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Evaluation of three Brazilian antivenom ability to antagonize myonecrosis and hemorrhage induced by *Bothrops* snake venoms in a mouse model

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Abstract

Despite preventing death after snakebites, there is little evidence that polyvalent antivenoms (PAVs) protect against myotoxicity and local damages. We evaluated antibothropic Brazilian PAVs from three manufacturers against the myotoxicity and hemorrhagic activity of *Bothrops jararacussu* and *B. jararaca* venoms, respectively, by using two protocols: preincubation of PAVs with venom, and i.v. pretreatment with PAVs, prior to the venom inoculation. In this investigation, we used doses of PAVs ranging from 0.4 to 4.0 mL/mg of venom equivalent up to 10 times the amount recommended by the producers for the clinical practice in Brazil. In our preincubation protocol *in vivo*, PAVs antagonized myotoxicity of *B. jararacussu* venom by 40–95%, while our pretreatment protocol antagonized myotoxic activity by 0–60%. Preincubation of antivenoms with *B. jararaca* venom antagonized its hemorrhagic activity by 70–95%, while pretreatment antagonized hemorrhagic activity by 10–50%. Although all PAVs demonstrated partial antagonism against both venoms, the magnitude of these effects varied greatly among the manufactures. The results suggest that the current clinical doses of these PAVs may have negligible antimyotoxic effect.

Keywords: *Bothrops* venoms; Antivenoms; Myotoxicity; Hemorrhage

1. Introduction

Snakebites are a health problem in many countries and the global incidence exceeds 2.5 million per year. Moreover, snakebites are estimated to cause

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many deaths each year worldwide and disproportionately affect rural populations in resource-poor settings. In Brazil, the annual morbidity is circa of 15 per 100,000 people and almost 80% of these snakebites are associated with *Bothrops* sp. envenomation (Kamiguti et al., 1986; Jorge and Ribeiro, 1990; Nishioka and Silveira, 1992; Chippaux, 1998). This envenomation causes mainly local tissue damage, hemorrhage, edema and myonecrosis. While supportive care alone may prevent death, the administration of the specific antivenom does not prevent local tissue damage, and resultant disabilities (Gutiérrez et al., 1990; Ownby, 1990; Sanches et al., 1992; Cardoso et al., 1993; Milani et al., 1997; Chippaux and Goyffon, 1998; Zamuner et al., 2004). The variability of venom composition in snakes poses special problems for the manufacture of antivenoms and undermines the commercial attractiveness of this class of therapeutic agent. In particular, it has been amply documented that both inter- and intra-specific variation in venom composition can affect the neutralization capacity of antivenoms (Fry et al., 2003). Polyvalent antivenoms (PAVs) have marked variability in pharmacokinetic properties, and depend on the type of neutralizing molecule. Most antivenoms are made of F(ab′)2 antibody fragments, obtained by pepsin digestion of whole immunoglobulin (IgG) molecules. Other products consist of whole IgG molecules purified by either ammonium sulfate or caprylic acid precipitation, and few manufacturers distribute Fab antivenoms, obtained by papain digestion of sheep IgG (Lalloo and Theakston, 2003; Quesada et al., 2006).

PAV can also be produced by injecting animals with the venoms from several snake species, or by mixing the serum from several animal species inoculated with different venoms. In Brazil, these polyvalent antivenoms are made by horse immunized against *B. jararaca*, *B. jararacussu*, *B. alternatus*, *B. cotiara*, *B. moojeni* and *B. neuwiedi* venoms (The Instituto Butantan, S. Paulo, SP), by adding *B. pradoi*, (The Instituto Vital Brazil, Niterói, RJ), or with *B. jararaca*, *B. jararacussu*, *B. alternatus*, *B. cotiara* and *B. moojeni* (The Fundação Ezequiel Dias, FUNED, Belo Horizonte, MG). All of the antivenoms producers ascribed that 1.0 mL of these antivenoms are able to neutralize 2.5 mg of the *B. jararaca* or *B. jararacussu* venom (World Health Organization, 1981; Ministério da Saúde, 1991; Dos santos et al., 1992; Chippaux, 1998; Heard et al., 1999; Camey et al., 2002). Components of the *Bothrops* venoms have previously been isolated and characterized, demonstrating proteolytic and phospholipase enzymatic activities (Mandelbaum et al., 1982; Homsi-Brandeburgo et al., 1988; Leite et al.,1992; Paine et al., 1992; Zingali et al., 1993; Bjarnason and Fox, 1994; Tu, 1996). Some venoms (e.g. *B. jararacussu*) may have more than one myotoxic agent (Homsi-Brandeburgo et al., 1988; Gutiérrez et al., 1991, 2003; Melo et al., 1993, 1994; Andrião-Escarso, 1981, 2000).

Previous works in our laboratory and others (Melo and Suarez-Kurtz, 1987; Melo et al., 1993) have shown that changes in plasma creatine kinase (CK) activity or the rate of CK release from isolated muscle can be used as a reliable indicator of the myotoxic effect of *Bothrops* venom and provide simple quantitative models for evaluating the effectiveness of antivenom against envenomation caused by snakebites. There are also data showing that plasma CK activity after intramuscular injection of venom can be used to evaluate the myotoxicity of many different snake venoms (Mebs et al., 1983; Gutiérrez et al., 1985; Melo and Ownby, 1999). In the present work, we compared the ability of three Brazilian PAVs to inhibit the myotoxicity of *B. jararacussu* venom and two isolated myotoxins, BthTX I and II, and compared the ability of these antivenoms to inhibit the hemorrhagic activity of *B. jararaca* venom. We also tested the ability of these PAVs to antagonize the venom's proteolytic and phospholipase activity. The findings of this study have important implications for the use of PAVs in clinical practice.

2. Materials and methods

B. jararaca and *B. jararacussu* venoms were obtained from the Instituto Vital Brazil, Rio de Janeiro. Bothropstoxin I and II were isolated from *B. jararacussu* crude venom as described by Andrião-Escarso et al. (2000). PAVs against the genus *Bothrops* were supplied from three different Brazilian producers: Fundação Ezequiel Dias, Instituto Vital Brazil and Instituto Butantan. Antivenoms were removed from the original ampoules and named PAV A, PAV B and PAV C to blind the source of antivenom. To quantify the neutralization of venom by each PAV, we expressed the amount of PAV (mL) in terms of venom (mg) and herein refer to the term PAV: venom ratio, where each milliliter of PAV is ascribed to neutralize 2.5–5.0 mg of the *Bothrops* crude venoms. The amount of PAV was

adjusted for each milligram of crude venom injected according to the producer's recommendations. Male Swiss mice (21.0 ± 1.0 g) were used for the study, receiving water and food "*ad libitum*" and being kept under a natural light cycle. We adhered to protocols approved by the Committee for the Evaluation of Animals Use in Research of our university (CAUAP-UFRJ). CK activity was determined using a kit (CK NAC).

2.1. Myotoxicity in vitro

In vitro CK assays were performed as described previously (Melo and Suarez-Kurtz, 1988; Melo et al., 1993). Briefly, mouse isolated *extensor digitorum longus* muscle was removed, weighed and superfused continuously with physiologic saline solution (PSS), which composition (mM) was: NaCl, 135; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; $NaHPO_4$, 1; $NaHCO_3$, 15 and dextrose, 11. The pH of this solution was equilibrated to 7.3 with 5% CO_2/95% O_2. During superfusion, muscles were exposed to *B. jararacussu* venom (25 μg/mL) or to the venom preincubated with PAV A, B or C at PAV:venom ratios ranging from 0.4 to 1.2 mL/mg and added to the perfusion solution. Perfusion batches were collected at 30 min intervals and replaced with fresh solution. The collected samples were stored at 4 °C and their CK activity determined according to the procedures previously described (Melo and Suarez-Kurtz, 1987, 1988). Muscle preparations were mounted in the sample collecting units for a minimum of 1 h prior to the experiment, when the basal release rate was defined as the enzyme loss from the muscles into the perfusion bath prior to the addition of venom. The CK release was subsequently determined prior to the addition of venom alone or the venom and each PAV. The loss of CK from the isolated muscles was expressed as enzyme units released into the medium per gram of muscle, per hour of collection (U/(g h)). Preincubation of venom with antivenom was performed at room temperature 30 min prior to addition to the perfusion bath solution. Enzyme activities were expressed as international units, where 1 U is the amount that catalyzes the transformation of 1.0 μmol of substrate at 25 °C/min.

2.2. Myotoxicity in vivo

We evaluated the myotoxicity of *B. jararacussu* venom or its myotoxins (BthTX I and II) by measuring the increase of plasma CK activity induced by intramuscular (i.m.) injection of venom alone, or PAV mixed with venom at ratios ranging from 0.4 to 4.0 mL/mg. The venom or the toxins were dissolved in PSS to a final volume of 0.1 mL (1.0–2.5 mg/kg of body weight) and they were injected into the back thigh of mice as described previously (Melo and Suarez-Kurtz, 1987, 1988; Melo et al., 1993, 1994). Negative controls consisted of mice injected with the same volume of PSS. We established the ratio of PAV:venom to the proportion of 0.4 mL/mg as the minimum neutralizing proportion, which was recommended for use in clinics by the producers. To evaluate the PAV antimyotoxic activity, two different protocols were used. Preincubation (protocol A): venom dissolved in PSS was first incubated with each PAV in individual vials for 30 min at room temperature prior to injection. Pretreatment (protocol B): the PAVs were injected intravenously 15 min before the i.m. injection of venom. We used PAV:venom rations of 0.4–4.0 mL/mg.

2.3. Hemorrhagic activity

The hemorrhagic lesions were induced by an intradermic injection of *Bothrops* venom and quantified as described previously (Melo et al., 1994). Two protocols were performed: in protocol A (preincubation), the venom was premixed with each PAV at PAV:venom ratios ranging from 0.4 to 4.0 mL/mg. The venom or the incubated mixtures were kept at room temperature for 30 min and then injected i.d. (0.1 mL) in the ventral abdominal area of mice. In protocol B (pretreatment), each PAV was injected i.v. at PAV:venom ratios ranging from 0.4 to 4.0 mL/mg, 15 min before the intradermic injection of venom. Negative control received 0.1 mL of PSS i.d. injection. 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 then fixed to a lucite base plate, and the whole area in the site of injection and the surrounding area were transilluminated using an incandescent light. Light transmitted over an area of 109 mm^2 was read and light transmission or the absorbance normalized by taking the mean values of the readings over skins injected with either PSS or the values of the absorbance induced by the hemorrhagic effect of *Bothrops* venoms studied as arbitrary units of absorbance, respectively.

2.4. Proteolytic and phospholipase activities

The proteolytic assay was made as described previously (Garcia et al., 1978). *Bothrops* venom preincubated at concentration of 10 μg/mL or with the antivenom venom mixture at ratios ranging from 0.02 to 1.2 mL/mg at room temperature during 30 min. We added the venom or PAV:venom mixtures in a solution containing azocasein (0.2%), $CaCl_2$ (20 mM), and Tris–HCl (0.2 M, pH 8.8). The reaction was performed for 90 min at 37 °C and stopped by the addiction of 0.4 mL of trichloracetic acid (15% v/v) and then centrifuged. We then took 1.0 mL of the supernatant and mixed it with 0.5 mL of NaOH (2.0 M). This final solution was analyzed by spectrophotometry at an absorbance of 420 nm. The readings for *B. jararaca* venom at the concentration of 10 μg/mL and to physiologic PSS were taken as 100% and 0% of proteolytic activity, respectively. Phospholipase A_2 activity was carried out by adapting the turbidimetric assay described previously (Marinetti, 1965). We prepared the substrate by shaking one chicken egg yolk in a solution of NaCl (150 mM) to a final volume of 100 mL and stored at 4 °C prior to the reaction. In each assay, we prepared several tubes by taking a final volume (0.33 mL) of a 10% dilution of the egg suspension and adding it into a solution containing NaCl (150 mM), $CaCl_2$ (10 mM), Taurocholic acid (0.01%) and Tris–HCl (5.0 mM, pH 7.4), until a spectrophotometrical absorbance between 0.62 and 0.65 at 925 nm, was achieved. The tubes were kept at 37 °C, under mild and constant agitation during the procedure. The reactions were started by adding the *B. jararacussu* venom alone at a final concentration of 10 μg/mL or by adding the preincubated PAV:venom mixtures at ratios ranging from 0.02 to 1.6 mL/mg.

3. Results

3.1. Inhibition of myotoxicity (in vitro)

Mouse *extensor digitorum longus* muscles exposed to the *B. jararacussu* crude venom (25 μg/mL) showed a time-dependent increase in the rate of CK release from the baseline levels: 0.27 ± 0.03 to 61.70 ± 8.27 U/(g h), ($n = 24$, $p < 0.05$) after 90 min of exposition. At the lowest ratio of antivenom/venom (0.4 mL/mg), PAV A showed a partial inhibition, and reached up to 93% of inhibition at 1.2 mL of PAV A/mg of venom (Fig. 1). At this ratio, the other antivenoms had reduced inhibitory effects (PAV B circa of 84% and PAV C circa of 59%).

3.2. Inhibition of myotoxicity (in vivo)

Intramuscular injections of *B. jararacussu* crude venom (1 mg/kg of body weight) induced a

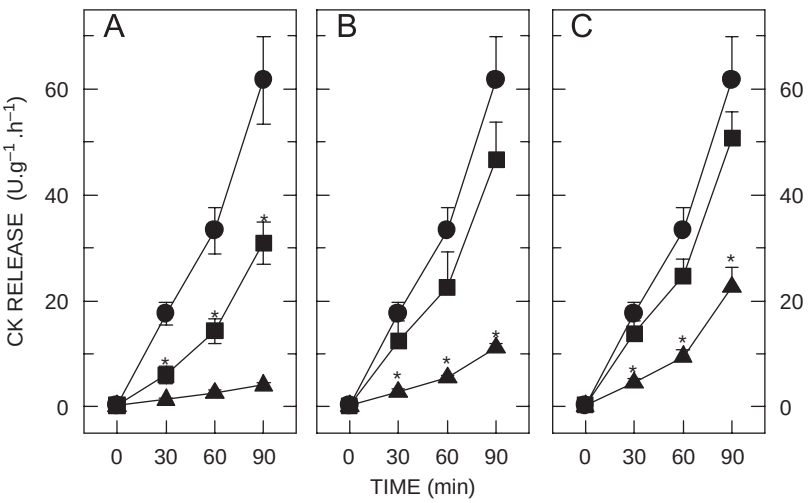

Fig. 1. Effects of polyvalent antivenoms on *B. jararacussu* venom myotoxic activity *in vitro*. Mouse EDL muscles exposed to *B. jararacussu* venom (25 μg/mL) alone (circles) or venom plus PAV A, B or C at PAV/venom proportions of 0.4 (squares) and 1.2 mL/mg (triangles). Data are plotted as the rate of CK release (U/(g h)), and the basal CK release at time zero (PSS) was 0.27 ± 0.03U/(g h) ($n = 24$). $^{*}p < 0.05$ for the difference between venom alone and PAV A, B or C at the same time of exposure (ANOVA). Each time point after zero represents the mean ± S.E. ($n = 9$).

significant increase of plasma CK activity above control levels (PSS: 169.5±3.7 U/L $n=27$ to 2854.68±109.08 U/L $n=9$) at 2 h after the injection and were defined as the 100% of venom effect. Pretreatment with PAV:venom ratios from 0.4 to 4.0 mL/mg for the three different PAVs protected against the increase of plasma CK induced by *B. jararacussu* venom (1 mg/kg) with differing grades of inhibition. PAV A significant inhibition of the increase of plasma CK activity was at two ratios, 1.2 or 4.0 mL/mg, and they were 28% or 67%, respectively, while PAV B and C showed significant inhibition only at the highest ratio, 4.0 mL/mg, circa of 53% and 26%, respectively (Fig. 2).

When preincubated with the venom, all three PAVs exhibited greater inhibition of increase of plasma CK activity when compared to the pretreatment protocol. However, only in PAV:venom ratio of 4.0 mL/mg the inhibition levels reached more than 90% (Fig. 3). In this protocol, our data suggested that the PAVs may abolish the myotoxicity of the crude venom in concentration lower than 4.0 mL/mg. In this preincubation protocol, the

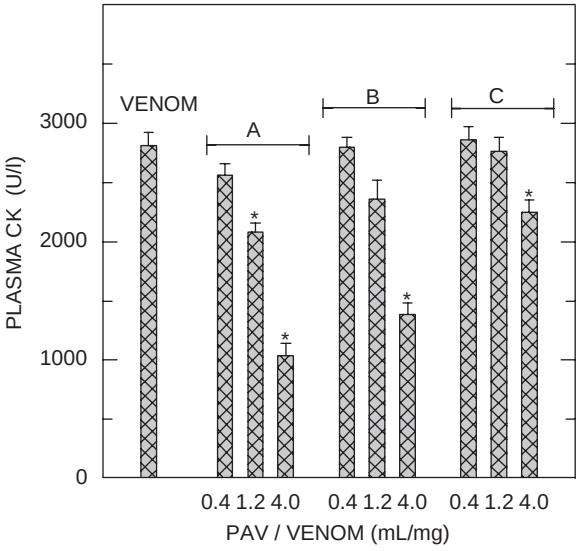

Fig. 2. Effect of pretreatment with polyvalent antivenom on myotoxic activity of *B. jararacussu* venom *in vivo*. *B. jararacussu* venom was injected (1.0 mg/kg, i.m.) 15 min after i.v. injection of polyvalent antivenom A, B or C at PAV/venom indicated ratios. The i.m. injection of PSS as a control increased the plasma CK activity up to 167.3±3.9 U/L ($n=18$). Plasma CK activity for venom alone was 2812.7±113.3 U/L ($n=9$). The values are expressed as means±S.E.M. ($n=9$). $^{*}p<0.05$ for the difference between each mixture and the venom alone.

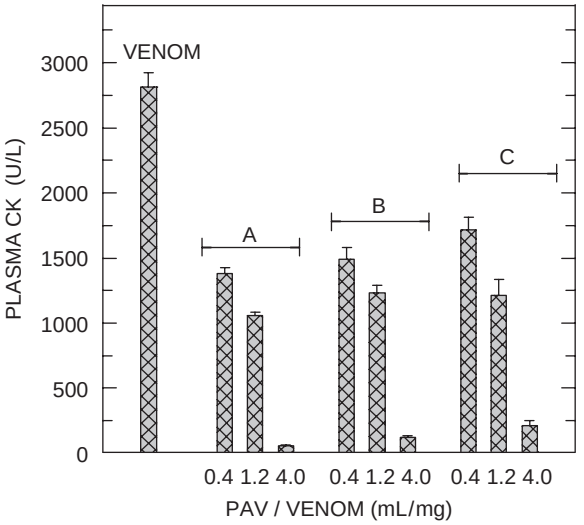

Fig. 3. Effect of preincubation with polyvalent antivenom on myotoxic activity of *B. jararacussu* venom *in vivo*. *B. jararacussu* venom at the dose of 1 mg/kg was preincubated with each PAV named A, B or C at PAV/venom ratios of 0.4, 1.2 and 4.0 mL/mg. The data express the plasma CK activity induced by *B. jararacussu* venom alone (2854.7±107.1 U/L; $n=9$), or the venom plus each PAV. The values are expressed as means±S.E.M. ($n=9$). All the inhibitory effects of PAVs were significant ($p<0.05$ for the difference between PAV A, B, or C and the crude venom).

PAVs also displayed great variability in their efficacies (Fig. 3). Intramuscular injections of *B. jararacussu* crude venom and its isolated toxins BthTX I and II (0.5–3 mg/kg) induced a dose-dependent myotoxic effect. At the same doses, BthTX I reached almost 80% of the myotoxic effect of the crude venom, while BthTX II reached less than 50% of values of plasma CK activity induce by the venom (Fig. 4A). Preincubation of BthTX I or BthTX II with PAV A at the ratio of 0.4 mL/mg of toxin decreased less than 20% of the myotoxic effect of BthTX I and more than 50% for BthTX II (Fig. 4B).

3.3. Antihemorrhagic activity of PAVs

Both *B. jararaca* and *B. jararacussu* venom induced a dose-dependent hemorrhagic effect in the mouse skin, and the *B. jararaca* effect was 20 times greater than that of *B. jararacussu* venom (Fig. 5A). Preincubation of 0.4 mL of PAV/mg of venom inhibited the hemorrhagic effect circa of 95% for PAV A, 87% for PAV B and 74% for PAV C (data not shown). The same profile of inhibition

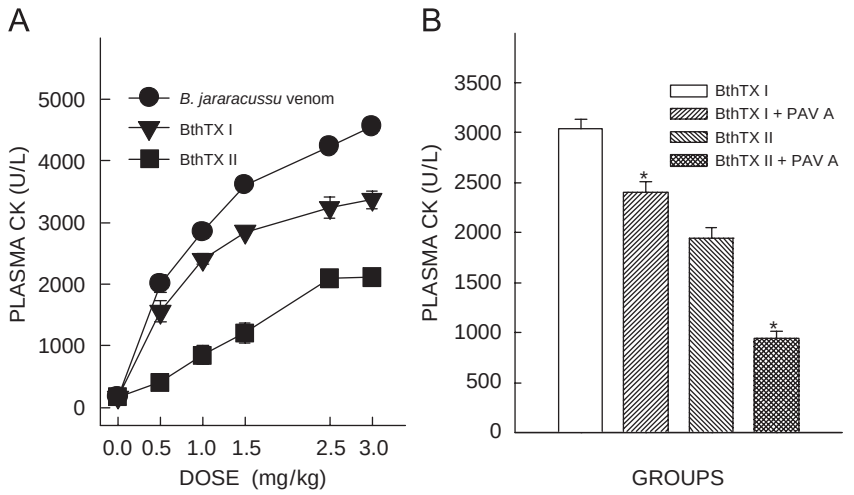

Fig. 4. Dose-response curve of the myotoxic effect of *B. jararacussu* venom and its myotoxins, BthTX I and BthTX II, *in vivo*: antagonism of the myotoxic effect of BthTX I or BthTX II by PAV A. Data show the increase of plasma CK activity 2 h after i.m. injection of *B. jararacussu* crude venom, BthTX I and II. Panel A shows the dose–response curve of *B. jararacussu* crude venom and BthTX I and II in mice after i.m. injections of 0.5–3.0 mg/kg. Panel B shows the antagonism of BthTX I or BthTX II (2.5 mg/kg) myotoxicity, after preincubation of each myotoxin with PAV A at the ratio of 0.4 mL of PAV A: 1.0 mg of myotoxin. Data are expressed as mean ± S.E.M. ($n = 9$), asterisk (*) indicates $p < 0.05$ (ANOVA).

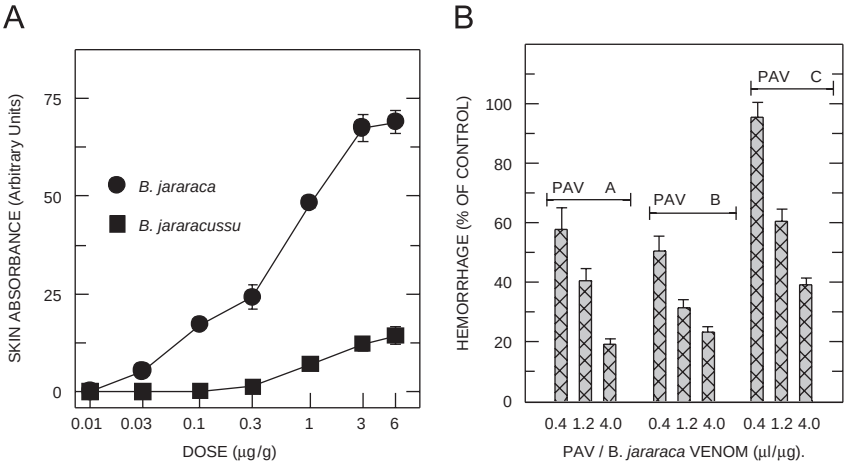

Fig. 5. Hemorrhagic activity of *B. jararaca* and *B. jararacussu* venom in mouse skin: effect of pretreatment with polyvalent antivenom. Panel A shows the dose–response curve of the hemorrhagic effect of *B. jararaca* and *B. jararacussu* venom in mouse skin. Panel B shows *B. jararaca* venom hemorrhagic effect, 15 min after intravenous injection of PAV A, PAV B or PAV C at PAV/venom ratios of 0.4, 1.2 and 4.0 mL/mg. Data were plotted as the hemorrhage induced by *B. jararaca* venom (1.0 mg/kg) by taking the mean values of the hemorrhage induced with either saline or the *Bothrops* venoms alone as arbitrary units of absorbance, as negative or positive control, respectively. The values are expressed as means ± S.E.M. ($n = 9$ for each dose or group).

was observed with higher amount of PAVs tested. Pretreatment with PAV A, PAV B and PAV C at the ratios of 1.2 and 4.0 mL/mg of venom inhibited significantly the hemorrhage induced by *B. jararaca* venom. There was no significant difference between the protections provided by pretreatment with PAV A or B (0.4–4.0 mL/mg). At all PAV:venom ratios PAV C was less effective than A or B, and had negligible effect on hemorrhage at the lowest PAV:venom ratio.

3.4. Effect of venom phospholipase and proteolytic enzymatic activities

In vitro assays revealed that *B. jararacussu* phospholipase and *B. jararaca* proteolytic activities were both inhibited by preincubation of 0.02–1.6 mL/mg of PAV A, B or C. The IC_{50} of *B. jararacussu* phospholipase A_2 activity was circa of 0.39 mL/mg. PAV IC_{50} of proteolytic activity of *B. jararaca* venom was circa of 0.22 mL/mg and a complete inhibition occurred at 1.0 mL/mg with any of the three PAVs investigated (not shown).

4. Discussion

In this study, we have demonstrated that three commercially available Brazilian PAVs antagonize the myotoxic effect of *B. jararacussu* and the hemorrhagic effect of *B. jararaca* crude venoms with different degrees of potency. These findings are in agreement with previous observations (Zamuner et al., 2004) that described only a partial inhibition of neurotoxicity and myotoxicity of *Bothrops* venom by antibothropic antivenom *in vitro*, as well as incomplete protection against myotoxicity from *B. Jararacussu* venom. Despite muscle protection subsequent to high concentrations of these PAVs, poor neutralizing activity from both preincubation and pretreatment protocols was observed at the concentrations of 0.4–1.2 mL/mg. The poor neutralizing effects could be due to the low amount of neutralizing antibodies in the three antivenoms against the venom myotoxins. Moreover, the extremely fast action of toxins limits the protection of muscle fiber by the antivenoms, even when we have preincubated the venom with the PAVs. Previous reports also described that mixtures of specific antivenoms did not inhibit some venoms effects, but antivenom efficacy could be greatly improved by adding specific antimyotoxins (Ownby et al., 1985, 1986; Lomonte et al., 1987). Our data revealed that BthTX I, a myotoxin devoid of PLA_2 activity, which is present in *B. jararacussu* venom, showed a greater myotoxic effect than BthTX II as well as reduced inhibition by the same amount of PAV A. Further experiments should be done in *in vitro* assay to clarify the neutralization of isolated myotoxins by antibodies. Neutralization of the whole venoms was only effective at the higher concentrations range close to 4.0 mL/mg, which is almost 10 times the amount clinically used in cases of *Bothrops* snake bites in Brazil (Ministério da Saúde, Brazil, 1991). This suggests that the prominent myotoxic effect induced by *B. jararacussu* crude venom is due, in part, to its polycationic myotoxins, such as BthTX I and BthTX II. Both myotoxins have phospholipase A_2 structure: BthTX II is an active enzyme Asp-49 PLA_2, while BthTX I is a Lys-49 PLA_2 devoid of enzymatic activity (Homsi-Brandeburgo et al., 1988; Cintra et al., 1993; Gutiérrez and Ownby, 2003). On the other hand, *B. jararacussu* venom's PLA_2 activity was completely abolished by PAVs *in vitro* at 1.6 mL/mg. Despite the complete inhibition of PLA_2 activity, at the same ratio, the preincubation of PAV A with *B. jararacussu* venom with this same venom's concentration did not avoid the muscle damage. It is possible that *B. jararacussu* myotoxins with phospholipase activity, like other viper venoms, might be not completely related to its myotoxicity, as suggested by some previous studies (Maria et al., 1998; Gutiérrez and Ownby, 2003).

All three PAVs demonstrated significant antagonism against venom-induced hemorrhagic activity during the preincubation protocol, with various degrees of efficacy. In contrast, there was only weak antagonism of the three PAVs demonstrated by the pretreatment protocol. It suggests that *B. jararaca* venom, which has several metalloproteinases and coagulation disturbing proteins that induce local hemorrhage and systemic coagulopathies, did not interact with the antivenoms (Kamiguti et al., 1986, 1991; Baramova et al., 1990; Paine et al., 1992; Zingali et al., 1993; Bjarnason and Fox, 1994; Fujimura et al., 1995). Previous studies described that these hemorrhagic proteases, Bothropasin and jararhagin, both members of the reprolysin family of zinc metalloproteinase, induce rapid degradation of sub-endothelial matrix proteins and disruption of the blood vessel, and are involved in the local lesions and systemic hemorrhage (Mandelbaum et al., 1982; Paine et al., 1992; Laing and Moura da Silva, 2005; Gutiérrez et al., 2005). Weak neutralization of hemorrhagic effect could be explained by poor penetration of tissues barriers by PAV, and thus its inability to reach the site of injury during the pretreatment protocol as previously suggested by other reports (Ownby, 1990; Furtado et al., 1991; Chippaux and Goyffon, 1998; Gutiérrez et al., 2003).

Our data are in agreement with previous studies that show *B. jararacussu* venom components are not a good immunogenic, as well as with others that have shown that the use of antivenoms does not

protect against the local effects of *Bothrops* venoms (Furtado et al., 1991; Rucavado and Lomonte, 1996; Picolo et al., 2002). Neutralization of the whole venoms in our study was only effective at the higher concentrations range (4.0 mL/mg), which is almost 10 times the concentration clinically used in cases of *Bothrops* snake bites in Brazil (Ministério da Saúde, Brazil, 1991). Furthermore, the amount of Igs in the antivenoms used in the medical practice against snakebites at present is already sufficient to establish serious collateral effects (Cardoso et al., 1993; Chippaux and Goyffon, 1998), and doses therefore cannot be further increased.

Gutiérrez et al. (2003) proposed that the heterogeneity in pharmacokinetic and mechanism of action of venom components requires a detailed analysis of each venom–antivenom system in order to determine the most appropriate type of neutralizing molecule for each individual case. Finally, research should be directed toward obtaining more diffusible antivenom compounds that can reach the tissues. An alternative strategy would be to examine some plants extracts or produce new synthetic derivatives based on existing indigenous remedies that could neutralize the different snake venoms and improve new treatments for snakebites or new polyanion compounds, which are able to complex with polycation toxins and improve the antivenom ability (Mors et al., 1989; Melo et al., 1993, 1994; Melo and Ownby, 1999; Calil-Elias et al., 2002; Arruda et al., 2002; Murakami et al., 2005).

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