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LOCAL TISSUE DAMAGE INDUCED BY *BOTHROPS* SNAKE VENOMS. A REVIEW

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ABSTRACT: This review focuses on the local effects induced by *Bothrops* venoms. These envenomations are characterized by myonecrosis, hemorrhage and edema which develop rapidly after venom inoculation. Myonecrosis is caused (a) directly, due to the direct action of myotoxins on the plasma membrane of muscle cells, and (b) indirectly, through the development of ischemia resultant from drastic alterations induced by these venoms on both microvasculature and intramuscular arteries. Regarding myotoxins, there is a group of closely related muscle damaging components that can be classified as ''myotoxins with phospholipase A_2 structure'', although some of them lack this enzymatic effect. These myotoxins have been purified from the venoms of *Bothrops asper, B. jararacussu,* and. *B. nummifer.* Local hemorrhage in bothropic envenomations results from the action of acidic metalloproteins of relatively high molecular weight which act directly on the capillary vessels inducing extravasation. Five hemorrhagic toxins have been isolated an characterized from the venoms of *B. jararaca* and *B. neuwiedi.* Local edema is also a typical feature after injection of *Bothrops* venoms. This effect is probably due to: (a) Direct action of venom components on the microvasculature, increasing the permeability of capillaries and venules, and (b) the effect of endogenous mediators released by venom components. *Among* these mediators are histamine, prostaglandins, kinins, C3a and C5a. Besides edema, inoculation of *Bothrops* venoms elicits a prominent cellular inflammatory infiltrate. The need of a comprehensive approach in the study of snake venom-induced local tissue damage is stressed.

KEY WORDS: *Bothrops* venoms, myonecrosis, myotoxins, hemorrhage, edema.

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INTRODUCTION

The large majority of snakebites in Latin America are inflicted by species classified in the genus *Bothrops* [5,8,65]. Despite the existence of evident intraspecific and interspecific variations in the composition and pharmacological activities of their venoms [3,15,30] they induce a qualitatively similar pathophysiological picture, characterized by: (a) Immediate and prominent local tissue damage, i.e. myonecrosis, hemorrhage and edema [17,65]; (b) cardiovascular alterations, especially hemorrhage, and hypovolemic shock[2,65]; (c) coagulation disorders, most frequently defibrination [2,5,65]; and (d) renal alterations which might evolve into acute renal failure[1,2,69].

If antivenom administration is initiated rapidly after envenomation, neutralization of systemic effects is usually achieved successfully, but neutralization of local tissue damage is a more difficult task. In a number of snakebite cases, lack of neutralization of local effects results in permanent sequelae, i.e. tissue loss. Due to the relevance of local effects in envenomations induced by snakes of the genus *Bothrops*, several research groups have studied this problem from different perspectives. In this work we will review their findings, and some conclusions regarding the pathogenesis of local tissue damage by these venoms will be presented.

LOCAL MYONECROSIS

Myonecrosis is a common consequence of envenomations by species of *Bothrops* [2,5,56,65]. Experimental studies have observed this activity in the venoms of *B. asper, B. nasuta, B. nummifer, B. godmani, B. lateralis, B. ophryomegas B. picadoi* and *B. schlegelii* from Costa Rica [14,16,28,44,68], and of *B. jararaca, B. neuwiedi* [44], *B. jararacussu* [58] and *B. alternatus* [57] from Brazil.

The development of muscle damage has been studied with the venoms of *B. asper* [16,19], *B. jararacussu* [58] and *B. alternatus* [57].

In the first two cases myonecrosis was evident from the beginning, and necrotic fibers were characterized by the presence of amorphous clumped masses of myofibrils alternating with empty spaces. In the case of *B. alternatus* venom, necrotic fibers presented a ''waxy hyaline appearance''.

Homma and Tu [28] proposed a classification of venom-induced myonecrosis based on the morphology of the necrotic fibers 24 hr after injection of the venoms. ''Myolytic'' type of necrosis was characterized by fibers where myofibrillar material appeared clumped and alternating with empty spaces in the cytoplasm. ''Coagulative'' type of necrosis, on the other hand, was typified by cells whose myofibrillar material had a more hyaline appearance and a homogeneous distribution. According to these authors, the venoms of *B. atrox* and *B. schlegelii* induced a ''myolytic'' necrosis, whereas those of *B. nasuta* and *B. picadoi* induced a ''coagulative'' myonecrosis; the venom of *B. nummifer* caused a ''mixed'' myonecrosis, i.e. presenting both myolytic and coagulative necrotic fibers. In 1980, Gutiérrez and Chaves [14] used these criteria to characterize myonecrosis induced by several Costa Rican crotaline venoms.

As further studies clearly demonstrated, this classification is an over-

simplification of a complex and dynamic pathologic phenomenon. For instance, when muscle tissue was taken at various time intervals after i.m. injection of *B. asper* venom, it was observed that the morphology of necrotic fibers changed [19]. In the first 3 hr there was a predominance of necrotic fibers with "clumped" myofibrillar material alternating with cytoplasmic spaces devoid of myofilaments ("myolytic fibers"). However, after the sixth hour, the large majority of necrotic fibers contained a more hyaline cytoplasm, with myofibrillar material distributed in a more uniform fashion in the cellular space [19]. Thus, the morphological type of necrosis is different depending upon the time of tissue sampling. Similar findings have been made recently with the venoms of *Naja naja, Crotalus viridis* and *Crotalus atrox* [51].

Despite the lack of validity of this "myolytic" vs "coagulative" classification of myonecrosis, experimental work performed with the venoms of *B. jararacussu* [58], *B. asper* [19] and *B. alternatus* [57] strongly suggested that there are, at least, two different ways by which *Bothrops* venoms affect muscle cells: (a) directly, by the action of "myotoxins" which probably affect the integrity of skeletal muscle plasma membrane [19,57]; (b) indirectly, through an ischemic condition that develops in skeletal muscle secondarily to the disruptive action of venoms on the vasculature [19,57]. Morphologically, cells affected by myotoxins initially presented clumping of myofibrils and subsequently became hyaline in appearance[19,27,58]. In contrast, other necrotic cells, probably affected by ischemia, presented a hyaline morphology from the beginning[19,57]. Similar observations were made by Ownby and Colberg[51], with the venoms of *Crotalus atrox* and *Crotalus viridis,* describing these cells as "moth-eaten" on the basis of their morphology in sections made from plastic-embedded tissue.

In the past, the study of myonecrosis induced by snake venoms was limited by the lack of reliable quantitative assays to estimate the extent of muscle damage. Moreover, the fact that only histology allowed these studies precluded many laboratories to isolate and characterize myotoxins. In recent years, several techniques have been used in the quantitation of venom-induced myonecrosis, such as: (a) quantitation of serum levels of the enzyme creatine kinase and, more specifically, of the isozyme CK-MM[10,16,18,44,47,54]; (b) quantitation of the release of creatine kinase *in vitro* from preparations of skeletal muscle incubated with venoms [23,45]; (c) quantitation of the residual content of creatine kinase in muscle injected with venoms or toxins [26]; and (d) quantitative histological estimation of muscle damage, by counting the number of necrotic and surviving cells [33,55].

MUSCLE DAMAGE DUE TO THE DIRECT ACTION OF MYOTOXINS

Five myotoxins have been purified to homogeneity from *Bothrops* venoms: *B. asper* myotoxin I [18], *B. asper* myotoxin II [35], *B. nummifer* myotoxin [22], and two myotoxins from the venom of *B. jararacussu,* one of which was named "bothropstoxin" [29]. They had molecular weights of 13,000-16,000 and basic isoelectric points, showing similarities in their amino acid composition. When analyzed by polyacrylamide gel electrophoresis under reducing and non-reducing conditions, it was observed that some of them were dimers whereas others were predominantly monomers [22,35].

A puzzling finding regarding these myotoxins has to do with their enzymatic activity. Two of them (*B. asper* myotoxin I and one of *B. jararacussu* myotoxins) showed phospholipase A_2 activity, whereas the rest of them lacked this effect. [18,22,29,35]

However, all of these myotoxins had biochemical characteristics very similar to those of phospholipases A_2. For instance, *B. nummifer* myotoxin and *B. asper* myotoxin II cross-reacted immunologically with both polyclonal and monoclonal antibodies raised against *B. asper* myotoxin I, a phospholipase A_2[22,35,36,39]. Moreover, the myotoxin from *B. jararacussu,* lacking this enzymatic activity, showed conspicuous homology in the amino acid sequence of the amino terminal region with toxic phospholipases A_2 from crotaline and elapid venoms [29]. Thus, all myotoxins isolated from *Bothrops* venoms may be tentatively classified in the group of "toxins with phospholipase A_2 structure" [31] on the basis of their evident biochemical similarities with toxic phospholipases.

Immunochemical data evidenced the presence of components that cross-react with *B. asper* myotoxin I in the venoms of *B. atrox* [18], *B. schlegelli* [37,39] *B. godmani, B. nummifer, B. picadoi* and *Agkistrodon bilineatus* [39]. Moreover, recent unpublished results obtained at the Instituto Clodomiro Picado indicated that there are cross-reacting proteins in the venoms of *B. jararacussu, B. neuwiedi, B. jararaca, B. moojeni, B. colombiensis, B. pictus, B. bilineatus, B. xanthograma* and *B. brazili.* Therefore, there seems to exist a "family" of closely related components in a variety of *Bothrops* venoms having a leading role in the development of muscle tissue damage in these envenomations.

Upon intramuscular injection in mice these myotoxins induced rapid degenerative changes in muscle cells leading to necrosis [18,19,27,29,35]. In the case of *B. asper* and *B. nummifer* myotoxins, the cells underwent early changes characterized by the presence of "delta lesions", followed by clumping of myofibrillar material into amorphous, dense masses which alternated with spaces in the cytoplasm devoid of myofilaments [18,19,27]. Afterwards, myofilaments redistributed in the cellular space and necrotic cells became more hyaline and homogeneous in appearance. An inflammatory infiltrate was observed after the sixth hour, reaching maximum levels by 48-72 hr [19,27,29]. Interestingly, after removal of necrotic debris by phagocytes, there was a normal and successful muscle regenerative process, with the formation of myotubes and regenerative muscle fibers [20,29]. The only morphological feature that distinguished these regenerative muscle cells from adult normal muscle cells was the presence of centrally-located nuclei[20,29]. Otherwise, there was no fibrosis nor proliferation of adipose cells substituting necrotic muscle fibers[20,29]. This pattern of regeneration contrasted with the one observed after myonecrosis induced by crude venoms which, by affecting muscle microvasculature, severely hampered the regenerative response [20,21,58].

The mechanism of action has been studied in the cases of *B. asper* myotoxin I and *B. nummifer* myotoxin. The following experimental findings strongly indicate that the first site of action of these toxins is the skeletal muscle plasma membrane: (a) The first morphological lesions detected were focal, wedgeshaped areas of degeneration in the periphery of muscle fibers [18,19,27], very similar to the "delta lesions" described in biopsy material

from patients with Duchenne muscular dystrophy [46]. Ultrastructural observations corroborated the fact that the plasma membrane was discontinuous or absent in these early lesions,[19] (b) Both *in vivo* and *in vitro* there was a rapid and drastic release of intracellular markers, such as creatine kinase and creatine, after addition of the toxins *18,22,23,27*, concomitantly with a rapid influx of calcium [18,27] (c) Immunohistochemical findings corroborated the binding of *B. asper* myotoxin I to skeletal muscle plasma membrane[7] (d) *B. nummifer* myotoxin disrupted multilamellar liposomes [27].

Interestingly, although *B. asper* myotoxin I exerted its phospholipolytic activity in muscle tissue[18], it induced the release of creatine kinase and creatine *in vitro* in conditions where phospholipase A_2 activity was inhibited, i.e. when calcium was eliminated and EDTA added to the bathing solution[23]. Similar observations were made with a myotoxic phospholipase fraction from the venom of *B. jararacussu* [62]. These findings pointed towards a dissociation between enzymatic and toxic activities in these toxins, as has been shown for other toxic phospholipases A_2[9,64]. Kini and Iwanaga[32] studied the amino acid sequences of several myotoxic phospholipases and concluded that a variety of myotoxins possess characteristic cationic and hydrophobic domains which are different from the catalytic site. Structural studies suggested that the "myotoxic segment" of these molecules is amphiphilic in nature and readily availabe for interaction with membranes [32]. In this regard, it might be relevant the observation that *B. nummifer* myotoxin behaves as an amphiphilic protein when tested by charge-shift electrophoresis [27].

In view of these findings, it is tempting to propose that the myotoxins isolated from *Bothrops* venoms form a group with strong homologies. If indeed myotoxicity depends on a molecular region different from the catalytic site, then the preservation of the catalytic domain is irrelevant from the point of view of myotoxicity. This hypothesis requires support from comparative studies on the amino acid sequences of these "myotoxins with phospholipase A_2 structure".

If enzymatic degradation of membrane phospholipids is not the basis of the myotoxic activity of these toxins, an alternative mechanism has to be proposed. On the basis of the findings discussed above, it is possible that they exert their myotoxicity by an interaction of the amphiphilic portion of the molecule with the phospholipid bilayer, inducing membrane leakiness that eventually would lead to irreversible muscle cell injury. The nature of the binding site, as well as the mechanism of membrane disorganization, remain unknown.

How important are these "myotoxins with phospholipase A_2 structure" in the total myonecrosis induced by crude *Bothrops* venoms? In the case of *B. asper* venom, where four myotoxin variants have been described[36], Lomonte *et al.*[37,38] observed that incubation of crude venom with horse and rabbit polyclonal antibodies neutralized approximately 75% of the myotoxic effect induced by the venom, a demonstration that this group of toxins are responsible for the major part of muscle damage induced by *B. asper* venom. This conclusion was supported by recent unpublished data in which a monoclonal antibody against myotoxin was able to fully neutralize myotoxicity induced by this venom.

MUSCLE DAMAGE DUE TO ISCHEMIA

Besides myotoxicity caused by the direct action of toxins on muscle cells, there is also muscle damage due to ischemia in bothropic envenomations[19,57]. Although there are no studies describing biochemical evidences of ischemia in muscle affected by venoms, it is likely that there is ischemia due to (a) drastic damage to the microvasculature leading to hemorrhage, and (b) alterations in larger vessels, particularly in intramuscular arteries.

Damage to the capillary network is due to the action of hemorrhagic components (see below). As a consequence of their action, capillaries collapse and blood leaks to the extravascular compartment[48]. This phenomenon causes an immediate effect on the blood supply to muscle cells, inducing ischemia. In this regard, it is relevant to point out that experimental inoculations of hemorrhagic components from the venom of *B. jararaca* resulted in immediate hemorrhage, followed by widespread muscle damage[59]. Queiroz *et al.*[59] suggested that damage to muscle fibers after injection of hemorrhagic toxins might be a consequence of the ischemia which developed after the drastic hemorrhagic effect. However, it is also possible that some hemorrhagic components exerted a direct myotoxic activity, as has been proposed for viriditoxin and hemorrhagic toxin *b*, from the venoms of *Crotalus viridis*[10,13] and *Crotalus atrox*[53] respectively. Studies on the action of hemorrhagic toxins on skeletal muscle *in vitro* are required in order to discriminate between direct myotoxicity and myotoxicity due to ischemia. Evidently, there is an urgent need of pathological studies with purified hemorrhagic components from *Bothrops* venoms.

Ischemic muscle damage can also occur as a consequence of alterations in intramuscular arteries. Homma and Tu[28] described the occurrence of arterial lesions in mice injected intramuscularly with the venoms of *B. atrox, B. nasuta, B. nummifer, B. picadoi* and *B. schlegelii*. These lesions included disintegration of endothelial cells, leucocytic infiltration beneath the endothelium, necrosis and disappearance of the smooth muscle fibers in the media, hemorrhage and insudation of a fibrin-like substance into the subendothelial and medial layers, and formation of mural thrombi. Queiroz and Petta[57] described hyaline necrosis of the media in arteries of muscle tissue obtained from mice injected with *B. alternatus* venom. Moreover, inoculation of high doses of *B. jararacussu* venom induced hyalinization of the media of some arteries, as well as loss of endothelium[58]. Little effort has been made on the isolation and characterization of factors affecting large vessels. However, it is interesting that the hemorrhagic component HF_2 of *B. jararaca* venom induced necrosis of intramuscular arteries [59]. Arterial necrosis and thrombosis may have dramatic effects in the blood supply to muscle tissue, originating ischemia in groups of muscle cells. This, in turn, might induce irreversible cell damage, also affecting the process of muscle regeneration[57].

An additional element which may impair the blood supply to muscle tissue is the elevation in the interstitial pressure of some muscle compartments, due to the accumulation of large volumes of fluid after the action of hemorrhagic and edemaforming toxins on the microvasculature. Although this phenomenon has received little attention at the experimental level, so-

me studies suggested that it may be a relevant component in the pathogenesis of local tissue damage in crotaline envenomations[12].

HEMORRHAGE INDUCED BY *BOTHROPS* VENOMS

Hemorrhage is one of the most characteristic effects induced by crotaline venoms (see reviews by Ohsaka[48] and Ownby[50]). The pioneer work made by Ohsaka and his group on the nature and mechanism of action of hemorrhagic components purified from the venom of *Trimeresurus flavoviridis*, a close relative of *Bothrops*[6], led to the proposal that these toxins induce hemorrhage by ''diapedesis'', i.e. by opening the intercellular junctions between endothelial cells in capillaries, with the consequent extravasation[48]. In contrast, Ownby *et al.*[52,53] presented strong evidence indicating that several hemorrhagic toxins from the venoms of *Crotalus atrox* and *Crotalus horridus* induce hemorrhage ''per rhexis'', i.e. by disrupting the integrity of endothelial cells, escaping the erythrocytes through gaps formed within these cells.

Many hemorrhagic components isolated from crotaline venoms are proteases[67], and this enzymatic activity may be relevant to the process of capillary damage. Several hemorrhagic toxins degrade collagen, and it is known that collagen type IV is a component of the basal lamina that surrounds endothelial cells in the capillaries[66] Ohsaka *et al.*[49] demonstrated that several hemorrhagic components from *Trimeresurus flavoviridis* venom released proteins and carbohydrates from isolated glomerular basement membrane. Thus, it might be that hemorrhagic toxins induce damage to the microvasculature by first affecting the integrity of the basal lamina, inducing the collapse of the capillary structure. However, a direct cytotoxic effect on endothelial cells cannot be ruled out at the present time. Further work is required to elucidate the mechanism of action of hemorrhagic toxins.

Five hemorrhagic toxins have been isolated and characterized from *Bothrops* venoms: HF_1, HF_2 and HF_3 from *B. jararaca*[41,43] and NHF_a and NHF_b from the venom of *B. neuwiedi*[42,43]. These five toxins have similar molecular weights (46,000-62,000), being all of them proteins with acidic pI. They were described as heat labile metalloproteins, inhibited by EDTA, EGTA and 1.10 phenanthroline[41,42,43]. Interestingly, many hemorrhagic components isolated from snake venoms were Zn^{2+} — containing proteases which lost their activity upon Zn^{2+} removal[4,67].

The hemorrhagic toxins isolated from *Bothrops* venoms displayed proteolytic activity on casein, with the exception of HF_3 from *B. jararaca*[43]. However, all of them hydrolyzed fibrinogen and the B-chain of oxidized insulin[43]. In this regard, there has been a controversy concerning the proteolytic activity of hemorrhagic toxins[4]. Since casein is not the best substrate for this enzyme, it is necessary to test this activity on other substrates such as dimethylcasein and collagen. It is likely that the large majority of hemorrhagic toxins are proteases with limited substrate specificity.

Besides these five bothropic hemorrhagic components, two additional proteases, bothropasin from *B. jararaca* venom and moojeni protease A from *B. moojeni* venom, induced hemorrhage, although at much higher doses[40,59]. These enzymes were highly active in terms of proteolysis, having a low hemorrhagic effect. Immunologically, these two proteases clearly

differed from hemorrhagic components[40]. On the other hand, polyclonal antibodies raised against hemorrhagic factors reacted with several *Bothrops* venoms, indicating that immunologically-related proteins are present in a variety of these venoms.

EDEMA AND INFLAMMATION

Bothropic envenomations are also characterized by the rapid development of edema and inflammation at the site of venom injection.[2,5,65]. Despite its clinical relevance, the study of venom-induced edema has received little attention, as demonstrated by the scarcity of reports on the isolation and characterization of edema-forming toxins.

When tested on the foot-pad assay described by Yamakawa *et al.*[70], the venom of *B. asper* induced a rapid edema which remained at a high level even at 24 hours[16]. The edema induced by Bothrops snake venoms is problably due to the action of a variety of substances: (a) Hemorrhagic toxins which disrupt the microvasculature inducing extravasation[48,50]. (b) Toxins acting directly on the endothelial cells of the capillaries and venules, increasing their permeability[48,50]. (c) Venom components (phospholipases or cytotoxins) which induce the release of histamine from mast cells. (d) Phospholipases A_2 that release arachidonic acid from phospholipids in cell membranes, initiating the pathway leading to the synthesis of prostaglandins[60] (e) Proteases that act on plasma kininogens, liberating kinins (e.g. bradykinin), as was demonstrated by Rocha e Silva *et al.*[61] back in 1949. Moreover, kallikrein can be activated by factor XII of the coagulation cascade, once this factor is activated after damage to the vasculature. (f) components of the complement cascade, particularly C3a and C5a, which participate in the inflammatory reaction[60]. Due to the presence of many pharmacologically active proteins in *Bothrops* venoms, it is likely that the development of edema is due to the combination of these elements.

Lomonte[34], Gutiérrez *et al.*[25] and Rojas *et al.*[63] observed that neutralization of edema-forming activity of Central American crotaline venoms by a polyvalent antivenom is difficult to achieve. Due to the relevance of edema in bothropic envenomations, it is necessary to identify and characterize edema-forming components in these venoms, as well as to study their mechanism of action, in order to design new therapeutic strategies to confront this significant local effect.

Besides edema, there is a conspicuous cellular component in the inflammatory reaction in bothropic envenomations. In the case of intramuscular injection of *B. asper* venom, inflammatory infiltrate started after the sixth hour and reached its highest level by 48-72 hours[24]. Initially, the predominant cell type was the polymorphonuclear neutrophil but, at later time periods, macrophages became more abundant.[24] It was proposed that degradation of myofibrillar proteins in necrotic muscle cells after injection of *B. asper* venom was accomplished by proteases from invading phagocytic cells.

FINAL REMARKS: THE NEED OF AN INTEGRATED VIEW

Muscle tissue, where local effects take place, is complex both anatomically and functionally. *Bothrops* venoms affect not only skeletal muscle cells,

but also intramuscular arteries, microvessels and nerves. Therefore, the study of local effects must include an integrated analysis of the effect of these venoms on the various cell types and extracellular structures forming a given muscle.

Until now there have been few studies in this direction. For instance, it is not known how relevant is the ischemia in the development of muscle damage. Furthermore, it is necessary to determine which phenomenon is more important in the development of ischemia, if damage to microvasculature by hemorrhagic toxins or lesions in intramuscular arteries. This particular point is especially relevant, since the design of new therapeutic avenues will depend on this information. For instance, if ischemia is basically due to microvessel damage, then there is a good point in using angiogenic substances to stimulate revascularization[11] which, in turn, would contribute to a more successful skeletal muscle regeneration. If, on the other hand, arterial damage predominates, the approach must be different.

Two additional points that need to be investigated are: (a) The role of axonal degeneration in local tissue damage, particularly in regard to the process of muscle regeneration; and (b) the effect of *Bothrops* venoms on the extracellular matrix, including the basal lamina surrounding muscle fibers and capillaries. Since this matrix is determinant in the maintenance of an adequate spatial relationship between cells, its alteration may have drastic effects in the muscle as a whole.

In conclusion, it is necessary to isolate and characterize additional components from *Bothrops* venoms which induce myonecrosis, hemorrhage and edema. It is also important to study the immunological relationships of these components, in order to have a more rational basis for selecting the venoms to be used in the production of antivenoms. Concomitantly, the integration of biochemical, physiological and pathological studies is essential, in order to gain a comprehensive knowledge of these complex and challenging phenomena.

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RESUMO: A revisão focaliza os efeitos locais induzidos pelo veneno de *Bothrops* caracterizados por mionecrose, hemorragia e edema, de evolução rápida. As miotoxinas classificadas como fosfolipase A_2 pela estrutura, embora algumas não possuam essa atividade enzimática; as hemorraginas caracterizadas como metaloproteínas acídicas, e os fatores edematogênicos, estão presentes nos venenos botrópicos. Várias miotoxinas e hemorraginas foram purificadas de venenos botrópicos.

UNITERMOS: Venenos de *Bothrops*, miotoxinas, mionecrose, hemorragia, edema.

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