Toxicon 184 (2020) 180–191

Contents lists available at ScienceDirect

Toxicon

journal homepage: http://www.elsevier.com/locate/toxicon

Disorders on cardiovascular parameters in rats and in human blood cells caused by *Lachesis acrochorda* snake venom

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ARTICLE INFO

Keywords:
Snake venom
Lachesis
platelet Aggregation
Vasodilation
Hypotension

ABSTRACT

In Colombia, *Lachesis acrochorda* causes 2–3% of all snake envenomations. The accidents promote a high mortality rate (90%) due to blood and cardiovascular complications. Here, the effects of the snake venom of *L. acrochorda* (SVLa) were analyzed on human blood cells and on cardiovascular parameters of rats. SVLa induced blood coagulation, as measured by the prothrombin time test, but did not reduce the cell viability of neutrophils and platelets evaluated by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay and by the lactate dehydrogenase (LDH) enzyme assay. In fact, SVLa increased the absorbance in tests made with platelets subjected to the MTT assay. SVLa induced platelet aggregation whose magnitude was comparable to that of the positive control adenosine diphosphate (ADP), and occurred earlier with increasing SVLa concentration. Acetylsalicylic acid (ASA, a cyclooxygenase inhibitor) or clopidogrel (an ADP receptor blocker) inhibited the aggregating effect of SVLa. Inhibition of SVLa-elicited platelet aggregation also resulted from the treatment with disodium ethylenediaminetetraacetate (Na_2-EDTA; metalloproteinase inhibitor) and with 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, serine protease inhibitor). In isolated right atrium of rats, SVLa increased slightly, but significantly, the magnitude of the spontaneous contractions and, in isolated rat aorta, SVLa relaxed KCl- or phenylephrine-induced contractions. In vivo, SVLa induced hypotension and bradycardia in rats, with detection of hemorrhage in pulmonary and renal tissues. Altogether, under experimental conditions, SVLa induced blood coagulation, platelet aggregation, hypotension and bradycardia. Part of the effects presented here may be explained by the presence of snake venom metalloproteinases (SVMPs) and snake venom serine proteases (SVSPs), constituents of SVLa.

1. Introduction

In South American countries, as in other tropical countries, ophidism is a public health problem. As a neglected tropical disease, snakebite envenomation annually reaches approximately 1.8–2.7 million people worldwide, with an estimation of 81,000–138,000 deaths (Gutiérrez et al., 2017a). Snakebite accidents occur most often in rural areas and the high mortality is due to the scarce availability of antivenoms (Gutiérrez et al., 2010). In Latin America, an estimation of 137,000–150,000 annual snakebite accidents has been reported, with 3400–5000 deaths (Gutiérrez et al., 2017b; Walteros and Paredes, 2014).

The *Lachesis acrochorda* snake, popularly known as "verrugosa" (warty), inhabits the Pacific region of Colombia and northern Ecuador (Madrigal et al., 2012). In Colombia, this species is responsible for 2–3% of snakebite accidents, which result in approximately 90% mortality. Clinical reports indicate the following most common symptoms

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https://doi.org/10.1016/j.toxicon.2020.06.009
Received 31 January 2020; Received in revised form 11 June 2020; Accepted 15 June 2020
Available online 23 June 2020

resulting from *Lachesis* bites: bleeding around the bite, profuse sweating, abdominal cramps, nausea, coagulation, diarrhea and vagal symptoms such as hypotension and bradycardia (Castrillón-Estrada et al., 2007; Madrigal et al., 2012). Electrocardiographic changes such as T wave elevation, cell death, interfibrillar edema and elevation of creatine kinase-MB (CK-MB) enzyme fraction has been reported (Angel-Camilo et al., 2016). Other reports also include vascular and cardiac effects (Ayerbe-González and Latorre-Ledezma, 2010; Otero et al., 1992).

The presence of enzymatic (snake venom metalloproteinase - SVSP, snake venom serine protease - SVMP, phospholipases A_2, L-amino acid oxidase, thrombin-like enzymes, kallikrein-like enzymes and fibrinogenases) and non-enzymatic (C-type lectins) proteins has already been reported in the composition of *Lachesis* venom (Madrigal et al., 2012; Pla et al., 2013). The clinical manifestations observed in *Lachesis* envenomation are attributed to the presence of these proteins in the venom. They include local tissue damage, hemostatic changes and potentially lethal systemic effects (Benvenuti et al., 2003; Gutiérrez et al., 2017a; Mora-Obando et al., 2014; Takeda et al., 2012). There is generally a high conservation in terms of the composition of venoms of species belonging to the *Lachesis* genus, although the literature on the biological effects caused by the venom of *L. acrochorda* is still scarce. Therefore, the present study experimentally addressed the direct effects of *L. acrochorda* venom on hemostatic and cardiovascular parameters.

2. Materials and methods

2.1. Lachesis acrochorda snake venom (SVLa)

Pooled SVLa used in the present study was a pool of samples obtained from 4 adult specimens of *L. acrochorda* from the Center for Biomedical Investigations – Bioterium of the University of Cauca (CIBUC) at Colombia. The extraction of SVLa was by manual stimulation following the biosafety protocols adopted by CIBUC-Bioterium. After collection, the pool of venom was lyophilized and stored at −20 °C until use.

2.2. Biochemical analysis of SVLa

SVLa was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Laemmli, 1970) using the VERT-i10 mini-gel system (Loccus, Cotia, SP, Brazil). The Low Molecular Weight Calibration Kit for SDS Electrophoresis (GE Healthcare, Chicago, IL, USA) was used as molecular mass reference on 12% T running gels. It was made of phophorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). Gels were run at 200 V for approximately 90 min, followed by protein staining for 1 h in a solution containing 0.1% Coomassie® R-250 in 40% ethanol, 10% acetic acid (Morrissey, 1981). Finally, the gel matrix background was reduced incubating the gel in the same solvent, excluding the dye.

The 10 most abundant Coomassie-stained SDS-PAGE bands from SVLa were excised and processed as previously described (Shevchenko, 1996), with minor modifications: gel bands were destained with 50% ascetonitrile in 25 mM ammonium bicarbonate pH 8.0, reduced in 65 mM DTT at 56 °C for 30 min, alkylated in 200 mM iodoacetamide at room temperature in the dark for 30 min and trypsinized (20 ng/μL) overnight at 37 °C. Peptides extracted from the gel using an ultrasonic bath were vacuum dried and dissolved in 12 μL of 1% formic acid. The peptides (4 μL) were loaded at 2 μL/min on a pre-column (2 cm × 100 μm i.d.) packed with 5 μm 200 Å Magic C18-AQ matrix (Michrom Bioresources), before sample fractionation on a 12 cm-long column (75 μm i.d.) with a laser-pulled tip, packed in-house with C18 beads (Reprosil-Pur 120 C18-AQ, 3 μm, Dr. Maisch). The elution gradient was 2–40% acetonitrile in 0.1% (v/v) formic acid in 32 min, at 200 nL/min. Acetonitrile concentration was raised to 80% in 4 min and the column was kept under this elution condition for 2 additional min. Data were acquired using a data-dependent strategy, in which up to seven most intense peaks of each MS1 spectrum were sequentially selected for high-resolution MS/MS in the orbitrap, following fragmentation by higher-energy collision-induced dissociation (HCD), with a normalized collision energy (NCE) of 45%, a fixed first mass of *m/z* 100 and an isolation window of 2.5 *m/z*. The spray voltage was set to 1.9 kV, with the capillary at 200 °C. The following parameters were used: MS1 (5E6 AGC, 500 ms IT, resolution 60,000 FWHM at *m/z* 400, survey scan 300–1700 m/z, centroid mode); MS2 (2E5 AGC, 250 ms IT, resolution 15,000 FWHM at *m/z* 400, centroid mode). Dynamic exclusion was set to 45 s and single charged precursors or those with unassigned charge states were excluded.

Protein identification was based on the peptide-spectral matching (PSM) approach, using the PatternLab for Proteomics computational environment (version 4.1.1.22, freely available at http://www.patternlabforproteomics.org), which adopts Comet algorithm as the database search engine. The "Generate Search DB" module was used to generate a target-reverse database made of "Serpentes" protein sequences retrieved from UniProt (taxon ID 8570; 156,362 protein entries downloaded April 10, 2020), in addition to common MS contaminants (*e.g.*, keratins, albumin and trypsin). Uninterpreted high-resolution MS/MS spectra were searched against this combined database using Comet default parameters. Enzyme (trypsin) specificity was fully specific, up to 2 missed cleavages were allowed, and the initial precursor mass tolerance was set to 40 ppm. Carbamidomethylation of cysteine (+57.02146 Da) was considered as fixed modification and variable modifications included methionine oxidation (+15.9949) and asparagine/glutamine deamidation (+0.9840). PSM results were filtered by the Search Engine Processor (SEPro) tool. The final post-processing step was adjusted to converge to reliable results showing ≤1% FDR at the protein level and ≤10 ppm mass error for MS1 and MS2 spectra.

2.3. Human blood samples

Human blood samples, were collected from healthy donors with the authorization of the Research Ethics Committee at the Federal University of Ceará, Fortaleza, Brazil [Certificate of Presentation for Ethical Appraisal (CPEA) No. 11590519.7.1001.5054 (report No. 3.355.242)]. For *in vitro* assays, human neutrophils were isolated from a by-product of human blood (buffy coat) kindly provided by Centro de Hematologia e Hemoterapia do Ceará (Fortaleza, Brazil). For the platelet aggregation study, human blood samples were collected from healthy voluntary donors after signing a Free and Informed Consent Form. The collected blood was placed in Vacuette® 3.5 mL coagulation tubes containing 3.2% sodium citrate, discarding the first tube from each donor to avoid possible activation of platelets by tissue residues. The blood was homogeneized 2–4 times by gentle inversion after collection.

2.4. Coagulation test in human plasma samples

Citrated blood samples were centrifuged at 3000 rpm for 15 min for assessment of prothrombin time in platelet-poor plasma. Subsequently, a tube containing 100 μL of plasma was incubated for 5 min at 37 °C with a commercial kit of Ca^{2+}-enriched tissue factor (thromboplastin) source (TP CLOT, Bios Diagnóstica, Sorocaba, SP, Brazil). Such procedure using the kit TP CLOT was also made in a second tube containing 100 μL of plasma in the presence of 0.5 IU/mL heparin. Other tubes containing 100 μL of plasma were incubated only with SVLa at 4, 8, 16 or 50 μg/mL and time for SVLa-induced coagulation was measured. All tubes were shaken slowly for one additional min, followed by the measurement of the clotting time, reported according to the international normalized ratio (INR). The experiments were performed in triplicate.

2.5. Platelet aggregation test

To obtain the platelet rich plasma (PRP), blood samples were

centrifuged at room temperature for 10 min at 1000 rpm, followed by transfer of PRP to Falcon tubes. PRP suspensions were preheated at 37 °C and then treated with ADP or SVLa (0.5–4 μg/mL) for 10 min with constant stirring at 1200 rpm, in the absence or presence of AAS (0.1 μM), clopidogrel (0.02 μM), 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF, 8 mM) or Na_2-EDTA (13 mM). ADP was used as a positive platelet aggregation control (Kamiguti et al., 1996). Platelet aggregation percentages were obtained by calculating the area under the curve (AUC) using the Aggrolink8 software (Chrono-log Corp., USA).

2.6. Effects of SVLa on platelets subjected to the MTT assay

The effect of SVLa was evaluated on platelets subjected to the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983). PRP was incubated with SVLa (0.5–4 μg/mL) and, after 30 min of incubation, the sediment was incubated in a new medium (200 μL) containing 5% MTT (5 mg/mL). Then, the cells were incubated for an additional period of 3 h. Finally, the supernatant was discarded and 150 μL of dimethyl sulfoxide (DMSO) was added. Subsequently, the plates were shake for 15 min and the absorbance was measured at 540 nm. The experiments were performed in triplicate.

2.7. Effects of SVLa on neutrophils

After obtaining the PRP, polymorphonuclear cells (PMN) were isolated from the remaining suspension of the remaining sample in 2.5% (w/v) gelatin solution at 37 °C for 15 min following the Henson method (1971). After erythrocyte sedimentation, the supernatant was treated with 0.83% Na_4Cl and additional centrifugation was performed. The obtained PMN were predominantly neutrophils (80–90%) after counting in Hanks medium. The effects of SVLa were evaluated on neutrophils subjected to the MTT assay (Mosmann, 1983). SVLa (0.5–4 μg/mL) was incubated (30 min of incubation) with 5×10^6 neutrophils/well and the experiments were performed in triplicate with absorbance monitored at 540 nm. The effect of SVLa was also analyzed by measuring lactate dehydrogenase (LDH) activity using the Liquiform kit (Labtest Diagnóstica, MG, Brazil).

2.8. Effect of SVLa on neutrophil myeloperoxidase activity

Human neutrophil suspension (5×10^6 cells/mL) maintained for 30 min at 37 °C in the presence or absence (Hanks gel, untreated cells) of indomethacin (100 μM) was treated with phorbol-12-myristate 13-acetate (PMA, 0.1 μM) for 15 min in the absence or in the presence of SVLa. After centrifugation for 10 min at 4 °C, the supernatant obtained was used to determine the myeloperoxidase (MPO) concentration with 3,3,3,5′-tetramethylbenzidine (TMB, 1.5 mM) and sodium acetate (1.5 M; pH 3.0), according to Úbeda et al. (2002). Absorbance was determined at 620 nm with standard curve obtained by adding MPO (0.125–3 U/mL) (Young et al., 1989).

2.9. Animals

Male adult *Rattus norvegicus* (Wistar, 260–300 g) were obtained from the institutional vivarium at the School of Medicine, Federal University of Ceará. The animals were maintained at room temperature (22 ± 0.5 °C) in polypropylene cages, with light/dark light cycles of 12 h each and with access to standard chow and water *ad libitum*. All procedures were conducted in accordance with the ethical principles of the National Council for Animal Experimentation Control (CONCEA, Brazil). The institutional animal ethics committee approved the experimental procedures (CEUA n° 9555140618).

2.10. Isolated organ experiments

After euthanasia of the animals (exsanguination following previous anesthetesia with 2,2,2-tribromoethanol - 250 mg/kg, i.p.), the thoracic aorta was quickly removed and kept in physiological solution at room temperature. The aorta was cut transversely into ring-shaped segments (1 × 5 mm), which were mounted on triangular pieces of steel wire (0.3 mm in diameter) suspended in an isolated organ bath chamber containing 5 mL of Krebs-Henseleit solution of the following composition: 118.0 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM KH_2PO_4, 25.0 mM $NaHCO_3$, and 10.0 mM glucose. The solution was maintained at 37 °C, continuously aerated with carbogenic mixture (5% CO_2 in O_2). The experiments were conducted in endothelium-denuded aortic preparations. To remove the endothelial layer, a gentle rubbing of the luminal surface of the aortic preparation with a stainless steel wire (0.3 mm diameter) was previously performed. Aortic rings were kept under basal tension of 1 g. Active tissue tension was recorded using an isometric force transducer (ML870B60/C–V, AD Instruments, Australia) connected to a data acquisition system (PowerLab^{TM} 8/30, AD Instruments, Australia). After a period for tissue stabilization (60 min), reference contractions were recorded after the addition of 60 mM KCl. The endothelium removal was confirmed by the lacking of a relaxant response following the addition of 1 μM acetylcholine on the steady state of a contraction induced by 0.1 μM phenylephrine. The right atrium of each animal was also mounted in an isolated organ chamber. Atrial preparations were not stimulated with 60 mM KCl as they show spontaneous activity. The frequency of the spontaneous contractions served as reference to express the effects caused by SVLa (1–1000 μg/mL). Basal tension in isolated atrial preparations was 0.5 g.

2.11. Effects of SVLa on rat blood pressure

Rats anesthetized with an intramuscular injection of ketamine (100 mg/kg) and xylazine (20 mg/kg) had the left femoral artery cannulated to allow recordings from blood pressure while the femoral vein was cannulated for injection of SVLa (0.5 and 1.5 mg/kg). The cannulas were previously filled with heparinized saline solution (10 IU/mL). The venom was administered intravenously (iv). Hemodynamic parameters were continuously monitored on a PowerLab data acquisition system (ADInstruments). Blood pressure was recorded using the MLT-0699/670 blood pressure transducer (ADInstruments), from which the heart rate signal was derived. After recording, surviving rats were euthanized with anesthetic overdose (Dias et al., 2016).

2.12. Histopathological analysis

After blood pressure assessment (i.e. 2 h after SVLa administration), the heart, lung, kidney, liver and intestine were isolated from rats and transferred to histology cassettes, which were immersed in formaldehyde 10% for 24 h. After subsequent fixation in 70% ethanol, the organs were prepared in an automatic processor, dehydrated in ethanol (70–100%) and finally paraffin embedding. Tissues were cut into 5 μm slices arranged on slides for subsequent hematoxylin-eosin (HE) staining and evaluation under optical microscope.

2.13. Statistical analysis

In the statistical analysis we followed a completely randomized experimental design. The data obtained was analyzed in terms of their adjustment to the normal curve and homogeneity of variances, allowing the choice of one-way ANOVA (parametric) or Kruskal-Wallis (non-parametric) inference tests, followed by a post-Hoc test. All analyses and dose-response curves were mapped out using the GraphPad-Prism 5.0 software. Results were expressed as mean ± standard error of the mean (S.E.M.). Statistical significance was considered at $p < 0.05$.

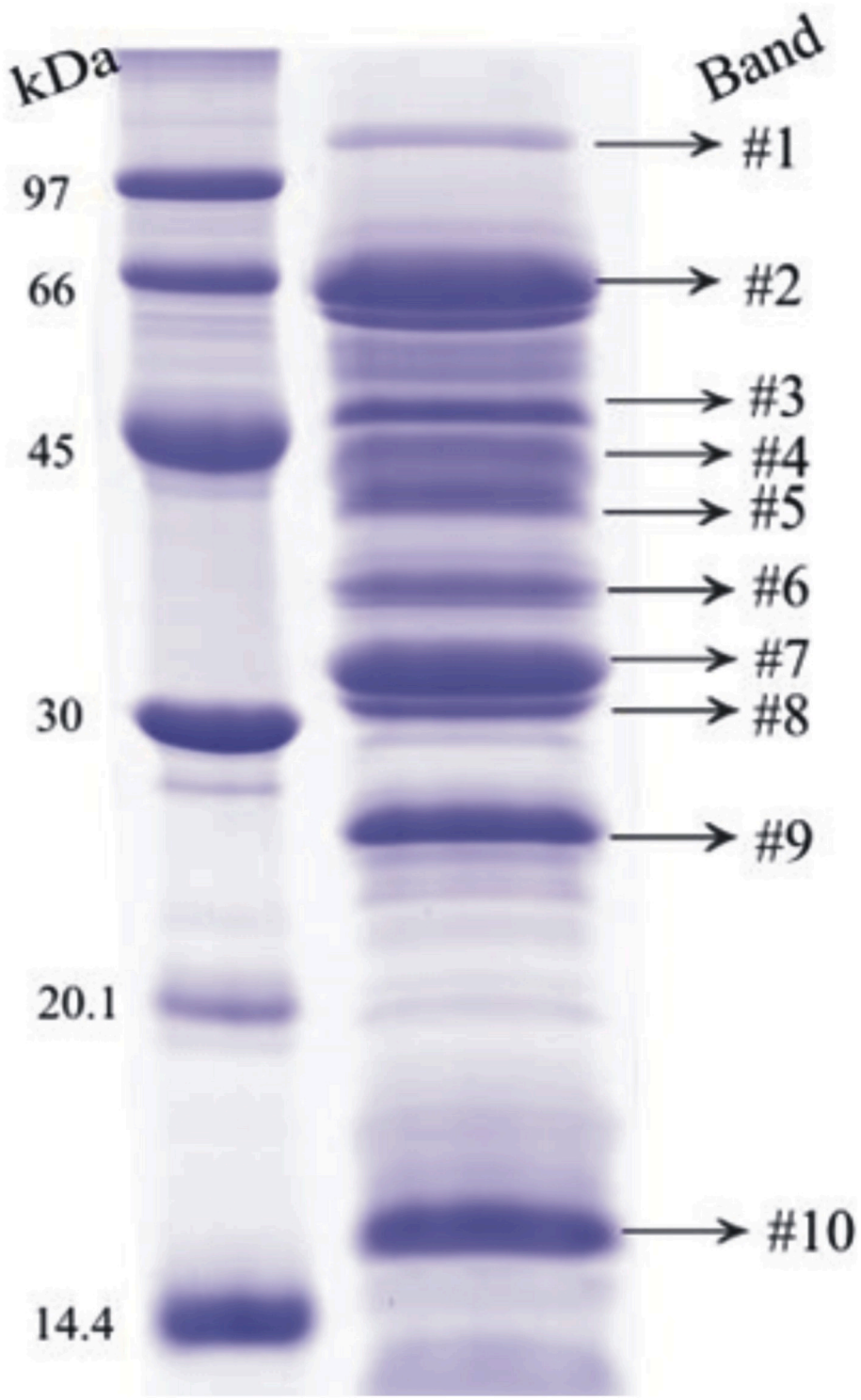

Fig. 1. SDS-PAGE (12% T) under reducing conditions of *L. acrochorda* snake venom. The left lane shows the molecular mass standards (kDa) and the right lane shows the protein profile of SVLa (20 μg of venom protein) stained by Coomassie blue R250. The most abundant Coomassie-stained SDS-PAGE bands from the venom were excised, followed by in-gel digestion and analysis by nLC-nESI-MS/MS. Based on the gel-based proteomic approach from LCMS/MS, the main protein families identified in the SVLa were: phosphodiesterase (gel band #1); L-amino acid oxidase (band #2); metalloproteinase (bands #3, #4, #5, #7 and #9); serine proteinases (bands #4, #6, #7 and #8); phospholipase B-like (band #4); C-type-lectin and phospholipase A_2 (band #10). Detailed data on protein identification for each gel digest are shown in Table 1, Supplementary material. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Biochemical analysis of SVLa

Analysis of the SVLa by SDS-PAGE under reducing conditions revealed the presence of protein bands with a wide range of molecular masses (Fig. 1). The 10 most intensely stained Coomassie bands were excised, followed by in-gel digestion and analysis by high-resolution MS/MS. Detailed identification results of the individual gel bands are shown in Table 1, Supplementary material. To reduce redundancy due to peptides matching multiple target sequences, identified proteins were grouped using the maximum parsimony criterion. For each gel digest, the counts of identified MS/MS spectra were used as rough estimates of protein abundances. Based on this gel-based proteomic approach, the main protein families identified in the SVLa were: phosphodiesterase (gel band #1); L-amino acid oxidase (band #2); metalloproteinase (bands #3, #4, #5, #7 and #9); serine proteinases (bands #4, #6, #7 and #8); phospholipase B-like (band #4); C-type-lectin and phospholipase A_2 (band #10).

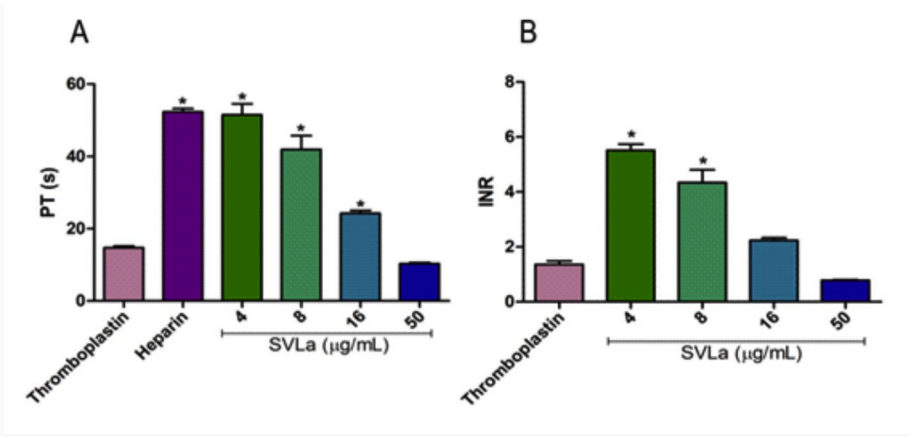

Fig. 2. Effect of the snake venom of *Lachesis acrochorda* (SVLa) on blood coagulation. In A, prothrombin time (PT) in seconds (s) measured in a tube containing plasma and a Ca^{2+}-enriched tissue factor source (thromboplastin) in the absence (first bar from the left) or in the presence (second bar) of 0.5 IU/mL heparin (heparin), an anticoagulant compound. The PT was also measured in tubes containing plasma and SVLa (4, 8, 16 or 50 μg/mL) without the addition of thromboplastin or heparin. In B, the same results for SVLa are shown according to the international normalized ratio (INR) values. Data are mean ± standard error of the mean (S.E.M.). *, $p < 0.05$ vs. thromboplastin (ANOVA followed by Dunnett test).

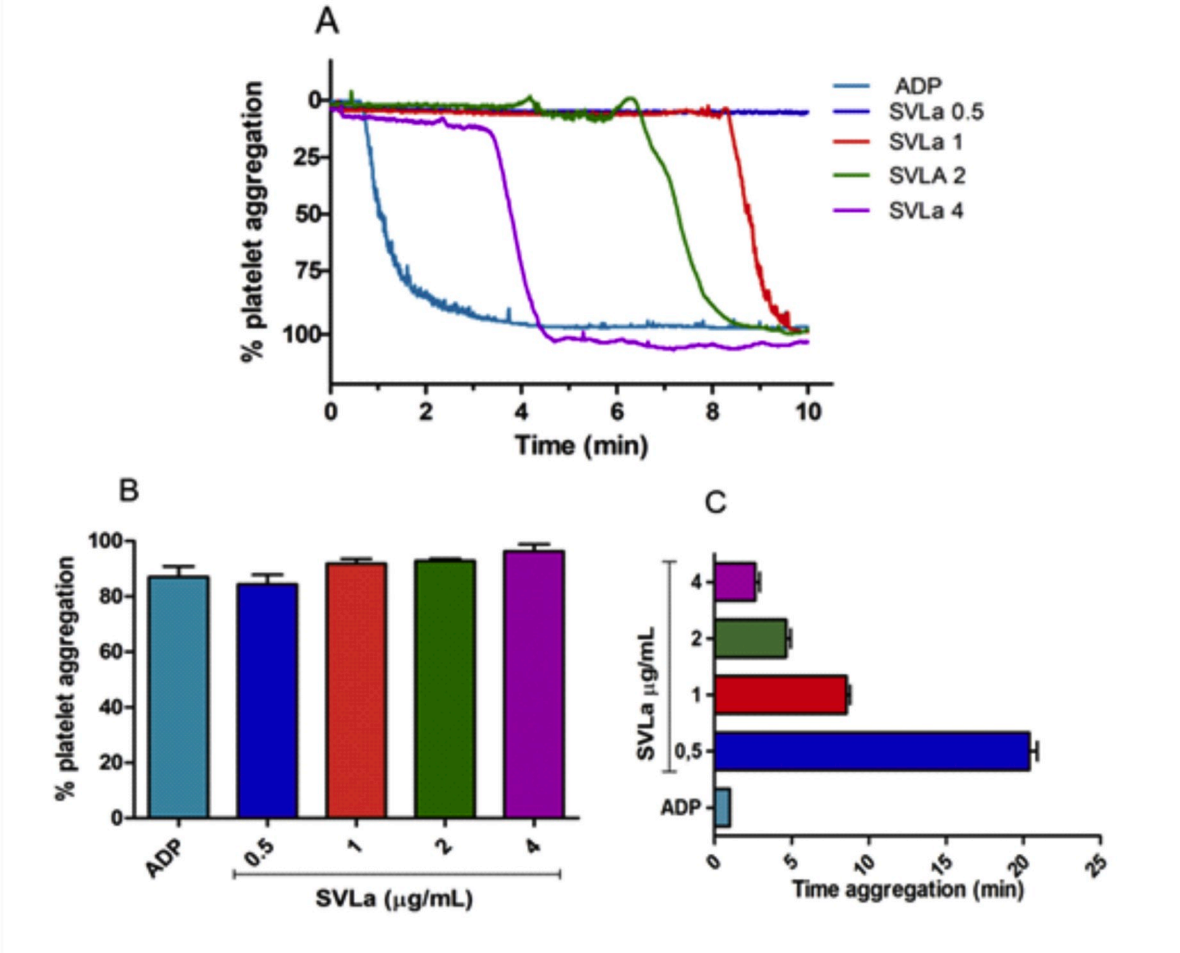

Fig. 3. Effect of the snake venom of *Lachesis acrochorda* (SVLa) on platelet aggregation. In A, experimental traces showing platelet aggregation (indicated by the downward deflection on the experimental trace) caused by 10 μM adenosine diphosphate (ADP) or SVLa at concentration varying from 0.5 to 4 μg/mL. Note that time in this figure was limited to only 10 min. In B, percentage of maximal platelet aggregation at different venom concentrations (data were obtained at 20 min of recording to include the result with 0.5 μg/mL SVLa). C, time (in min) to trigger platelet aggregation at each SVLa concentration or ADP. In B and C, data are mean ± standard error of the mean (S.E.M.).

3.2. Role of SVLa in prothrombin time

Fig. 2A shows that a tissue factor source (thromboplastin) triggered coagulation with a prothrombin time of 14.7 ± 0.6 s. This coagulation time was significantly increased when thromboplastin was added in the presence of heparin (0.5 UI/mL; 52.3 ± 2.1 s; $p < 0.05$, Dunnett test). Added to plasma samples alone, SVLa (4–50 μg/mL) was also able to trigger coagulation. At the lowest concentration (4 μg/mL), the prothrombin time corresponded to 51.3 ± 2.3 s, which was significantly higher in comparison with the plasma subjected only to the stimulus

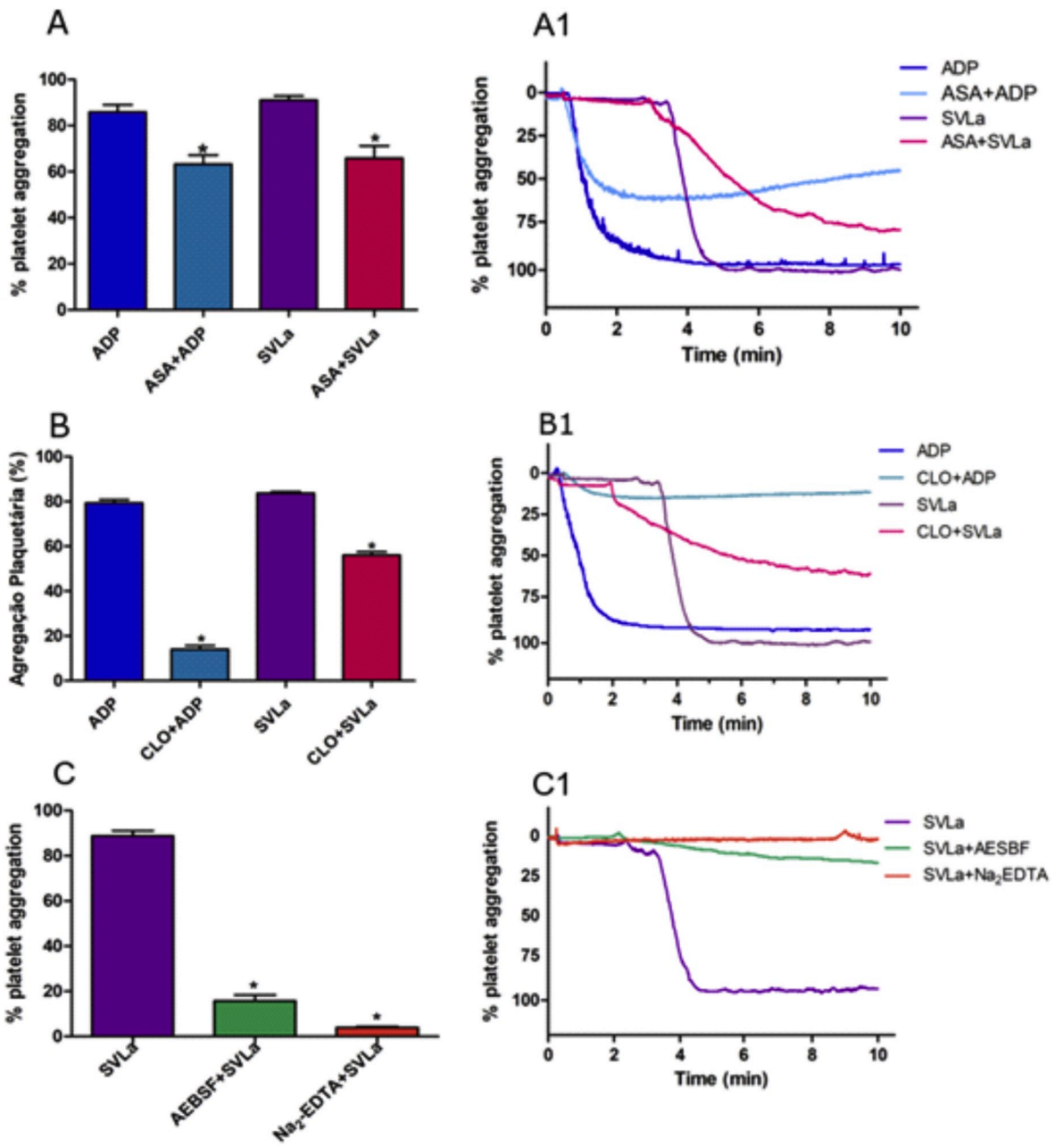

Fig. 4. Effect of platelet aggregation inhibitors on the effects of the snake venom of *Lachesis acrochorda* (SVLa) on platelet-rich plasma (PRP). Treatment of PRP with 50 μM acetylsalicylic acid (ASA; panels A/A1) or 4.4 mM clopidogrel (CLO, B/B1) significantly reduced the aggregating properties of 10 μM adenosine diphosphate (ADP) or 4 μg/mL SVLa. Similarly, treatment of PRP with 8 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) or 13 mM disodium ethylenediaminetetraacetate (Na_2-EDTA) (panels C/C1) decreased the % of platelet aggregation caused by 4 μg/mL SVLa. On the left for all panels, graphs with the percentage of platelet aggregation measured at the time 10 min. On the right, original traces obtained in each experimental protocol. Data are mean ± standard error of the mean (S.E.M.) *, $p < 0.05$, ANOVA test vs. SVLa or ADP in the absence of a given inhibitor, Kruskal-Wallis test.

with thromboplastin ($p < 0.05$). At 50 μg/mL, SVLa induced a prothrombin time (10.3 ± 1.2 s) that did not differ from the value measured in plasma samples stimulated with thromboplastin ($p > 0.05$). Fig. 2B shows that treatment of human plasma with SVLa resulted in international normalized ratio (INR) values that were initially higher than in the tube containing plasma stimulated with thromboplastin, but that diminished at higher concentrations of SVLa ($p < 0.05$, Dunnett test).

3.3. Aggregating effect of SVLa on human platelets

In samples of human PRP, SVLa (0.5–4 μg/mL) induced platelet aggregation, as revealed by the downward deflection in the experimental tracing reported in Fig. 3A. At all tested concentrations of SVLa, the magnitude of the aggregation was comparable to that induced by ADP (10 μm; Fig. 3B), but the time to trigger aggregation was inversely related to the concentration of SVLa (Fig. 3C).

Fig. 4 shows that the aggregating effect induced by 4 μg/mL SVLa was significantly ($p < 0.05$) reduced when PRP was previously treated with acetylsalicylic acid (ASA, 50 μM) or clopidogrel (CLO, 4.4 mM), which reduced the magnitude of aggregation to 65.8 ± 5.3% ($n = 6$) (Figs. 4A) and 56.0 ± 1.5% ($n = 8$) (Fig. 4B), respectively. Both AAS and clopidogrel were used at concentrations able to significantly reduce the aggregating effect of ADP. The magnitude of SVLa-induced platelet aggregation was also significantly reduced ($p < 0.05$, Kruskal-Wallis test)

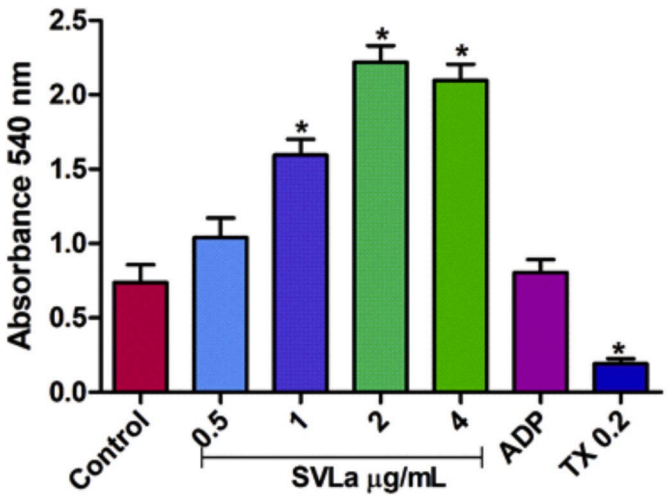

Fig. 5. Effect of the snake venom of *Lachesis acrochorda* (SVLa) on the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay in human platelets. Values of absorbance at 540 nm in samples of platelet-rich plasma (PRP) under treatment with SVLa (0.5–4 µg/mL). Note that 10 µM adenosine diphosphate (ADP) did not change whereas 0.2% (v/v) Triton X-100 significantly reduced the absorbance values in the MTT assay. Data are mean ± standard error of the mean (S.E.M.) of absorbance, *$p < 0.05$ vs. Control, ANOVA followed by Tukey test.

to 15.7 ± 2.6% (n = 12) and 4.0 ± 0.5% (n = 9) when the test was performed in the presence of 8 mM AEBSF or 13 mM Na_2-EDTA (Fig. 4C), respectively. Such values were significantly lower than 88.8 ± 2.3% (n = 9) in the control. In the MTT assay (Fig. 5), treatment of PRP with SVLa resulted in increased absorbance values (from 0.5 ± 0.1 in the PRP group (n = 17) to 2.2 ± 0.1 (n = 15) at a concentration of 2 µg/mL SVLa). In contrast, absorbance values did not differ when PRP was maintained in the presence of 10 µM ADP, although they were significantly reduced to 0.2 ± 0.03 (n = 14) in the presence of 0.2% (v/v) Triton X −100 ($p < 0.05$, Tukey test; comparison vs. PRP).

3.4. Effect of SVLa on the MPO enzyme activity in neutrophils

In neutrophils, SVLa (0.5–4 µg/mL) changed neither the absorbance values in the MTT test (Fig. 6A) nor the activity levels of LDH enzyme (Fig. 6B), parameters which were significantly ($p < 0.05$) decreased (Fig. 6A) or increased (Fig. 6B), respectively, when the test was performed in the presence of 0.2% (v/v) Triton X-100. The effects of SVLa were tested on the MPO enzyme activity, but Fig. 6C shows that even at 4 µg/mL SVLa did not change the MPO levels in human PMA-stimulated neutrophils ($p > 0.05$).

3.5. Effects of SVLa on isolated tissues

In rat aorta, SVLa (3–1000 µg/mL) induced relaxation of contractions induced by 60 mM KCl or 1 µM phenylephrine. The relaxing effect was concentration-dependent ($p < 0.001$, ANOVA) and, at the highest concentration tested (1000 µg/mL), SVLa-induced relaxation correspondent to 94.6 ± 12.9% (n = 5) and 61.3 ± 11.6% (n = 4) of the contraction caused by KCl or phenylephrine, respectively (Fig. 7A). In isolated rat right atrium preparations, SVLa (1–1000 µg/mL) increased the frequency of spontaneous contractions. The effect was significant ($p < 0.05$) from the concentration of 10 µg/mL SVLa and it was maximal at 300 µg/mL corresponding to 24.3 ± 4% of the basal heart rate before the addition of the venom (Fig. 7B).

3.6. Effects of SVLa on rat blood pressure

The effect of SVLa (0.5 and 1.5 mg/kg) was evaluated on blood pressure after intravenous injection in rats (Fig. 8). At a dose of 0.5 mg/kg, immediate hypotension occurred with a maximum peak reached after 3 min of injection, accompanied by partial recovery of blood pressure after 10 min, and recovery of blood pressure to pre-venom levels after 60 min of injection (Fig. 8 A/A1). At 120 min, there was a trend of hypotension, but not statistically confirmed ($p > 0.05$). At 1.5 mg/kg, SVLa induced immediate hypotension that was maximal after 3 min without recovery to pre-venom blood pressure levels and death of all animals after 120 min (Fig. 8 A/A1). No significant change in heart rate at the dose of 0.5 mg/kg was detected after injection of SVLa. In contrast, transient bradycardia was seen after 3 min of the injection at the dose of 1.5 mg/kg (Fig. 8B). In animals that received only saline i.v., no significant change occurred in the hemodynamic parameters.

3.7. Histological analysis

Histological analysis showed that rats subjected to intravenous injection of SVLa (0.5 or 1.5 mg/kg) did not reveal detectable lesions under the microscope in organs such as liver, intestine, and heart. In contrast, animals treated with SVLa 0.5 mg/kg showed lung tissues possessing zones of intra-alveolar hemorrhage and erythrocyte sedimentation, inflammatory infiltrate with presence of neutrophils (Fig. 9A1). The kidneys showed preserved glomerular structure, but with marked swelling and vacuolization of the tubular epithelium, dilated ducts containing proteinaceous luminal eosinophilic material, degeneration of tubular epithelial cells, ectasia and small foci of inflammatory cells Fig. 9B1). Animals subjected to 1.5 mg/kg SVLa, in addition to the marked intra-alveolar hemorrhage, also depicted signs of edema and diffuse neutrophilic inflammatory infiltrate were evident in the pulmonary tissue (Fig. 9A2). In renal tissues (Fig. 9B2), marked swelling and vacuolization of tubular epithelium, dilated ducts, intratubular accumulation of proteinaceous material, degeneration of epithelial cells, ectasia and foci of inflammatory cells were seen.

4. Discussion

The present study reported, under experimental conditions, the extensive biological effects caused by SVLa. They include cardiovascular effects such as vasodilation and hypotension, platelet aggregation and blood coagulation. In vivo, intravascular injection of SVLa revealed tissue damage such as pulmonary and renal hemorrhage. In vitro tests showed no evidence of decreased cell viability in platelets and neutrophils, at least at the concentrations adopted in this study.

Hemostatic alterations such as mild bleeding around the bite or coagulation are among the common symptoms reported in virtue of *Lachesis* accidents (Castrillón-Estrada et al., 2007; Madrigal et al., 2012). In the present study, hemostatic changes in response to SVLa were evidenced *in vitro* by the values of TAP and INR in human blood, methods traditionally adopted to evaluate the extrinsic coagulation pathway (Stettler et al., 2019). Such findings indicate that SVLa has coagulant properties, at least under the conditions tested herein.

In vitro, SVLa proved to be a potent aggregator of human platelets. This effect was observed with 0.5 µg/mL of SVLa, a lower concentration than that reported for *Bothrops jararaca*, which aggregated platelets at concentrations between 20 and 80 µg/mL (Antunes et al., 2010; Davey and Lüscher, 1965; Rosa et al., 2019). Platelet aggregation inhibitors such as ASA and clopidogrel reduced aggregation in response to SVLa, indicating that the aggregating activity of the venom involved the participation of TXA_2 and ADP. Such findings differentiate SVLa from the *B. colombiensis* venom, which was able to induce platelet aggregation at low concentrations (~0.2 ng/mL) without influence of ASA or clopidogrel (Arteaga-Vizcaíno et al., 2011). In platelets, while ASA inhibits the synthesis of TXA_2, a metabolite that acts on target receptors to induce aggregation (Hamilton, 2009), clopidogrel is a blocker of ADP receptors, an important target of the aggregator ADP under physiological conditions (Falcão et al., 2013). TXA_2 and ADP act synergistically

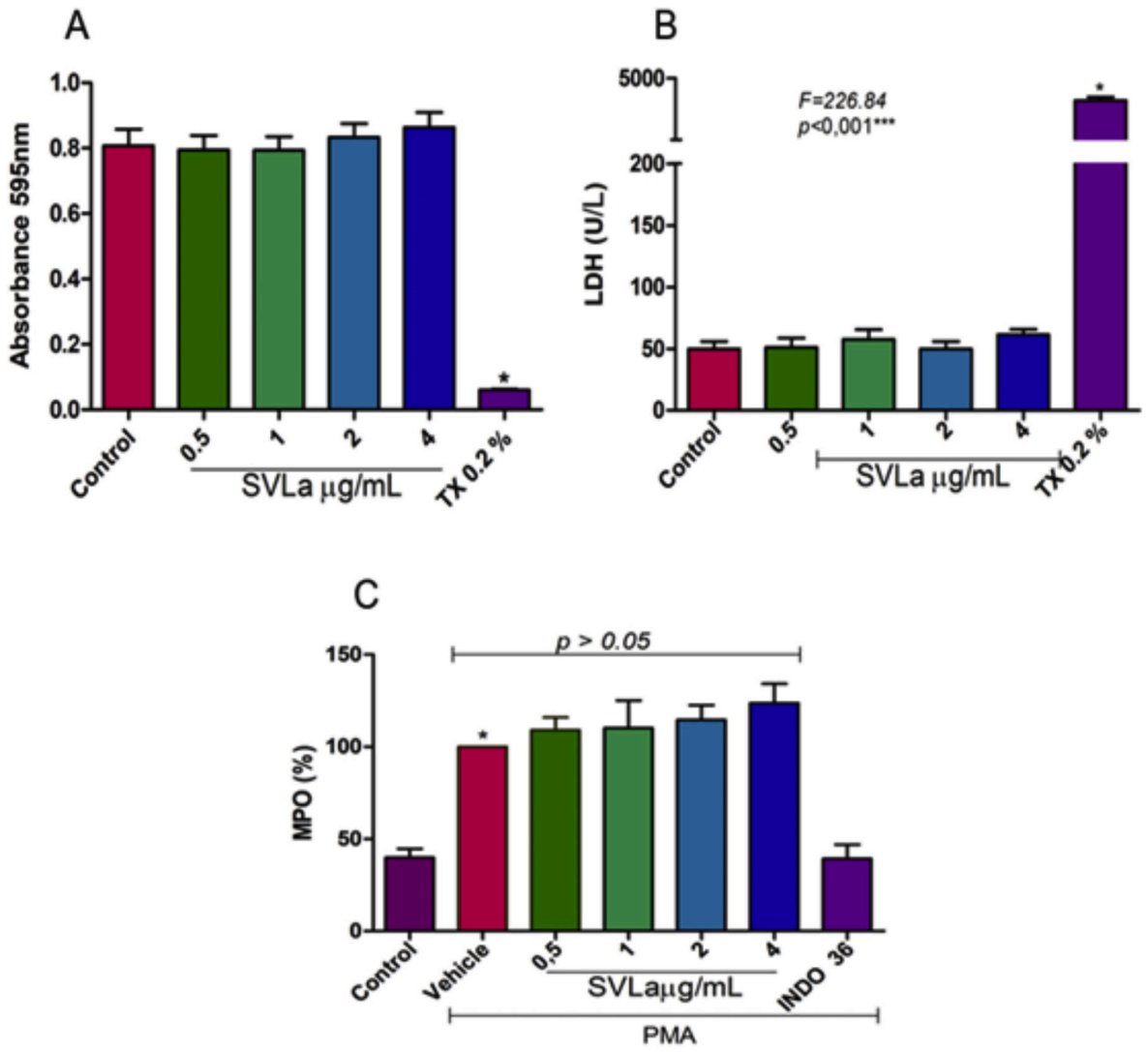

Fig. 6. Effects of the snake venom of *Lachesis acrochorda* (SVLa) on neutrophils. The absorbance at 595 nm in the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay (A) or the activity of lactate dehydrogenase (LDH) were evaluated on neutrophils maintained in the absence or in the presence of SVLa (0.5–4 µg/mL). Note that the venom was inert to produce significant changes in both assays, whereas 0.2% (v/v) Triton X-100 (TX 0.2%) significantly reduced or increased the experimental values in panels A and B, respectively. In C, the myeloperoxidase (MPO) levels were significantly increased in neutrophils subjected to stimulation with phorbol 12-myristate 13-acetate (PMA) in comparison with the control. Note that SVLa did not change the increased levels of MPO in comparison with the group treated with PMA alone. In contrast, 100 µM indomethacin (INDO) reduced the MPO levels to values that did not differ from the control group. Data are mean ± standard error of the mean (S.E.M.) *, $p < 0.05$, ANOVA and Bonferroni test.

during the platelet activation process (Hamilton, 2009).

Hemostatic disorders caused by snake venoms may be due to the action of proteolytic enzymes such as SVSP and SVMP and recent proteomic analyzes indicate these are abundant protein families in both crotalid and viperid venoms (Yamashita et al., 2014). In this study, we reported that the irreversible serine protease inhibitor AEBSF and the metalloprotease inhibitor Na_2-EDTA clearly inhibited platelet aggregation caused by SVLa. Early findings report that *L. muta* venom-induced platelet aggregation was abolished in rabbit platelets treated with phenylmethanesulfonyl fluoride (PMSF, an SVSP inhibitor) or Na_2-EDTA (SVMP inhibitor) (Francischetti et al., 1998). These results reinforce the hypothesis that both SVSP and SVMP contribute to the aggregating activity of SVLa. It is noteworthy that these proteins have already been reported as constituents of SVLa (Madrigal et al., 2012). On the other hand, SVMP and SVSP do not appear to participate in the platelet aggregation and thrombocytopenia processes observed in vivo by *B. jararaca* venom (Rosa et al., 2019; Yamashita et al., 2014).

SVLa increased the absorbance of samples containing platelets in the MTT assay in a concentration-dependent manner, which suggests an increase in MTT salt reduction to yield formazan. Similar result was reported in RAW 264.7 cells maintained in the presence of the viperid venom of *Bothropoides insularis* (Menezes et al., 2016). Traditionally adopted as a measure of cell viability, the results of MTT assay may suggest venom-elicited cell proliferation or increase in the metabolic activity. In platelets, blood elements without nucleus, the hypothesis of increased metabolic activity seems to relate well to the platelet activator effect induced by SVLa, which resulted in platelet aggregation. This hypothesis is reinforced by the results obtained with neutrophils when, in the presence of SVLa, absorbance changes were evidenced neither in the MTT assay nor in the LDH levels, thus precluding the occurrence of cell membrane damage to explain the effects in platelets. These findings differentiate the effects of SVLa from other venoms, such as the *Bothrops leucurus*, which is clearly cytotoxic for platelets as it reduced cell viability by 90% (Bustillo et al., 2009; Francischetti et al., 1998).

In neutrophils, SVLa did not increase the action of PMA on the activity of MPO enzyme. Although unable to exert direct stimulation of the

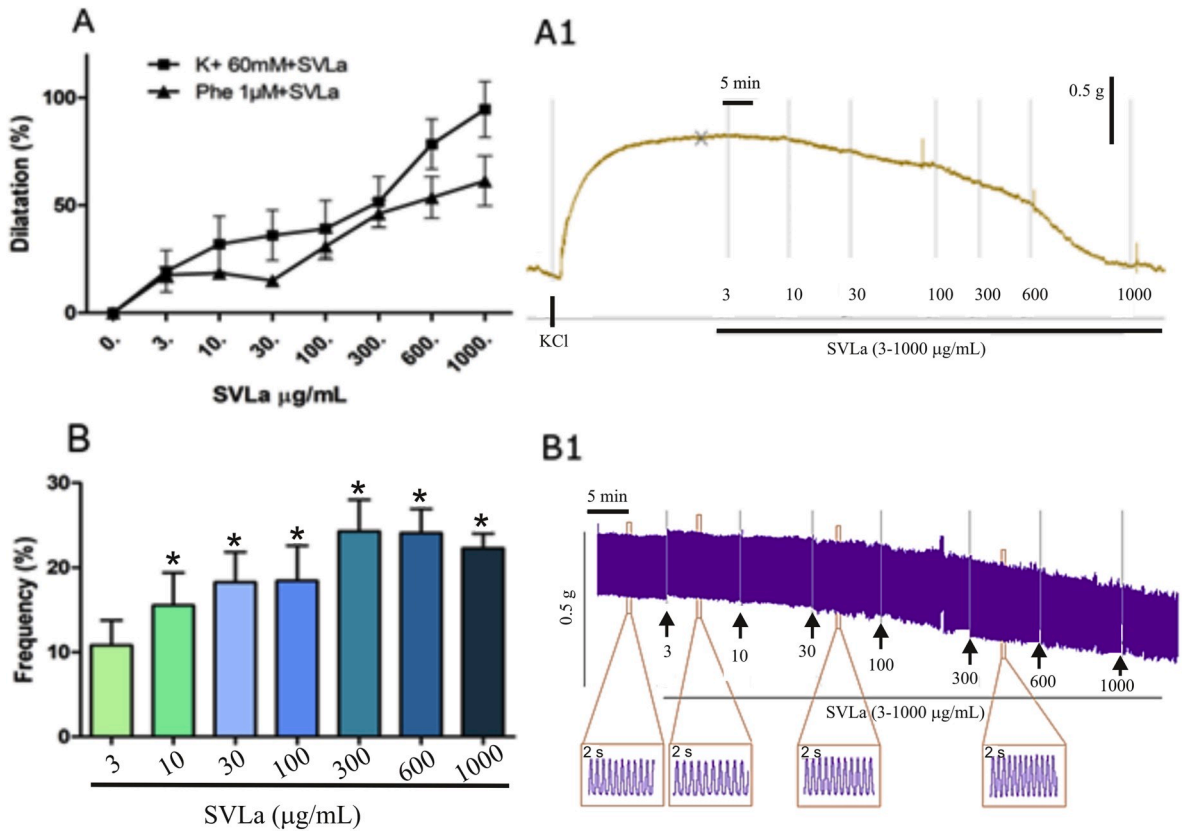

Fig. 7. Vasodilatory effect of the snake venom of *Lachesis acrochorda* (SVLa; 1–1000 μg/mL) on isolated preparations of rat aorta or in the frequency of spontaneous contractions of isolated rat atrium. In A, graph with the mean values of the relaxation induced by the cumulative addition of SVLa on the steady state of a contraction induced by 1 μM phenylephrine or 60 mM KCl. A typical experimental trace of a contraction induced by KCl with the relaxing effect induced by SVLa is showed in panel A1. In B, graph showing the increasing effect induced by SVLa (3–1000 μg/mL) on the frequency of the spontaneous contractions recorded in right atrium preparations. In panel B1, note that the magnitude of the contractions did not change, but the frequency increased in response to SVLa treatment. Data are mean ± standard error of the mean (S.E.M.) *, $p < 0.05$, ANOVA and Dunnett's test.

neutrophil activity, in the present study we observed the presence of neutrophils in tissues of animals intravenously inoculated with SVLa, especially kidneys and lung. Neutrophil recruitment can occur because of the regenerative role of these cells in venom-damaged tissues (Teixeira et al., 2003). *Bothriopsis bilineata* venom, other viperid, induced inflammation and pronounced neutrophil infiltration associated with *Bothrops* envenomation (Porto et al., 2007). Similarly to the effect induced by SVLa, the venom of *B. bilineata* did not affect neutrophil viability *in vitro*, indicating low venom toxicity in this cell type (Setubal et al., 2013).

Cardiovascular alterations were evaluated in the present study. Intravenously administered, SVLa induced prolonged hypotension, an effect that seems to be caused by a vasodilatory response. In fact, isolated rat aorta preparations relaxed in response to SVLa. In these experiments, the preparations were previously contracted by phenylephrine or by high KCl concentration. In common, these contractile agents increase the intracellular concentration of Ca^{2+}, phenylephrine by activating G protein-coupled α1-adrenergic receptors (Consolini and Ragone, 2017; Martínez-Salas et al., 2010), and KCl by depolarizing transmembrane electrical potential (Ratz et al., 2005; Webb, 2003). As the vasodilatory effect of SVLa was similar for phenylephrine and KCl, its activity is likely to be nonspecific or may be explained by an interference with Ca^{2+} influx pathways in the smooth muscle cells. In fact, L-type Ca^{2+} channel blockade by SVMP enzymes (independent of their enzymatic actions) has been suggested to produce vasodilatory effects. A class P-III metalloproteinase isolated from *Trimeresurus stejnegeri* venom revealed these properties in mouse aorta, resulting in a potent vasorelaxant effect (Zhang et al., 2009). However, the hypotensive effect caused by the venom does not seem to depend solely on the effects on the smooth muscle, as the vasodilator effect was caused only at high SVLa concentrations.

Exposure to SVLa increased the frequency of spontaneous beats in isolated right atrium preparations. Although small in magnitude (~25%), the *in vitro* accelerating effect was statistically significant, but does not seem to be related to the bradycardia observed after intravenous administration of SVLa. It is possible that the systemic effects of SVLa involves parasympathetic neural pathways. In fact, individuals bitten by Lachesis snakes may have a syndrome involving nausea, vomiting, abdominal cramps, diarrhea, sweating, hypotension, bradycardia, and shock, possibly of autonomic origin (Otero-Patiño, 2011). Compared to other venoms, studies reported biphasic effects for the venom of *Vipera lebetin* (Fatehi-Hassanabad and Fatehi, 2004), *Bothrops jararacussu* (Rodrigues, 2010) and *Cerastes vipera* (Alzahaby et al., 1995). Exposure of the rat right atrium to these venoms induced transient increase, followed by a sustained reduction in the amplitude and frequency of the spontaneous contractions. Our results also agree with the literature reporting hypotensive effects on other viperid species such as *L. muta* (Dias et al., 2016), *B. jararacussu* (Sifuentes et al., 2008) and *B. atrox* (Glusa et al., 1991) which produce a rapid reduction in blood pressure. In contrast, the venom of *V. lebetina* did not induce hypotension (Fatehi-Hassanabad and Fatehi, 2004). These differences in the hypotensive response to venoms may be influenced by their proteomic composition (Diniz and Oliveira, 1992; Marsh et al., 1997; Pla et al., 2013; Soares et al., 2005; Yarleque et al., 1989).

From the present evidence, it can be concluded that SVLa is able to, under experimental conditions, manifest effects related to many

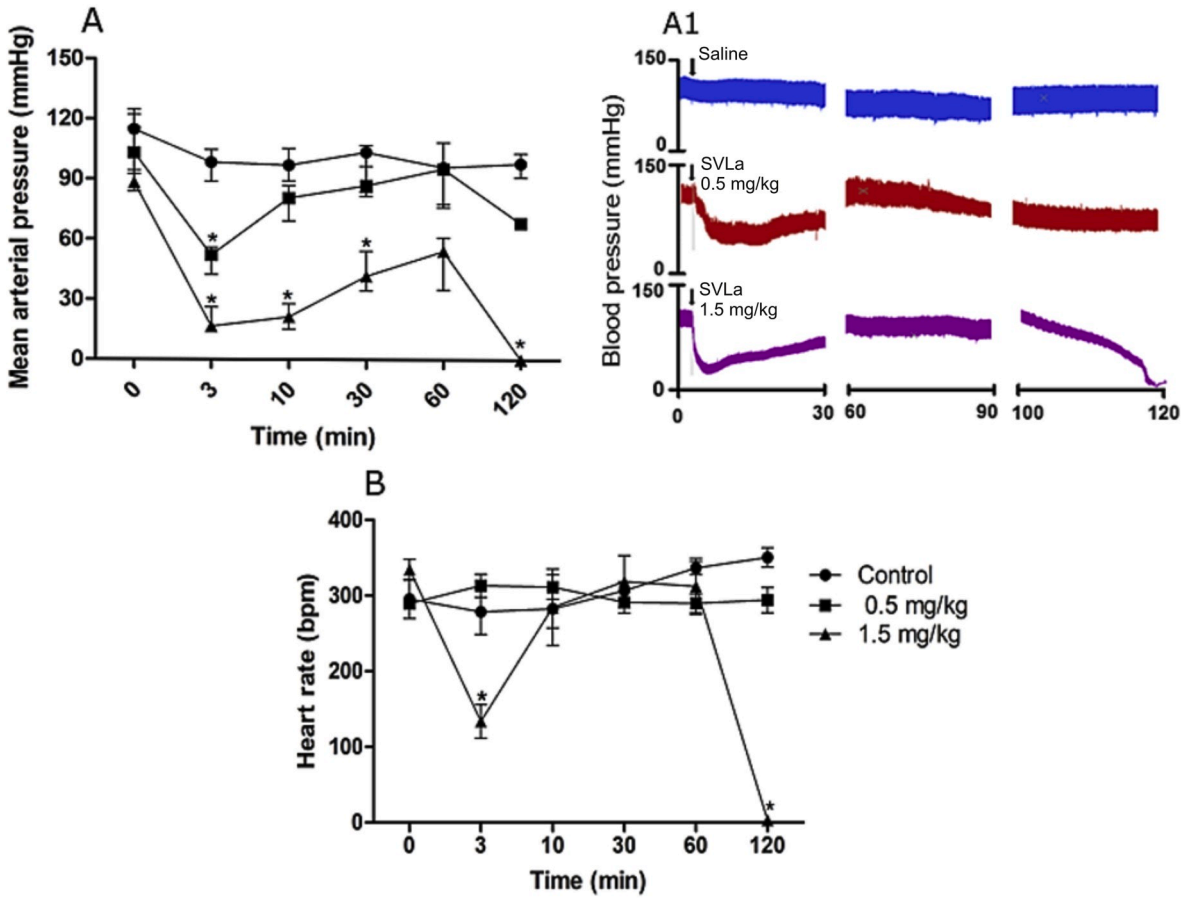

Fig. 8. Effect of the snake venom of *Lachesis acrochorda* (SVLa) on blood pressure and heart rate of rats. In A, values of the mean arterial pressure (MAP) measured on different times after treatment of rats with SVLa (0.5 or 1.5 mg/kg, i.v.). In panel A1, typical traces obtained from the hemodynamic recordings. In B, values of heart rate derived from pressure pulse signals. In experiments with SVLa (1.5 mg/kg), the fall in MAP and heart rate (HR) at 120 min indicates that all animals died. Data are mean ± standard error of the mean (S.E.M.) *, $p < 0.05$, vs. values of each parameter before the injection of SVLa, ANOVA and Bonferroni).

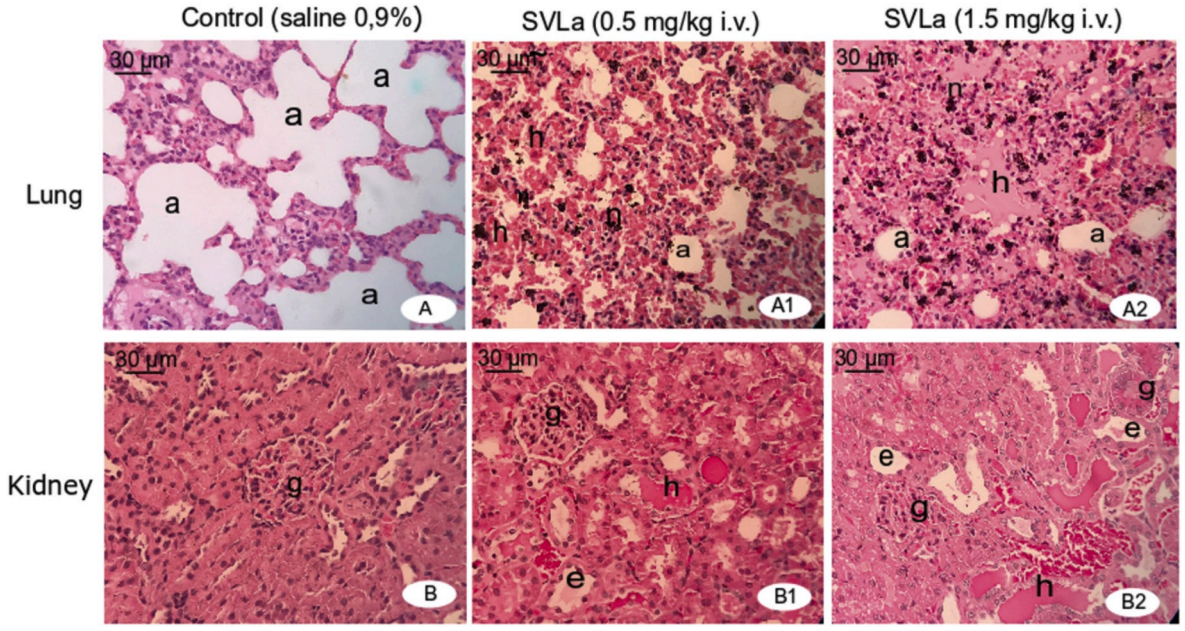

Fig. 9. Histological evaluation of the effecs caused by the snake venom of *Lachesis acrochorda* (SVLa) in lung (panels A) and kidney (panels B) tissues under hematoxylin and eosin staining. Anesthetized rats were injected with SVLa (0.5 [panels 1] or 1.5 mg/kg [panels 2], i.v.) or vehicle (0.9% saline solution, panels A or B). Hemorrhage (h), inflammatory infiltrate (neutrophils, n), ectases (e) are highlighted in the images. Alveoli (a); Glomeruli (g). HE staining. Scale bars: 30 µm in all panels.

symptoms presented by victims of snake bites of the Lachesis genus such as coagulation, hypotension and bradycardia. Given the complex nature of SVLa chemical composition, the effects were diverse and occurred at cellular, tissue and systemic levels. The effects induced by SVLa can be observed at small concentrations or doses, revealing the considerable potency when compared with reports in the literature about the effects caused by venoms of other species under similar experimental approaches. Part of the effects presented here appear to result from the presence of SVMPs and SVSPs in the composition of SVLa.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Karen Leonor Angel-Camilo: Conceptualization, Methodology, Investigation, Writing - review & editing. **Jimmy Alexander Guerrero-Vargas:** Conceptualization, Investigation, Writing - review & editing. **Emanuella Feitosa de Carvalho:** Methodology, Investigation. **Karine Lima-Silva:** Methodology, Investigation, Writing - review & editing. **Rodrigo José Bezerra de Siqueira:** Methodology, Investigation, Writing - review & editing. **Lyara Barbosa Nogueira Freitas:** Methodology, Investigation, Methodology, Investigation. **João Antônio Costa de Sousa:** Methodology, Investigation. **Mario Rogério Lima Mota:** Resources, Writing - review & editing. **Armênio Aguiar dos Santos:** Methodology, Resources, Writing - review & editing. **Ana Gisele da Costa Neves-Ferreira:** Conceptualization, Resources, Writing - review & editing. **Alexandre Havt:** Methodology, Resources, Writing - review & editing. **Luzia Kalyne Almeida Moreira Leal:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision.

Acknowledgments

The study received financial support provided as scholarships from the Brazilian agencies Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Finance code 001), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). This study resulted from the Organization of American States (OAS) program with the Center of Biomedical Investigations of the University of Cauca (CIBUC-Bioterio) – Colombia and Federal University of Ceará – Brazil. The authors are indebted to Prof. Dr. Gerly Anne de Castro Brito for the valuable contribution to perform the histological evaluation of this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2020.06.009.

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