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# Antithrombotic activity of Batroxase, a metalloprotease from *Bothrops atrox* venom, in a model of venous thrombosis

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ABSTRACT

*Background:* Snake venoms are great sources of bioactive molecules, which may be used as models for new drugs. Toxins that interfere in hemostasis have received considerable attention over the years.
*Objectives:* This study aimed at the evaluation of the antithrombotic activity of Batroxase, a P-I metalloprotease from *Bothrops atrox* venom, in an animal model of venous thrombosis.
*Methods:* The antithrombotic activity of Batroxase was tested *in vivo* in a model based on two factors of the Virchow's *Triad*: blood flow alterations (partial stenosis of the inferior vena cava), and vessel wall injury (10% ferric chloride for 5 min), in comparison with sodium heparin (positive control) and saline (negative control). Bleeding/clotting time was assessed by a tail bleeding assay. The immunogenicity of Batroxase was also analyzed.
*Results:* Batroxase (12 mg/kg) reduced thrombus formation in 81%, similarly to heparin (100 U/kg), which reduced it in 85% in comparison with the saline group. Both Batroxase and heparin increased bleeding/clotting time in approximately 3 fold. Immunizations of rabbits with Batroxase do not result in detectable levels of antibodies against this metalloprotease.
*Conclusion:* Batroxase presents antithrombotic activity *in vivo*. Moreover, its lack of immunogenicity increases the interest on its possible therapeutic potential over thrombogenic disorders.


## 1. Introduction

The approach on toxinology research involves the study on toxins from plants, animals and microbial sources, in order to understand their characteristics, metabolism, and functions [1]. These investigation efforts intend not only to prevent and treat their effects on envenomation, but also their actions on different scenarios, *e.g.* the activation or inhibition of the hemostatic system [2–5].

*Bothrops* snake venom metalloproteases (SVMPs) are the main class of toxins responsible for triggering hemostasis events, inducing blood coagulation disorders, and leading to hemorrhage, as they are capable of degrading proteins of vessel membranes allowing blood extravasation, or acting on coagulation factors activators [6–10]. Another feature involving some SVMPs concerns the capacity to act directly on fibrin, degrading clots and preventing the formation of new clots. The therapeutic potential of fibrin(ogen)olytic SVMPs is recently being explored for the treatment of patients with cardio- and cerebrovascular disorders, as described for Fibrolase and its recombinant analog Alfimeprase [11,12].

Our research group purified and biochemically characterized a neutral (pI 7.5) P-I class (~25 kDa) metalloprotease from *Bothrops atrox* snake venom: Batroxase, which is capable of degrading components of the extracellular matrix, such as type IV collagen and fibronectin, as well as components of the coagulation cascade, as fibrinogen and fibrin [13]. This enzyme had its fibrin(ogen)olytic activity inhibited by α2-macroglobulin [14]. Most importantly, Batroxase presented a dose-dependent *in vivo* thrombolytic activity, similar to the clinically relevant drug Alteplase (tissue-type plasminogen activator), without, however, affecting bleeding/clotting time of the studied animals [14]. The inactivation of the metalloprotease by α2-macroglobulin may reduce its

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activity, but also its potential side effects, as seen for bleeding time [14].

In order to continue the exploitation of Batroxase's therapeutic potential for the future development and production of new drugs with effects over hemostasis, the present study aimed at assessing its antithrombotic activity and also its immunogenicity.

## 2. Methods

The equipment and other materials were described in the course of the methodology, and unspecified reagents used were of analytical grade.

### 2.1. *Isolation* of *Batroxase from Bothrops atrox venom*

Batroxase was obtained through chromatographic fractionation of *Bothrops atrox* venom, acquired from Centre of Extraction of Animal Toxins (Morungaba-SP, Brazil), as previously described [14,15]. Briefly, two consecutive chromatographic steps on resins obtained from GE Healthcare (Chicago, IL, USA) were performed. First a size exclusion chromatography on Sephacryl S-200, followed by anion exchange chromatography on DEAE Sepharose. Latter, the fraction containing the metalloprotease was ultrafiltered in concentrator tube with polyethersulfone membrane with cut-off of 3000MWCO, Vivaspin® 20 (Sartorius, Goettingen, Germany), and the isolated protein was quantified using Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer instructions, separated in aliquots of 1 mg/tube, lyophilized and stored at −20 °C until its use in the experiments.

### 2.2. Animals

Male Wistar rats (250–270 g) and adult female New Zealand White rabbits were obtained from the Central Animal Facility of USP (Ribeirão Preto-SP), and maintained under controlled conditions of temperature (24 °C) and brightness (12 h light/dark cycles), with free access to food and water. In the previous day from the venous thrombosis experiments, rats had their food removed overnight, to allow the emptying of their intestine and facilitate its handling during the surgical procedure. All experiments involving animals were performed according to the Brazilian College of Animal Experimentation (COBEA) guidelines and experimental protocols were approved by the Ethics Committee on Animal Use of Ribeirão Preto campus, University of São Paulo, protocol number 12.1.1809.53.2.

### 2.3. Assessment of the antithrombotic activity of Batroxase on venous thrombosis

In order to analyze the antithrombotic activity of Batroxase in comparison to the antithrombotic drug of clinical use (sodium heparin, Hepamax-S®, Blausiegel Ltd, Brazil), a model of partial blood stasis and vascular damage of the inferior vena cava was used [16].

Wistar rats (250–270 g, N = 3–5/group) were anesthetized with an association of ketamine (80 mg/kg) and xylazine (10 mg/kg) IP, and laid down in supine position. The abdomen was opened by incision along the linea alba toward the sternum, followed by the inferior vena cava exposure. The partial stasis was induced by tying a cotton thread just below the junction of the vena cava with the left renal vein. A blind 21G needle was placed between the node and the inferior vena cava, which was then removed to allow partial blood flow and patterned formation of partial stasis. The tested drug was injected through the left femoral vein of the animal over 2 min before the induction of the thrombus, which was done by a filter paper (5 × 5 mm) saturated with 10% ferric chloride solution (10 μL), which remained in contact with the vein for 5 min, causing vascular damage and enabling the induction of the thrombus

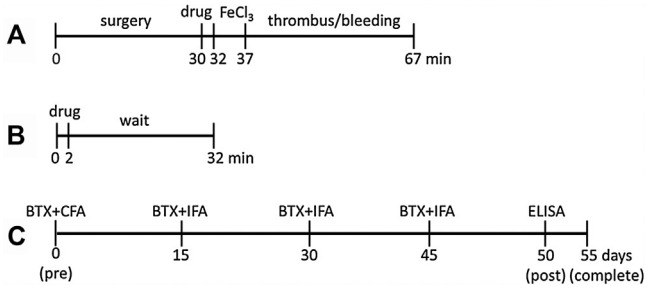


**Fig. 1.** Schematic protocols. Panel A: Antithrombotic test *in vivo*. It was first performed the surgery for cannulation of the left femoral vein, isolation and partial stenosis of the inferior vena cava (approximately 30 min). Then, stimuli (saline, heparin or Batroxase) were injected through the left femoral vein, during the 2 min that precede the red thrombus induction by a piece of filter paper saturated with ferric chloride solution at 10% placed in contact with the vascular wall for 5 min. After, a period of 30 min was waited in order to allow the formation/stability of thrombus. During this period of wait, the bleeding/clotting time of the animals was accompanied for up to 30 min. Panel B: Evaluation of Batroxase effects over biochemical analysis of rats' blood. For the biochemical analysis of the rats' blood, animals were anesthetized, and stimuli (saline, heparin or Batroxase) were injected through their tail vein over a 2 min period, as for the antithrombotic study protocol. Another 30 min was waited before cardiac puncture, in order to mimic the bleeding time evaluation period. Panel C: Rabbits immunization protocol: Immunogenicity of Batroxase was tested in rabbits. Blood samples were collected before the immunization protocol starts (pre). In the first day, Batroxase (BTX) emulsified with complete Freund adjuvant (CFA) was subcutaneously injected in the neck region of the rabbits. The immunization was repeated every 15 days for 3 more times, using incomplete Freund's adjuvant (IFA) instead of CFA. After 5 days of the fourth immunization, another blood sample was collected through the ear vein for ELISA analyses, and 5 days later, animals were anesthetized for complete blood collection by cardiac puncture.

(Fig. 1A). After the 5 min of induction with ferric chloride, a 30 min period was waited in order to allow the formation of the red thrombus. During this period, the bleeding/clotting time of the animals was assessed.

Different doses of sodium heparin (40, 90, 100 and 120 U/kg) and a dose of Batroxase (12 mg/kg) were evaluated and compared with a saline group. The Batroxase dose was chosen based on the dose that had previously shown thrombolytic activity in this venous thrombosis model [14].

### 2.4. Evaluation of Batroxase changes over bleeding/clotting time of animals

For the analysis of the bleeding/coagulation time [17], after the drug administration, the tail of anesthetized rats was heated in water (40 °C) for 1 min, and after drying, a small cut at the tip of the tail was performed with the use of a razor (Laser®, Laser Shaving Ltd, United Kingdom). Bleeding time starts when the first blood drop touches the filter paper, and it was recorded at 30 s intervals, accompanied until bleeding stopped or to a maximum of 30 min.

After the end of the experiments, the inferior vena cava was excised for immediately weighing of the thrombus formed (wet thrombus). A thrombus of the saline group, fixed in 10% formol, was prepared and stained using hematoxylin and eosin for the histological analyses of the venous thrombus characteristics.

### 2.5. *Evaluation of Batroxase effects over biochemical analysis of rats' blood*

For the biochemical analysis of the rats' blood, animals (N = 4/group) were anesthetized and drugs (saline, heparin 100 U/kg and Batroxase 12 mg/kg) were injected through their tail vein over a 2 min period, as for the antithrombotic study protocol. Another 30 min period was waited before cardiac puncture, to mimic the thrombus formation/bleeding time evaluation period.