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Comparative analysis of local effects caused by *Bothrops alternatus* and *Bothrops moojeni* snake venoms: enzymatic contributions and inflammatory modulations

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ABSTRACT

Bothropic envenomation is characterised by severe local damage caused by the toxic action of venom components and aggravated by induced inflammation. In this comparative study, the local inflammatory effects caused by the venoms of *Bothrops alternatus* and *Bothrops moojeni*, two snakes of epidemiological importance in Brazil, were investigated. The toxic action of venom components induced by bothropic venom was also characterised. Herein, the oedema, hyperalgesia and myotoxicity induced by bothropic venom were monitored for various lengths of time after venom injection in experimental animals. The intensity of the local effects caused by *B. moojeni* venom is considerably more potent than *B. alternatus* venom. Our results also indicate that metalloproteases and phospholipases A_2 have a central role in the local damage induced by bothropic venoms, but serine proteases also contribute to the effects of these venoms. Furthermore, we observed that specific anti-inflammatory drugs were able to considerably reduce the oedema, the pain and the muscle damage caused by both venoms. The inflammatory reaction induced by *B. moojeni* venom is mediated by eicosanoid action, histamine and nitric oxide, with significant participation of bradykinin on the hyperalgesic and myotoxic effects of this venom. These mediators also participate to inflammation caused by *B. alternatus* venom. However, the inefficient anti-inflammatory effects of some local modulation suggest that histamine, leukotrienes and nitric oxide have little role in the oedema or myotoxicity caused by *B. alternatus* venom.

1. Introduction

Snake venom is a rich source of proteins and peptides that induce a large variety of pharmacological and toxic effects. Bothropic envenomation is characterised by intense local inflammatory manifestation associated with oedema, myonecrosis, pain and haemorrhage (Teixeira et al., 1994; Brasil, 2009; Gutiérrez et al., 2009). The pathologies caused by snakebites are considered a public health challenge. Nevertheless, the mechanisms of action of the components involved in the local effects are not well understood (Gutiérrez, 2012). Moreover, these effects are not effectively neutralised by conventional antivenom serum therapy, which can contribute to an increase in the number of victims with permanent incapacity (Gutiérrez et al., 1998; Isbister et al., 2009; Queiroz et al., 2008).

The venomic composition of *Bothrops alternatus*, a representative venomous snake widespread in Brazil, revealed that proteolytic enzymes play important roles in the pathological events of envenoming by these snakes (Cardoso et al., 2010). The *B. alternatus* venom is also a rich source of cytotoxins that influence the severe haemorrhagic disturbances, tissue damage and

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inflammatory response (Ohler et al., 2010). *Bothrops moojeni*, another important Brazilian snake, is responsible for most ophidian accidents reported in the Minas Gerais State, South East Region of Brazil (Nishioka & Silvera, 1992). The envenomation caused by these snakes is characterised by severe local oedema and myonecrosis, which are frequently associated with a vast array of active enzymes present in these venoms (Lima et al., 2009; Nascimento et al., 2010).

Bothropic venoms contain multiple components that induce tissue lesion and inflammation (Bonavita et al., 2006; Moura-da-Silva et al., 2007; Olivo et al., 2007; Teixeira et al., 2009; Zychar et al., 2010; Wanderley et al., 2014). Snake venom metalloproteases (SVMPs) cause direct damage to the microvessels, with consequent increments in permeability and extravasation, leading to vascular disturbances such as oedema and haemorrhage (Fox & Serrano, 2008; Markland Jr. & Swenson, 2013). SVMPs are also able to activate specific inflammatory cells and mediators frequently associated with hyperalgesia (Teixeira et al., 2005; Lopes et al., 2009; Zychar et al., 2010). The inflammatory effects induced by phospholipases A_2 (PLA_2) from snake venoms are primarily related to enzymatic activity on the membrane phospholipids and the release of eicosanoid precursors (Dennis et al., 2011).

The damages caused by the actions of these toxins, associated with the inflammatory process, can evolve for the appearance of necrosis and, often, determine the loss of function or amputation of the affected limbs (Cardoso et al., 2003; Gutiérrez et al., 2006). The myonecrosis can be caused by the direct action of myotoxic PLA_2 on the membrane of muscle cells, by alterations caused by SVMPs or by other myotoxic proteins that interfere on the ion control of the muscle fibres (Harris, 2003; Gutiérrez et al., 2009). Another characteristic of this process is the presence of leukocyte infiltrate at the lesion site. The activated leukocytes release inflammatory mediators such as eicosanoids and cytokines, which amplify the recruitment of phagocytic cells and contribute to the necrosis process (Voronov et al., 1999).

The local effects induced by bothropic venoms are the result of multifactorial and synergistic actions of toxins, which are as yet poorly understood. Thus, in this work, we investigated the oedematogenic, hyperalgesic and myotoxic effects induced by *B. alternatus* and *B. moojeni* venoms. We also evaluated the contribution of proteases and PLA_2 to the local effects caused by these venoms. In addition, the involvement of inflammatory mediators was also characterised.

2. Material and methods

2.1. Animals

The experimental animals, male Wistar rats (200–250 g) and Swiss mice (20–25 g), were obtained from the Federal University of Uberlândia (UFU – Brazil). The animals were housed in a temperature-controlled room (23 °C) on an automatic 12 h light/dark cycle (6:00 a. m. – 6:00 p. m. of light phase). Food and water were freely available until the beginning of the experiments. The experiments were performed using 3 animals per group (n = 3/group). After the tests, the animals were euthanased by an overdose of ketamine/xylazine, according to ethical parameters approved by the Ethics Committee on Animal Experimental of the Federal University of Uberlândia (CEUA/UFU - Protocol number 108/12).

2.2. Venom and chemicals

The crude venoms from *B. alternatus* ($Balt_v$) and *B. moojeni* ($Bmoo_v$) were acquired from Bioagents Serpentarium (Brazil). The venoms were dissolved in sterile saline (1.0 mg/mL) at the time of the each experimental procedure. The protein concentrations of the venoms were determined according to Bradford (1976). Carrageenan (inflammatory agent), promethazine (H1 histamine receptor antagonist), indomethacin (cyclooxygenase inhibitor), nordihydroguaiaretic acid (dual cyclooxygenase and lypooxygenase inhibitor) and 2,4'-dibromoacetophenone (BAP - phospholipases A_2 inhibitor) were purchased from Sigma–Aldrich (United States). N^G-Monomethyl-L-arginine acetate (L-NMMA - nitric oxide synthase inhibitor) and Bradykinin D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8] (HOE-140 - selective B_2 bradykinin receptor antagonist) were purchased from Tocris Bioscience (United Kingdom). Phenylmethanesulfonyl fluoride (PMSF - serine proteases inhibitor) and 1,10-phenanthroline (Phe - metalloprotease inhibitor) were purchased from AMRESCO Life Science Research (United States).

2.3. Analysis of local inflammatory effects

2.3.1. Chemical treatments

The following enzymatic activities were determined in order to establish the inhibition of the specific classes of enzymes from snake venoms. SVMP activity was evaluated by the local haemorrhagic effect. The crude or treated venoms (30–60 μg/0.1 mL) were injected intradermally (i.d.) into the dorsal skin of mice. After 3 h the animals were euthanased, the skins were removed and the area of haemorrhage on the internal surface of the skin was measured with the aid of a low-pressure pachymeter (Nikai et al., 1984; Borkow et al., 1997). The snake venom serine proteases (SVSPs) activity was characterised by the capacity of coagulating the fibrinogen solution up to 120 s (Smolka et al., 1997). Clotting times were determined by mixing the crude or treated venoms (30–60 μg/0.1 mL) with 200 μL of fibrinogen solution (3 mg/ml) at 37 °C. The fibrinogen-clotting formation was monitored by a coagulometer (CLOTIMER, Brazil). The PLA_2 activity was evaluated in vitro by indirect haemolysis in agar containing erythrocytes and egg yolk induced by crude or treated venoms (30–60 μg/0.1 mL) (Gutiérrez et al., 1988). After 48 h incubation, at room temperature, the diameter of the haemolytic spot was measured by a low-pressure pachymeter. All experiments were carried out in triplicate. Sterile saline was used as negative control.

The inhibition of specific enzymatic activities of the snake venoms was evaluated by chemical treatments. Samples of venoms (1.0 mg/mL) were incubated with 10 mM Phe for SVMPs inhibition, 20 mM PMSF for SVSPs inhibition or 20 mM BAP for PLA_2 inhibition for 1 h at 37 °C (Lundblad, 1971; Borkow et al., 1997; Diaz-Oreiro & Gutierrez, 1997). After the chemical pretreatment, the venoms were administered to animals and the progress of oedema, hyperalgesia and myotoxicity were monitored as described below. In these tests, an equal volume of each inhibitor was injected into the contralateral paw for control purposes.

2.3.2. Pharmacological treatments

The participation of some inflammatory pathways in the local effects induced by venoms was analysed by pharmacological treatments. The doses of the drugs used were chosen from relevant published reports of inflammatory activities (Faria et al., 2001; Chacur et al., 2001, 2003; Barbosa et al., 2003). The oedematogenic and hyperalgesic responses induced by carrageenan (250 μg/paw) were used as the positive control groups for assessing the efficacy and dosage of the pharmacological treatments (Van Arman et al., 1965). Groups of rats were pre-treated with specific inhibitors or receptor antagonists of inflammatory mediators. To investigate the involvement of arachidonic acid metabolites, the animals received 8 mg/kg of indomethacin, a nonselective cyclooxygenase −1 and −2 (COX-1/COX-2) inhibitor and 100 mg/kg