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ACUTE HEPATOTOXICITY OF *Crotalus durissus terrificus* (SOUTH AMERICAN RATTLESNAKE) VENOM IN RATS

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ABSTRACT: Venom of the South American rattlesnake, *Crotalus durissus terrificus* (Cdt), presents myotoxic and neurotoxic outcomes, but reports on its effects on the liver are scarce. This study examined the hepatotoxicity resulting from Cdt venom administration (100, 200 and 300 µg/kg) in male Wistar rats. Animals were studies at 3, 6, 9 and 12 hours after venom injection. The hepatotoxicity was assessed through serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), gamma glutamyl transferase (GGT), bilirrubin and also by histopathological evaluation. All the different concentrations of Cdt venom resulted in increased levels of hepatic enzymes, when compared with the control group, except for the 100 µg/kg dose, which presented normal levels at 9 and 12 hours after venom administration. Bilirrubin levels remained unchanged by Cdt venom. Histological analysis revealed endothelial damage, inflammatory cell infiltration, as well as sinusoidal and portal congestion. Based on these observations, we may conclude that Cdt venom causes dose- and time-dependent hepatic damage in rats, characterized by elevated hepatic enzyme levels and histological alterations.

KEY WORDS: *Crotalus durissus terrificus*, hepatotoxicity, histology, AST, ALT, rattlesnake, liver enzymes, venom.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

The South American rattlesnake, *Crotalus durissus terrificus*, presents a wide distribution throughout southeastern Brazil (1). Envenomations by this species account for 6 to 8% of all registered bites by venomous snakes in the country, with an approximate mortality rate of 1.8%, the highest among venomous species (2). *C. d. terrificus* venom contains a variety of toxins, including convulxin (3), crotamine (4), crotoxin – its main neurotoxin, which accounts for nearly 50% of the venom dry weight (5) – and gyroxin (6-8), as well as several enzymes common to other snake venoms (9, 10).

Experimental and clinical envenomations by *C. d. terrificus* venom result mainly in myotoxicity and neurotoxicity, while the most effective treatment is administration of anticrotalic antivenom (11). Whereas some consequences of *C. d. terrificus* venom have been extensively studied – such as myonecrosis (12-17), coagulation disturbances (7, 8, 18-20), neuromuscular effects (21, 22), renal involvements (14, 23) and homeostatic problems (20) – considerably less attention has been paid to its effects on the liver.

In humans, envenomation by *C. d. terrificus* causes hepatic lesions involving necrosis, effects attributed to release of pro-inflammatory cytokines (24-27). Also, hepatic steatosis and increased serum aspartate aminotransferase (AST) levels were reported by Azevedo Marques *et al*. (14) in patients bitten by *C. d. terrificus* and extensive hepatic necrosis by Barraviera *et al*. (24). In addition, Barraviera *et al.* (25) showed increased serum levels of AST (86%) and ALT (66%), by hepatocyte electron microscopy that showed mitochondrial damage, suggesting that liver injury may occur partly in the cytoplasm and partly in the mitochondria. Bancher, Rosa and Furlanetto (28) proved that, in mice, the venom could be detected in hepatic tissue.

The liver is a multifunctional organ, responsible for vital functions and for maintenance of energy balance in the organism. The release of intracellular enzymes into circulation is frequently used as an indicator of hepatocyte damage (29), although the release of some enzymes like AST and ALT – also present in tissues other than liver – needs to be evaluated by other injury markers to confirm any hepatic damage. Therefore, in the present study, we examined the liver damage induced by *C. d. terrificus* venom in rats through enzymatic markers of liver function and also by histological analysis.

MATERIALS AND METHODS

Venoms and Reagents

The kits for the quantification of serum enzymes and bilirubin were from Labtest Diagnóstico (Brazil). The reagents for histology were purchased from Synth (Brazil). *C. d. terrificus* venom was obtained by manual milking of snakes (both sexes) collected in the region of São José dos Campos and maintained in the snake house of the Nature Study Center, Vale do Paraíba University. The venom was lyophilized and stored at 4°C. Stock solutions of venom were prepared in sterile 0.9% NaCl immediately before use.

Animals

Male Wistar rats (180-220 g) obtained from Bem-te-vi Farm (Indaiatuba, SP, Brazil) were maintained in a 12-hour-light/dark cycle at 23 ±2°C with free access to food and water. Animal procedures described herein were approved by the institutional Animal Research Ethics Committee of UNIVAP (protocol number L035/2004/CEP) and performed in accord with the guidelines of the Brazilian College for Animal Experimentation (COBEA).

Experimental Protocols

To study the hepatotoxicity of *C. d. terrificus* venom, rats (283 per group) received an intramuscular injection of sterile saline solution only or venom (100, 200 or 300 µg/kg) with saline solution in a final volume of 100 µL. The rats were subsequently killed with an overdose of ether at 3, 6, 9 or 12 hours after saline or venom injection (7 rats/time interval in each group) and blood was collected from the inferior vena cava. Samples of hepatic tissue were also obtained for histological analysis.

Serum Enzyme Activities and Bilirubin Levels

Serum alanine aminotransferase (ALT), alkaline phosphatase (AP), aspartate aminotransferase (AST) and γ-glutamyl transferase (GGT) activities were determined spectrophotometrically, as described by Reitman and Frankel (30), Szasz (31) and Roy (32). Bilirubin tests were made by a chosen diazo-coupling reagent and by a spectrophotometric method, according to Winsten and Cehelyk (33).

Histomorphometrical Analysis

Liver fragments from rats killed at different times after venom administration were fixed in 10% formaldehyde overnight and then dehydrated in an increasing alcohol series, clarified with xylol and embedded in paraffin. Sections of 5-µm-thick were cut by a microtome (American Optical Co., USA) and stained with hematoxylin and eosin, for subsequent examination and documentation by an Olympus CX-41® microscope (Olympus Optical, USA) fitted to an Olympus PM10SP Automatic Photomicrographic® system (Olympus America Medical, USA). Twenty fields were analyzed for each slide. Using a 100-point grid with a known area (10,000 μm^2 at 1,000x magnification) attached to the microscope lens the number of points contacting the Kupffer cells and leukocytes were counted within a total area (10,000 μm^2). The density of congested blood vessels, endothelial area and degenerated hepatocytes were counted and the values adjusted to the total tissue area (10,000 μm^2). The density of Kupfer and mononuclear cells, as well as the density of congested blood vessels, endothelial area and degenerated hepatocytes were expressed as number of cells/mm^2 of tissue. For a qualitative histological evaluation, the following criteria were used to determine the histologic score (scale 0-10): 0 = negative; 0.5 to 2.0 = mild; 2.5 to 5.5 = moderate; 6.0 to 8.0 = severe; 8.5 to 10.0 = very severe (34).

Statistical Analysis

The results were expressed as mean ± SEM and statistical comparisons were performed by Student's t-test or one-way of variance (Anova) followed by the Tukey-Kramer test. The p value was considered significant ($p < 0.05$); very significant ($p < 0.01$) and extremely significant ($p < 0.001$).

RESULTS

Serum Liver Enzyme Activities and Bilirubin Levels

Figure 1A shows the changes in AST levels at various time intervals following the injection of different doses of *C. d. terrificus* venom. The administration of 100 µg/kg of the venom resulted in increased AST activity at 3 hours when compared to the control group ($p < 0.05$), but no differences after 6, 9 and 12 hours ($p > 0.05$). Regarding the 200- and 300-µg/kg venom doses, we observed an increased AST activity at all times (3, 6, 9 and 12 hours) relative to the control group ($p < 0.05$).

Figure 1B reveals the changes in ALT levels at diverse intervals after administration of different concentrations of *C. d. terrificus* venom. Concerning the 100-µg/kg dose, no changes in ALT activity was found, when compared with the control group at 3, 6, 9 and 12 hours ($p > 0.05$). The 200-µg/kg concentration of *C. d. terrificus* venom augmented ALT activity only at 9 and 12 hours relative to the control group ($p < 0.05$). At the dose of 300 µg/kg of *C. d. terrificus* venom, the ALT activity was greater than in the control group at 3, 6, 9 and 12 hours ($p < 0.05$).

AP activity increased progressively after the administration of all venom doses, although a significant change was seen only with the highest dose (300 µg/kg) at 12 hours post-venom (Figure 1C). GGT levels showed a peak increase 6 hours after administration of the lowest dose, with a return to the pre-venom levels at 12 hours post-venom. A moderate and persistent rise in GGT activity was registered with the intermediate dose (200 µg/kg), while the highest one produced a progressive increase during the 12 hours after venom administration (Figure 1D). Figure 1E shows that total bilirubin levels were not changed by any venom dose or time interval.

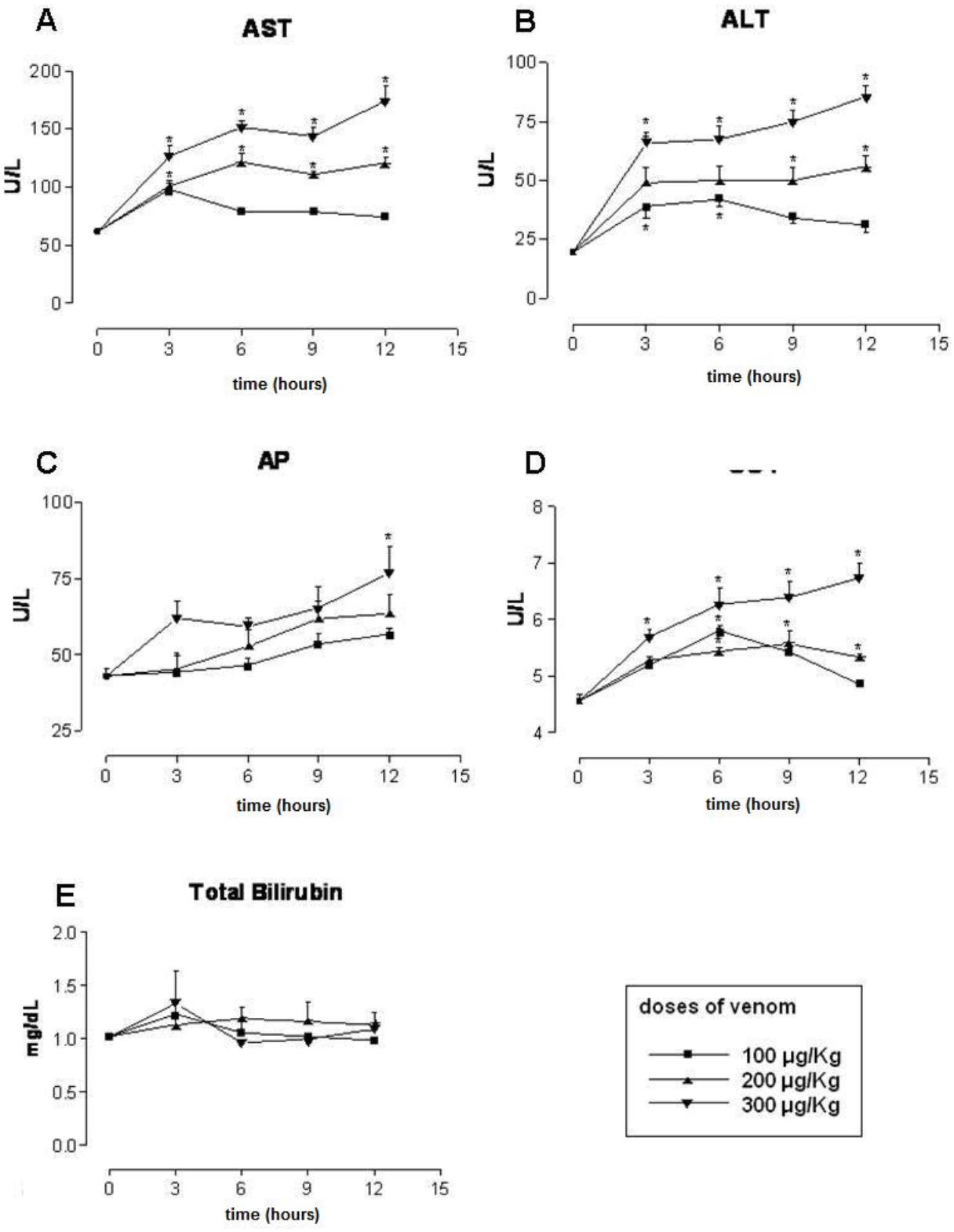

Figure 1. Serum enzyme activities and bilirubin levels. Enzymes were collected from rat sera 3, 6, 9 and 12 hours after administration of 100, 200 and 300 µg/kg of Cdt venom. (**A**) Alanine aminotransferase (ALT); (**B**) aspartate aminotransferase (AST); (**C**) alkaline phosphatase (AP); (**D**) gamma glutamyl transferase (GGT) and (**E**) total bilirubin concentration. Data expressed as mean values ± S.E.M for 7 rats per group; *p < 0.05 when compared to the control group and analyzed by one-way Anova, followed by Tukey-Kramer multiple comparisons test.

Histological Analysis

Sections of hepatic tissue from saline-treated (control) rats showed normal hepatocytes with strongly stained nuclei and protein-rich cytoplasm. Transversal sections showed intact sinusoids, portal spaces and a lobular central vein (Figure 2A). In contrast, rats injected with 100 μg/kg showed damage caused by an inflammatory infiltrate 3 and 6 hours after venom injection (Figure 2B). At 9 and 12 hours post-venom, the appearance of the tissue had returned to normal (Figure 2C). However, the morphometric analysis did not show statistical differences between the control group and 100 μg/kg group. At the venom dose of 200 μg/kg, copious cellular infiltrate and the extent of damage augmented with time (Figure 2D). The morphometric analysis showed that at 200 μg/kg at 12 hours, the densities of Kupfer cells, endothelium and degenerated hepatocytes were increased when compared with the control group. The density of congested blood vessels at 9 hours was higher than in the control group. At the highest venom dose (300 μg/kg), the hepatocytes showed marked disorganization, with swelling, necrosis and a progressive increase in damage up to 12 hours after venom administration (Figure 2E). Moreover, the histomorphometric findings demonstrate in Table 1 that at 9 hours, the density of Kupfer cells, congested blood vessels, endothelium densities and degenerated hepatocyte were greater than in those of control group. At 12 hours, all parameters were elevated when compared with the control group.

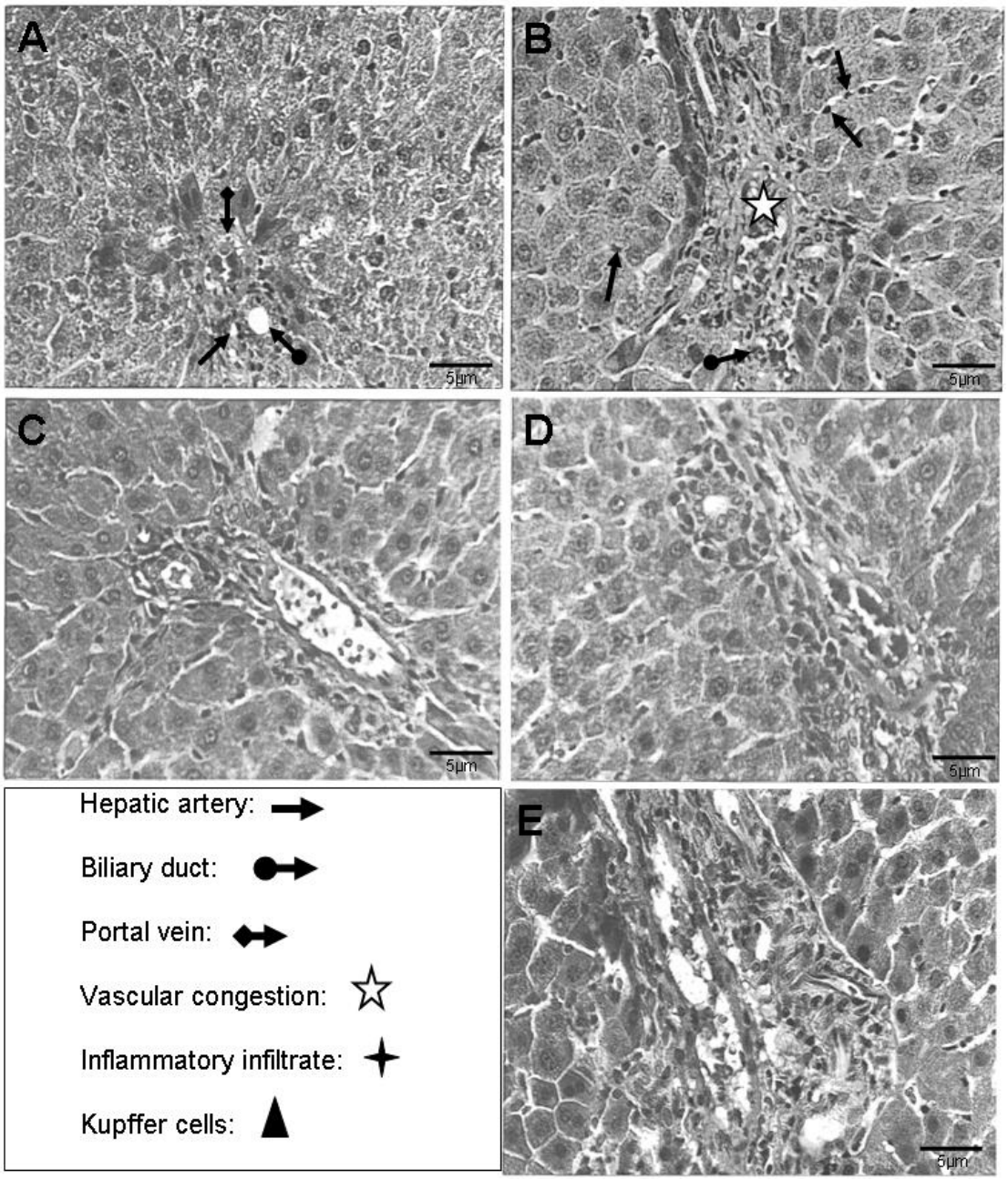

Figure 2. Histological analysis. **(A)** Photomicrograph of hepatic tissue from the control group (HE, 400x). See hepatic artery (→), biliary duct (●→), portal vein (◆→). **(B)** Photomicrograph of hepatic tissue 6 hours after venom injection (100 µg/kg) (HE, 400x). Observe vascular congestion (☆), inflammatory infiltrate (●→) and increased density of Kupffer cells (→). **(C)** Photomicrograph of hepatic tissue 12 hours after venom injection (100 µg/kg) (HE, 400x). A few inflammatory cells and plate hepatocytes. **(D)** Photomicrograph of hepatic tissue 6 hours after venom injection (200 µg/kg) (HE, 400x). Dense inflammatory infiltrate and portal vein congestion. **(E)** Photomicrograph of hepatic tissue 12 hours after venom injection (300 µg/kg) (HE, 400x). Damaged hepatic vascular system showing high inflammatory infiltrate; hepatocyte degeneration.

Table 1. Morphometric analysis of liver histological aspects

		Kupffer cells/mm^2	Inflammatory infiltrate/mm^2	Blood vessel congestion/mm^2	Endothelial density/mm^2	Hepatocyte degeneration/mm^2
Control		1.00±0,78	1.68±1.23	0.63±0.47	0.16±0.14	0.26±0.32
Venom 100 µg/kg	3h	2.47±0.92	6.00±1.22***	2.95±1.16*	0.68±0.34	1.42±0.37
	6h	2.74±0.76	7.37±1.36***	3.16±0.81*	1.63±1.02	1.84±0.92
	9h	1.53±0.71	2.37±0.69	1.26±0.54	0.68±0.78	1.010±0.43
	12h	2.06±1.34	2.05±0.83	0.95±0.39	0.26±0.20	0.79±0.84
Venom 200 µg/kg	3h	2.47±0.71	5.58±1.26***	2.21±0.51	1.16±0.47	1.84±0.81
	6h	4.53±0.82***	4.90±1.54***	4.95±1.05***	1.63±0.69	3.90±0.66***
	9h	4.10±0.99***	4.74±1.43**	5.16±1.92***	1.95±1.05*	4.00±1.44***
	12h	6.47±1.60***	4.95±0.94***	3.58±2.26**	3.68±1.12***	4.05±1.05***
Venom 300 µg/kg	3h	3.21±0.62*	4.79±1.06**	5.21±1.06***	3.37±0.91***	3.95±1.05***
	6h	4.21±1.18***	5.58±1.81***	6.53±1.37***	3.47±1.60***	3.95±1.05***
	9h	6.05±1.61***	5.26±0.98***	8.11±1.88***	5.16±1.14***	5.63±0.91***
	12h	6.74±1.76***	6.10±1.66***	9.58±0.37***	7.74±1.54***	6.00±0.78***

Morphometric analysis of liver histological aspects found in the control group and Cdt venom group (100 µg/kg; 200 µg/kg and 300 µg/kg) 3, 6, 9 and 12 hours after administration (7 rats per group). Data expressed as intensity score scale from 0 to 10: 0 = negative; 0.5 to 2.0 = mild; 2.5 to 5.5 = moderate; 6.0 to 8.0 = severe; 8.5 to 10.0 = very severe. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ *vs.* shocked rats treated with saline (Kruskal-Wallis test).

DISCUSSION

The present study showed that the *Crotalus durissus terrificus* venom augments activity of hepatic enzymes and also produces histological alterations, suggesting hepatotoxicity.

The liver maintains the organism's energy supply for many functions, such as producing substances that break down fats, converting glucose to glycogen, producing urea, synthesizing certain amino acids, filtering harmful substances and maintaining a proper blood glucose level. There are some intracellular enzymes that are released into the circulation and are frequently used as an indicator of damage to hepatocytes. The principal marker enzymes include alanine (ALT) and aspartic (AST) aminotransferases, which catalyze the transfer of α-amino groups from alanine and aspartate to the α-keto group of ketoglutaric acid to produce pyruvic acid and oxaloacetic acid, respectively (29). ALT is a more specific marker of hepatocellular injury, and occurs in the liver, kidneys, heart, skeletal muscle and pancreas, whereas AST has a wider distribution, occurring in organs such as heart, liver, skeletal muscles, kidneys and pancreas. In the liver, AST occurs predominantly (80%) in mitochondria and 20% in the cytoplasm, whereas ALT is confined to the cytoplasm (29, 35). Other enzymes such as alkaline phosphatase (AP) and γ-glutamyl transferase (GGT) may also be used as markers of hepatic dysfunction, although they are found in the liver, bone, the kidneys and its enhancement is related to production elevation or excretion deficit, such as obstructive jaundice and cholestasis. GGT is present in the liver, kidney and pancreas. Bilirubin is a pigment derived from the catabolism of the heme radical in hemoproteins such as hemoglobin, while increased serum levels of this pigment can indicate cholestatic processes and hepatocellular damage.

The measurement of serum enzyme activities in clinical studies, after *C. d. terrificus* venom administration, showed increased levels of creatine kinase (14, 16, 18, 36), creatine phosphokinase (14), lactate dehydrogenase (14, 16, 36), aspartate aminotransferase (36) and glutamic-oxaloacetic transaminase (14), primarily as consequences of venom myotoxic action (11), as well as injury markers of cardiac muscle disturbance similar to those of skeletal muscles (7, 36). Serum levels of alkaline phosphatase and alanine aminotransferase were also elevated in dogs treated with *C. d. terrificus* venom (1 mg/kg, IM) (20). Approximately half of the

patients bitten by *C. d. terrificus* show coagulation disorders primarily mediated by thrombin-like enzymes present in this venom (8, 18).

A study in dogs revealed that *C. d. terrificus* venom causes marked alterations in hematological, hemostatic and biochemical parameters that may also be seen, through histological examination, as intravascular coagulation in cardiac and hepatic tissues (20). These authors also showed a progressive increase of serum levels of myoglobin, creatine kinase and aspartate aminotransferase demonstrated that animals developed rhabdomyolysis. On the other hand, AP did not increase expressly. Probably, this is related to cholestatic diseases, which is plausible because, like AP, bilirubin concentration did not increase over time and, as outlined in the preceding sections, is also related to cholestatic process (35-37).

As proven by the current study, the administration of *C. d. terrificus* venom to rats resulted in increased circulating levels of enzymes (ALT, AST and GGT) that are frequently employed as indicators of hepatic damage and cholestasis. The increased AST levels observed in the present work are similar to elevated serum activity of the same enzyme after human envenomations (24, 11). Changes in serum levels of other enzymes, including creatine kinase (CK) and lactate dehydrogenase (LDH), have also been observed in human envenomations (11). However, there are differences in kinetic profiles of these alterations. For example, CK and LDH levels present their peaks respectively before and after other enzymes. The hepatic damage caused by the smallest venom dose (100 µg/kg), assessed by enzyme release and histological analysis (Table 1), appeared to be minimal and reversible. Higher venom doses (200 and 300 µg/kg) resulted in greater damage that was not reversible within the time scale of the present experiments. In part, this hepatotoxicity is probably mediated by PLA_2 component of crotoxin, which presents the ability to interfere in hepatic mitochondrial respiration (38). The presence of inflammatory infiltrates associated with severe damage indicates that *C. d. terrificus* venom and its components are capable of affecting macrophage function and stimulating cytokine production (39, 40). These findings agree with the demonstrations of acute phase inflammatory reactions in humans bitten by *C. d. terrificus* (26).

The venom components responsible for the hepatotoxicity are still undetermined, but they probably involve PLA_2 action on cell membrane lipids and mitochondrial respiration (38), as well as various cell types such as lymphocytes (41, 42), macrophages (39, 40, 43, 44), mast cells (45), and platelets (3, 46-49). In support of

this conclusion, crotoxin – which presents all PLA_2 activity of *C. d. terrificus* venom – has been implicated in numerous effects, including neurotoxicity (22), myotoxicity (12, 50, 51), renal toxicity (52), edema (45, 51), inhibition of macrophage activity (40, 44), immunosuppression (42), stimulation of the hypothalamo-pituitary-adrenal axis (including increased plasma glucose, ACTH, corticosterone and TNF-α levels) and of corticotropin-releasing hormone (CRH) and of arginine vasopressin pathways (53), and cytostatic (54) and cytotoxic (55) effects in cancer cell lines.

Coagulation disturbances (19, 20) leading to fibrin deposition and ischemia, mainly through the action of the thrombin-like enzyme (gyroxin) of this venom (8, 9, 19) could also contribute to the hepatic damage registered in the present study. In addition, considering the inter- and intrasubspecific variation in the composition and biological activities of this venom (51, 56-61), there may be differences in hepatic effects produced by venoms obtained from *C. d. terrificus* specimens from other regions, and among other subspecies like *C. d. cascavella*, *C. d. collilineatus*, *C. d. durissus* and *C. d. ruruima*. Finally, diverse susceptibilities of animal strains and species (18) may also contribute to variations in hepatic effects of this venom.

The present study provides evidence that *Crotalus durissus terrificus* venom induces hepatotoxicity in rats, by increasing blood liver enzymes, probably derived from liver, since histological analysis revealed augmented endothelium density and hepatic damage.

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