and in the liver were carried out. Blood counts revealed anemia and serological tests showed high levels of blood urea nitrogen, creatinine, aspartate aminotransferase, creatine phosphokinase, total bilirubin and indirect bilirubin; which indicates liver, kidney and skeletal muscle damages. At necropsy, the animal presented hepatopathy and nephropathy. Histological examination revealed renal and hepatic features that may imply copper intoxication. Copper levels were 237.8 µg/g in the liver and 51.2 µg/g in the kidneys. Although the amount of metal found in both organs was below the level that can cause death, according to the literature, anatomopathological signs were suggestive of copper intoxication. Therefore, the hypothesis of metal toxicosis during the hyperimmunization process became more consistent than the crotalic envenomation one.

KEY WORDS: differential diagnosis, copper toxicosis, ovines, crotalic venom, hyperimmunization.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Usually, snakebite treatments include heterologous sera that are normally obtained from equines (8, 9, 17) and ovines (10, 14, 27) previously hyperimmunized with snake venoms (18, 19). Although poorly immunogenic, *Crotalus durissus terrificus* (*C.d.t*.) venom is clearly toxic (29, 31). The production of anticrotalic serum is hindered by the presence of immunosuppressant components in the venom that results in low humoral immune responses (3, 7, 27, 29, 31). Additionally, injuries suffered by serum-producer animals after crude venom injection contribute to a small production of antivenom (25, 28, 30).

In Brazil, sheep husbandry records expressive growth. Currently, about 15 million animals are distributed throughout the country; and São Paulo State, specifically, keeps a flock of approximately 300 thousand sheep (13).

Ferreira Junior *et al.* (11) evaluated the possibility of using young sheep for the production of anticrotalic serum and observed that hyperimmunization process was successfully accomplished though the development of specific antibodies against *Crotalus durissus terrificus* venom. These findings brought the possibility of employing ovines in the commercial production of anticrotalic serum, which may be used to treat human and animal envenomation. The production costs, in this case, may be reduced by subsequent use of hyperimmunized sheep for human consumption.

The present study aimed to investigate the death of a young ovine during hyperimmunization process with *Crotalus durissus terrificus* venom for antiserum production.

CASE REPORT

During a hyperimmunization experiment, with young sheep, that intended to produce anticrotalic serum for inoculation of *Crotalus durissus terrificus* venom, one of the ovines presented anorexia and icteric/brownish mucosae for two days.

The animal had received four venom inoculations (respectively, 0.5 mg; 1.0 mg; 1.0 mg and 1.5 mg) every 14 days. Symptoms started on the 60th day, and out of the 16 sheep, this was the only one that developed those symptoms. Ten days before these manifestations, the animals had their Purina® ovine food, whose composition does not include copper, substituted for Nutrimin®, which contains 3 ppm of copper.

All the studied sheep presented clinical and laboratory similar profiles, which indicates that the action of crotalic venom did not cause debility and nutritional deficiency.

After extensive clinical examination and laboratory tests, some hypotheses were formulated, including intoxication by *Crotalus durissus terrificus* venom; copper toxicosis; rupture of the urinary bladder, urethra or ureters; leptospirosis; and clostridial infection.

According to physical examination, the young sheep presented: quadrupedal attitude, icteric/brownish mucosae, malnutrition, 22.3 kg, 38.6°C, respiratory frequency of 20 mpm, heart rate of 80 bpm, no ruminal movements, ocular secretion (bilateral), slight cachexia, 8% dehydration, arrhythmic heart sound with II-IV systolic murmur, tachycardia, anorexia, neck abscesses, coarse crackles and increased sounds in the tracheobronchial region (bilateral), anuria (diagnosed by the catheter use), and swelling of the prescapular lymph nodes.

The animal was hospitalized for diagnostic evaluation. Initial diagnostic tests, performed in the first day, presented: hematocrit levels of 14%; serum protein levels of 9.0 g/dL and fibrinogen levels of 1,000 mg/dL.

On the second day, the lamb presented slight appetite, anuria, hard mucous feces, 38.2°C, heart rate of 106 bpm, respiratory frequency of 32 mpm, no ruminal movements, tachycardia with splitting of the second heart sound, brownish mucosae, strong pulse, bladder and vermiform appendix were normal on palpation, both preputial and penile mucosae were brownish. Concerning treatment, initially, the ovine received: intravenous (IV) mannitol, 1 g/kg (100 mL); IV furosemide, 4 mg/kg; intramuscular (IM) broad-spectrum antibiotic (penicillin), 20,000 UI/kg (3 mL); and IV fluid therapy with 2.0 L of Lactated Ringer's solution. Subsequently, the animal was submitted to a blood transfusion (500 mL) and to a second fluid therapy (1.5 L of Lactated Ringer's solution). Results from hemogram, biochemical tests and peritoneal effusion are presented in Tables 1, 2 and 3, respectively.

Table 1. Hemogram of a young ovine previously hyperimmunized with *Crotalus durissus terrificus* venom, 12 hours after hospitalization

Variable	Values	Normal values (14)
Red blood cells	5,306,000/µL	9-15 x 10^3/µL
Hemoglobin	6.0 g/dL	9-15 g/dL
Hematocrit	18%	27-45%
Serum protein	8.8 g/dL	6-7.5 g/dL
Fibrinogen	Reading was not possible due to the serum color	0.1-0.5 g/dL
Platelets	808,000/µL	2.5-7.5 x 10^3/µL
Metarubricytes	18/100 leukocytes	Rare
Leukocytes	45,025/µL	4-12 x 10^3/µL
Segmented neutrophils	28,816/µL	0.7-6 x 10^3/µL
Lymphocytes	9,455/µL	2-9 x 10^3/µL
Eosinophil	6,304/µL	0-1 x 10^3/µL

Note: Neutrophils were hypersegmented and serum was intensely hemolyzed.

Table 2. Biochemical tests of a young ovine previously hyperimmunized with *Crotalus durissus terrificus* venom, 12 hours after hospitalization

Variable	Values	Normal values (15)
Urea	220.4 mg/dL	8-20 mg/dL
Aspartate aminotransferase	1,120.0 UI/L	49-123.3 UI/L
Total serum protein	11.0 g/dL	5.9-7,8 UI/L
Globulin	2.5 g/dL	3.2-5 g/dL
Creatine phosphokinase	6,947.3 UI/L	7.7-101 UI/L
Creatinine	10.0 mg/dL	0.9-2 mg/dL
Gamma glutamyl transferase	84.0 UI/L	19.6-44.1 UI/L
Albumin	7.5 g/dL	2.7-3.7 g/dL
Total bilirubin	7.4 mg/dL	0.01-0.47 mg/dL
Indirect bilirubin	6.8 mg/dL	0.04-0.44 mg/dL
Direct bilirubin	0.6 mg/dL	0.03-0.16 mg/dL

Table 3. Peritoneal effusion of a young ovine previously hyperimmunized with *Crotalus durissus terrificus* venom, 12 hours after hospitalization

Variable	Values	Normal values (15)
Color	Brownish	Colorless
Aspect	Turbid	Clear
Density	1030	1001-1013
pH	Determination was not possible due to coloration	6,5-7,5
Proteins	+++ (4.6 g/dL)	20-40 mg/dL
Glucose	Determination was not possible due to coloration	35-70 mg/dL
Occult blood	+++	+
Erythrocytes	5,500/µL	5,000/µL
Eukaryotic cells	440.0/µL	absent
Creatinine	80 mg/dL	0,5-1,5 mg/dL
Urea	374 mg/dL	17,08 ± 3,08 mg/dL
Fibrinogen	< 100 mg/dL	50-100 mg/dL

Note: clotting, reactive lymphocytes and erythrophagocytosis were positive.

Cytology: whole erythrocytes; predominance of segmented neutrophils (31%), followed by lymphocytes (30%), macrophages (23%), mesothelial cells (10%) and erythroblasts (6%).

On the third day, the animal showed anuria, absence of feces, 38.3°C, heart rate of 112 bpm, respiratory frequency of 20 mpm, no ruminal movements, tachycardia with splitting of the second heart sound, brownish mucosae, strong pulse, brownish preputial and penile mucosae. Given that the young sheep was unable to urinate, the vermiform appendix was removed to allow catheterization and infusion of physiologic solution, but to no avail. The urine remained dark brown (oliguria) with grumes and some calculi. Urea and creatinine values were 194.3 mg/dL and 10.0 mg/dL, respectively.

Microscopic serum agglutination test for leptospirosis was carried out by the Zoonosis Diagnostic Service (in the Veterinary Hospital of the Veterinary Medicine and Animal Husbandry School, UNESP) and it was negative for the disease.

Several clinical and surgical procedures were carried out with the objective of treating the acute renal failure, however they were ineffective. Considering the irreversible

clinical outcomes and that the animal probably had urine in its abdominal cavity, euthanasia was suggested.

The young ovine was killed according to the norms from the Ethics Committee of the Veterinary Medicine and Animal Husbandry School, UNESP, and later underwent necropsy. The post-mortem examination was carried out by the Veterinary Pathology Service of the Veterinary Medicine and Animal Husbandry School, UNESP (ID: 127378; necropsy: 374/04) and the stated cause of death was “sacrifice *in extremis*”.

In the course of the corpse inspection, we were able to observe: hydrothorax, hydroperitoneum, right and left upper lobe pneumonia, hydropericardium, subcutaneous edema in the ventral region, splenomegaly, hepatic degeneration, dark kidneys (Figure 1), unilateral ureteral rupture, catarrhal ileitis, brain congestion, brownish ocular mucosa due to the massive hemolysis caused by copper (Figure 2).

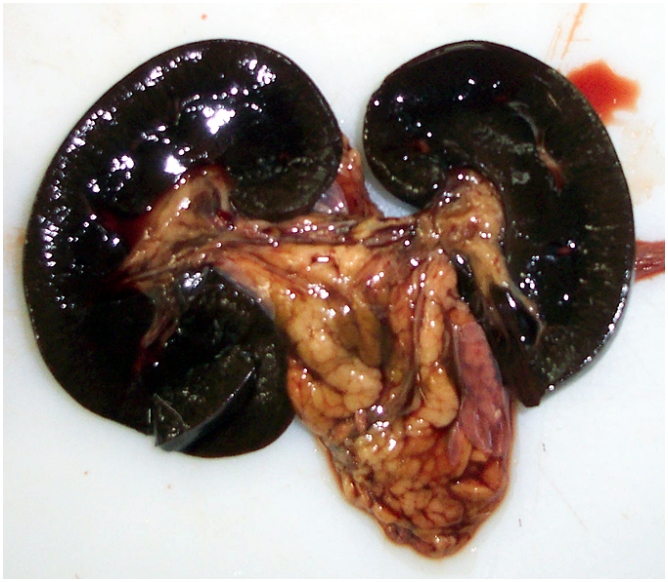

Figure 1. The dark kidneys are markers of nephropathy.

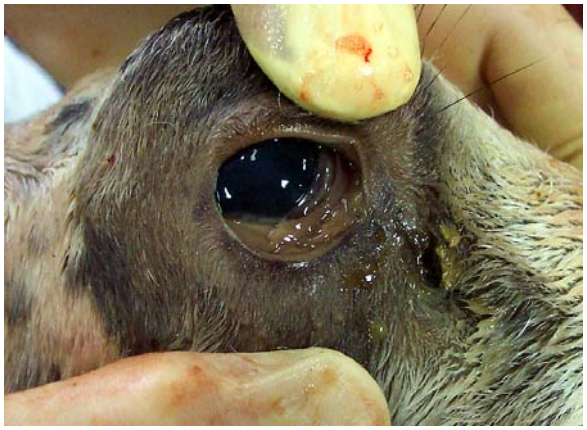

Figure 2. Brownish ocular mucosa due to the massive hemolysis caused by copper.

Histological findings showed congested kidneys, hemorrhage, glomerular hypercellularity, proteins in the tubular lumen and in the Bowman's space, degeneration and necrosis of the tubular epithelium, glomerular sclerosis, glomerular synechia, proliferation of parietal cells and tubular dilatation (Figures 3 and 4). The liver presented diffuse droplet degeneration , diffuse polymorphonuclear inflammatory infiltrate, discrete congestion and cholestasis (Figure 5).

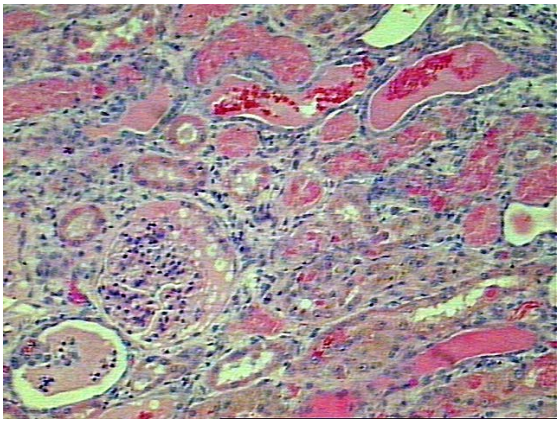

Figure 3. Histological section of the kidney: glomerular hypercellularity; Bowman's capsule with high amount of proteins; proximal renal tubules with high amount of proteins and erythrocytes; and tubular degeneration (hematoxylin and eosin stain, 200x).

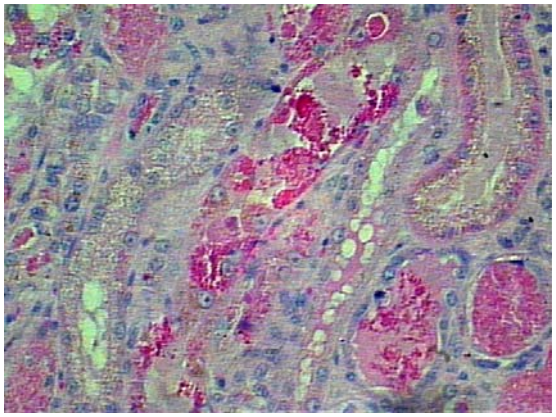

Figure 4. Histological section of renal tubules: tubular hydropic degeneration and necrosis; and necrotized cells in the lumen (hematoxylin and eosin stain, 400x).

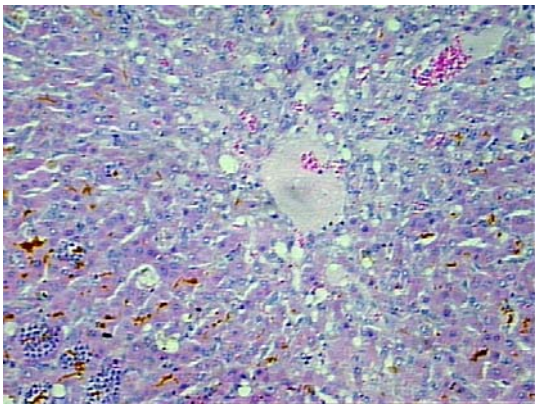

Figure 5. Histological section of the liver: lobe central vein with droplet degeneration (steatolysis); numerous areas of cholestasis (bile); mononuclear glomerular infiltrate; and areas of congestion (hematoxylin and eosin stain, 200x).

Histological section of intestine showed a little damaged mucosa, which ruled out the possibility of clostridial infection, while histological section of skeletal muscle demonstrated that the snake venom had no systemic action; therefore, the hypothesis of envenomation by crotalic venom employed for immunization was also excluded.

The determination of heavy metal levels (copper) in liver and kidney samples was carried out by means of atomic absorption spectroscopy and the results were 237.8 µg/g and 51.2 µg/g, for liver and kidney, respectively.

DISCUSSION

The composition of the crotalic venom is complex; it consists of enzymes, toxins and peptides, as well as presents myotoxic, nephrotoxic, neurotoxic, hematological and hepatotoxic actions (2).

In crotalic poisoning, myoglobinuria and occult hematuria may also be observed. Usually, the venom myotoxicity is assessed by its ability to release myoglobin into the blood.

The definitive diagnosis of rhabdomyolysis is made by muscle biopsy, by the evaluation of increased levels of myoglobin in blood and in the urine or by the evolution of serum levels of creatine kinase (CK), lactic dehydrogenase (LDH) and aspartate aminotransferase (AST) (3, 24).

Renal changes that may be induced by myoglobin action on renal parenchyma can be registered. Moreover, it is believed that the venom has a direct action on injury production. Furthermore, myoglobinuria due to rhabdomyolysis also appears to contribute to the genesis of kidney lesions; among the mechanisms proposed to explain it are the tubular obstruction by myoglobin cylinders and direct toxic injury on the tubules by myopigment. High levels of serum indirect bilirubin often indicate liver impairment and are also likely in severe hemolytic anemia.

Other factors – such as dehydration, hypotension, metabolic acidosis and shock – may be associated with rhabdomyolysis and contribute to kidney damage (4, 6).

Meanwhile, ovines are considered the most susceptible animals to copper intoxication, which may occur in the following forms: acute, subsequent to ingesting high doses of this metal; primary chronic, after eating copper-containing food; phytogenous chronic, after grazing in fields with normal levels of copper but reduced amount of molybdenum; and hepatogenous chronic, when there is copper accumulation due to hepatic lesions induced by plants containing pyrrolidines (11, 26). The analysis of copper levels in the kidney and liver are employed as a tool to detect chronic copper intoxication in sheep (15, 20, 21, 23).

Some authors believe that copper concentration in the liver is the most sensitive indicator of exposure to copper-laden diets. The levels of this metal in ovines that had hemolytic crisis in the present study varied from 369 to 854 ppm of dry matter (DM) in the liver and from 152 to 679 ppm DM in the kidney. Levels equal to or higher than 500 ppm DM in the liver and 80 ppm DM in the kidney suggest intoxication (12, 22).

However, low levels of the metal in the liver (247 ppm DM) had already been observed in copper-intoxicated sheep. Determination of copper in renal cortex is the most reliable procedure to diagnose intoxication in ovines, since hepatic copper levels tend to decrease after hemolytic crises (1, 5, 16).

CONCLUSIONS

The necropsy findings, from anatomopathological signs, were suggestive of copper intoxication. Additionally, histological tests presented renal and hepatic features that were also indicative of copper intoxication, although the amount of metal found in the liver and the kidney was below the levels that could cause death, according to the literature.

Despite the low amount of copper in ovine food, intoxication could possibly be the consequence of urolithiasis that led to anuria and ureteral rupture. Therefore, the hypothesis of envenomation by crotalic venom during the hyperimmunization process was rejected.

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