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Efficient muscle regeneration after highly haemorrhagic *Bothrops alternatus* venom injection

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ABSTRACT

Bothrops alternatus snake venom is particularly characterized for inducing a prominent haemorrhage and affecting hemostasis as a consequence of 43.1% of metallo-proteinases and less than 10% of PLA_2 (almost all non-myotoxic phospholipases) in its venomics. In addition, myonecrosis is the major local effect in viper envenoming which might lead to permanent sequela. Then, the rebuilding of the microvasculature at the local injured site acquires significance since represents one of the pivotal stages for subsequent skeletal muscle regeneration either at morphological or functional aspects. Due to the significance played by vasculature in this process, it is important to study by histology and immunohistochemical techniques, the muscular damage and the sequence of skeletal muscle reconstruction (degree of damage, reconstitution of muscle fibres and capillaries). In this work, we injected intramuscularly 50 or 100 μg *per* mouse of *B. alternatus* venom in gastrocnemius muscles. We provided a complete description and characterization of the different stages of myogenesis after mild (50 μg) and severe (100 μg) local injury induced by *B. alternatus* venom toxins. The regeneration was evaluated 24 h, 3, 7, 14 and 28 days after receiving venom injection. Finally, both doses induced an extended necrosis at the site of injection where, when critical steps in the regenerative process are taking place, an efficient tissue rebuilding is achieved. *B. alternatus* venom is characterized by the high percentage of exclusively class P-III metalloproteinases, and by the lack of class P-I metalloproteinases in its venom composition. This could explain the effectiveness of muscle regeneration after venom injection despite the severity of the initial phase of envenoming.

1. Introduction

Snakebites are well-known medical emergencies in many parts of the world, especially in rural areas. Agricultural workers and children are the most affected. Early in 2009, snake-bite was finally included in the WHO's list of neglected tropical diseases (Harrison et al., 2009) confirming the experience that snake-bite is a common occupational hazard of farmers, plantation workers and others, resulting in thousands of deaths each year and many cases of chronic physical handicap (WHO, 2007; Williams et al., 2010). In South America, *Bothrops alternatus* is a medically relevant species. Definitively, in Argentina this specie is included in Category 1, which consists of highly venomous snakes, which are common or widespread and cause numerous snakebites, resulting in high levels of morbidity, disability or mortality (WHO, 2008).

Although *Bothrops* envenoming is characterized by local tissue damage, edema, necrosis, haemorrhage and an inflammatory response associated with a prominent cellular infiltration (Gutiérrez et al., 2006; Vracko and Benditt, 1972) the myotoxic PLA_2 responsible for myonecrosis are largely absent from this venom (Gutiérrez et al., 2006 and 2010, Warrell, 1996). Therefore,

Abbreviations: PLA_2, phospholipase A_2; i.m., intramuscular administration; svMPs, snake venom metallo-proteinases; i.p., intraperitoneal administration; K49 PLA_2, Lysine- 49 variant phospholipase A_2; LS, longitudinal section; TS, transversal section.

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B. alternatus venom contains a high concentration of svMPs (only P-III) that explain the local and systemic hemorrhagic effects and hemostatic disturbances observed after envenomation by this viper (Öhler et al., 2010). Snake venom metalloproteinases (svMPs) constitute the main enzyme venom's family responsible for this effect in addition with degradation of extracellular matrix components and impairment of regeneration of affected skeletal muscle (Gutiérrez and Rucavado, 2000). Moreover, these hemorrhagic toxins degrade basement membrane and adhesion proteins to endothelial cells, thus weakening the capillary wall. Therefore, transmural pressure acting on the weakened capillary wall causes distention and loss of its integrity with the consequent extravasation (Gutiérrez et al., 2005). Under these circumstances, the persistence of a structurally intact basement membrane around necrotic muscles is also important for the demarcation of the space where myoblast replication and fusion occurs (Hernández et al., 2011).

Muscle regeneration is the process by which damaged skeletal, smooth or cardiac muscle undergoes biological repair and formation of new muscle in response to death (necrosis) of muscle cells. The success of the regenerative process depends upon the extent of the initial damage and many intrinsic and environmental factors (Grounds, 2011). An important part of investigating snake envenoming is the assessment of muscle regeneration. When venoms affect both skeletal muscle and microvasculature, the regenerative process is impaired, with substitution of muscle tissue by fibrosis in some areas, and with the presence of regenerating fibres of reduced diameter (Gutiérrez et al., 1984; Queiroz et al., 1984; Arce et al., 1991; Salvini et al., 2001). In the case of *B. alternatus* snakebites, the process of myotoxicity was already described (Garcia Denegri et al., 2010; Mamede et al., 2013). However, the process of myogenesis after injury was not studied.

According with *B. alternatus* venom composition, it would be expected a deficient regenerative scenario based on the microvascular damage provoked by hemorrhagic snake venom metalloproteinases (svMPs). Moreover, it is still unknown the contribution of this particular venom that also lacks myotoxic PLA_2 and svMPs class I (P-I). In this study, we aim to assess the sequence of muscle recovery after intramuscular injection of *B. alternatus* venom, regardless antiserum administration and considering that venom's proteome displays, among PLA_2 enzymes only acidic non-myotoxic, among svMPs only metalloproteinases class III (P-III) and exerts predominantly hemorrhagic and coagulopathic activities.

2. Materials and methods

2.1. Venom

Crude venom was obtained from a collection of adult *B. alternatus* specimens from Corrientes (Argentina), maintained at the Serpentarium of CEPSAN. Immediately after collection, venom was lyophilized and kept frozen at −20 °C. Venom solutions were prepared in ammonium bicarbonate (1 M, pH 8.0) immediately before use.

2.2. Biological model

Our muscle regeneration experiments were performed on adult Swiss CF-1 mice weighing 20 g ± 5 g. The animal services unit of the Veterinary Faculty of National University from the Northeast supplied these animals. Mice housed in controlled conditions as a constant temperature of 20 °C on a 12 h light/dark cycle received food and water *ad libitum*.

2.3. Ethics statements

The Ethics and Biosafety Committee of the Faculty of Veterinary Science of National University from the Northeast, Argentina, approved the experimental protocol involving the use of animals in this study (Protocol No056).

2.4. Determination of median lethal dose (LD_{50}) via intramuscular (i.m.)

In order to evaluate the maximum challenge dose an animal could survive, intramuscular lethality of *B. alternatus* venom was assayed. Swiss CF-1 mice were injected in the gastrocnemius with different amount of venom: 50, 71, 100, 142 and 200 μg *per* mouse of 20 g ± 5 gr. Six mice were injected *per* dose and the survivors were counted 48 h after injection. Sperman-Karber method (Hamilton et al., 1977) was used for estimating the medial lethal dose that causes the death of 50% of the tested animals. The Median Lethal Dose value was calculated by probit analyses at 95% confident.

2.5. Creatine kinase activity in plasma

Once fixed the highest challenge dose previously estimated in item 2.4., it was set as challenge doses 50 and 100 μg of crude venom in order to evaluate myotoxicity within 24 h post-intoxication. Serum creatine kinase values were measured in venom-injected mice to evaluate muscle damage. After 3, 6 and 24 h of venom injection, mice were anesthetized with chloral hydrate (300 mg kg^{-1}i.p.). Blood samples were obtained from the abdominal aorta without coagulant to obtain sera. Thus, the resulting plasma was submitted to creatine kinase assays (Sigma-Aldrich, USA). CK activity was expressed in International Units *per litre*, where one unit was defined as the amount of enzyme that transfers 1.0 mM of phosphate from creatine kinase to ADP *per* min at pH 7.4 at 30 °C. In the course of the envenomation the local symptoms resulted in a rapid development of internal bleeding and a noticeable inflammation at the site of the injury. These external signs were observed and recorded both, clinically (Acosta et al., 1996a,b and 1997) and experimentally (this paper). No haemorrhage was observed in the control samples.

2.6. Muscle injury and regeneration evaluation. Histological and immunohistochemical assessment

Once fixed the highest challenge dose previously estimated in item 2.4., it was set as challenge doses 50 and 100 μg of crude venom in order to evaluate both the degree of muscle injury and regeneration. Groups of six mice received an i.m. injection at the upper two-thirds of the gastrocnemius muscle of 50 or 100 μg of crude venom dissolved in 0.1 ml of PBS, pH 7.2 (phosphate buffered saline). Control groups were treated with PBS as negative controls. For histological examination, mice were sacrificed at various time intervals (6 and 24 h, and 3, 7, 14 and 28 days) carefully chosen as stated previously by Gutiérrez et al. (1984) and Arce et al. (1991). The injected gastrocnemius muscles were dissected and photographed. Then fixed in Bouin's fluid for 12 h, and wax-embedded in paraffin. Samples were submitted for a graded ethanol series. Sections about 1–3 μm thick collected on slides were pretreated with silane (3-amino-propyltriethoxysilane; Sigma Chemical, St Louis, MO, USA), were allowed to dry overnight, then de-waxed and hydrated. When samples were Hematoxylin-eosin (HE) stained and immunohistologically stained with the primary rabbit monoclonal antibody anti-CD31 (PECAM-1) (Novocastra™ Labs, NCL-CD31-1A10), immunostaining analysis were carried out. All incubations