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Suramin counteracts the haemostatic disturbances produced by *Bothrops jararaca* snake venom

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Abstract

Snakebite accidents produced by *Bothrops jararaca* typically results in haemostatic changes including pro- and anticoagulant disturbs as well as interference with platelets. Suramin is a hexasulfonated naphthylurea derivative that was recently characterized as a thrombin inhibitor (Monteiro et al., 2004. Suramin interaction with human alpha-thrombin: inhibitory effects and binding studies. Int. J. Biochem. Cell Biol. 36(10), 2077–2085). Here, we evaluated the ability of suramin to counteract some of the haemostatic disturbs produced by *B. jararaca* venom. *In vitro* assays showed that suramin inhibited venom-induced hydrolysis of a number of synthetic substrates: S-2238, S-2266, S-2302 and S-2288, being this ability more prominent towards the thrombin substrate S-2238 ($IC_{50} = 4.3\,\mu M$). It was also observed that suramin impaired the fibrinogen clotting induced by *B. jararaca* venom ($IC_{50} = 124\,\mu M$). Accordingly, increasing concentrations of suramin progressively delayed venom-induced plasma clotting, with complete inhibition attained at concentrations above 1.0 mM. In addition, the platelet-aggregating properties of *B. jararaca* venom were inhibited by suramin in a dose-dependent fashion ($IC_{50} = 127\,\mu M$). Suramin showed no effect in the *in vivo* hemorrhagic effect of venom in mouse skin. The *in vivo* effect of suramin was further tested using a previously established venous thrombosis model in rats induced by intravenous administration of *B. jararaca* venom combined with stasis. Venom doses of 100 μg/kg produced 100% of thrombus incidence (10.6 ± 1.7 mg). On the other hand, previous administration of suramin partially inhibited thrombus formation. Thus, 12.5 or 25 mg/kg of suramin decreased thrombus weight by 24% and 40%, respectively. Remarkably, co-administration of 3 μL/kg of antibothropic serum (which has no effect on thrombus formation) and 12.5 mg/kg of suramin decreased thrombus weight by 75%, suggesting a synergic effect. Altogether, we demonstrate here that suramin inhibits *in vitro* and *in vivo* haemostatic changes caused by *B. jararaca* venom. At this point, this drug could be of potential interest for association with conventional antiserum therapy.

Keywords: Suramin; *Bothrops jararaca* venom; Venous thrombosis; Antibothropic serum

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1. Introduction

Venoms from *Viperidae* snakes are rich sources of factors that interfere with the haemostatic system (Markland, 1998; Castro et al., 2004; Serrano and Maroun, 2005). These include a variety of proteins and peptides that may be classified as pro- or anticoagulant, pro- or antiplatelet aggregating as wells as fibrinolytic factors (Marsh, 1994). In Brazil, *Bothrops* genus is responsible for more than 90% of the registered snakebite accidents (Cardoso, 1990). The signs and symptoms presented by patients include local (pain, swelling, ecchymosis and myonecrosis) and systemic (blood incoagulability, hemorrhage) manifestations (Kamiguti et al., 1986, 1991; Maruyama et al., 1990). In fact, envenomation by these snakes generally results in persistent bleeding due to excessive degradation of fibrinogen and consumption of coagulation factors, thus preventing clot formation (Kamiguti et al., 1986; Maruyama et al., 1990; White, 2005).

The current treatment for ophidic accidents consists almost entirely of serum therapy, which has a number of drawbacks limiting in its use, such as poor availability in remote regions, the need for refrigerated storage and the occurrence of allergic reactions in many individuals (Chippaux and Goyffon, 1998; Moran et al., 1998; Heard et al., 1999). Moreover, a clinical study has demonstrated a high incidence of immediate hypersensitivity (anaphylactic) reactions in patients bitten by *Bothrops jararaca* treated with three distinct commercial antivenoms (Cardoso et al., 1993). Nevertheless, alternative or adjuvant treatments have been proposed (Soares et al., 2005).

In vitro studies showed that heparin inhibits some venom components (Nahas et al., 1975; Melo et al., 1993). Therefore, the ability of heparin to counteract the myotoxic effects of *Bothrops* venoms on isolated mammalian skeletal muscles has been attributed to the formation of acid–base complexes between the basic myotoxins of the venoms and the polyanionic glycosaminoglycan (Melo and Suarez-Kurtz, 1988). A similar mechanism of action was proposed for the polysulfonated naphtylurea suramin, which inhibits the myotoxic properties from some *Crotalidae* venoms (Arruda et al., 2002; De Oliveira et al., 2003). This hypothesis has been confirmed by crystallographic data obtained for a complex between a venom-derived myotoxic PLA_2 and suramin (Murakami et al., 2005).

We have recently demonstrated that glycyrrhizin, a plant-derived thrombin inhibitor (Francischetti et al., 1997), counteracts the *in vitro* and *in vivo* procoagulant activities of *B. jararaca* venom (Assafim et al., 2006). Of interest, *in vivo* studies were carried out using a venous thrombosis model in rats that combines intravenous administration of crude venom associated with stasis.

Based on the recently described property of suramin as a thrombin inhibitor (Monteiro et al., 2004), in this study we evaluated the counteracting properties of this drug against *B. jararaca* venom-induced haemostatic disturbs. Suramin effectively prevented venom-induced clot formation both *in vitro* and *in vivo*. Also, it showed a synergistic effect when combined with antibothropic serum. Our data indicate that this drug may display a potential antiophidic activity which could be further evaluated as an adjuvant in antiserum therapy.

2. Materials and methods

2.1. Material and drugs

Lyophilized *B. jararaca* venom and polyvalent horse antibothropic serum were kindly provided by Instituto Butantan (São Paulo, SP, Brazil). All other reagents were of analytical grade. Suramin (hexasodium *sym*-bis [*m*-aminobenzoyl-*m*-amino-*p*-methylbenzoyl-1-naphtylamino-4,6,8-trisulfonate] carbamide) was from Sigma Chemical Co. (St. Louis, MO); Human fibrinogen was from Calbiochem (La Jolla, CA); H-D-Phe-Pip-Arg-*p*-nitroanilide (S-2238), H-D-Val-Leu-Arg-*p*-nitroanilide (S-2266), H-D-Ile-Pro-Arg-*p*-nitroanilide (S-2288), and H-D-Pro-Phe-Arg-*p*-nitroanilide (S-2302) were from Chromogenix (Mölndal, Sweden). Anasedan (Xylazin) and Dopalen (Ketamin) were from Agribrands (Rio de Janeiro, Brazil). Silicone tubing (0.9×25 mm) BD Insyte^{TM} was purchased from Dickinson Ind. Cirúrgicas (Minas Gerais, Brazil).

2.2. Animals

Adult male Swiss mice (21.0 ± 1.0 g) and Wistar rats (both sexes, weighing 200–250 g) were housed under controlled conditions of temperature ($24 \pm 1\,^{\circ}C$) and light (12 h light starting at 07:00 h), and all experiments were conducted in accordance with standards of animal care defined by an Institutional Committee (Center of Medical Sciences, Federal University of Rio de Janeiro).

2.3. Assay for chromogenic substrate hydrolysis

Hydrolysis of the synthetic substrates by snake venom was measured in 10 mM HEPES, 100 mM NaCl, 0.1% PEG 6000, pH 7.4 using a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA) equipped with a microplate mixer and heating system as described (Francischetti et al., 1999). *B. jararaca* venom (1.5 μg/mL for S-2238 and S-2302 and 6.0 μg/mL for S-2288 and S-2266) was incubated for 5 min at 37 °C with various concentrations of suramin and reaction was started by addition of chromogenic substrate (100 μM, final concentration). The total volume of the reactions was 100 μL. The initial rate of the *p*-nitroaniline release was determined by the increase in the Abs_{405} at 6 s intervals.

2.4. Assay for fibrinogen clotting

Fibrinogen clotting by snake venom was measured in 10 mM HEPES, 100 mM NaCl, 0.1% PEG 6.000, pH 7.4 using a Thermomax Microplate Reader. *B. jararaca* venom (12.5 μg/mL) was incubated for 5 min at 37 °C with various concentrations of suramin and reaction was started by addition of human fibrinogen (2 mg/mL, final concentration). The total volume of the reactions was 100 μL. The initial rate of fibrinogen clotting was determined by the increase in the Abs_{405} at 6 s intervals.

2.5. Venom-induced plasma clotting

The ability of suramin to counteract venom-induced plasma clotting was measured on an Amelung KC4A coagulometer (Labcon, Heppenheim, Germany). Briefly, 70 μL of *B. jararaca* venom (12.5 μg/mL in PBS buffer, final concentration) was incubated for 2 min at 37 °C with 70 μL suramin at various concentrations. Reaction was started by addition of 100 μL of citrated human plasma (3.8% sodium citrate, 1:9, v/v) and time for clot formation was then recorded.

2.6. Platelet aggregation assays

Washed rabbit platelets were obtained from blood anticoagulated with 5 mM EDTA. Platelets were isolated by centrifugation and washed twice according to Zingali et al. (1993) with calcium-free Tyrode's buffer, pH 6.5, containing 0.1% glucose, 0.2% gelatin, 0.14 M NaCl, 0.3 M $NaHCO_3$, 0.4 mM NaH_2PO_4, 0.4 mM $MgCl_2$, 2.7 mM KCl, and 0.2 mM EGTA. Washed platelets were resuspended in a modified Tyrode's buffer, pH 7.4, containing 2 mM $CaCl_2$ at 300,000–400,000 cells/μL. Assays were performed at 37 °C using a Chronolog Aggregometer (Havertown, PA). Aggregation in the volume of 300 μL was induced by *B. jararaca* venom (65 μg/mL). Inhibition of platelet aggregation was tested by adding suramin, at various concentrations, 1 min before induction with venom. The inhibition was calculated using the maximum peak height tracing, which was compared with control values obtained in the absence of the drug.

2.7. Hemorrhagic activity

The hemorrhagic lesions were induced in mice by an intradermic injection of *B. jararaca* venom and quantified as previously described (Melo et al., 1994). *B. jararaca* venom (1 mg/kg) was injected alone or premixed with suramin (5.0 mg/kg). Samples were kept at room temperature for 30 min and then injected i.d. (0.1 mL) in the ventral abdominal area of mice. Two hours after venom injection, animals were euthanized by inhalation of ether, the skin covering the abdomen removed, stretched and dried at room temperature for 72 h. The skin was fixed to a lucite base plate, and the area surrounding the site of injection was transilluminated using an incandescent light. Light transmitted over an area of 1.09 mm^2 was read and light transmission or the absorbance normalized by taking the mean values of the readings over skins injected with either physiological saline solution (PSS) or the values of the absorbance induced by the hemorrhagic effect of *Bothrops* venom as arbitrary units of absorbance, respectively.

2.8. Stasis-induced thrombosis after injection of snake venom

We have previously described a deep venous thrombosis model in rats induced by a combination of stasis and hypercoagulability produced by intravenous administration of *B. jararaca* venom (Assafim et al., 2006). Rats were anesthetized with xylazin (16 mg/kg, intramuscularly) followed by ketamin (100 mg/kg, intramuscularly). The abdomen was surgically opened and after careful dissection the vena cava was exposed and dissected free from surrounding tissue. Two loose ligatures

were prepared 1 cm apart on the inferior vena cava just below the left renal vein. *B. jararaca* snake venom at 100 μg/kg body weight was injected into the vena cava (below the distal loose suture) and stasis was immediately established by tightening the proximal suture. Tightening of the distal suture was performed 20 min after administration of venom solution and the ligated segment was removed. The formed thrombus was detached from the segment, rinsed, blotted on filter paper, dried for 1 h at 60 °C, and weighed. Suramin and/or antibothropic serum were administered intravenously (below the distal loose suture) 5 min before thrombosis induction. The protocol received official approval with regard to the care and use of laboratory animals. Data presented represent mean ± SD of 5–10 animals.

2.9. Statistics

All data presented represent mean ± SD. Differences in mean values were analyzed using Student's *t*-test. When more than one group was compared with one control, significance was evaluated using one-way analysis of variance (ANOVA). $P<0.05$ was taken as statistically significant.

3. Results

In order to determine whether suramin could antagonize the activity of *B. jararaca* venom, we first tested the drug using *in vitro* enzymatic assays. Fig. 1 shows that increasing suramin concentrations caused a dose-dependent inhibitory effect upon venom-induced hydrolysis of all synthetic substrates tested (S-2238, S-2302, S-2288, S-2266). It is clear, however, that the venom's enzymes responsible for cleavage of S-2238 and S-2302 were more sensitive (Fig. 1A), with IC_{50} of 6.4 μg/mL (4.3 μM) and 42.5 μg/mL (28.5 μM), respectively. On the other hand, the maximum inhibitory effect towards S-2266 was ~30% while IC_{50} for S-2288 hydrolysis was 1680 μg/mL (1126 μM) (Fig. 1B). At this point, it seems that suramin displays reasonable specificity towards thrombin-like enzymes although other enzymes might be affected. This is in agreement with the observation that suramin display potent inhibitory effect towards thrombin (Monteiro et al., 2004) and neutrophil enzymes (Cadene et al., 1997).

We further evaluated the ability of suramin to counteract venom-induced fibrinogen clotting (Fig. 2). Again, it was observed a dose-dependent inhibitory pattern, with an IC_{50} of ~185 μg/mL

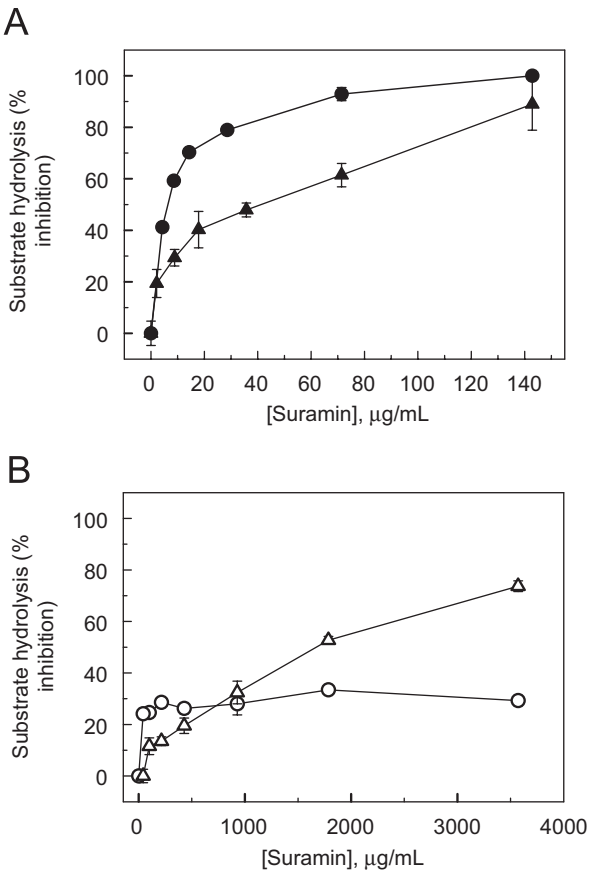

Fig. 1. Effect of suramin on *B. jararaca* venom-induced hydrolysis of chromogenic substrates. (A) Effect of increasing suramin concentrations in the hydrolysis of S-2238 (●) or S-2266 (▲) (100 μM, final concentrations) induced by *B. jararaca* venom (1.5 μg/mL). (B) Effect of increasing suramin concentrations in the hydrolysis of S-2302 (○) or S-2288 (△) (100 μM, final concentrations) induced by *B. jararaca* venom (6 μg/mL). Ordinates, Vi/Vo, initial rate of substrate hydrolysis in the presence of suramin/initial rate in its absence. Data represent mean ± SD of three independent determinations.

(124 μM). We also tested the effect of suramin on venom-induced plasma clotting. Fig. 3 shows that suramin efficiently prevented the procoagulant action of *B. jararaca* venom. In fact no plasma coagulation has been observed for suramin concentrations above 1.6 mg/mL (~1.0 mM).

It has been reported that suramin inhibits platelet aggregation and Ca^{2+} mobilization induced by thrombin and other agonists (Siafaka-Kapadai et al., 1998). Consistent with this observation, suramin decreased venom-induced platelet aggregation in a dose-dependent manner, with IC_{50} ~190 μg/mL (127 μM) (Fig. 4).

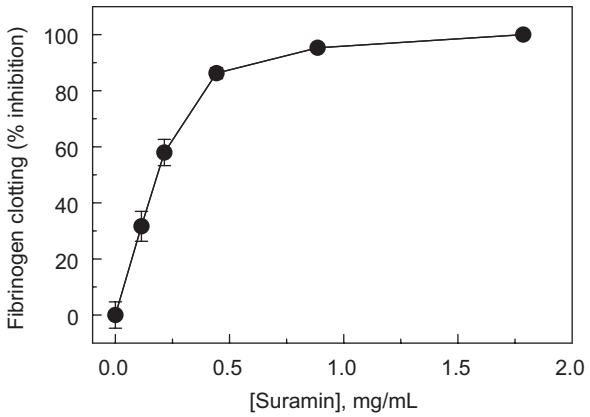

Fig. 2. Inhibitory effect of suramin on fibrinogen clotting induced by *B. jararaca* venom. Effect of increasing suramin concentrations on fibrinogen (2 mg/mL) clotting induced by *B. jararaca* venom (12.5 μg/mL) was assayed as described in Materials and methods section. Experiments were performed at 37 °C in 10 mM HEPES, 100 mM NaCl, 0.1% PEG 6.000, pH 7.4. Data represent mean ± SD of three independent determinations.

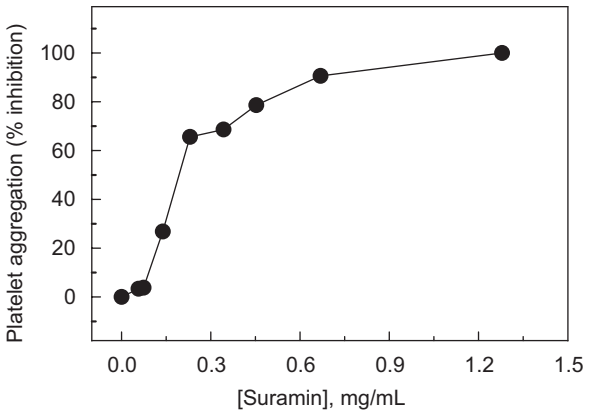

Fig 4. Suramin inhibits venom-induced platelet aggregation. Effect of increasing suramin concentrations on venom-induced aggregation was assayed with washed rabbit platelets. Maximum aggregation was induced by *B. jararaca* venom (65 μg/mL) in the absence of suramin. Experiments were performed as described in the Materials and methods section. Data represent mean ± SD of three independent determinations.

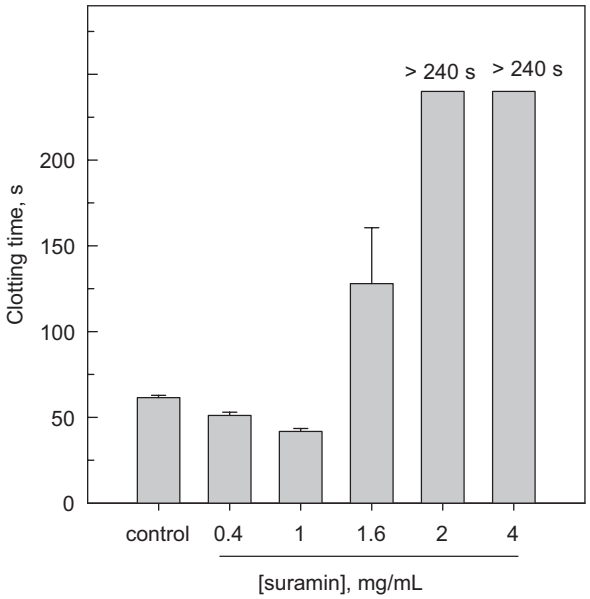

Fig. 3. Counteracting effect of suramin on venom-induced plasma clotting. Plasma clotting produced by *B. jararaca* venom was measured as described in the Materials and methods section. Venom diluted in PBS buffer (12.5 μg/mL, final concentration) was incubated for 2 min at 37 °C with various concentrations of suramin. Reaction was started by addition of citrated human plasma and time for clot formation was then recorded.

The ability of suramin to interfere with the *in vivo* hemorrhagic activity produced by *B. jararaca* venom was further tested in mice. In fact, no significant differences in the skin hemorrhagic lesions were observed in groups treated with venom alone or venom pre-treated with suramin (Fig. 5).

For evaluation of the *in vivo* antiophidic effect, we have used a previously established venous thrombosis model in rats induced by *B. jararaca* venom administration combined with stasis (Assafim et al., 2006). Venom doses of 100 μg/kg caused 100% of thrombus incidence (10.6 ± 1.7 mg, Fig. 6A). On the other hand, previous i.v. administration of suramin 12.5, 25 and 50 mg/kg produced a significant decrease on thrombus weight. This effect, however, was limited to ~40% inhibition for doses of 25 or 50 mg/kg. We further tested the association between suramin and antibothropic serum. As seen in Fig 6B, antibothropic serum doses of 3 μL/kg showed no effect on thrombus formation. However, co-administration of 12.5 mg/kg of suramin with 3 μL/kg of antibothropic serum decreased thrombus weight by 75% (2.5 ± 0.9 mg). Therefore, this result strongly indicates that suramin and antibothropic serum have a synergistic effect towards *in vivo* procoagulant action of *B. jararaca* venom.

4. Discussion

The massive activation of blood coagulation upon accidental or experimental envenomation by *B. jararaca* is widely described (Maruyama et al., 1990; Kamiguti et al., 1991). This phenomenon is mainly attributed to the presence of venom components that

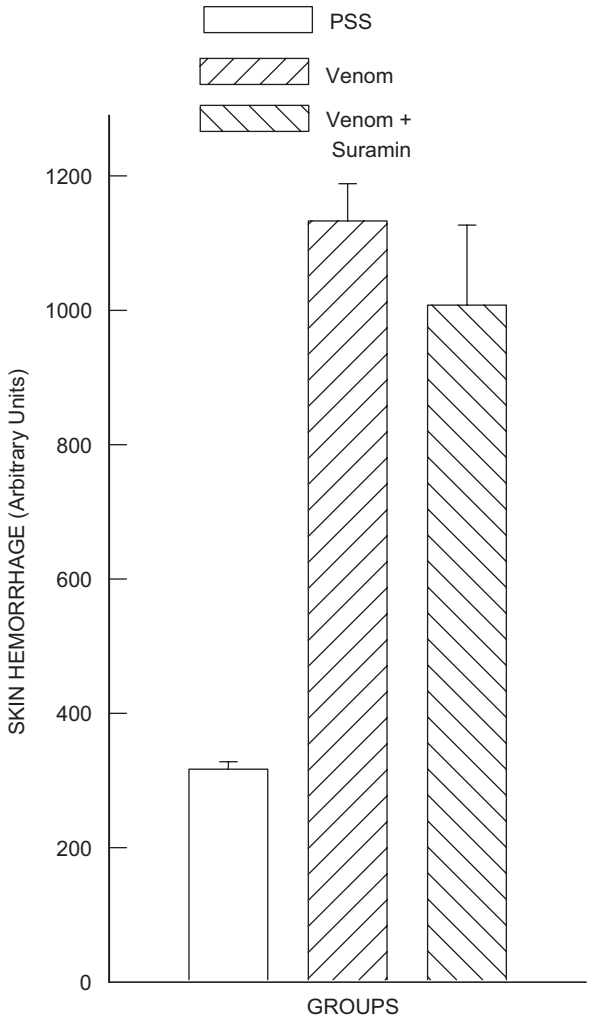

Fig 5. Hemorrhagic activity of *B. jararaca* venom in mouse skin: effect of suramin. Data show the hemorrhagic effect of *B. jararaca venom* in mouse skin, after intradermic injection of venom (1.0 mg/kg; 0.1 mL) alone, or the venom associated with suramin (5.0 mg/kg). The control group received intradermic injection of PSS in the same volume. On ordinates the data show the absorbance in arbitrary units and the values are expressed as mean ±SD ($n = 6$ for each dose or group).

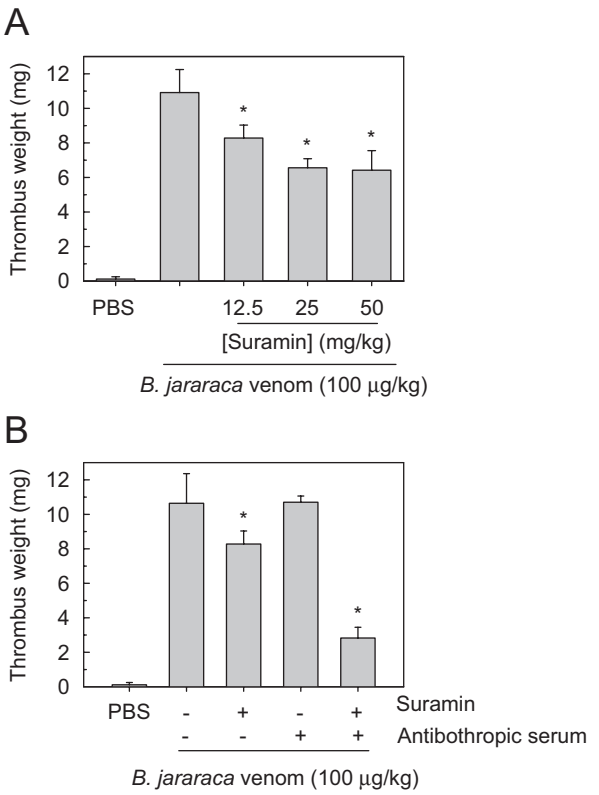

Fig. 6. Effect of suramin and antibothropic serum on stasis-induced venous thrombosis after administration of *B. jararaca* venom in the rat. (A) Suramin at the indicated doses was administered intravenously 5 min before the induction of thrombosis by venom (100 µg/kg) combined with stasis. (B) Suramin and/or antibothropic serum were co-administrated intravenously 5 min before the induction of thrombosis by venom (100 µg/kg) combined with stasis. Each point represents mean ± SD of 5–10 animals. $^{*}P<0.05$ was taken as statistically significant.

directly activate blood clotting enzymes (Nahas et al., 1979). In fact many components of the venom act as procoagulants, causing *in vivo* activation of the coagulation system which results in hemorrhagic states followed by consumption of the coagulation factors rather than massive thrombosis and/or consequent embolic disease (White, 2005; Markland, 1998). A striking feature is that venom's enzymes are poorly affected by natural coagulation inhibitors which difficult the pharmacological treatment of the haemostatic abnormalities elicited upon envenomation. For instance, structural differences between thrombin and venom-derived thrombin-like enzymes impair the inhibitory action of the heparin–antithrombin complex as well as other coagulation inhibitors such as hirudin (Castro et al., 2004).

Suramin is a polysulfonated naphthylamine-amine-benzamide derivative that has been extensively used in the treatment of onchocerciasis and trypanosomiasis (Voogd et al., 1993). Interestingly, it is reported that long-term *in vivo* administration of suramin causes a significant increase in plasma heparin-like glycosaminoglycans, which induces an anticoagulant state (Horne et al., 1988). In addition, it has been also shown that suramin inhibits several blood clotting proteins including factors V, VIII,

IX, X, XI, and XII (Horne et al., 1992) as well as thrombin (Monteiro et al., 2004).

Some studies have demonstrated that suramin may interfere with some snake venom pharmacological effects, in some cases by acting on specific molecules, such as the neurotoxic effect of β-bungarotoxin (Lin-Shiau and Lin, 1999), the myotoxic effect of bothropstoxin-I (Alzahaby et al., 1995), some of the pharmacological effects of *Cerastes vipera* venom (Oliveira et al., 2003) as well as the myotoxic properties from some *Crotalidae* venoms (Arruda et al., 2002; De Oliveira et al., 2003).

In the present study we demonstrate that suramin inhibits several *in vitro* haemostatic activities produced by crude *B. jararaca* venom, including cleavage of thrombin chromogenic substrate, fibrinogen clotting, plasma coagulation and platelet aggregation. On the other hand, suramin showed no effect upon the *in vivo* hemorrhagic activity of the venom. These observations indicate that suramin interfere with some specific venom proteins, possibly thrombin-like enzymes, which are involved in many of the *in vivo* haemostatic abnormalities produced by the ophidic accident.

We have previously described an *in vivo* model of thrombosis in rats that combines i.v. administration of crude venom and stasis. Rather than being a model that simulates a snakebite accident, this animal model allows rapid detection of venom procoagulant action resulting in a detectable thrombus formation (Assafim et al., 2006). By using this model it was possible to demonstrate that suramin exerts a statistically significant but limited effect against *B. jararaca* venom-induced thrombus formation, with ~40% inhibition for doses of 25 and 50 mg/kg. Most strikingly, the association of a low effective dose of suramin (12.5 mg/kg) with an ineffective dose of antibothropic serum in the thrombosis model decreased thrombus formation by ~75%, suggesting a synergistic effect. Similarly, we have previously demonstrated that the association of a plant-derived thrombin inhibitor, glycyrrhizin, and low doses of antibothropic serum display a synergistic action against *B. jararaca* venom-induced thrombus formation (Assafim et al., 2006).

Altogether our study demonstrates that the association of antibothropic serum with suramin strongly decreases the *in vivo* thrombus formation produced by *B. jararaca* venom in an animal model. At this point, suramin as well as other drugs, especially those already used in the clinical practice, may be further evaluated as potential adjuvants for antivenom therapy.

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