INFLUENCE OF THE PRESENCE OF CROTAMINE IN THE COMPOSITION OF THE *CROTALUS DURISSUS TERRIFICUS* VENOM FACE TO CROTALIC ANTIVENOM, A HISTOLOGICAL APPROACH

INFLUÊNCIA DA PRESENÇA DE CROTOXINA NA COMPOSIÇÃO DO VENENO DE *CROTALUS DURISSUS TERRIFICUS* FRENTE AO SORO ANTICROTÁLICO: UMA ABORDAGEM HISTOLÓGICA

INFLUENCIA DE LA PRESENCIA DE CROTOXINA EN LA COMPOSICIÓN DEL VENENO DE *CROTALUS DURISSUS TERRIFICUS* FRENTE AL SUERO ANTICROTÁLICO: UN ENFOQUE HISTOLÓGICO

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ABSTRACT
The main fractions isolated from *Crotalus durissus terrificus* venom include crotamine, gyrotoxin, crotoxin, convulxin and phospholipase A2. The antivenom is produced by a pool from *Crotalus durissus* ssp venoms, which do not always express crotamine, which may reflect on the treatment. The objective was to evaluate, by light microscopy, the effectiveness of antivenom in cellular injuries induced by *C. d. terrificus* venom with (Vcrot+) and without (Vcrot-) crotamine. The study design was *in vitro* using routine histological procedures on donated neuromuscular preparations under Ethics Committee 201/2021, exposed to 1) control; 2) Vcrot+; 3) Vcrot-; and treated with antivenom in 4) preincubation and 5) post-venom models. The results were myotoxicity index (MI) in control preparations of 42.2 % ± 2.6 cells; Vcrot+ 95.2 % ± 1.8; Vcrot- 89.6 % ± 2.3. In the preincubation model, the antivenom significantly decreased the lesions to 74.1 % ± 3.8 of Vcrot+ and 71.9 % ± 4.5 of Vcrot-. In the post-venom model, the results were Vcrot+ → 10', 98.0 % ± 0.1; → 30', 84.8 % ± 0.1; and → 60' 85.6 % ± 0.1, while for Vcrot- were 84.7 % ± 0.1; 83.6 % ± 0.01 and 91.9 % ± 0.09, respectively. In conclusion, the antivenom neutralizes a major amount of constituents in the venom in the preincubation model, while in the post-venom model, it was not able against the myotoxic effects of both venoms, but allowed to show the sequence of pathological events in an *ex vivo* model.

Keywords: Antivenom. *Crotalus durissus terrificus*. Histology. Phrenic Nerve-Diaphragm Preparation. Snake Venom.

RESUMO
As principais frações isoladas do veneno de *Crotalus durissus terrificus* incluem crotamina, girotoxina, crotoxina, convulxina e fosfolipase A2. O soro anticrotálico é produzido a partir de um pool de venenos de *Crotalus durissus* ssp., que nem sempre expressam crotamina, o que pode refletir na eficácia do tratamento. O objetivo deste estudo foi avaliar, por microscopia de luz, a eficácia do soro anticrotálico nas lesões celulares induzidas pelo veneno de *C. d. terrificus* com (Vcrot+) e sem (Vcrot−) crotamina. O delineamento experimental foi realizado in vitro, utilizando procedimentos histológicos de rotina em preparações neuromusculares doadas, sob aprovação do Comitê de Ética nº 201/2021, expostas a: 1) controle; 2) Vcrot+; 3) Vcrot−; e tratadas com soro anticrotálico nos modelos de 4) pré-incubação e 5) pós-veneno. Os resultados demonstraram índice de miotoxicidade (IM) nas preparações controle de 42,2 % ± 2,6 células; Vcrot+ 95,2 % ± 1,8; e Vcrot− 89,6 % ± 2,3. No modelo de pré-incubação, o soro anticrotálico reduziu significativamente as lesões para 74,1 % ± 3,8 no Vcrot+ e 71,9 % ± 4,5 no Vcrot−. No modelo pós-veneno, os resultados para Vcrot+ foram: → 10', 98,0 % ± 0,1; → 30', 84,8 % ± 0,1; e → 60', 85,6 % ± 0,1; enquanto para Vcrot− foram 84,7 % ± 0,1; 83,6 % ± 0,01 e 91,9 % ± 0,09, respectivamente. Conclui-se que o soro anticrotálico neutraliza grande parte dos constituintes do veneno no modelo de pré-incubação, enquanto no modelo pós-veneno não foi capaz de neutralizar os efeitos miotóxicos de ambos os venenos, permitindo, contudo, demonstrar a sequência de eventos patológicos em um modelo ex vivo.

Palavras-chave: Soro Anticrotálico. *Crotalus durissus terrificus*. Histologia. Preparação Nervo Frênico-Diafragma. Veneno de Serpente.

RESUMEN
Las principales fracciones aisladas del veneno de *Crotalus durissus terrificus* incluyen crotamina, girotoxina, crotoxina, convulxina y fosfolipasa A2. El suero anticrotálico se produce a partir de un pool de venenos de *Crotalus durissus* ssp., que no siempre expresan crotamina, lo que puede influir en la eficacia del tratamiento. El objetivo de este estudio fue evaluar, mediante microscopía óptica, la eficacia del suero anticrotálico frente a las lesiones

celulares inducidas por el veneno de *C. d. terrificus* con (Vcrot+) y sin (Vcrot−) crotamina. El diseño experimental fue in vitro, utilizando procedimientos histológicos de rutina en preparaciones neuromusculares donadas, bajo aprobación del Comité de Ética nº 201/2021, expuestas a: 1) control; 2) Vcrot+; 3) Vcrot−; y tratadas con suero anticrotálico en los modelos de 4) preincubación y 5) post-veneno. Los resultados mostraron un índice de miotoxicidad (IM) en las preparaciones control de 42,2 % ± 2,6 células; Vcrot+ 95,2 % ± 1,8; y Vcrot− 89,6 % ± 2,3. En el modelo de preincubación, el suero anticrotálico redujo significativamente las lesiones a 74,1 % ± 3,8 en Vcrot+ y 71,9 % ± 4,5 en Vcrot−. En el modelo post-veneno, los resultados para Vcrot+ fueron: → 10', 98,0 % ± 0,1; → 30', 84,8 % ± 0,1; y → 60', 85,6 % ± 0,1; mientras que para Vcrot− fueron 84,7 % ± 0,1; 83,6 % ± 0,01 y 91,9 % ± 0,09, respectivamente. En conclusión, el suero anticrotálico neutraliza gran parte de los constituyentes del veneno en el modelo de preincubación, mientras que en el modelo post-veneno no fue capaz de neutralizar los efectos miotóxicos de ambos venenos, aunque permitió demostrar la secuencia de eventos patológicos en un modelo ex vivo.

Palabras clave: Suero Anticrotálico. *Crotalus durissus terrificus*. Histología. Preparación Nervio Frénico-Diafragma. Veneno de Serpiente.

1 INTRODUCTION

Snakebites represent a significantly overlooked public health concern in numerous tropical and subtropical nations. Annually, approximately 5.4 million snakebites transpire, leading to 1.8 to 2.7 million instances of poisoning. The associated mortality ranges from 81,410 to 137,880 deaths, with nearly threefold incidents of amputations and other enduring disabilities recorded each year (World Health Organization, 2018a).

The venomous snakes of public health concern in Brazil are represented by two genera of the Elapidae family, *Micrurus* and *Leptomicrurus*, and three genera of the Viperidae family, *Bothrops*, *Crotalus*, and *Lachesis* (Brazil, 2020). According to data from the epidemiological Bulletin of the Ministry of Health, 27,515 snakebite incidents were reported to the Notification of Aggravations Information System (SINAN) in Brazil in the year 2022. Of these, 19,044 cases (69.2%) belong to the *Bothrops* genus with 82 deaths (0.4%), 2,237 cases (8.1%) belong to the *Crotalus* genus with 15 deaths (0.6%), and 3,697 cases (13.4%) which the snake species was not identified (Brazil, 2022).

The *Crotalus* genus in Brazil comprises only one species, *Crotalus durissus*, and six subspecies*: C. d. terrificus*, *C. d. cumanensis*, *C. d. collilineatus*, *C. d. cascavella*, *C. d. ruruima*, and *C. d. marajoensis*. Snakes of the *Crotalus* genus are found in the arid and semi-arid regions of the Northeast, as well as in fields and open areas of the South, Southeast, and North (Melgarejo et al., 2009). These snakes are terrestrial and nocturnal, commonly known as rattlesnakes or South American rattlesnakes. They are robust and not very agile, with a rattle at the end of their tails (Pinho and Pereira, 2001; Pereira, 2011; Da Silva-Junior et al., 2020).

The venom of venomous snakes is composed of a mixture of proteins and peptides that bind to receptors. When interaction with tissue occurs, it induces a series of modifications in the organism's homeostasis, disrupting the nervous and cardiovascular systems with neurotoxic, myotoxic, and coagulant effects (Vonk et al., 2011).

The toxic effects resulting from the bite of *C. d. terrificus* are due to the toxins present in the venom (Brazil, 2001; Pinho and Pereira, 2001). The rattlesnake venom is composed of various substances, including four main toxins: crotoxin (Slotta and Fraenkel-Conrat, 1938; Vital Brazil and Excell, 1971; Hörtnagel and Hanin, 1992, Sampaio et al., 2010; Fusco et al., 2015), gyroxin (Kruse; Van Wimersma; Wieda, 1977; Barrabin et al., 1978; Alexander et al., 1988; Barros et al., 2011), convulxin (Prado-Franceschi et al., 1981; Oliveira, 2014), and the myotoxin crotamine (Gonçalves and Vieira, 1950; Ownby; Aird; Kaiser, 1988; Lourenço et al., 2013; Lima et al., 2018).

Crotamine, capable of inducing muscle spasms, was isolated in 1947 from the venom of the Brazilian rattlesnake from the central and southern regions (Gonçalves and Vieira, 1950). It is a polypeptide consisting of 42 amino acid residues with low molecular weight, including 11 basic amino acids, 9 lysines, and 2 arginines. Additionally, it has 6 cysteines interconnected by disulfide bridges, providing significant conformational stability (Gonçalves, 1956; Laure, 1975; Hampe; Vozari-Hampe; Gonçalves, 1978). Crotamine possesses antimicrobial, antifungal, and antitumor properties. Its three-dimensional structure is comparable to peptides belonging to the family of mammalian β-defensins, which are rich in arginine and lysine residues and play a role in the immune system (Siqueira et al., 2002; Pereira, 2011; Yamane, 2013; Da Cunha et al., 2018).

There are various perspectives on the action of crotamine on skeletal muscle tissue. Concerning voltage-dependent sodium channels, its depolarization is antagonized by tetrodotoxin or reduced Na^+ concentration, leading to increased permeability of Ca^{2+} ions (Cheymol et al., 1971). Additionally, it inhibits voltage-dependent potassium channels (Peigneur et al., 2012). There is no consensus on its mechanism of action, with acceptance both on voltage-dependent potassium channels and sodium channels, causing electrophysiological changes and structural damage to muscle fibers (Da Cunha et al., 2018; Lima et al., 2018; Silvestrini et al., 2019). This polypeptide (Figure 1) can easily traverse the cell membrane and has been employed as a carrier for pharmacological molecules (Cell Penetrating Peptides - CPPs) (Oguiura; Boni-Mitake; Rádis-Baptista, 2005; Rodrigues et al., 2012; Oliveira, 2014).

Differences in the venom composition based on the snake's gender, developmental phase and living conditions were described by Lourenço et al. (2013). Biological activity was analysed in comparison with the Brazilian reference venom in 60 adults (captive males and females, as well as newly captured) and 18 newborns. Crotamine was found in 39.7% of the samples, and the concentration significantly differed between adults (75%) and newborns (60%). Venom with a yellow color was found in 10.2% of the samples, of which 1.3% were crotamine-positive, a rare occurrence. These results demonstrate that venoms vary significantly in terms of activity and protein concentration, even when originating from the same species and region. This variation should be considered in the production of antivenoms for treating snakebites (Lourenço et al., 2013).

The presence or absence of crotamine can impact antivenom production, particularly in selecting the pool of crotalic venoms, as crotamine-positive and crotamine-negative venoms may have different mechanisms of action (Tasima et al., 2020). When present, crotamine represents about 10 to 25% of its dry weight (Lima et al., 2018) and can lead to

severe accidents in animals such as cattle (Lago et al., 2000). Its presence can be determined through the traditional lethality test in mice via intraperitoneal (i.p.) administration of venom, protein electrophoresis on polyacrylamide gel (SDS-PAGE), and high-performance liquid chromatography (HPLC) using a photodiode array (PDA) detector (de Oliveira et al., 2015).

Serotherapy is the official treatment recommended by the WHO (Gutiérrez et al., 1998; Brazil, 2001), of which Brazil is a signatory. Antivenom heterologous sera are concentrated immunoglobulins obtained through hyperimmunization of horses. In Brazil, the laboratories that produce these immune derivatives for the public health system are Butantan Institue (São Paulo), Ezequiel Dias Foundation (Minas Gerais), Vital Brazil Institute (Rio de Janeiro), and Immunobiological Production and Research Center (Paraná), except for the antilatrodectic serum (SALatr), which has been imported from Argentina. The sera are produced in liquid form. The ampoules should be stored in the refrigerator at a temperature of 4 to 8 degrees Celsius, avoiding freezing, with a general self-life of two to three years (Brazil, 2001).

The crotalic venoms used in the hyperimmunization of horses come from two subspecies – *C. d. terrificus* (50%) and *C. d. collilineatus* (50%), and the serum produced with crotamine-negative venom will not recognize this molecule (Teixeira-Araújo et al., 2017). Furthermore, it is known that this toxin induces myonecrosis (Cameron and Tu, 1978; Toyama et al., 2003), paralysis of hind limbs, and spontaneous, irregular contractions in the diaphragms of rats, mice, and rabbits (Gonçalves, 1956; Lima et al., 2018) by acting on sodium and potassium ion channels (Brazil and Fontana, 1993; Peigneur et al., 2012; Lima et al., 2018).

This study aims to assess the efficacy of antivenom on cellular damage induced by venoms with and without crotamine exposure in *ex vivo* preparations through histological analysis. This evaluation aims to elucidate the specific impact of crotamine in venom, providing valuable insights for public health authorities responsible for antivenom production. Utilizing a consistent experimental model employed by previous researchers (Toyama et al., 2003; Lima et al., 2018) facilitates result comparisons and informs evidence-based decision-making in the field.

2 MATERIAL AND METHODS

2.1 VENOM AND ANTIVENOM

The venoms of *C. d. terrificus* with (Vcrot+) and without (Vcrot-) were kindly provided by Prof. Dr José C. Cogo from the University of Brazil, São Paulo (SP), and certified by Prof. Dr Stephen Hyslop/Kristian A. Torres-Bonilla from the Faculty of Medical Science at the State

University of Campinas (FCM/Unicamp) and assayed in the study of De Souza et al. (2022). They were stored in a freezer at -20 ºC. The study involving the venoms was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge – SisGen, under the code A7DF1D4, on 16/02/2022. The anticrotalic-IgG serum (Instituto Vital Brazil, S.A., L. 135202 MS:1.0407.008.001-8, expiration date 10/16) was donated by ERSA-Piracicaba (SP), after its expiry for human use, as a condition for its utilization in scientific research. The antivenom concentration in this study was determined according to the manufacturer's recommendation.

2.2 EXPERIMENTAL GROUPS

The biological preparations were divided into different groups: control (nutrient solution), venom with crotamine (Vcrot+ 10 µg/mL), and venom without crotamine (Vcrot- 30 µg/mL), a preincubation model with antivenom (AV) named Vcrot+:AV and Vcrot-:AV, and a post-venom model by adding AV at different time intervals after venom exposure resulting in Vcrot+ →AV (10', 30", 60') and Vcrot- →AV (10', 30", 60').

2.3 HISTOLOGICAL ANALYSIS

The preparatios underwent electrical and indirect stimulation (approved by the Committee on Ethics in Animal Use - CEUA of the University of Sorocaba - SP, with protocol number 201/2021). Subsequently, the preparations were fixed in 10 % formalin for 24 h, followed by immersion in 70 % ethanol until routine processing and embedding in paraffin. From these processed samples 5 µm sections were obtained. These sections were stained with hematoxylin and eosin (HE) for histological observation. The analysis of all samples was performed using a light microscope (Nikon Alphaphot-2YS2-H), and the best-cut fragments were selected for examination. Photos were taken with an Android smartphone equipped with a 48Mp + 5Mp camera with a resolution of 4000 x 3000 pixels. The photos adhered to a standard magnification of 400X (40X objective, bar 1 cm=40 µm). Two examiners, employing a double-blind approach, independently analyzed selected slides, following established criteria for cell type and lesion counting. Each evaluator defined three random and virtual lines for slide analysis. The assessment included quantifying (Figure 2) the normal cells (N, panel 1), and cell damage which was evaluated based on a scoring system that considered delta injury (D, panel 2), myofibril condensation (MC, panel 3), vacuoles (V, panel 4) edema (E, panel 5), myonecrosis and ghost cells (M and G, panel 6) (Ferraz et al., 2014).

2.4 STATISTICAL ANALYSIS

Statistical results were expressed as mean and ± standard error of the mean using the One-way ANOVA system followed by the Tukey test for multiple comparisons, where SIG is equal to 0, it means that the difference between the means is not significant; for SIG equal to 1, it means that there is a significant difference between the means. The Origin 9.5 software (OriginLab Corporation, Northampton, MA, USA) was used, where the confidence interval was 95% and $p< 0.05$.

3 RESULTS

In this study, a comparative analysis of the effectiveness of antivenom on cellular injuries induced by rattlesnake venom (with and without crotamine) was carried out in two pharmacological models (preincubation and post-venom) using the histology approach, which has not yet been described in the literature.

Figure 3 shows in A, a representative control maintained in nutritive solution after 2 h of indirect stimulation, as in all other protocols, which in itself causes slight disorganization of the skeletal muscle cells. Each cell has myofibrils grouped into bundles and the normal cellular organelles, not visible by light microscopy. Note the presence of many nuclei peripherally located just beneath the cell membrane. B and C show representative sections of preparations exposed to crotalic venom with (B, Vcrot+) and without (C, Vcrot-) crotamine. Note the presence of myofibril condensation (MC) as a result of hypercontracted myofibrils densely clumped, which could lead to vacuoles formation (V) as a result of the dilatation of the sarcoplasmic reticulum, and subsequent myonecrosis (M). Ghost cells (G) arise as a result of the destruction of cytoplasm, characterized by optically empty spaces, leaving scattered nuclei in this space. Figure 3D shows the quantitative histological analysis, influenced by the high presence of myofibril condensation (Vcrot+, 95.2 % ± 1.8; Vcrot-, 89.6 % ± 2.3) including in the control preparations (42.2 % ± 2.6).

The pre-incubation model (Figure 4) of the antivenom with Vcrot+ and Vcrot- proved to be significantly (#, $p<0.05$ in comparison to respective venom) effective in protecting the cells as shown in Fig. 4B, reducing the cell damage to 74.1 % ± 3.8 and 71.9 % ± 4.5, respectively.

Post-venom model (Figure 5) with Vcrot+ acting during 10´ rapidly showed swollen cells (see inserted histological section in Fig. 5A), in which the antivenom was unable to counteract, to trigger subsequently, myonecrosis (M) and ghost cells. The addition of antivenom at 30 min, however, protected against the majority of score injuries. The effect at 60´ was similar to that observed at 10´. In terms of myotoxicity index, the antivenom was

unable to avoid the cell damage induced by Vcrot+ as shown in Fig. 5B, which counting was 98.0 % ± 0.1 (10 min); 84.8 % ± 0.1 (30 min) and 85.6 % ± 0.1 (60 min).

Figure 6 shows the post-venom model with Vcrot-. Antivenom decreased significantly edema induction, but myonecrosis did remain with antivenom addition after 60 min. The small quantity of normal cells seems to be decisive in the slow protection by antivenom, which did not happen at any time (Fig. 6A). The myotoxicity index showed no protection of antivenom in the post-venom model, which counting was 84.7 % ± 0.1 (10 min); 83.6 % ± 0.01 (30 min) and 91.9 % ± 0.09 (60 min).

4 DISCUSSION

The snake venom-induced muscle damage is less well-understood compared to other acute effects of envenoming (Thakshila et al., 2022). This becomes even more difficult when the venom does not cause local, but systemic effects, as is the case with the South American rattlesnake *Crotalus durissus terrificus* (Azevedo-Marques et al., 1987). In contrast to venoms from other viperid species, *Crotalus* venoms exhibit a notable absence of substantial inflammatory reactions at the site of the bite in both animals and humans (Rosenfeld, 1971; Azevedo-Marques et al., 2003).

According to Mebs and Onwby (1990) “to detect and evaluate myotoxicity of a venom component, histological examination of the muscle tissue affected is superior to methods such as assaying serum creatine kinase levels”, and it was the method of choice in this study.

The pioneer studies of Cameron and Tu (1978) clearly showed myovacuolization after injection of 50 µg of crotamine in 0.1 mL of 0.9 % NaCl into the medial aspect of the thigh muscles of Swiss Webster white mice. Mice were killed 72 h after injection and myotoxicity was evaluated by light microscope histology. These findings were also confirmed by other researchers (Gutiérrez and Cerdas, 1984; Mebs and Ownby, 1990; Toyama et al., 2003). Thus, we focused the discussion based on data from literature addressed histological evaluation, with the presence or absence of crotamine occupying the central question. There is no histological study evaluating the influence of absence of the crotamine in crude venom.

Isolated mice neuromuscular preparations were submitted to *C. d. terrificus* with or without crotamine and assayed with antivenom according to the recommendation of a manufacturer by De Souza et al. (2022), using the myographic technique, and two validated pharmacological models (Camargo et al., 2010). The resulting muscles were saved for further histological analysis, as mentioned in Material and Methods. The effects of the venom without crotamine (Vcrot-) decreased the neurotoxicity at least three times more than the venom with crotamine (Vcrot+) in those pharmacological protocols carried out by De Souza et al. (2022),

needing the adjustment for the triple of Vcrot-. It may explain the highly damaged cells in both venoms (Fig. 3 B, C, and D).

The preincubation method involves incubating the snake venom with the antivenom usually for 30 min since it allows an interaction between both, before the mixture is added into the bath containing the neuromuscular preparation. Thus, in theory, only the remaining constituents not enough neutralized by antivenom will be evaluated. Our results showed a major number of normal cells and a decrease in the edema induction (Fig. 4A) resulting in significative protection by antivenom (Fig. 4 B). If edema showed to be the main remaining effect not neutralized by the antivenom in the preincubation model, the question arises to what extent the venom-edema induction is responsible for myonecrosis. In a documented instance, an edematogenic response triggered by *C. d. terrificus* venom displayed a non-dose-dependent pattern and manifested swiftly with a fast and transient course (Cupo et al., 1990). Certain investigations have highlighted that the induction of edema by phospholipase A_2 (PLA_2) may occur, with the impact in some instances being contingent on the binding of PLA_2 to particular membrane proteins (Iglesias et al., 2005).

The use of *ex vivo* preparations is scientifically robust to represent the ophidian accidents (Franco and Belo, 2017; Zanetti et al., 2018), is an alternative to the use of *in vivo* animals (Nepal and Jeong, 2020), and allows to carry out also post-venom model, in a simulated condition of envenoming. Interestingly, Vcrot+ rapidly induced edema, and antivenom added into the bath after 10 min did not avoid its progression (Fig. 5A). Taken together with the myotoxicity index, antivenom did fail in protecting the cell damage of both venoms. On the other hand, when Vcrot- was submitted to the same model, antivenom decreased significantly the edema induction, which is curious since the triple of protein (including PLA_2s) was put into the bath, except the crotamine. This finding leads us to wonder what role crotamine plays in the induction of edema. In addition to its role in edema induction, various studies have underscored the involvement of PLA_2s in the myotoxic effects associated with crotalic envenoming. A substantial portion of this impact is due to crotamine and crotoxin (Cameron and Tu, 1978; Kouyoumdjian et al., 1986; Mebs and Ownby, 1990; Carvalho et al., 2019). The CB subunit, functioning as a PLA_2, is responsible for the toxicity induced by crotoxin (Kouyoumdjian et al., 1986), which is classified as a neurotoxic and myotoxic PLA_2 (Aird et al., 1985; 1990; Montecucco et al., 2008). Crotamine, in turn, is essentially a myotoxin, whose main property is inducing myotoxicity. All these points lead to a mathematical understanding of the Vcrot+ and Vcrot- to explain our results, i.e., is the inefficacy of antivenom to combat the edema-induction of Vcrot+, but not in Vcrot-.

Mebs and Onwby (1990) suggested the sequence of pathological events induced by myotoxins and PLA_2, among others, with rich concepts of delta-lesion (wedge-shaped clear areas within the muscle clear of organelles), myofibril hypercontraction, vacuolation, edema, myonecrosis, which are followed by researchers nowadays. A hypothesis using microscopic examination reveals that the initial observable change in cells involves the dilatation or enlargement of elements within the sarcoplasmic reticulum (SR). This phenomenon could stem from either a direct or indirect impact of the toxin on SR membranes. For instance, if the toxin were to influence the plasma membrane at the cellular level, opening Na^+ channels and permitting an unimpeded influx of Na^+ into the cell, it could trigger the accumulation of water within the cell due to osmotic gradients. It is established that the endoplasmic reticulum, specifically the SR in this case, acts as a reservoir for water uptake from the cytosol. Consequently, as the SR fills with water, it undergoes swelling, a process documented in other mammalian cells (Ginn et al., 1968). Alternatively, it is plausible that the toxin penetrates the cell and directly interacts with SR membranes, potentially inducing swelling. However, the specific mechanism driving this effect remains unclear. Authors have proposed convergent mechanisms of action for the several snakes of the world and the literature is abundant (Gutiérrez and Cerdas, 1984; Gutiérrez and Lomonte, 1995; Lomonte et al., 2003; Montecucco et al., 2008; Carvalho et al., 2019) which does not encourage us to explore this field in our study.

The antivenom significantly neutralized constituents in the venom in the preincubation model, demonstrated by the increased quantity of normal cells and inactivation of edematogenic components in both venoms. In contrast, in the post-venom model, the antivenom showed ineffectiveness against the myotoxic effects of both venoms and was unable to prevent edema induction in Vcrot+. Interestingly, it successfully prevented edema in Vcrot-, highlighting the significance of edema in triggering myonecrosis in the *ex vivo* model.

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DECLARATION OF INTEREST STATEMENT

No potential conflict of interest was reported by the authors.

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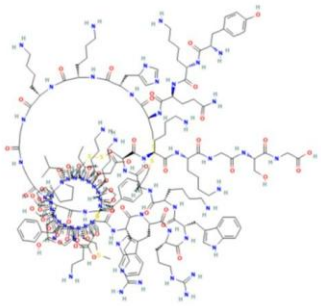
APPENDIX

Figure 1

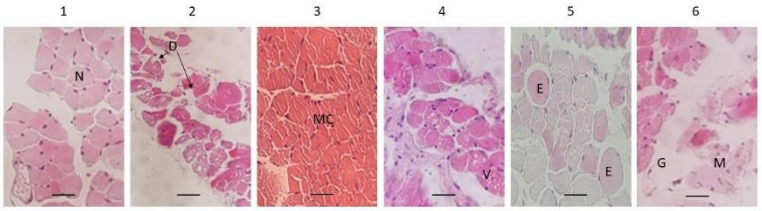
2D representation of the chemical structure of crotamine (MW = 4,884)



Source: (PubChem (https://pubchem.ncbi.nlm.nih.gov/compound/Crotamin#section=2D-Structure)

Figure 2

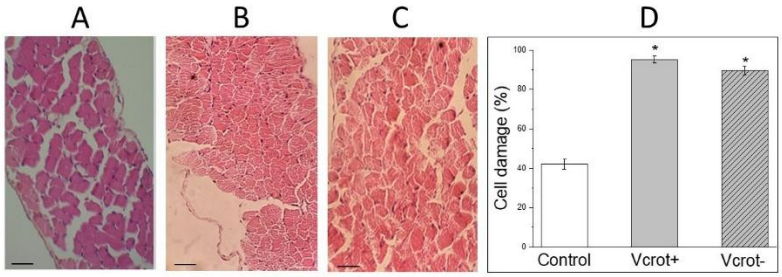
The score is used to quantify the cell morphology. (1) N, normal cell. (2) D, delta lesion. (3) MC, myofibril condensation. (4) V, vacuoles. (5) E, edema. (6) M, myonecrosis. G, ghost cells.



Source: Elaborated by the author.

Figure 3

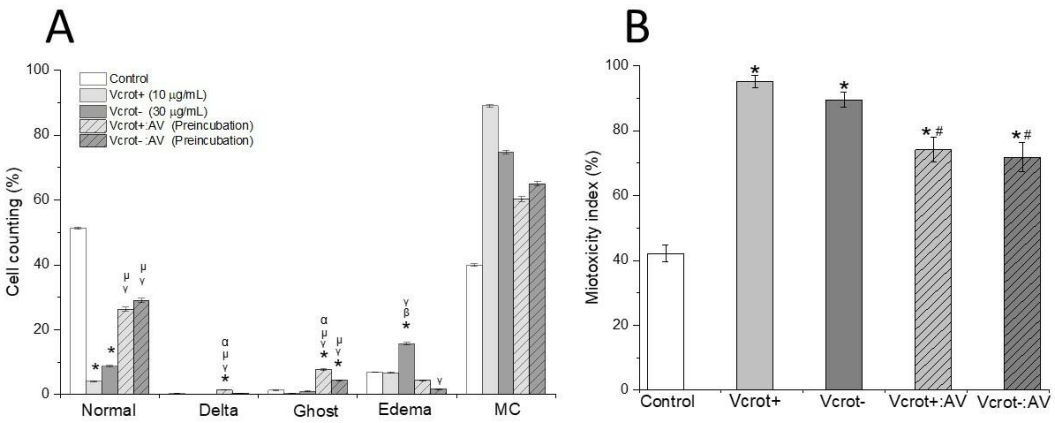
*Cell damage. **A**, control, showing N, normal cells with polygonal aspect, peripheral nuclei, and organized fiber bundles. **B**, Vcrot+, showing MC, myofibril condensation; V, vacuoles, and ghost cells. **C**, Vcrot-, showing MC; M, myonecrosis. **D**, quantitative comparison between control and Crotalus durissus terrificus venom with crotamine (Vcrot+, 10 µg/mL) and without crotamine (Vcrot-, 30 µg/mL)*



Source: Elaborated by the author.

Figure 4

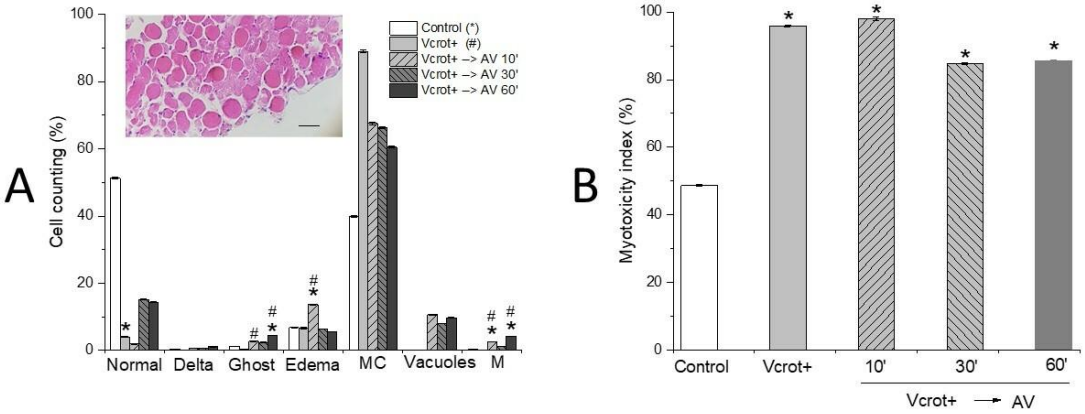
*Preincubation model. (**A**), quantitative analysis by score (normal, delta lesions, ghost cells, edema and myofibril condensation, MC). It is possible to verify the major content of normal cells when preparations were treated with antivenom (Vcrot+:AV and Vcrot-:AV). Antivenom failed against delta lesions induced by Vcrot+, and ghost cells induced by both venoms, but did protect against edema formation of both venoms. However, the number of myofibrils hypercontracted did remain high. Crotalus durissus terrificus venom with (Vcrot+) and without (Vcrot-) crotamine. AV, antivenom. *, p < 0.05 compared to control. α, p < 0.05 compared to Vcrot-:AV. γ, p<0,05 compared to Vcrot+. μ, p<0.05 compared to Vcrot-. β, p < 0.05 compared to Vcrot+:AV. (**B**), Miotoxicity index of treated groups. The preincubation model showed a significant decrease in total cell damage. *, p<0.05 compared to control. #, p<0.05 compared with the respective Vcrot+ or Vcrot-*



Source: Elaborated by the author.

Figure 5

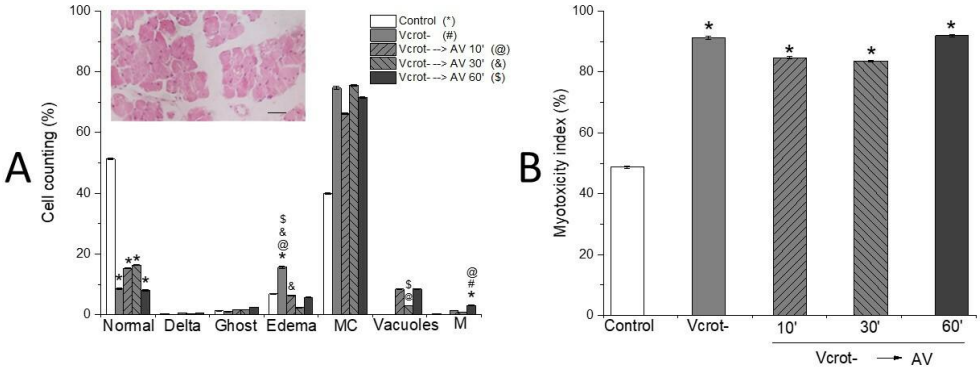
*Post-venom model. **A**, quantitative analysis by score (normal; delta lesions; ghost cells; edema; myofibril condensation, MC; vacuoles, and myonecrosis, M). Antivenom was added after 10, 30 and 60 min. Inserted photography stained with HE, showing the early edema induction induced by Vcrot+, in which antivenom was added after 10 min. Bar: 1 cm = 40 μm. **B**, myotoxicity index showing no protection of antivenom when added after the venom. Crotalus durissus terrificus venom with crotamine (Vcrot+). AV, antivenom. *, p < 0.05 compared to control. #, p<0.05 compared to Vcrot+*



Source: Elaborated by the author.

Figure 6

*Post-venom model. **A**, quantitative analysis by score (normal; delta lesions; ghost cells; edema; myofibril condensation, MC; vacuoles, and myonecrosis, M). Antivenom was added after 10, 30 and 60 min. Inserted photography stained with HE shows a decreased edema formation induced by Vcrot-, in which antivenom was added after 10 min. Bar: 1 cm = 40 μm. **B**, myotoxicity index showing no protection of antivenom when added after the venom. Crotalus durissus terrificus venom without crotamine (Vcrot-). AV, antivenom. *, p < 0.05 compared to control. #, p<0.05 compared to Vcrot-*



Source: Elaborated by the author.