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HISTOLOGICAL AND BIOCHEMICAL EFFECTS INDUCED BY SUBLETHAL DOSES OF *Bothrops jararacussu* VENOM IN MICE

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ABSTRACT: Snake venom is characterized by hemorrhagic, coagulant, proteolytic and myotoxic activities which in *Bothrops jaracussu* venom are related to intraspecific variations. In the present study, female Swiss mice were divided into two groups: treated with 25μg or 50μg venom. These were subdivided into three groups of six animals each, according to blood collection: 2, 4 or 24h after venom injection. Animals were anesthetized using diethyl-ether inhalation and 1ml of blood was collected by heart puncture. Then, the following organs were removed: spleen, skeletal muscle, kidneys, liver and lungs; histological sections were obtained and stained with hematoxylin-eosin (HE). The following biochemical parameters were analyzed: aspartate aminotransferase (AST/GOT), alanine aminotransferase (ALT/GPT), total lactate dehydrogenase (LDH), glucose, creatinine and urea levels, and total protein content. Results showed significant alterations in AST, LDH, glucose and urea levels, and total protein content, as well as important tissue alterations in the liver, kidneys and lungs. It could be concluded that, even using sublethal doses of venom, there were significant changes in almost all the tested biochemical parameters as well as tissue alterations in the kidneys and lungs.

KEY WORDS: *Bothrops jararacussu*, snake venoms, sublethal doses, *in vivo* studies, biochemical parameters, histopathological alterations.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

In the Brazilian territory, snakes of the *Bothrops* genus account for a total of twenty species and are responsible for 90% of the snakebite accidents. According to the Ministry of Health, more than 17000 bothropic accidents occur every year in the country, with a fatality rate of approximately 0.6% treated cases.

Bothropic venom is very complex. It contains twenty or more different compounds and over 90% of its dry weight is constituted of proteins encompassing a large variety of components such as lecithin, metalloproteinases, serine proteinases, disintegrins, phospholipases and peptides such as bradykinin and angiotensin (7). Non-protein fractions are represented by carbohydrates, lipids, metals, and biogenic enzymes (10).

The venom from *Bothrops* snakes presents a complex mixture of toxins with different toxic or enzymatic properties. Proteases apparently act by degrading tissue proteins in a non-specific manner (26) and by cleaving plasmatic proteins through hydrolysis, interfering in the homeostasis of the organism (16, 21). Such substances can have hemorrhagic, coagulant, proteolytic and myotoxic activities that result in inflammatory process and tissue destruction during damage to blood vessel walls or in pain, edema, ecchymosis, abscess formation, and necrosis (12, 18, 20, 23, 27).

The aim of the present work was to study in mice the local and systemic effects of sublethal doses of venom from *B. jararacussu* specimens commonly found in tropical forests of Santa Catarina, Brazil. Histological alterations as well as changes in serum levels of several enzymes and metabolites were analyzed.

MATERIALS AND METHODS

Venom

Venom was obtained from an adult *B. jararacussu* in the animal facility of the Regional University of Blumenau, Santa Catarina, Brazil. It was preserved at –20°C until use, when the lyophilized venom was dissolved in phosphate buffered saline (PBS), pH 7.2, to obtain the doses 25μg and 50μg.

Animals

Female Swiss mice (20–25g) were divided into two groups: treated with 25μg or 50μg venom; these sublethal doses were also used by Feitosa Neto (8). Groups were

subdivided into three subgroups of six animals each. The fasted animals were intraperitoneally (i.p.) injected with venom and anesthetized through diethyl-ether inhalation after 2, 4 or 24h for blood collection (about 1ml) by cardiac puncture. Control animals received 100μl PBS, i.p. The present experiment was approved by the Committee of Ethics in Research with Animals 009/04.

Histological Analysis

Animals were sacrificed by ether inhalation and the following organs were removed: spleen, skeletal muscle, kidneys, liver and lungs, which were then fixed in 10% formalin, dehydrated and included in paraffin wax for 5μm sections that were stained with HE and observed under light microscope.

Biochemical Analysis

After blood collection, serum was separated by centrifugation and the following tests were carried out: AST and ALT levels determination, which was based on the formation of 2,4-dinitrophenyl phosphate; LDH levels determination, which was based on the formation of NADH (one unit results in the formation of 1μmol NADH per minute); total protein content determination using the Biuret method; glucose levels determination, which was based on the formation of quinoneimine dye; creatinine levels determination using the creatinine picrate method; and urea levels determination, which was based on the formation of indophenol blue, using a BioSystems BTS 310 photocolorimeter and Standard BioSystems reagents.

Statistical Analysis

Data of normal distribution were expressed as means ± S.E.M. The values obtained were evaluated by analysis of variance (ANOVA), followed by sufficient *post hoc* tests. Each treatment was considered an independent variable. In all cases, the considered statistical significance level was $p<0.05$. The Graph Pad Prism® program, version 3.0, was used for obtaining graphs and statistical analysis of the results.

RESULTS

Histological Findings

Figure 1 shows some tissues of organs from treated animals, compared with those from control animals. Cellular alteration was verified on the lungs, kidneys and liver, but not in the spleen or skeletal muscle. Figure 1A shows a histological section of liver 4h after injection of 25µg venom. There are leukocyte aggregations near blood vessels and evident vascular congestion. Figure 1B displays a histological section of liver from a control mouse. The center-lobe vein has normal morphological characteristics. Figures 1C and 1D present lung sections 4h after injection of 25µg venom. Cellular alterations were noticed due to the presence of inflammatory cells in the inter-alveolar spaces and inside pulmonary alveoli as well as abnormal accumulation of erythrocytes and leukocytes. Control group presented normal lung characteristics (Figure 1E).

Figures 1F and 1G show the cortical region of kidneys 4h after injection of 50 and 25µg venom, respectively. Evident glomerular alterations were noticed as renal glomeruli necrosis and vascular congestion (Figure 1F). A renal corpuscle with leukocyte infiltration was observed in the glomerular capillaries and, more discretely, in the periarterial areas and between the renal tubules (Figure 1G). The renal corpuscle and the renal tubule in the control group (Figure 1H) were normal.

Venom-Induced Biochemical Alterations

An increase in AST activity was observed 4h after injection of 25µg venom, compared with the control group; 2h after injection of 50µg venom, it was also significant (Figure 2). On the other hand, ALT levels did not change significantly with venom doses at each time interval (results not shown). Serum levels of LDH were high at all studied periods and 50µg venom caused significant increase, whereas 25µg caused significant changes only at 2h (Figure 3).

Regarding the enzymatic determination of total proteins, the main increases were observed with 50µg at 4h and 24h and were considered extremely significant (Figure 4). Serum levels of glucose with 50µg at 2h were considered very significant compared with the control group (Figure 5).

Creatinine levels were not significant in relation to the control group (results not shown). Urea levels at 2h and 4h with 50μg venom were extremely significant compared with control (Figure 6).

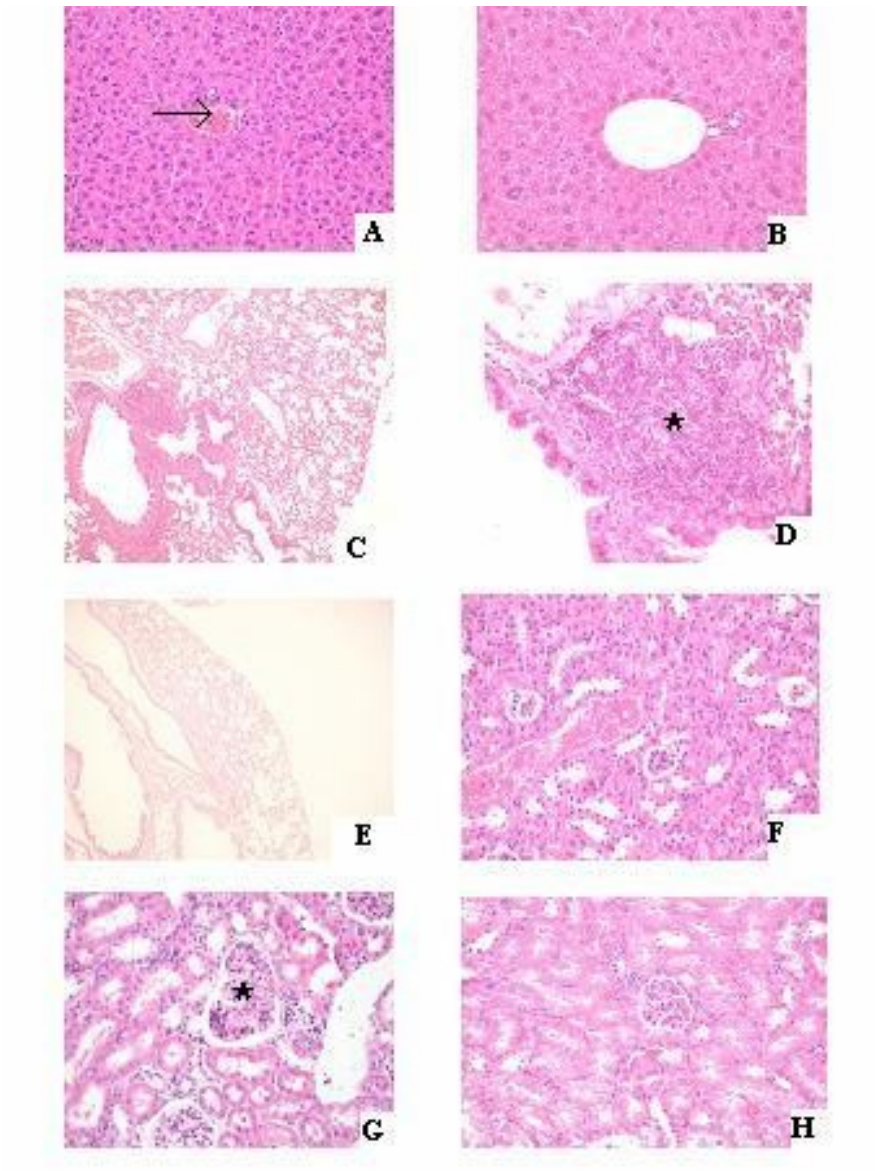

Figure 1. A: Center-lobe vein in the liver of an animal treated with 25μg venom (4h); B: Liver of a control animal (4h); C and D: Alveolar spaces and pulmonary alveoli in the lungs of animals treated with 25μg venom (4h); E: Lung of a control animal; F and G: Cortical region in the kidneys with glomeruli of animals treated with 50μg and 25μg venom, respectively (4h); H: Kidney of a control animal (4h). 400X magnification.

→: Vascular congestion.

∗: Inflammatory cells.

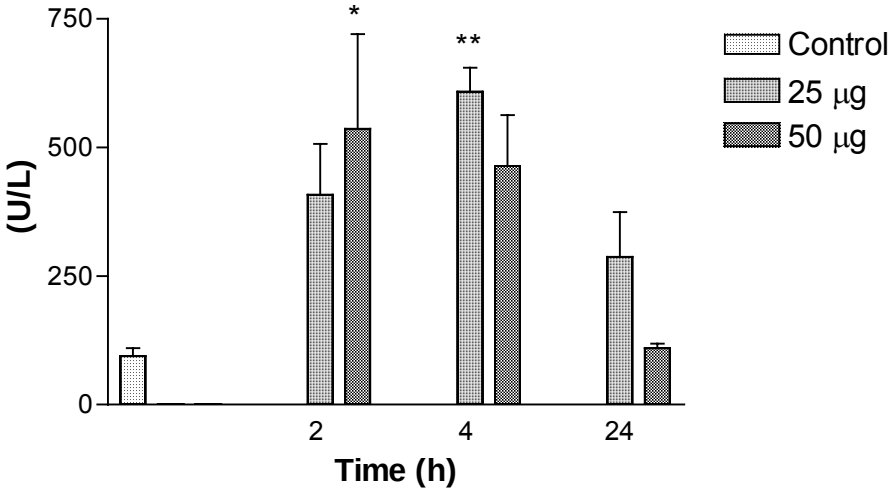

Figure 2. Variations in aspartate aminotransferase (AST/GOT) levels in mice intraperitoneally injected with 25 or 50μg of *Bothrops jararacussu* venom diluted with 0.1ml PBS. Each bar represents the mean and vertical lines, the standard error of mean (S.E.M.) of results obtained from 6 animals.

Asterisks denote the significance levels when compared with control group: * $p<0.05$, ** $p<0.01$.

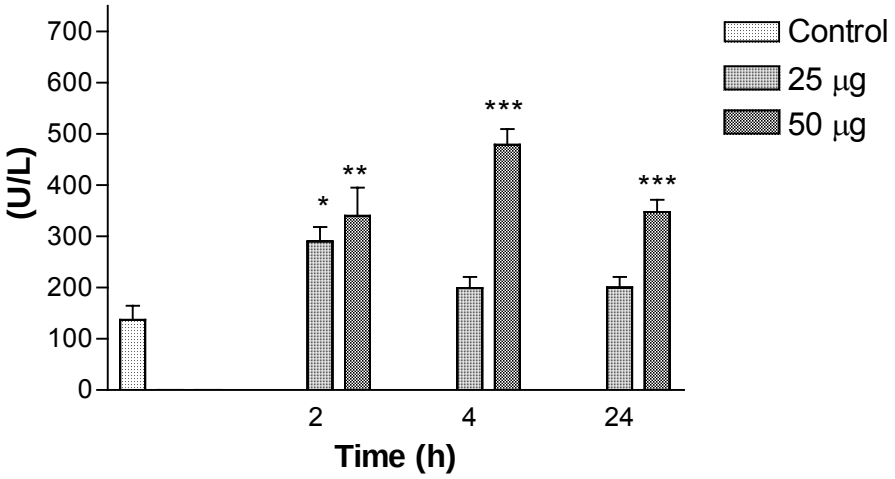

Figure 3: Variations in lactate dehydrogenase (LDH) levels in mice intraperitoneally injected with 25 or 50μg of *Bothrops jararacussu* venom diluted with 0.1ml PBS. Each bar represents the mean and vertical lines, the standard error of mean (S.E.M.) of results obtained from 6 animals.

Asterisks denote the significance levels when compared with control group: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

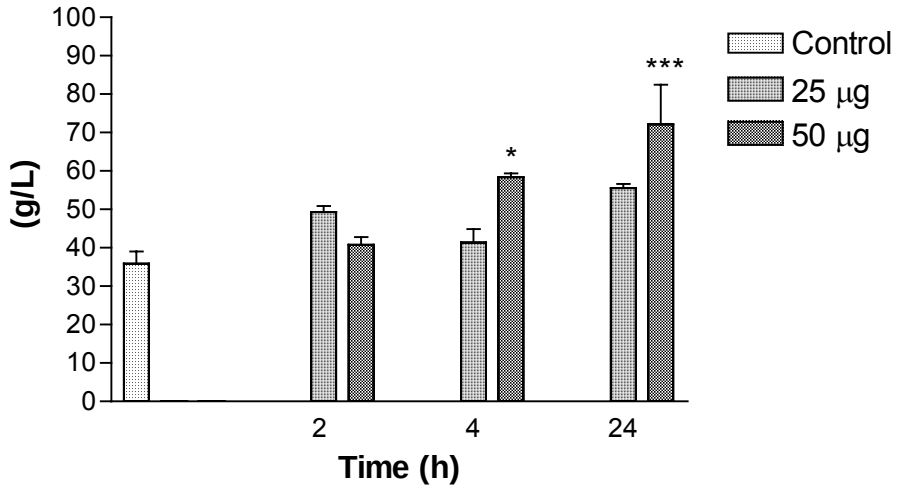

Figure 4. Variations in total protein levels in mice intraperitoneally injected with 25 or 50μg of *Bothrops jararacussu* venom diluted with 0.1ml PBS. Each bar represents the mean and vertical lines, the standard error of mean (S.E.M.) of results obtained from 6 animals.

Asterisks denote the significance levels when compared with control group: * $p<0.05$, *** $p<0.001$.

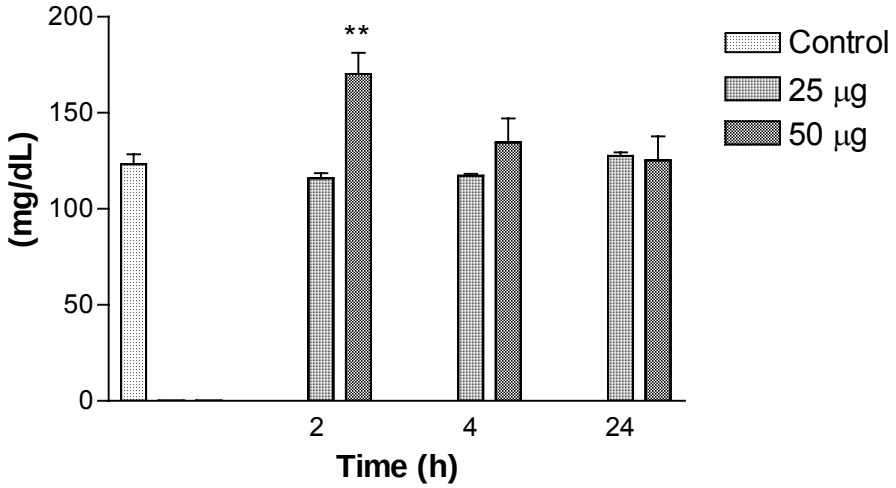

Figure 5. Variations in glucose levels in mice intraperitoneally injected with 25 or 50μg of *Bothrops jararacussu* venom diluted with 0.1ml PBS. Each bar represents the mean and vertical lines, the standard error of mean (S.E.M.) of results obtained from 6 animals.

Asterisks denote the significance levels when compared with control group: ** $p<0.01$.

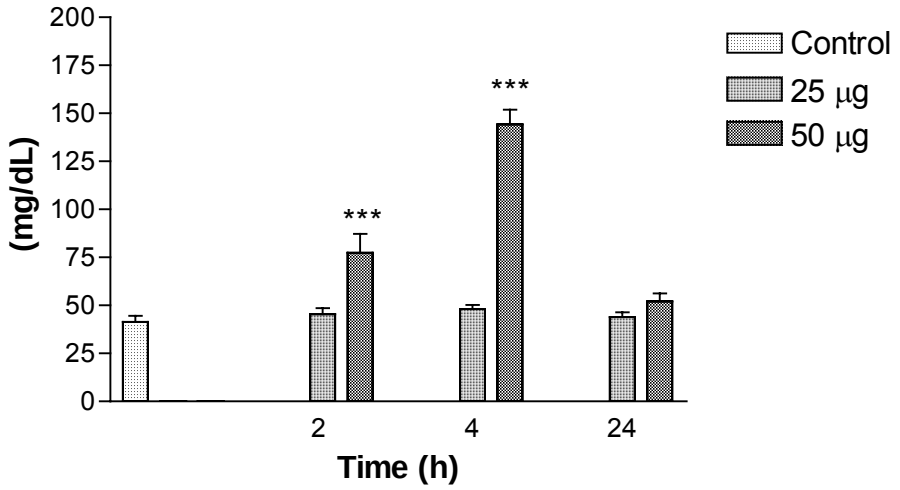

Figure 6: Variations in urea levels in mice intraperitoneally injected with 25 or 50μg of *Bothrops jararacussu* venom diluted with 0.1ml PBS. Each bar represents the mean and vertical lines, the standard error of mean (S.E.M.) of results obtained from 6 animals.

Asterisks denote the significance levels when compared with control group: *** $p<0.001$.

DISCUSSION

Bothrops jararacussu has one of the most lethal venoms among *Bothrops* species. In a review of 29 snakebite accidents caused by *B. jararacussu*, several patients developed shock and oliguria few hours after the bite and three victims died (18).

Pathological alterations (local effects provoked by snakebites) have been extensively reported due to the large variety of enzymes and other compounds present in snake venoms. However, pathophysiological alterations, the role of enzymes, and the systemic complications induced by sublethal doses are not very clear for B. *jararacussu* venom.

Figures 1A–H present important information about tissue alterations in some organs of animals treated or not with venom, corroborating the obtained serum data; however, such alterations were not dose and time-dependent. In Figure 1A, tissue alterations in the liver are visible as there is high concentration of perivascular leukocytes, indicating an inflammatory process, besides intense vascular congestion in the center-lobular vein.

Alterations in striated muscle were not visible, probably because the chosen envenomation route (i.p.) used a site with many capillaries carrying venom for all the

system, not restricting it to only one site. A snakebite accident is more similar to subcutaneous injection of venom (15, 17), allowing the observation of systemic signs and modified tissue characteristics in the liver and pulmonary parenchyma, together with renal hemorrhage. Zamunér *et al*. (28) also reported effective myonecrosis by *B*. *jararacussu* and *B. moojeni* venoms.

Hemorrhagic toxins and other components impair or increase the vascular permeability of endothelial cells and the basal membrane, allowing the blood to escape towards the neighboring tissues (2, 25). Liver cells presented altered metabolic activity, which was verified by blood biochemical analysis, suggesting hepatocyte death. Inflammatory focus was observed in all the liver parenchyma, principally in the perivascular region.

The metalloproteinases found in the venom presented hemorrhagic effect and were capable of inducing the release of inflammation mediators such as cytokines, intensifying the inflammatory response (25), which may justify the occurrence of a large quantity of defense cells in the pulmonary parenchyma. This effect collaborates to coagulation in microcirculation, promoting disseminated intravascular coagulation, which leads to hemorrhage and pulmonary edema.

In lung histological sections, morphological alterations were evident with 25μg venom, which caused increased inflammatory cells and intra-alveolar erythrocytes as well as edema, indicating compromised pulmonary functions (Figure 1D). The increase in pulmonary damage depends on the local lesion but its evolution is not frequent in human envenomation by *B. jararacussu* (4).

In animals treated with 25 or 50μg of venom, histological analysis of the kidneys confirmed the diagnosis of increased renal workload resultant from the high protein levels observed in serum analysis, which can lead to cellular and/or tissue alterations that compromise the organ function and cause renal glomeruli destruction, vascular congestion, microvascular hemorrhagic lesion, and leukocyte aggregation.

Renal failure is the major complication in envenomation by *B. jararacussu* and other *Bothrops* species (18). Cortical renal necrosis can be related to intravascular coagulation or directly to renal endothelium or even vasospasm toxic effect (1).

In the liver, alterations were not clearly visible under the microscope and were only verified by leukocyte aggregation and intravascular coagulation. Figure 2 shows that serum levels of AST significantly increased, indicating hepatic damage (24) as

observed in hepatitis, other hepatic diseases associated with necrosis after administration of some classes of medication or after myocardial infarction, diseases of the muscular-skeletal system, acute pancreatitis, hemolytic diseases, and others (5, 11).

The levels of ALT were also altered, although not significantly. The fact that AST levels increased more with dose than ALT levels indicates not only cell destruction, but also mitochondrial disruption (3). The results obtained for AST and ALT levels were similar to those obtained by Chavez *et al*. (6) with *B. asper*, which in turn were different from those reported by Teibler *et al*. (27) with *B. alternatus*: all two parameters significantly changed.

Regarding LDH, the increase in its activity was significant at 2, 4 and 24h with 50μg and only at 2h with 25μg venom; in the study of Chavez *et al*. (6), it differed only at 6h. LDH activity is increased in hepatic diseases, renal alterations, myocardial infarction, and progressive muscular dystrophy and in any case of hemolysis (5, 11). These alterations and ALT levels indicated skeletal-muscle (6) and, possibly, myocardial damage (3), which was also reported by Benvenuti *et al*. (4) about a woman bitten by *B. jararacussu*. The glucose levels observed in mice were within the normal range of 62–175mg/dl (13).

Total protein content presented dose and time-dependent alterations and urea levels significantly increased, showing intense protein degradation, increased renal workload and liver necrosis. Renal damages with different types of lesions (glomerular, tubular, interstitial or vascular) increased urea serum levels (19).

Results suggested that the increase in total protein content and urea levels was related to the myotoxic (14, 18, 22, 23) and proteolytic activities (4, 9) of *B. jararacussu* venom and to the decrease in the liver activity. Milani *et al*. (18) described 29 snakebite accidents caused by this species, in which several patients developed shock and oliguria few hours after the bite. This also explains the data found in the present study.

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