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Differential coagulotoxicity of metalloprotease isoforms from *Bothrops neuwiedi* snake *venom* and consequent variations in antivenom efficacy

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ABSTRACT

Bothrops (lance-head pit vipers) venoms are rich in weaponised metalloprotease enzymes (SVMP). These toxic enzymes are structurally diverse and functionally versatile. Potent coagulotoxicity is particularly important for prey capture (via stroke-induction) and relevant to human clinical cases (due to consumption of clotting factors including the critical depletion of fibrinogen). In this study, three distinct isoforms of P-III class SVMPs (IC, IIB and IIC), isolated from *Bothrops neuwiedi* venom, were evaluated for their differential capacities to affect hemostasis of prey and human plasma. Furthermore, we tested the relative antivenom neutralisation of effects upon human plasma. The toxic enzymes displayed differential procoagulant potency between plasma types, and clinically relevant antivenom efficacy variations were observed. Of particular importance was the confirmation the antivenom performed better against prothrombin activating toxins than Factor X activating toxins, which is likely due to the greater prevalence of the former in the immunising venoms used for antivenom production. This is clinically relevant as the enzymes displayed differential potency in this regard, with one (IC) in particular being extremely potent in activating Factor X and thus was correspondingly poorly neutralised. This study broadens the current understanding about the adaptive role of the SVMPs, as well as highlights how the functional diversity of SVMP isoforms can influence clinical outcomes.

Key Contribution: Our findings shed light upon the hemorrhagic and coagulotoxic effects of three SVMPs of the P-III class, as well as the coagulotoxic effects of SVMPs on human, avian and amphibian plasmas. Antivenom neutralised prothrombin-activating isoforms better than Factor X activating isoforms.

1. Introduction

A paradigm of snake venom evolution is that the venom gland promiscuously secretes proteins that were historically endogenous proteins with physiological function elsewhere in the body. When such expression in the venom gland confers an advantage, then this trait becomes fixed and amplified in the venom gland, with secondary specific duplication and diversification of the proteins, leading to multigene families with venom-gland specific expression. Toxins within such families neofunctionalise through random mutation, with additional evolutionary selection pressures operating upon their evolution. Consequently, within a single toxin family there may be diverse bioactivities. However, while such molecular and functional diversity provides a rich source of novel compounds of interest for drug design and development, they also greatly complicate treatment of the envenomed patient due to variable response to antivenom. Thus, snake venoms and their arsenals of toxins could be useful not only to trace the relevance of the venom phenotype for snake biology, but also to understand the clinical outcomes of human patients envenomed as recently demonstrated for *Bothrops atrox*, a pit viper species responsible for many snakebites in the Amazon region (Monteiro et al., 2020).

New World pit vipers in the genus *Bothrops* are responsible for the majority of snake bites in South America (Chippaux, 1998; Warrell, 2013). In humans, their venom induces preeminent local effects

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(oedema, necrosis, blisters and hemorrhage) and devastatingly procoagulant actions upon the clotting cascade (França and Málaque, 2009) associated with systemic bleeding (far from bite site) in severe cases. Hemorrhage is a very common and serious pathologic event observed during *Bothrops* envenoming, whereby the venom hydrolyses the microcirculatory basement membrane proteins (Freitas-de-Sousa et al., 2017). On the other hand, the procoagulant action generates large amounts of endogenous thrombin, resulting in stroke in prey items and consumptive coagulopathy in human bite victims (Isbister, 2010; Zdenek et al., 2019). Thus, studies on functional diversity of snake venoms and their major components can be medically important, as they help to understand the differences in venom toxicity, which may reflect antivenom efficacy and pathological effects in humans.

Many of the pathological effects, including the hemorrhage and consumptive coagulopathy, induced by snake venoms from the genus *Bothrops* are related directly or indirectly to the action of snake venom metalloproteinases (SVMPs). This toxin class is the most abundant toxin class in the proteomes of these venoms (Sousa et al., 2013; Andrade-Silva et al., 2018). These versatile enzymes are zinc-dependent multidomain proteins, belonging to the M12 subfamily of the reprolysin proteins (metalloproteases) (Fox and Serrano, 2005). According to the organization of its structural domains, they are classified in three classes (P-I, P-II and P-III) and several subclasses. After post-translational processing, the SVMPs can be found in different isoforms in the venoms, either only with the catalytic domain (P-I), or in combination with the catalytic domain, with the additional disintegrin domain (P-II) or disintegrin-like/cysteine-rich domain (P-III) (Fox and Serrano, 2008; Moura-da-Silva et al., 2016).

Biosynthesis and post-translational processing are two essential steps for the diversity generation in SVMPs (Moura-da-Silva et al., 2016). Such toxin diversification has important implications for the ecology of snakes (Bernardoni et al., 2014) and for the envenomated patient, as multiple hemostatic mechanisms and physiological targets can be affected by these toxins (Bjarnason and Fox, 1994; Andrews et al., 2001; Moura-da-Silva et al., 2007; Berger et al., 2008). For example, SVMPs interact with a wide range of biological targets including platelets (de Queiroz et al., 2017; Kini and Koh, 2016) and coagulation cascade factors (Hofmann and Bon, 1987a, 1987b; Silva et al., 2003; de Albuquerque Modesto et al., 2005; Senis et al., 2006), cleaving important zymogens such as Factor X (FX) and prothrombin into their activated forms FXa (activated FX) and thrombin, respectively (Takeya et al., 1992; de Albuquerque Modesto et al., 2005). Since FXa in turn activates even more prothrombin into thrombin through positive feedback loops, the net physiological state is a massive thrombin overdose, leading to Venom-Induced Consumptive Coagulopathy (VICC), clinically characterized by hypofibrinogenemia (drop in fibrinogen levels due to fibrinogen consumption) and prolongation of the aPTT and PT coagulation tests (White, 2005; Isbister, 2009). Other SVMPs may directly hydrolyse products of the coagulation cascade, such as fibrinogen and fibrin (Swenson and Markland, 2005; Moura-da-Silva et al., 2007; Sajevic et al., 2011; Kini and Koh, 2016), and also components of the basal membrane of blood capillaries (Freitas-de-Sousa et al., 2017; Tanjoni et al., 2003; Baldo et al., 2010). In blood capillaries, the hemorrhagic SVMPs produce catalytic activity upon specific regions of key basal membrane components, thereby destabilizing the basement membrane (BM) structure of the vascular wall and exacerbating the hemorrhagic effect (Escalante et al., 2006, 2011a, 2011b; Baldo et al., 2008, 2010). Consequently, SVMPs play a major role in haemostatic disturbances (consumptive coagulopathy and hemorrhage) induced during the envenomation (Sajevic et al., 2011; Berling and Isbister, 2015), and this fact can have important implications for the management of envenomed patients (Silva de Oliveira et al., 2017).

SVMPs represent approximately 50 % of the proteome in *Bothrops neuwiedi* venoms, of which 22 % are P-III class SVMP, 14 % P-II class, and 14 % P-I class (Sousa et al., 2013). In a previous study, our group used a pool of *B. neuwiedi* venoms to isolate several isoforms of SVMPs (P-I and P-III classes) capable of activating both FX and prothrombin, and inducing coagulation in plasmas of humans, birds and small rodents (Bernardoni et al., 2014). This suggests that the abundance and functional diversity of SVMPs in *Bothrops* venoms would reflect the importance of this class of toxins for the ecology of the snakes and for human envenomation. However, while the first aspect (ecological implication) has already been addressed at least partially (Bernardoni et al., 2014), the efficacy of antivenoms to neutralize coagulotoxic activities induced by these toxins still remains uncertain. Moreover, this type of analysis also enriches the current understanding on the global implications of the functional diversity of SVMPs, one of the most abundant toxin class in snake venoms.

In the present study, we used *in vitro* and *in vivo* techniques to investigate the hemorrhagic and coagulotoxic effects of three SVMPs of the P-III class (termed IC, IIB and IIC) isolated from *B. neuwiedi* venom, as well as examined their reactivity with Polyvalent Bothrops Antivenom (BAV). In addition, we compared toxin activity on plasmas from multiple taxa to aid ecological interpretation of toxin-specific activity. Overall, our results advance the ecological and pathological understanding of SVMPs, and our findings inform clinical management strategies for snakebite envenomations.

2. Material and methods

2.1. Animal model

Swiss mice of both sexes, weighing 18–20 g, were obtained from the central animal house of the Instituto Butantan. All experiments involving mice were approved by the Ethical Committee for Animal Research of the Instituto Butantan (CEUAIB), São Paulo, Brazil (identification number 1028/13).

2.2. Plasmas

Human plasma was provided by the Australian Red Cross (Research agreement #18-03QLD-09; University of Queensland Human Ethics Committee Approval #2016000256). Avian (domestic chicken, Gallus domesticus) and amphibian (cane toad, *Rhinella marina*) plasmas were obtained under the University of Queensland Animal Ethics approval SBS/019/14/ARC. All plasma was prepared to a 3.2 % citrated stock, pooled and aliquoted into 1 mL tubes, which were snap frozen in liquid nitrogen, and stored at −80 °C until required, at which time an aliquot was defrosted in a 37 °C water bath for 10 min. All plasma work was undertaken under University of Queensland Biosafety Approval #IBC134BSBS2015.

2.3. Antivenom

The Polyvalent Bothrops Antivenom (BAV) (batch: 1305077, ED: 05/16), produced by Instituto Butantan, Brazil, from horses immunised with a pool containing venoms of the species: *B jararaca*, (50 %), *B. neuwiedi* (12.5 %), *B. jararacussu* (12.5 %), *B. alternatus* (12.5 %), and *B. moojeni* (12.5 %). The antivenom solution, consisting of soluble IgG F (ab')2 fragments, was centrifuged (12,000 RCF, 10 min, 4 °C), the supernatant removed, and aliquoted into 1 mL tubes, and stored at 4 °C until required.

2.4. SVMPs from Bothrops neuwiedi venom

Three P-III type SVMPs (IC, IIB, and IIC) were isolated from a pool of venoms from *B. neuweidi* snakes (subspecies: *B. n. pauloensis*, *B. n. matogrossensis*, *B. n. neuwiedi*, *B. n. marmoratus* and *B. n. diporus*) by molecular exclusion and ion exchange chromatographies using an Äkta system (GE Healthcare). For that, we used the same protocol, columns and chromatographic system, previously employed by our group, in the

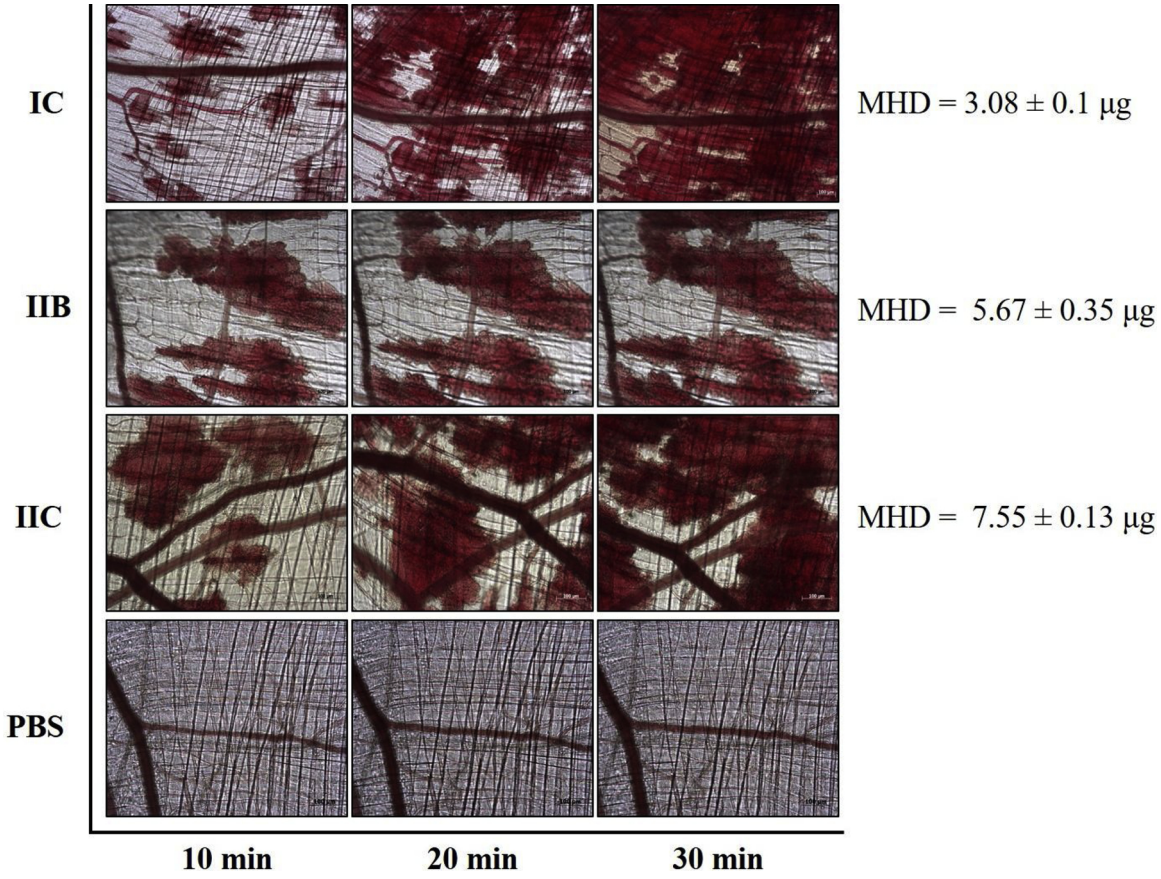

Fig. 1. Hemorrhagic spots in microvasculature of mice, evaluated by Intravital Microscopy (IVM) and Minimum Hemorrhagic Dose (MHD). In the IVM analyses, the cremaster muscle of mice was subject to the intrascrotal topical application of the fractions (10 µg/20 µL), and their actions on microvasculature were evaluated for 30 min. The representative panel shows the development of bleeding at 10, 20 and 30 min. Hemorrhagic potential was evaluated through MHD, 3 h after intradermal application of different amounts of the SVMPs (0.2, 0.5, 1, 3 and 5 µg/50 µL of PBS) in mice dorsal skin (three animals/dose). Each animal was injected once only. MHD data represent mean and SE values obtained in three independent experiments.

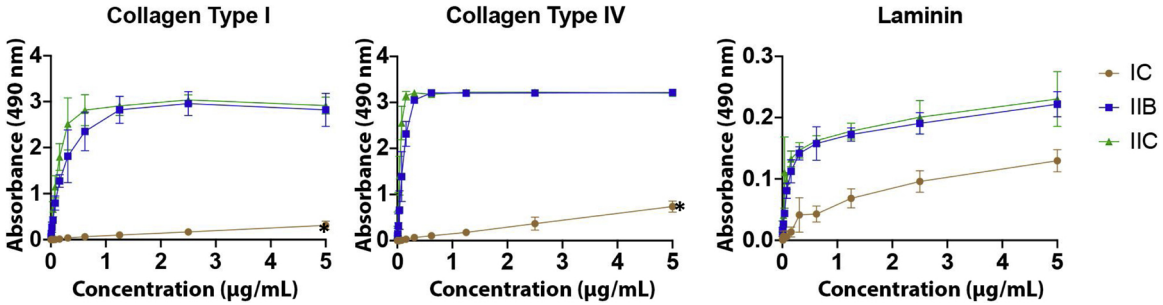

Fig. 2. Binding of the SVMPs to extracellular matrix components. Microtiter plates were coated with collagen type I, collagen type IV, or laminin (10 µg/mL) and incubated for 18 h at 4 °C. Next, samples of the fractions were added to the plates, which were incubated with the anti-jararagin rabbit polyclonal antibodies, followed by peroxidase-conjugated anti-rabbit IgG and the enzyme substrate (OPD). X-axes are concentration (µg/mL) and Y-axes are absorbance (490 nm). Note the Y-axis scale difference for laminin.

first characterization of these toxins (Bernardoni et al., 2014). Briefly, samples of 50 mg lyophilized crude venom were dissolved in buffer (200 µL of 20 mM Tris, 1 mM $CaCl_2$ and 150 mM NaCl, pH 7.8) and insoluble material was removed by centrifugation at 10,000*g* for 10 min at room temperature. Proteins of the supernatant were applied to a Hiprep 16/60 S-200 column (GE Healthcare) and subjected to isocratic elution at a flow rate of 0.5 mL/min. The fractions presenting metalloproteinase activities (hemorrhagic and/or fibrinolytic) were pooled according to major peaks and dialyzed against 20 mM Tris, 1 mM $CaCl_2$, pH 7.8 and applied to a Mono-Q HR 5/5 column (GE Healthcare) using the same buffer. Next, proteins from size exclusion peaks were eluted using different gradient of salt (ranging of 0–1 M of NaCl) at a flow rate of 1 mL/min. After the fractioning, the obtained fractions were evaluated by SDS-PAGE 12.5 %, under non-reducing conditions to evaluate their homogeneity, lyophilized and kept frozen (−80 °C) until use.

2.5. Minimum hemorrhagic dose (MHD)

The minimum hemorrhagic doses of the SVMP rich-fractions were calculated as described by Kondo et al. (1960) with modifications. Samples of each SVMP were diluted to different doses (0.2, 0.5, 1, 3 and 5 µg/50 µL of PBS) and injected by intradermal route into the dorsal skin of male Swiss mice, being each animal injected a single time. Three hours after inoculation, the animals were euthanized in a CO_2 chamber,

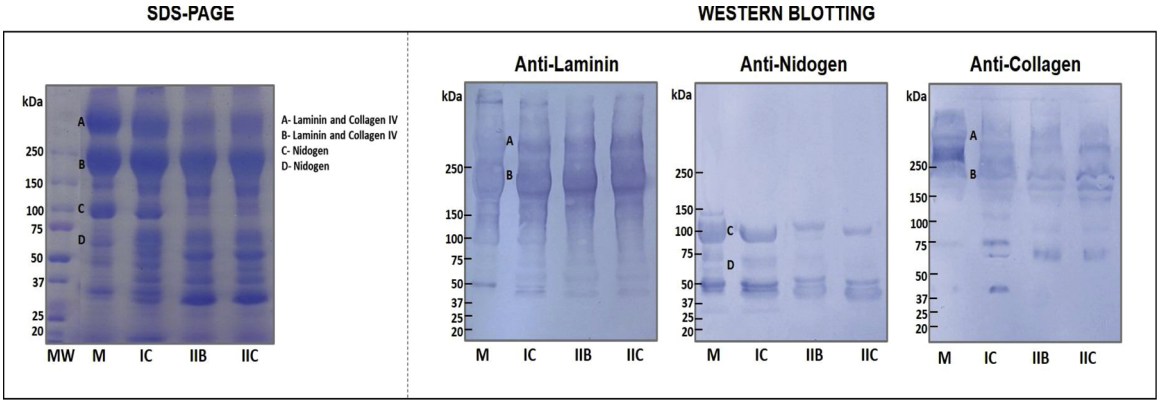

Fig. 3. Hydrolysis of extracellular matrix proteins by the SVMPs. Matrigel was incubated with each fraction in a 1:10 ratio of enzyme:substrate (w:w) for 3 h. The reactions were interrupted by adding sample buffer with reducing agent. Next, the reaction mixtures were separated in 5-15 % polyacrylamide gradient gel (SDS-PAGE). For western blotting, proteins present in the gels were transferred to nitrocellulose membranes, incubated with anti-laminin (0.35 µg/mL), anti-nidogen (0.3 µg/mL) or anti-collagen (10 µg/mL) for 18 h at room temperature, followed by incubation with peroxidase-labelled anti-rabbit IgG (1:1000 - for collagen and laminin) and peroxidase-labelled anti-goat IgG (1: 500 - for nidogen) for 2 h at 37 °C. Reactive bands were detected with 4-chloro-α-naphtol and H_2O_2. The control (**M**) containing only matrigel, incubated without SVMPs, shows mostly bands corresponding to laminin (~400 and 225 kDa - A and B, respectively), collagen IV (~400 and 225 kDa - **A** and **B**, respectively) and nidogen (~100 and 50 kDa - **C** and **D**, respectively). Molecular weight markers (MW).

the dorsal skin was removed, and the hemorrhagic area (mm^2) was calculated considering the product of the longest diameter by the diameter perpendicular to it. The MHD was as defined as the amount of protein (µg) that produced hemorrhages with a mean diameter of 10 mm after 3 h. Groups of 3 animals/dose were tested, and a control group was injected with PBS only. Results are expressed as mean ± SE of 3 independent experiments.

2.6. Intravital microscopy analyses

The effects induced by SVMPs samples, in the microcirculatory network of Swiss mice, was determined using intravital microscopy (IVM), as described by Umekita et al. (2001) with some modifications. The IVM was performed on an upright microscope (Axio Lab, Carl Zeiss, Oberkochen, Germany) with an immersion objective in saline (SW40 / 0.75 numerical aperture, Zeiss, Jena, Germany) coupled to a camera (AxioCam icc1, Carl Zeiss AG, Oberkochen, Germany) using a 10/0.3 lens longitudinal distance objective/numerical aperture and 1.6 optovar (Carl Zeiss, Oberkochen, Germany). To visualize the capillaries for analysis, was used the cremaster muscle of the mice as the main tissue because it is thin and transparent. Mice were anesthetized with an intraperitoneal injection of 2% Xylazine (Calmiun®, Agener Union, São Paulo, SP) and 0.5 g/kg of ketamine (Holliday-Scott SA, Buenos Aires, Argentina), and after 10 min the cremaster was exposed for microscopic examination *in situ*. Next, the mice were maintained on a special board that was thermostatically controlled at 37 °C and included a transparent platform on which the tissue was placed to be transilluminated. Postcapillary venules, with a diameter 25–40 µM, were chosen to evaluate the bleeding inducing by a topical application of 20 µL of SVMPs samples (10 µg) for 30 min. The negative control was subjected to the topical application of a PBS solution (20 µL). The results are representative of three independent experiments.

2.7. Binding to the extracellular matrix (ECM) proteins

The adhesion of the SVMPs samples (10 µg) to ECM components was investigated using microtiter plates coated with type I collagen, type IV collagen or laminin (10 µg/mL in carbonate buffer, pH 9.6) incubated for 18 h at 4 °C. Next, different dilutions of SVMPs samples were then added to the plates and the binding was detected (A490 nm) after incubation with rabbit anti-jararagin polyclonal antibodies (1:200), followed by anti-rabbit IgG peroxidase conjugates (1:2000) and the enzyme substrate.

2.8. Hydrolysis of the ECM proteins

Analyses of hydrolysis of ECM proteins were carried out as described by Escalante et al. (2006) with some modifications. For that, samples containing Matrigel TM (ECM gel, from Engelbreth Holm-Swarm mouse sarcoma - Sigma) were incubated with SVMPs samples using a 1:10 ratio of enzyme:substrate: 10 µg of SVMP samples to 100 µg of matrigel. The incubation of the samples (SVMPs and matrigel) was performed for 3 h at 37 °C. Reactions were stopped by the addition of reducing agent (β-mercaptoethanol at 58.3 mM). A sample of matrigel, incubated under identical conditions, but without SVMPs, was used as control. The products of hydrolysis were boiled for 5 min and analyzed by SDS-PAGE and western blotting. For SDS-PAGE analysis, the samples were added into 5–15 % Tris-HCl polyacrylamide gradient gels and submitted to electrophoresis, using the dual color molecular mass standard (Bio-Rad Laboratories, CA, USA). Next, bands were visualized with Coomassie Brilliant Blue R-250.

For western blotting, proteins present in the gradient gels (5–15 % polyacrylamide) were electro-transferred to nitrocellulose membranes, which were immersed in a blocking solution (5% non-fat milk in Tris-saline for 2 h at room temperature). Next, membranes incubated with rabbit polyclonal anti-collagen IV at 10 µg/mL (Fitzgerald, Acton, MA, USA), rabbit polyclonal anti-laminin at 0.35 µg/mL (Novus Biologicals, Littleton, CO, USA) or goat polyclonal anti-nidogen 1 at 0.3 µg/mL (R& D Systems, Minneapolis, MN, USA) for 18 h at room temperature. After washing with Tris-saline, the membranes were incubated with anti-rabbit IgG labelled with peroxidase (1:1000 – for collagen and laminin) and anti-goat labelled with peroxidase (1:500 – for nidogen) (Sigma-Aldrich, St. Louis, MO, USA) for 2 h at 37 °C and reactive bands detected by incubation with 4-chloro-α-naphtol and H_2O_2.

2.9. Cleavage of fibrinogen

Fibrinogen cleavage studies were conducted using 1 mm SDS-PAGE gels (12 %), and the effects upon fibrinogen chains produced by the fractions were quantified by ImageJ, as previously described (Coimbra et al., 2018; Dobson et al., 2018).

2.10. Coagulation analyses

Procoagulation and neutralization assays were undertaken using a STA-R Max coagulation analyser robot (Stago, Asnières sur Seine, France), as previously described (Sousa et al., 2018). In the

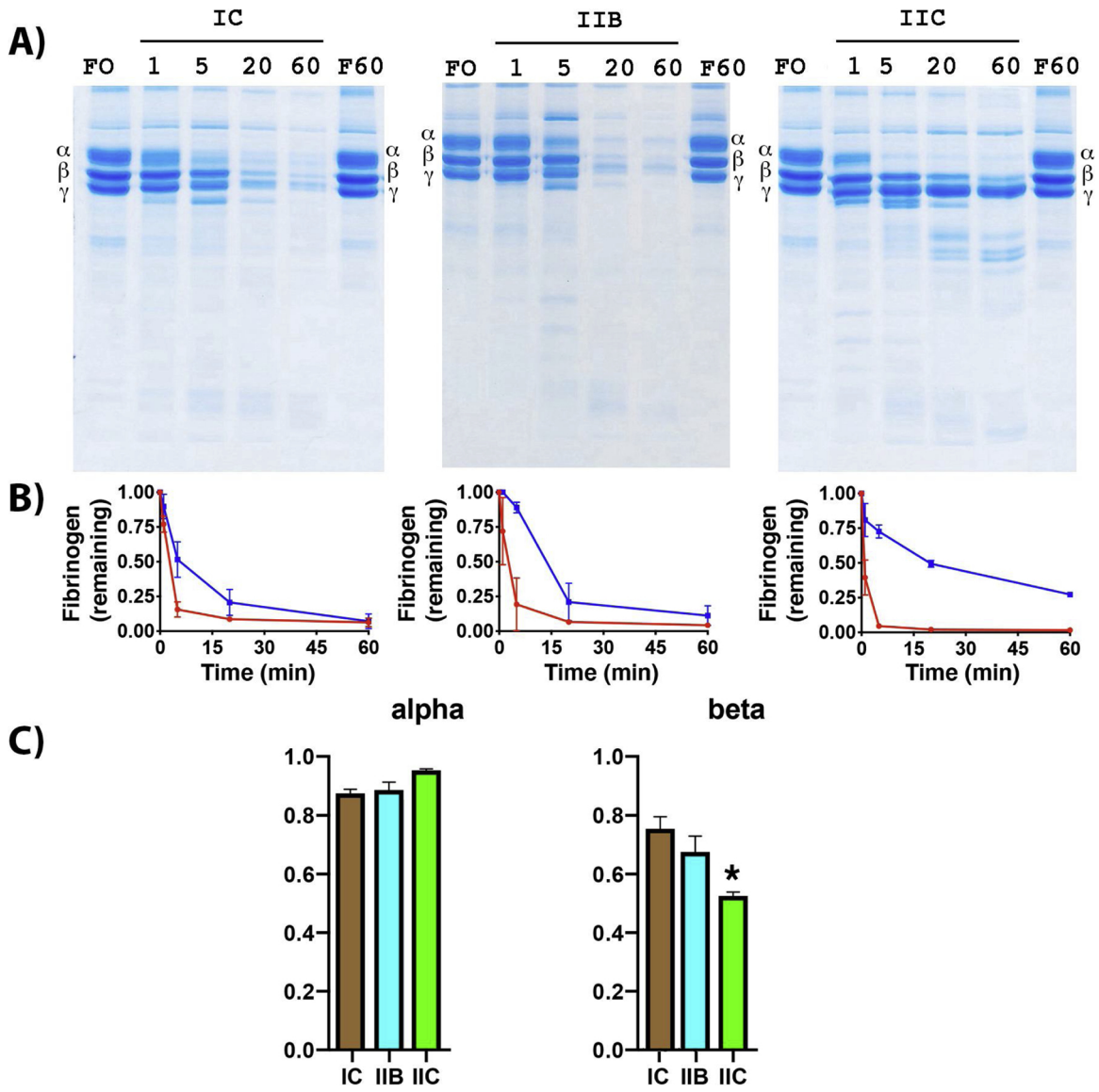

Fig. 4. 1D SDS-PAGE of fibrinogenolytic effects upon Aalpha and Bbeta fibrinogen chains. A) Each condition was run in triplicate and representative gels are shown. F0 = time 0 min fibrinogen control; F60 = time 60 min fibrinogen control; 1, 5, 20, and 60 = experimental time periods. B) The quantification of the effects upon fibrinogen chains are shown in line (red = Aalpha; blue = Bbeta) graphs with y-axis smaller numbers indicating less fibrinogen remaining, and C) bar graphs of the relative cleavage of fibrinogen, whereby 1 = complete cleavage. Data points are n = 3 means and standard deviations. The comparisons are between fractions. * = differences considered significant ($p \leq 0.05$). Fibrinogen α, β, and γ chains are noted for each gel panel.

neutralization protocols, the antivenom efficacy against the procoagulant activity of the fractions was investigated using the BAV diluted to 5% in the OK buffer (Owren Koller Buffer, Stago, Asnières sur Seine, France) of the assay, resulting in a final antivenom dilution of 1:200 (1.25 µL antivenom in 250 µL total cuvette volume). For the cofactor dependency tests, OK buffer replaced the cofactor of interest in the assay, thus maintaining the same total volume.

2.11. Factor X and prothrombin activation activity

The FX/prothrombin activation activity by the fractions, and the neutralization of these activities by antivenom, were performed in the STA-R Max coagulation analyser. As previously described by Sousa et al. (2018), the fractions were accompanied in the assay with a specific colorimetric substrate (Catalog 00311, Stago, Asnières sur Seine, France), and with or without: BAV, FX (at 0.01 µg/µL), or Prothrombin (at 0.1 µg/µL). Prothrombin was added at 10x the concentration as FX, as it is present in the blood stream at 10x the concentration (Palta et al., 2014).

2.12. Thromboelastography analyses

To determine the strength of clot induced by the fractions in human, avian and amphibian plasmas, viscoelastic assays also were undertaken, as previously described (Oulion et al., 2018; Coimbra et al., 2018). Briefly, 72 µL of $CaCl_2$ (25 mM), 72 µL phospholipids, 20 µL Owren-Koller (OK) buffer and 7 µL of SVMP samples at 0.1 mg/mL were added together into specific cups, before adding plasma. Next, 189 µL of plasma (human, avian or amphibian plasma) was added, the whole solution, pipette-mixed, and the test immediately started. The main parameters of the clots (SP = split point the time at which clot formation starts; R = time to reach 2 mm amplitude; MA = maximum amplitude) were analysed for 30 min. Seven microliters of human FXa (0.01 mg/mL) or human Thrombin (0.1 mg/mL) in replacement of the fractions worked as positive controls of the experiments.

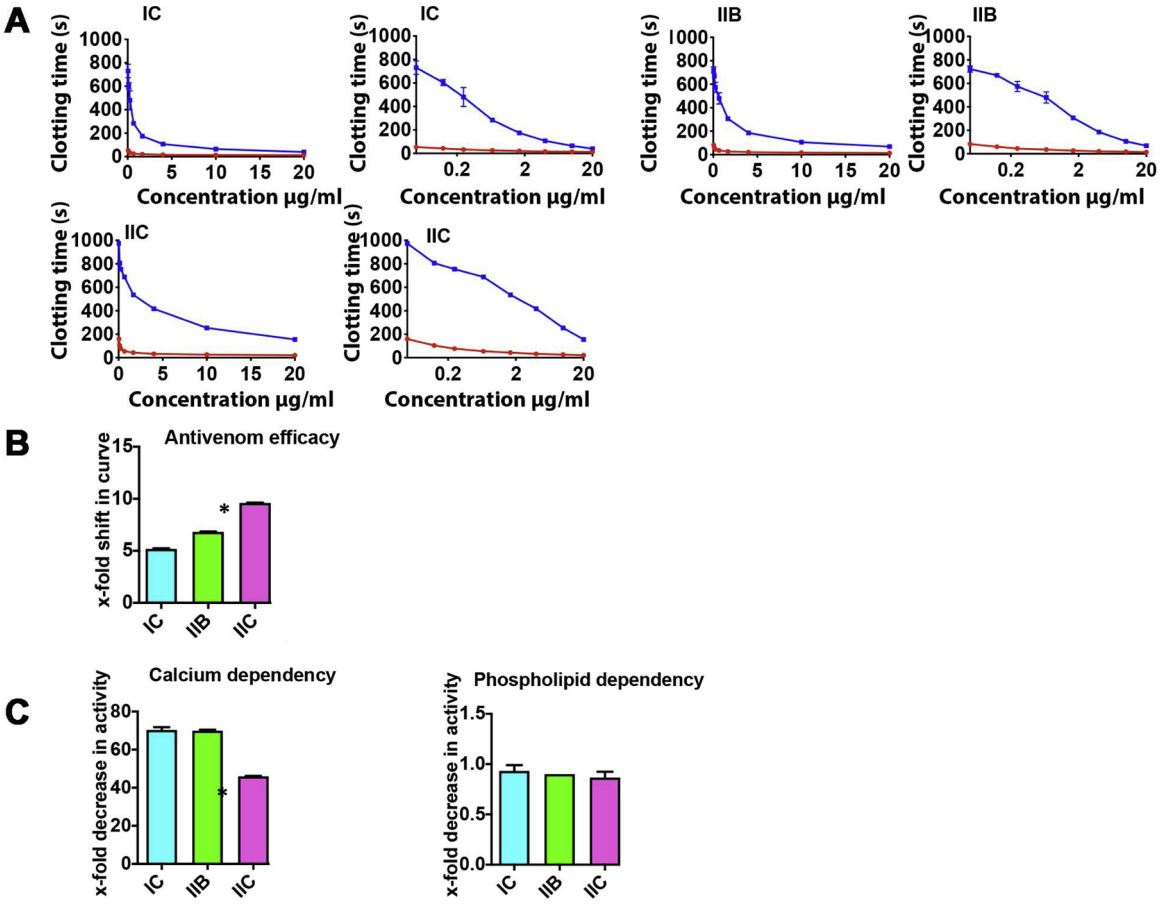

Fig. 5. Clotting speed and cofactor dependency measured on a Stago STA-R Max coagulation analyzer. A) 8-point dilutions of venom effects upon human plasma are shown in linear and logarithmic presentations. Red = venom only; blue = venom + antivenom; control = 512 +/- 34 s. **B)** Antivenom efficacy tests, whereby x-fold shift was calculated by dividing the venom + antivenom curve AUCs by the venom curve AUCs, then subtracting 1 so that a zero indicates no shift by antivenom; larger numbers indicate greater shift. **C)** Cofactor dependency studies: larger numbers indicate greater dependency; no change in the absence of a cofactor would have a value of 0. The comparisons are between fractions, and the differences were considered significant if $p \leq 0.05$. All data points are n = 3 means and standard deviations.

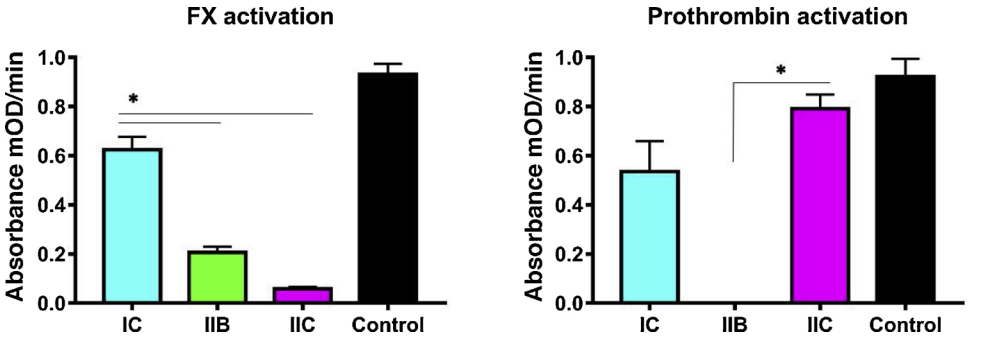

Fig. 6. Relative ability of SVMP fractions to activate the clotting zymogens Factor X and prothrombin, measured on a Stago STA-R Max coagulation analyzer. Activation studies: larger numbers indicate greater activity as indicated by higher rate of absorbption. Data points are n = 3 means and standard deviations, normalised against the respective controls (FXa and thrombin). The comparisons are between fractions, and the differences were considered significant if $p \leq 0.05$. Data represent mean + SD of the absorbance values, obtained from experiments performed in triplicates.

2.13. Statistical analyses

In some analyses, differences among the means were evaluated by one-way Analysis of Variance (ANOVA), followed by Tukey post-test (for multiple comparisons), and in this case the results represent mean and standard deviation or standard error, as appropriate, and the level of significance was set at $p \leq 0.05$. All dose-response curves, as well as cofactor dependency tests, were conducted in triplicate and the results are shown as mean and standard deviation. Data were analysed using Prism 7.0 software (GraphPad Software Inc., La Jolla, CA, USA, version 7, 2017, La Jolla, CA, USA).

3. Results and discussion

We performed a comparative study of three SVMPs of P-III class (IC, IIB and IIC), from *Bothrops neuwiedi venom, investigating* the ability of these toxins to affect hemostasis (inducing hemorrhage in mice and/or coagulotoxic effects in different plasma types), and their neutralization by the antivenom used in the serum therapy of human envenomations by *Bothrops* snakes. This study documented significant variation not only in hemorrhagic and procoagulant effects of the SVMPs but also revealed striking variations in the possible hemorrhagic mechanisms and relative efficacy of the antivenom against each toxin.

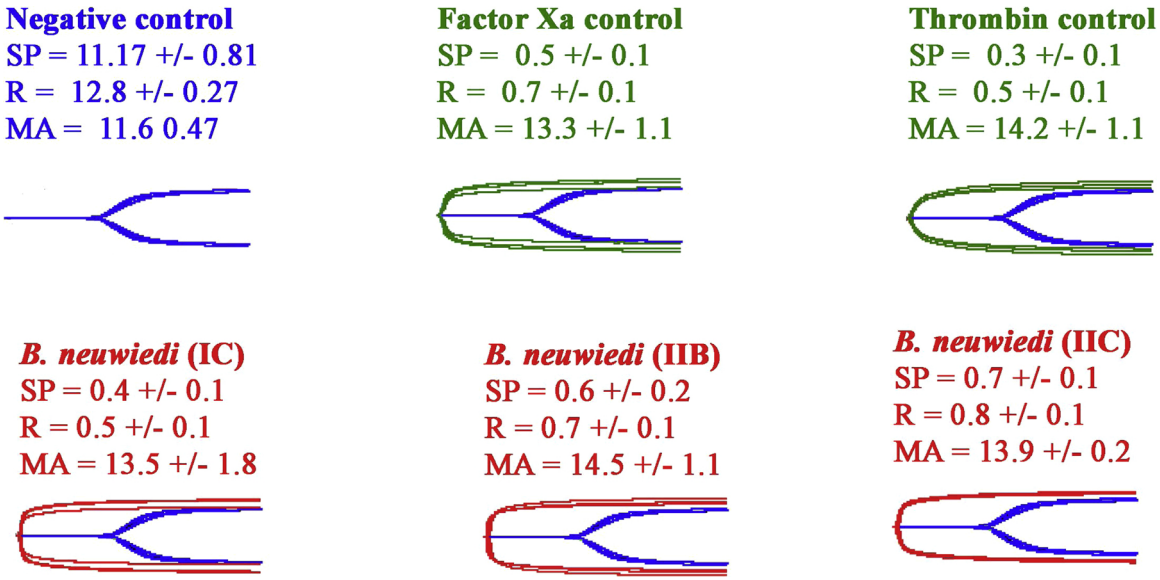

Fig. 7. Clot strength in human plasma produced by fractions, measured on Haemonetics TEG5000 Thromboelastography analysers for 30 min. In this analysis, 72 µL of $CaCl_2$ (25 mM), 72 µL of phospholipid, 20 µL of OK buffer, and 7 µL of the fractions at 0.1 mg/mL (0.7 µg/test), were initially added together in specialised cups, before adding plasma (189 µL) to the reaction mixture, with measurements beginning immediately. The main parameters of the clots (SP, R, and MA) were analysed for 30 min. For visualisation purposes, the human Factor Xa control (0.01 mg/mL), human thrombin control (0.1 mg/mL), and fraction experimental traces are overlaid with the spontaneous clotting negative control. Wider traces indicate stronger clots, and vice versa. All traces are n = 3. Values are n = 3 means and standard deviation. SP = split point—the time at which clot formation starts; R = time to reach 2 mm amplitude; MA = maximum amplitude.

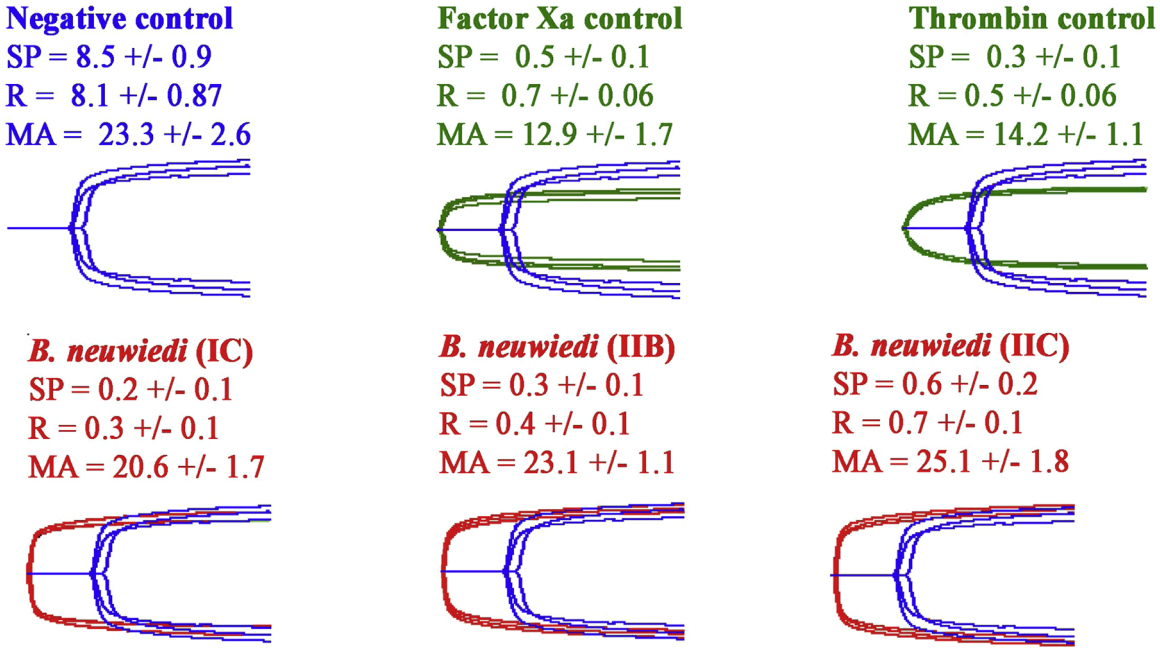

Fig. 8. Clot strength in avian plasma produced by fractions, measured on Haemonetics TEG5000 Thromboelastography analysers for 30 min. See Fig. 7 caption for assay details. For visualisation purposes, the human Factor Xa control (0.01 mg/mL), human thrombin control (0.1 mg/mL), and fraction experimental traces are overlaid with the spontaneous clotting negative control. Wider traces indicate stronger clots, and vice versa. All traces are n = 3. Values are n = 3 means and standard deviation. SP = split point—the time at which clot formation starts; R = time to reach 2 mm amplitude; MA = maximum amplitude.

3.1. Hemorrhagic activity

We performed intravital microscopy (IVM) to analyze the hemorrhagic action of the SVMPs and their effects on mice microcirculation. By ten minutes after the sample application, all SVMPs induced hemorrhage, to varying degrees, as verified by the presence of hemorrhagic spots in microvasculature of the mice (Fig. 1). However, although the hemorrhage induced by fraction IC was less intense in the first time period, it increased considerably after 20 min, resulting in the most intense blood extravasation during the 30 min observation time compared to other P-III fractions. In contrast, the hemorrhage induced by fraction IIB was evident in the first 10 min and did not increase in the longer period. These differences in hemorrhagic extent between the SVMPs were corroborated by distinct minimal hemorrhagic dose (MHD) values obtained: 3.08 ± 0.1 µg for IC, 5.67 ± 0.35 µg for IIB and 7.55 ± 0.13 µg for sample IIC.

It is known that hemorrhage is one of the most prominent effects of SVMPs in *Bothrops* envenomation (França and Málaque, 2009), and that

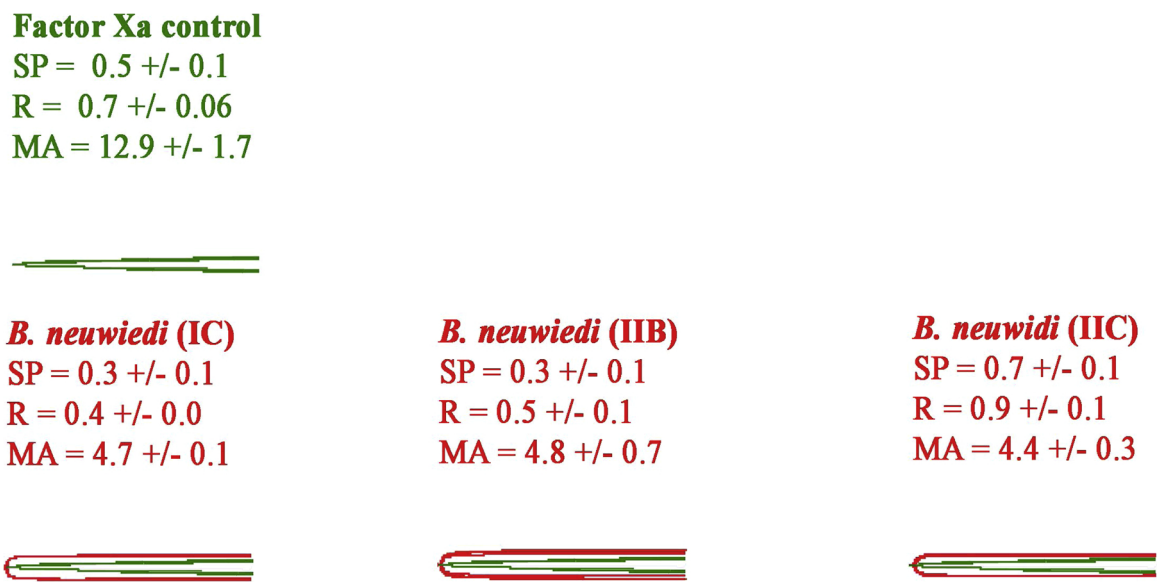

Fig. 9. Clot strength in amphibian plasma produced by fractions, measured on Haemonetics TEG5000 Thromboelastography analysers for 30 min. See Fig. 7 caption for assay details. The main parameters of the clots (SP, R, and MA) were analysed for 30 min. For visualisation purposes, the fraction experimental traces are overlaid with the bovine Factor Xa control. Wider traces indicate stronger clots, and vice versa. All traces are n = 3. Values are n = 3 means and standard deviation. SP = Split Point- the time at which clot formation starts; R = time to reach 2 mm amplitude; MA = maximum amplitude.

the accumulation of hemorrhagic SVMPs in the basement membrane (BM) leads to hydrolysis of its proteins and other elements involved in stability of the capillary (Herrera et al., 2015; Baldo et al., 2010; Freitas-de-Sousa et al., 2017). After envenomation, the capillary suffers distension due to the hemodynamic forces, normally operative in circulation, for example the pressure in the vessel wall, leading to a disrupted integrity and the blood extravasation (Gutiérrez et al., 2005). These events are caused mostly by PIII-class SVMPs, due to the presence of non-catalytic domains that facilitate binding of these toxins to the basement membrane proteins, mainly collagen type IV (Herrera et al., 2015; Freitas-de-Sousa et al., 2017; Baldo et al., 2010).

3.2. Binding to extracellular matrix and hydrolysis of the ECM proteins

We then analysed the action of the SVMPs from *B. neuwiedi* venom on extracellular matrix (ECM), investigating their capacity to bind and cleave the main components of the ECM (Type I collagen, Type IV collagen, and laminin) (Figs. 2 and 3). The Fig. 2 shows that the isoforms IIB and IIC presented a low affinity to the laminin but were able to strongly bind to collagen I and collagen IV. On the other hand, when the ECM protein hydrolysis (Fig. 3) was investigated by western blot, all P-III toxins cleaved collagen, although the IC isoform showed slightly smaller effects. Moreover, this toxin also did not hydrolyse laminin nor nidogen (Fig. 3). Thus, the results obtained suggest that the binding/hydrolysis of BM components, especially collagen IV, could be a key factor for the induction of hemorrhage by isoforms IIB and IIC. However, interestingly, the isoform IC was much less effective at binding to the BM proteins (Fig. 2) investigated in this study, although produced the smallest MHD values (Fig. 1) and are thus more hemorrhagic.

Reports suggest that direct binding to BM components is not a requirement for hydrolysis to occur. Freitas-de-Sousa et al. (2017) recently reported that Atroxlysin-Ia, a P-I class SVMP isolated from *Bothrops atrox* venom, has a high catalytic activity and induces a strong hemorrhage in mice dorsum skin, although being unable to bind to BM proteins. In the same study, the authors suggested that the potent hemorrhagic action of Atroxlysin-Ia is correlated to its high catalytic activity. In our study, the isoform IC is an SVMP of P-III class and has thus additional structural domains that would facilitate its binding to BM components, since the presence of additional domains—more specifically the C-terminus of disintegrin-like domain—may help to direct the hydrolysis of basal membrane components (Jia et al., 1997; Tanjoni et al., 2003). Notwithstanding, as well as observed with Atroxlisin-Ia, the binding of isoform IC to the major ECM proteins also appears unnecessary to promote hydrolysis and induce hemorrhage. This suggests a functional diversity of SVMPs, whereby toxins within the same class (P-III) can utilise different mechanisms of action.

Differences in P-III class SVMPs hemorrhagic activity are not new to the literature. For example, Silva et al. (2003) isolated a P-III fraction from *Bothrops erythromelas* snake venom (Berythractivase) devoid of hemorrhagic activity, while Paes Leme et al. (2012) isolated a P-III from *B. jararaca* snake venom (HF3) that is highly hemorrhagic. Nevertheless, our results demonstrate that differences in the hemorrhagic potential between SVMPs belonging to the same class can be observed even within a same venom, suggesting the importance of factors such as the primary sequence and post-translational modifications for the functional diversity of P-III class SVMPs.

3.3. Fibrinogen cleavage

Due to the abundance of P-III class SVMPs in the *B. neuwiedi* venom (Sousa et al., 2013) and their potential to induce consumptive coagulopathy to varying degrees in snakebite victims, we performed fibrinogen gel analyses (1D- SDS-PAGE) to quantify cleavage specificity of fibrinogen chains (Aalpha and Bbeta) by the three SVMPs isoforms (IC, IIB, and IIC). All P-III SVMPs presented a faster and more potent effect upon the Aalpha chain compared to the Bbeta chain (Fig. 4), while action upon the Bbeta chain was more variable. Although the majority of fibrinogenolytic SVMPs may cleave the Aalpha and Bbeta chains, frequently their actions are stronger upon the Aalpha chains (Swenson and Markland, 2005; Coimbra et al., 2018), as observed in our fibrinogen analyses (Fig. 4).

3.4. Coagulation analyses

We investigated the procoagulant activity of the fractions and the basic biochemistry underpinning this activity. The SVMPs showed wide variation in their clotting times on human plasma, as well as the basic biochemistry underpinning this activity. When testing the time in seconds until clot formation by the fractions at 20 μg/mL concentration, we observed variable clotting times (s) for each SVMP:

IC = 12.0 ± 0.1; IIB = 14.1 ± 0.2; IIC = 21.3 ± 0.2. Thereafter, 8-point dilution curves were undertaken to provide more comprehensive comparisons (Fig. 5A) and better evaluate the procoagulant potential of each toxin. Area under the curve values confirmed variation between the P-III class SVMP isoforms, with fraction IC exhibiting the highest activity (IC = 30.2 ± 0.7; IIB = 39.1 ± 0.3; IIC = 58.90 ± 0.7) (Fig. 5A). There was no correlation between the procoagulant potency and cofactor dependence, with all P-IIIs presenting much higher dependence (40−70x) on calcium ions than on phospholipids (PPL) (Fig. 5C). This reinforces the critical inclusion of both cofactors when conducting coagulation assays, as their omission can otherwise dramatically skew results (Sousa et al., 2018; Zdenek et al., 2019).

3.5. Antivenom efficacy

The neutralizing efficacy of the antivenom was variable and inversely related to procoagulant potency (Fig. 5B). Antivenom efficacy was also dependent on the ability of SVMPs to activate FX (Fig. 6): the SVMP that most potently activated FX (isoform IC) was the least neutralised. This result provides further support to the previously advanced theory that the *Bothrops* venoms with the highest levels of FX activating toxins are not only the most potent, but also the least neutralised (Sousa et al., 2018). On the other hand, the prothrombin activation activity, induced mainly by isoform IIC, was well neutralized by the antivenom. In the previous work performed by Bernardoni and co-workers (2014), the SVMPs IC and IIC already had been characterized as activators of FX and prothrombin, respectively; however, the neutralization of these activities, as well as their procoagulant actions on human and amphibian plasmas had not been evaluated yet. In present study, our results not only confirm the previous report, but also corroborate the finds of Sousa and co-workers (2018), which showed a lower relative efficacy of the Bothrops antivenom against the venoms of *B. neuwiedi* and a population of *B. atrox* from Santarém (Pará State, at Brazilian Amazon), both with strong FX activation activities (Sousa et al., 2018).

Taken together, these results indicate that the *Bothrops* antivenom better neutralizes prothrombin activating toxins than Factor X activating toxins. Thus, the driving variable regarding antivenom efficacy in *Bothrops* venom is not the relative potency of the venom per se, but rather the contribution to the potency by FX activating toxins (which are however more potent than prothrombin activating toxins). Rather than FX activating toxins being inherently difficult to neutralise, the differential neutralisation pattern may be simply due to the immunising venoms used in antivenom production being more rich in prothrombin activating toxins than FX activating toxins, yielding more prothrombin activating antibodies in the serum (Sousa et al., 2018). Thus, a proportionally larger amount of antivenom may be needed for venoms which are richer in Factor X activating toxins.

3.6. Thromboelastography

To conclude our analyses, thromboelastography assays were undertaken. Results confirmed the ability of all SVMPs to generate endogenous thrombin, by activation of FX or prothrombin, resulting in strong stable clots in avian and human plasmas (Figs. 7 and 8). Nevertheless, a particularly interesting find of our study was the species-specific difference observed in coagulation activation of the avian plasma. While the controls (FXa and thrombin, human) did not activate avian coagulation factors to the same extent (MA parameter = clot strength) that they activate native human plasma (Fig. 8). All SVMP isoforms strongly activated the avian plasma, generating clots with similar strengths ($IC_{MA\ value}$ = 20.6 ± 1.7; $IIB_{MA\ value}$ = 23.1 ± 1.1; $IIC_{MA\ value}$ = 25.1 ± 1.8) as the endogenously-generated thrombin, corresponding to the spontaneous clotting of the avian plasma (Negative control $_{MA\ value}$ = 23.3 ± 2.6). Also noteworthy is that, although all SVMP isoforms analysed in this study also clotted amphibian plasma, their effects on this plasma were equally lower than on chicken or human plasmas, with the positive control also weaker for amphibian plasma than chicken or human, suggesting that amphibian plasma inherently forms weaker clots (Fig. 9).

There are some important differences in the clotting systems of mammals (human), avian and amphibian, most of which are associated with the kallikrein-kinin system (FXII, FXI, prekallikrein, and kininogen) (Gentry, 2004). In frogs, FXI is absent and most of the coagulation factors are found in lower concentrations compared to human plasma, though fibrinogen is an exception (Doolittle, 2009). There is also a higher concentration of antifibrinolytic agents (Tentoni et al., 2010), which is associated with longer fibrinolysis times compared to mammal plasmas (Gentry, 2004). On the other hand, the chicken plasma is deficient in the factors XI and XII (Doolittle, 2009), and have longer clotting times compared to human plasma (Tentoni et al., 2010). Thus, it is likely that these findings are associated with phylogenetic variations underlying the clotting system in mammals (human) and earlier diverging vertebrates (chicken and toad) included in our study. The differential effect of the positive controls (human) on human versus avian or amphibian plasma indicates a specifies-specific variation in the clotting factors. Consequently, in an ideal scenario, only homologous proteins (for example thrombin from avian and amphibian) should be used as positive controls for each type of plasma, although this can be problematic to obtain.

Notwithstanding, similar thromboelastographic patterns were obtained with SVMP isoforms and the whole venom from *B. neuwiedi* (Sousa et al., 2018) on avian and amphibian plasmas. These results demonstrate a major role of SVMPs in *Bothrops* venoms, including for prey subjugation. Similarly, Chacón and co-workers (2015) reported that mice injected with doses corresponding to 4 LD_{50} of *Bothrops asper* venom died as a result of several pathophysiological alterations including systemic hemorrhage, plasmatic extravasation, hemoconcentration and coagulopathy, resulting in hypovolemia and cardiovascular collapse. These effects were attributed to a combined action of SVMPs and still unidentified components (Chacón et al., 2015). Thus, SVMPs appear to play important adaptive roles regarding immobilization, killing, and digestion of prey, as previously suggested (Gutiérrez et al., 2010; Bernardoni et al., 2014; Sousa et al., 2018). In this regard, taxon-specific venom effects on animal plasmas were also reported previously for human, rat, and chicken plasmas using the SVMPs analysed in our study (Bernardoni et al., 2014). However, while Bernardoni and co-workers (2014) found IC and IIB to be inactive on human plasma, we found these to be potent in this study. This is due to the inclusion of calcium and phospholipid in the assays conducted in the current study, which reinforces the importance of including both co-factors in order to replicate physiological conditions and thus properly assess toxin bioactivity. Similarly, the isoform IIC was more potent in this study than in the Bernardoni et al. (2014) study. These incongruencies between studies are consistent with the high dependence upon calcium by isoforms (Fig. 5).

4. Conclusion

This study investigated overall hemostatic disorders (hemorrhagic, fibrinogenolytic and procoagulant effects, as well the activation of blood coagulation zymogens), and neutralization by antivenom, of three SVMP P-III isoforms (IC, IIB and IIC) from the venom of *B. neuwiedi*, a medically relevant species in Brazil. In general, our results not only confirmed some previous findings reported by our group (Bernardoni et al., 2014) but differential results underscored the tremendous importance of including calcium and phospholipid cofactors in assay design in order to replicate physiological conditions and thus properly ascertain toxin bioactivity. This research also confirmed previous work (Sousa et al., 2018) that suggested taht due to the composition of venoms used in the immunising process, the *Bothrops* antivenom produced by Instituto Butantan is excellent in neutralizing prothrombin activating toxins, but performs not as well in neutralising

Factor X activating toxins found in *Bothrops* venoms, and thus proportionally more antivenom may be needed to neutralise envenomations by specimens with venoms rich in Factor X activating toxins In summary, our results highlight the great functional diversity of SVMPs within *Bothrops* venoms, shed light upon the coagulotoxic effects of these toxins on human, avian and amphibian plasmas, and identified areas of relatively deficient antivenom efficacy. Our study thus advances the current knowledge about the SVMP functional diversity and their potential ecological and clinical implications.

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CRediT authorship contribution statement

Leijiane F. Sousa: Conceptualization, Methodology, Resources, Data curation. **Juliana L. Bernardoni:** Conceptualization, Methodology, Data curation, Writing - review & editing. **Christina N. Zdenek:** Methodology, Writing - review & editing. **James Dobson:** Methodology. **Francisco Coimbra:** Methodology. **Amber Gillett:** Resources. **Mônica Lopes-Ferreira:** Conceptualization, Methodology, Resources. **A.M. Moura-da-Silva:** Conceptualization, Resources, Data curation, Writing - review & editing. **Bryan G. Fry:** Conceptualization, Resources, Data curation.

Declaration of Competing Interest

The authors report no declarations of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxlet.2020.08.009.

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