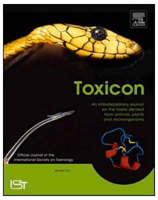
Toxicon 234 (2023) 107301

Contents lists available at ScienceDirect

Toxicon

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



Neutralization, by a polyspecific antivenom, of the coagulopathy induced by the venom of *Bothrops asper*: Assessment by standard coagulation tests and rotational thromboelastometry in a murine model

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ARTICLE INFO

Handling Editor: Denise Tambourgi

Keywords:
Venom-induced consumption coagulopathy
Bothrops asper venom
Polyvalent antivenom
Thrombocytopenia
Rotational thromboelastometry

ABSTRACT

Venom-induced consumption coagulopathy and thrombocytopenia are common and potentially severe manifestations of viperid snakebite envenoming since they contribute to local and systemic hemorrhage. Therefore, the assessment of the efficacy of antivenoms to neutralize coagulopathic and thrombocytopenic toxins should be part of the preclinical evaluation of these drugs. To evaluate the efficacy of the polyvalent (Crotalinae) antivenom produced in Costa Rica, in this study we have used a mouse model of coagulopathy and thrombocytopenia induced by the venom of *Bothrops asper*, based on the bolus intravenous (i.v.) injection of venom. When venom and antivenom were incubated before injection, or when antivenom was administered i.v. immediately after venom injection, venom-induced hemostatic alterations were largely abrogated. We also studied the recovery rate of clotting parameters in conditions where antivenom was administered when mice were coagulopathic. Some parameters recovered more rapidly in antivenom-treated mice than in control envenomed animals, but others showed a spontaneous recovery without antivenom. This is due to a rapid clearance of plasma venom levels in these experimental conditions. This implies that models based on the bolus i.v. injection of venom have limitations for assessing the effect of antivenom in the recovery of clotting alterations once coagulopathy has developed. It is suggested that alternative models should be developed based on a slower systemic absorption of venom. Overall, our findings provide a protocol for the preclinical evaluation of antivenoms and demonstrate that the polyvalent antivenom is effective in neutralizing the toxins of *B. asper* venom responsible for coagulopathy and thrombocytopenia.

1. Introduction

Envenomings by viperid snakes are often characterized, among other clinical manifestations, by a venom-induced consumption coagulopathy, associated with drastic alterations in laboratory coagulation parameters (Warrell, 2010; White, 2005). This effect is predominantly caused by procoagulant enzymes present in these venoms, i.e., metalloproteinases (SVMPs) and serine proteinases, which activate several clotting factors or, in the case of thrombin-like enzymes (also known as pseudo-procoagulant enzymes), generate feeble fibrin clots (Kini, 2005; Swenson et al., 2021). In addition, viperid venoms induce thrombocytopenia and platelet hypoaggregation (Otero-Patiño, 2009; Sano-Martins et al., 1997; Santoro et al., 2008). Such hemostatic alterations contribute to the local and systemic hemorrhage induced by these venoms by potentiating the action of hemorrhagic SVMPs that disrupt the integrity of microvessels by cleaving critical components of the basement membrane (Escalante et al., 2011). Thus, hemostatic alterations are key aspects of viperid snakebite envenoming.

In southern Mexico, Central America, and northern regions of South America, *Bothrops asper* is responsible for most snakebite cases and for

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https://doi.org/10.1016/j.toxicon.2023.107301
Received 6 August 2023; Received in revised form 10 September 2023; Accepted 20 September 2023

the most severe ones (Gutiérrez, 2021; Otero-Patiño, 2009). A high percentage of affected patients develop venom-induced consumption coagulopathy, as reflected by alterations in the laboratory tests (Barrantes et al., 1985; Otero-Patiño et al., 2012; Peña Chavarría et al., 1970). Recovery of coagulation parameters is regularly used to monitor the efficacy of antivenom therapy in these envenomings (Otero-Patiño et al., 2012). *B. asper* venom-induced coagulopathy is caused by a SVMP that activates prothrombin (Loría et al., 2003) and a thrombin-like serine proteinase (Pérez et al., 2008). At the experimental level, the procoagulant action of this venom has been demonstrated *in vitro* (Bourke et al., 2021; Gené et al., 1989; Nielsen et al., 2017). Likewise, intravenous (i.v.) administration of *B. asper* venom in mice induces incoagulability (Gené et al., 1989; Segura et al., 2010), as well as alteration of classical clotting tests and rotational thromboelastrometry (Rucavado et al., 2022). In addition to coagulopathy, *B. asper* venom induces drops in platelet counts, i.e., thrombocytopenia, owing to the action of a C-type lectin-like protein (Rucavado et al., 2005). Thrombocytopenia has been associated with a higher risk of bleeding in *Bothrops* sp envenomings in humans (de Oliveira et al., 2020).

Owing to the relevance of coagulopathy in the overall pathophysiology of viperid snakebite envenoming, the assessment of the ability of antivenoms to neutralize this effect is a relevant test in the preclinical evaluation of antivenoms (Gutiérrez et al., 2017; World Health Organization, 2017). Neutralization of the procoagulant action of venoms can be studied *in vitro* by a variety of assays based on venom-induced coagulation of citrated plasma (Bourke et al., 2021; Gené et al., 1989; O'Leary and Isbister, 2010; Theakston and Reid, 1983). More recently, methods based on the viscoelastic properties of plasma, i.e., thromboelastography and rotational thromboelastometry, have been used to assess antivenom efficacy *in vitro* (Bailey et al., 2022; Oguiura et al., 2014). On the other hand, the ability of antivenoms to neutralize coagulant venom enzymes *in vivo* has been mainly based on the evaluation of the assessment of defibrinogenating activity in whole blood (Gené et al., 1989; Segura et al., 2010). It is necessary to introduce novel methodologies that would provide a more detailed assessment of the neutralization of venom-induced consumption coagulopathy in murine models *in vivo*. Moreover, antivenoms' efficacy in neutralizing venom-induced thrombocytopenia in murine models must be incorporated in the preclinical evaluation of these immunobiologicals.

A murine model of venom-induced consumption coagulopathy and thrombocytopenia was recently described in which these hemostatic alterations are assessed by using classical coagulation assays (prothrombin time, activated partial thromboplastin time and fibrinogen concentration), rotational thromboelastometry, and platelet counts, thus allowing an in-depth evaluation of hemostatic disturbances in an *in vivo* system, including those induced by *B. asper* venom (Rucavado et al., 2022). In the present study we have used this experimental platform to analyze the efficacy of a polyspecific antivenom used in Central America and some countries in South America to abrogate the diverse set of hemostatic alterations induced by the venom of *B. asper* in mice.

2. Materials and methods

2.1. Venom and antivenom

Venom was obtained from adult specimens of *Bothrops asper* from the Pacific versant of Costa Rica, which were kept at the Serpentarium of Instituto Clodomiro Picado, University of Costa Rica. A pool of venom from more than 20 specimens was lyophilized and stored at −20 °C. Venom was diluted immediately before the experiments using PBS (0.12 M NaCl, 0.04 M phosphates, pH 7.2) as solvent. The polyvalent antivenom of Instituto Clodomiro Picado, University of Costa Rica (Polival-ICP®), San José, Costa Rica (batch 632-10-19 POLQ; expiry date: October 2022; protein concentration: 6.12 g/dL) was used. This is a whole IgG antivenom obtained by caprylic acid fractionation of the plasma of horses immunized with a mixture of the venoms of *Bothrops asper, Crotalus simus* and *Lachesis stenophrys* from Costa Rica (Rojas et al., 1994).

2.2. Animals, blood samples, and coagulation tests

CD-1 mice of both sexes (20–22 g body weight) were used throughout the study. The experimental protocols involving the use of animals were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUA) of the University of Costa Rica (approval number CICUA-062-2021). As previously described (Rucavado et al., 2022), in all the experiments, the animals were bled by cardiac puncture using inhaled isofluorane anesthesia at the time intervals detailed below. Blood samples were mixed with anticoagulant (3.8% sodium citrate; citrate: blood volume ratio of 1:9). Citrated blood samples from four mice of each experimental group were used for rotational thromboelastometry determinations, using a ROTEM Delta 4000 equipment according to the manufacturer's instructions (Tem Innovations, GmbH, Munich, Germany). Determination of the following parameters was carried out: Extem, Intem, and Fibtem clotting time (CT), clot formation time (CFT), and amplitude-clot strength at 20 min (A20). Extem and Intem tests provide an assessment of the extrinsic and intrinsic coagulation pathways, respectively, whereas Fibtem evaluates the contribution of fibrinogen to clot formation in conditions where platelets are inhibited (Cannata et al., 2021). CT is the time lapse (in sec) until a clot amplitude of 2 mm is reached. CFT is the time lapse (in sec) between 2 mm clot amplitude and 20 mm clot amplitude. A20 reflects the clot firmness (in mm amplitude) 20 min after CT (Cannata et al., 2021). Plasma from other citrated blood samples were obtained by centrifuging at 1300×*g* for 15 min. Prothrombin time (PT), activated partial thromboplastin time (aPTT) and fibrinogen concentration were determined in pools of plasma obtained from two to three individuals using a STA R Max 2 coagulation analyzer (Stago, Paris, France). Determination of platelet counts was done in citrated blood samples using an automated hematology analyzer (VetsCan HM5, Abaxis Global Diagnostics, USA).

2.3. Experiments with preincubation of venom and antivenom

Groups of mice received an intravenous (i.v.) injection of 7.5 μg venom preincubated with antivenom for 30 min at 37 °C, at venom/antivenom ratio of 1 mg venom/mL antivenom. The dose of 7.5 μg venom corresponds to 1.5 Minimum Defibrinogenating Doses (1.5 times the minimum dose necessary to render the blood unclottable in all injected mice 1 h after injection). One hour after injection mice were bled as described above to determine classical clotting tests (PT, aPTT and fibrinogen concentration), platelet counts and various parameters of rotational thromboelastometry. Mice which received only PBS or venom were used as controls.

2.4. Experiments in which antivenom was administered after venom injection

Groups of mice received an i.v. injection of 7.5 μg venom, dissolved in 100 μL of PBS. Then, either immediately or 20 min after venom injection, 100 μL of antivenom diluted 1:2 in PBS, were administered i.v. Mice which received the antivenom immediately after venom were bled 1 h after injection, while in the case of animals in which antivenom was administrated 20 min after venom, samples were collected at 1, 3, 5, 12 at 24 h after envenoming. Blood samples were collected as described above to carry out the tests related to clotting and platelet parameters. Mice injected with PBS or venom only were used as controls. PBS-injected controls were bled 1 h after injection, while venom controls were bled at 1, 3, 5, 12, and 24 h. In another experiment, the same protocol was followed, but the dose of venom was increased to 15 μg and a dose of 100 μL of undiluted antivenom was administered i.v. 20 min after venom. Three hours after venom injection, mice were bled as described and laboratory tests were carried out. Controls included mice injected with PBS alone or 15 μg venom alone.

2.5. *Intramuscular and intraperitoneal injection of sublethal doses of venom*

In order to assess whether a consumption coagulopathy develops when venom is injected by the intramuscular (i.m.) or the intraperitoneal (i.p.) routes, groups of three mice received an i.m. injection, in the right thigh, of 100 μg venom, dissolved in 50 μL PBS, or an i.p, injection of 31 μg venom, dissolved in 100 μL PBS. This i.p. dose corresponds to 0.5 LD_{50} of venom. Mice were bled at 1, 2, 3 and 5 h as described and approximately 50 μL were placed in glass tubes without anticoagulant and left undisturbed for 20 min. Then, tubes were gently tilted to observe whether blood was clotted or not. In the case of mice injected with venom i.m., PT, aPTT and fibrinogen concentration were also determined in samples collected 1 h after injection.

2.6. *Detection of venom in mouse plasma by immunoassay (ELISA)*

Following the same procedure described above, plasma samples were obtained from groups of three mice injected i.v. with either 7.5 μg or 15 μg of venom, dissolved in 100 μL PBS. Blood samples were collected 1 min, 20 min, 1, 3, and 5 h after injection, and plasma was prepared from citrated blood for venom quantification. To assess the effect of antivenom administration on the plasma concentration of venom, groups of three mice were injected with either 7.5 μg or 15 μg of venom, dissolved in 100 μL PBS. Then, 20 min after venom injection, mice received either 100 μL of antivenom diluted 1:2 in PBS or undiluted, respectively. Mice were bled 1 h after venom injection and plasma was obtained as described. For the quantification of venom in plasma, a sandwich ELISA was performed using horse IgG anti-*B. asper* venom antibodies as the capturing antibodies, and biotinylated horse IgG anti-*B. asper* antibodies for venom detection, followed by streptavidin conjugated with horseradish peroxidase (Calbiochem, San Diego, CA, USA). The reaction was developed using *o*-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, St. Louis. MO, USA) as substrate, and the absorbance was measured at 492 nm. Samples of plasma were diluted in 1% BSA/PBS, at dilutions ranging from 1:2 to 1:20, depending on the sampling time point. A standard curve of various concentrations of *B. asper* venom (1000, 333, 111, 37 and 12 ng/mL) was prepared for venom quantification, diluted in normal mouse serum. Sample diluent (1% BSA/PBS) was used as blank, and plasma of control mice injected with PBS was used as the negative control.

2.7. *Statistical analyses*

Results were expressed as mean ± SEM. The significance of the differences between the mean values of experimental groups were assessed by one-way ANOVA, followed by Tukey-Kramer post-hoc test to analyze differences between values of samples of control mice, envenomed mice and envenomed mice that received antivenom. P values < 0.05 were considered significant.

3. Results

3.1. *Experiments with preincubation of venom and antivenom*

A consumption coagulopathy and thrombocytopenia developed in mice 1 h after i.v. injection of a dose of 7.5 μg venom, as previously described (Rucavado et al., 2022), and as judged by classical clotting tests (Fig. 1), as well as by various parameters of rotational thromboelastometry (Figs. 2 and 3). When venom and antivenom were incubated for 30 min at 37 °C before injection, at venom/antivenom ratio of 1 mg venom/mL antivenom, a complete neutralization of the coagulopathy was observed, as judged by classical clotting tests (PT, aPTT and fibrinogen concentration) (Fig. 1), as well as by the various parameters of rotational thromboelastometry (Figs. 2 and 3). Likewise, platelet counts in mice receiving the mixture of venom and antivenom were higher than in envenomed mice not receiving antivenom and did not differ significantly from counts in control mice injected with PBS (Fig. 1).

3.2. *Experiments in which antivenom was administered after venom injection*

When 100 μL antivenom diluted 1:2 were administered i.v. immediately after i.v. injection of 7.5 μg venom, most of the alterations induced by the venom in clotting parameters and platelet counts were abrogated when blood samples were collected 1 h after envenoming (Figs. 1 and 2). However, in some cases, although there was a significant neutralization of the effect, the values of a few parameters analyzed were significantly different from those of control mice receiving PBS, thus indicating a significant, but not complete, inhibition of the effects. These parameters were: plasma fibrinogen concentration, Fibtem A20, and platelet counts (Figs. 1 and 2). In the case of Fibtem CFT, there was no neutralization when antivenom was administered immediately after venom (Fig. 2). Overall, administration of antivenom immediately after venom largely inhibited venom-induced coagulopathy and thrombocytopenia.

A set of experiments was then carried out in which 100 μL antivenom diluted 1:2 was administered 20 min after i.v. injection of 7.5 μg venom.

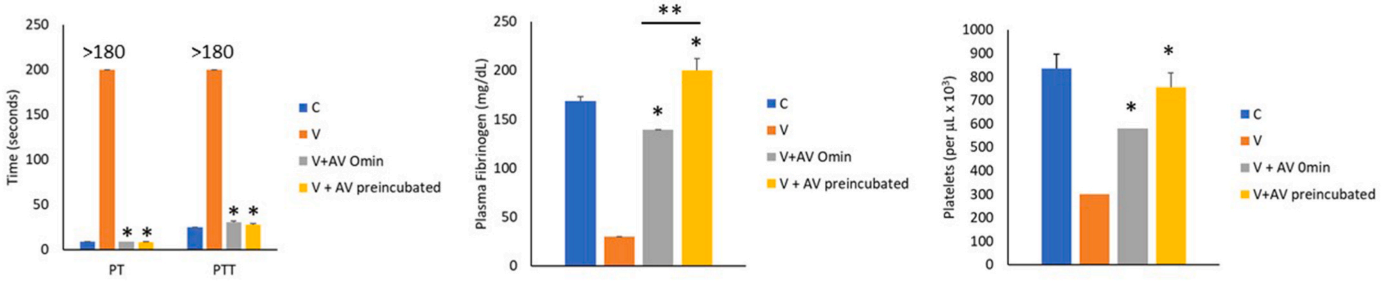

Fig. 1. Neutralization by antivenom of the coagulopathy and thrombocytopenia induced by *B. asper* venom in mice. In some experiments (V + AV preincubated), venom was incubated with antivenom at a ratio of 1 mg venom/mL antivenom for 30 min, and then aliquots containing 7.5 μg venom were injected i.v. In other experiments (V + AV 0 min), mice received an i.v. injection of 7.5 μg venom, immediately followed by the i.v. administration of 100 μL antivenom diluted 1:2 with PBS. Controls included mice injected with either venom alone (7.5 μg) (V) or PBS alone (C). One hour after venom or PBS injection, mice were bled under isofluorane anesthesia and blood was added to citrate anticoagulant for determination of prothrombin time (PT), activated partial thromboplastin time (PTT), fibrinogen concentration, and platelet counts (see materials and methods for details). Results are presented as mean ± SEM (n = 4). *$p < 0.05$ when compared to venom control; **$p < 0.05$ when comparing V + AV preincubated and V + AV 0 min.

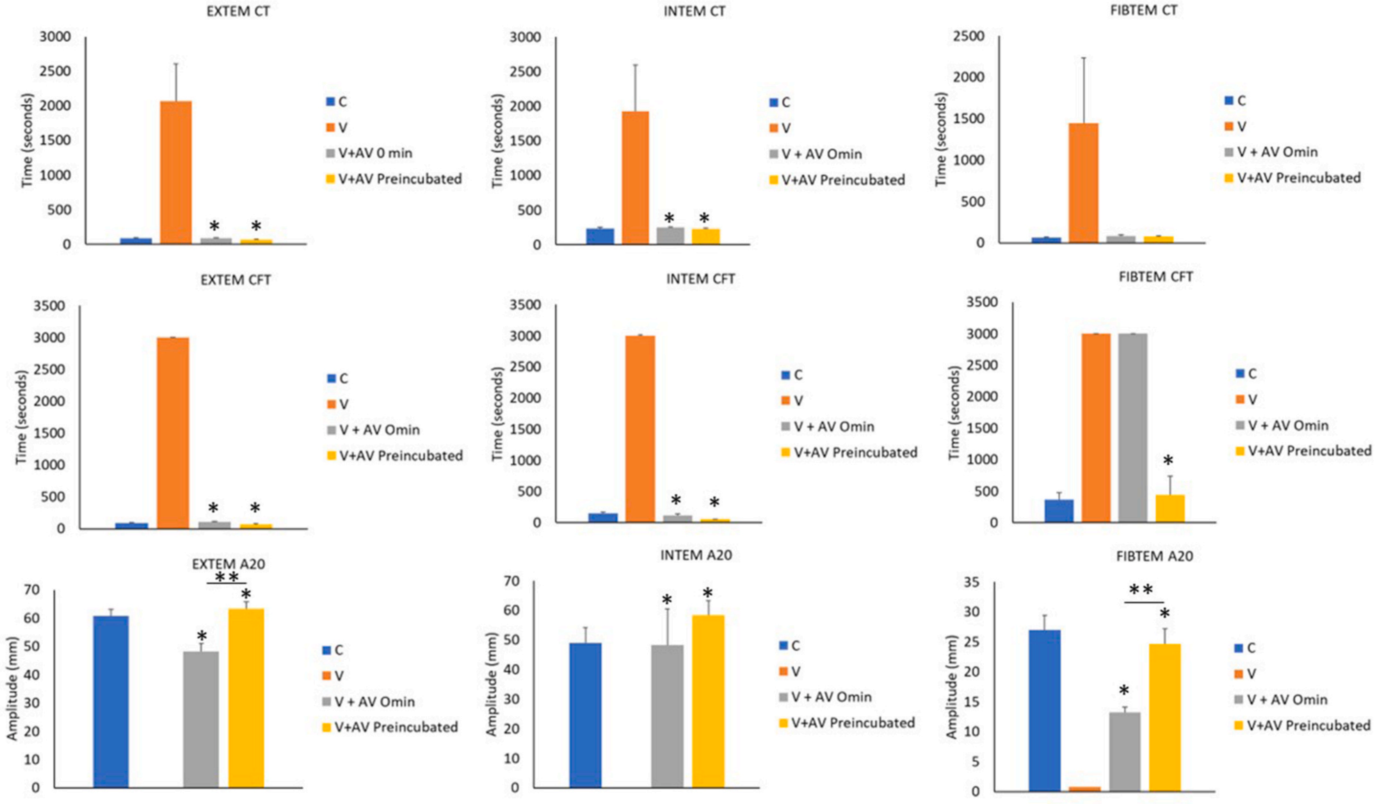

Fig. 2. Neutralization by antivenom of coagulopathy induced by *B. asper* venom in mice, as judged by rotational thromboelastometry parameters. In some experiments (V + AV preincubated), venom was incubated with antivenom at a ratio of 1 mg venom/mL antivenom for 30 min, and then aliquots containing 7.5 μg venom were injected i.v. In other experiments (V + AV 0 min), mice received an i.v. injection of 7.5 μg venom, immediately followed by the i.v. administration of 100 μL antivenom diluted 1:2 with PBS. Controls included mice injected with either venom alone (7.5 μg) (V) or PBS alone (C). One hour after venom or PBS injection, mice were bled under isofluorane anesthesia and blood was added to citrate anticoagulant for determination of Extem, Intem and Fibtem parameters [clotting time (CT), clot formation time (CFT) and A20 (see materials and methods for details)]. Results are presented as mean ± SEM (n = 4). $^{*}p < 0.05$ when compared to venom control; $^{**}p < 0.05$ when comparing V + AV preincubated and V + AV 0 min.

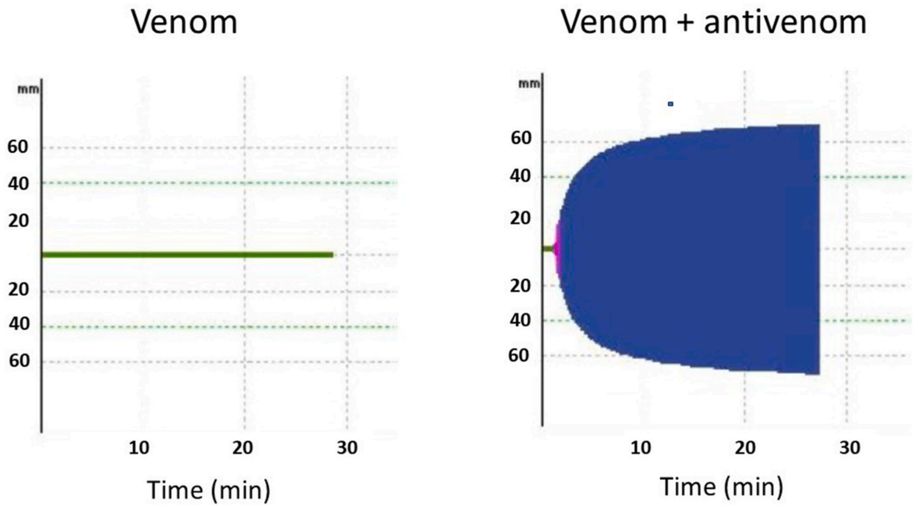

Fig. 3. Extem tracings from mice injected with the venom of *B. asper* previously incubated with PBS (left tracing) or with antivenom (right tracing). Mice were bled by cardiac puncture 1 h after injection under isofluorane anesthesia, added to sodium citrate solution and evaluated by rotational thromboelastometry, as described in Materials and Methods. A complete absence of clot formation is observed in the left tracing, whereas normal clotting is observed in the right tracing.

At this time interval after venom injection, all clotting and platelet parameters were drastically affected, identically as in samples collected 1 h after venom injection (not shown). Thus, this experimental protocol was developed to simulate a situation when antivenom is provided after coagulopathy has developed, in order to assess the effect of antivenom in the recovery of the clotting tests. In some parameters, i.e., PT and aPTT (at 1 h), fibrinogen concentration (at 12 h), Extem CFT (at 3 and 5 h), Intem CFT (at 3 h), Extem A20 (at 3 and 5 h), and platelet counts (at 1, 3 and 5 h), there was a greater extent of recovery of the parameters in mice receiving antivenom as compared to control envenomed mice (Figs. 4

and 5). However, in other clotting parameters, there were no significant differences at various time intervals between mice that received antivenom and those which did not (Figs. 4 and 5).

At the venom dose used, i.e., 7.5 μg, in the case of mice not treated with antivenom, several of the parameters were corrected spontaneously after the third hour, as previously described (Rucavado et al., 2022). Exceptions were fibrinogen concentration and Fibtem A20, whose correction was slower, and Fibtem CFT, which was not corrected at all (Figs. 4 and 5). These findings suggest that, when using a venom dose of 7.5 μg, the rapid spontaneous correction of most coagulation parameters in the absence of antivenom precludes the study of the effect of antivenom in accelerating such correction.

To extend the coagulopathic condition in mice in order to have a better model to assess whether antivenom accelerates the recovery of clotting parameters, various experimental models were attempted. Intramuscular and intraperitoneal injections of sublethal doses of venom (100 μg i.m. and 31 μg i.p.) were tested. Injection by the i.m. route did not induce blood incoagulability at any time intervals assessed. This finding was corroborated by the demonstration that no alterations were observed in PT (envenomed mice: 100% activity, identical to mice injected with PBS), and only a partial increment occurred in aPTT (envenomed mice: 67 ± 7 s; mice injected with PBS: 34 ± 3 s) and a partial drop in fibrinogen concentration (envenomed mice: 128 ± 17 mg/dL; mice injected with PBS: 158 ± 21 mg/dL) 1 h after i.m. injection. In the case of i.p. injection, blood incoagulability was observed only at 3 h. On the other hand, when using a dose of 15 μg by the i.v. route, the coagulopathy was observed not only at 1 h but also at 3 h and, therefore, it was feasible to assess whether antivenom promotes a recovery of clotting tests at that time interval when administered 20 min after venom. As shown in Fig. 6, several parameters improved at 3 h in envenomed mice receiving antivenom compared to control envenomed mice. In contrast, other parameters did not differ between the two groups. In particular, no differences were observed in fibrinogen concentration and Fibtem parameters, which depend on fibrinogen concentration.

3.3. *Time-course of changes in plasma venom concentrations*

Upon i.v. injection of venom doses of 7.5 μg and 15 μg, there was a rapid drop in plasma venom concentration. By 20 min after injection, venom concentration significantly dropped compared to the concentration in samples collected 1 min after injection. By 1 h, venom levels decreased even further (Fig. 7A). Venom concentration in plasma remained at very low levels throughout 5 h. When antivenom was administered 20 min after venom, and venom in plasma was quantified 1 h after injection, there was an almost complete elimination of venom in mice that received antivenom as compared to those receiving PBS instead (Fig. 7B).

4. Discussion

The preclinical assessment of the efficacy of antivenoms to neutralize coagulopathic effects induced by snake venoms has been based on a combination of *in vitro* and *in vivo* assays. The former evaluate the ability of antivenoms to neutralize the procoagulant effect of venoms on citrated plasma, by using several methodologies (Alsolaiss et al., 2023; Bourke et al., 2021; Gené et al., 1989; Oguiura et al., 2014; Segura et al., 2010) while the latter analyze the ability of antivenom to neutralize the defibrinogenating activity of venoms in laboratory animals based on whether the blood clots or remains unclottable (Gené et al., 1989; Segura et al., 2010). Despite the usefulness of coagulation *in vitro* assays, a thorough preclinical evaluation of antivenom efficacy to control coagulopathy should also involve *in vivo* models. However, up to now, most *in vivo* tests for antivenom efficacy have been based on the evaluation of defibrinogenation as judged by the coagulability of whole blood, highlighting the need for more sensitive assays.

In the present study we have used a combination of classical clotting tests and rotational thromboelastometry to carry out a more detailed analysis of the neutralizing efficacy of a polyspecific antivenom when confronted with the venom of *B. asper*. The introduction of thromboelastographic and thromboelastometric methods allows a more in-depth

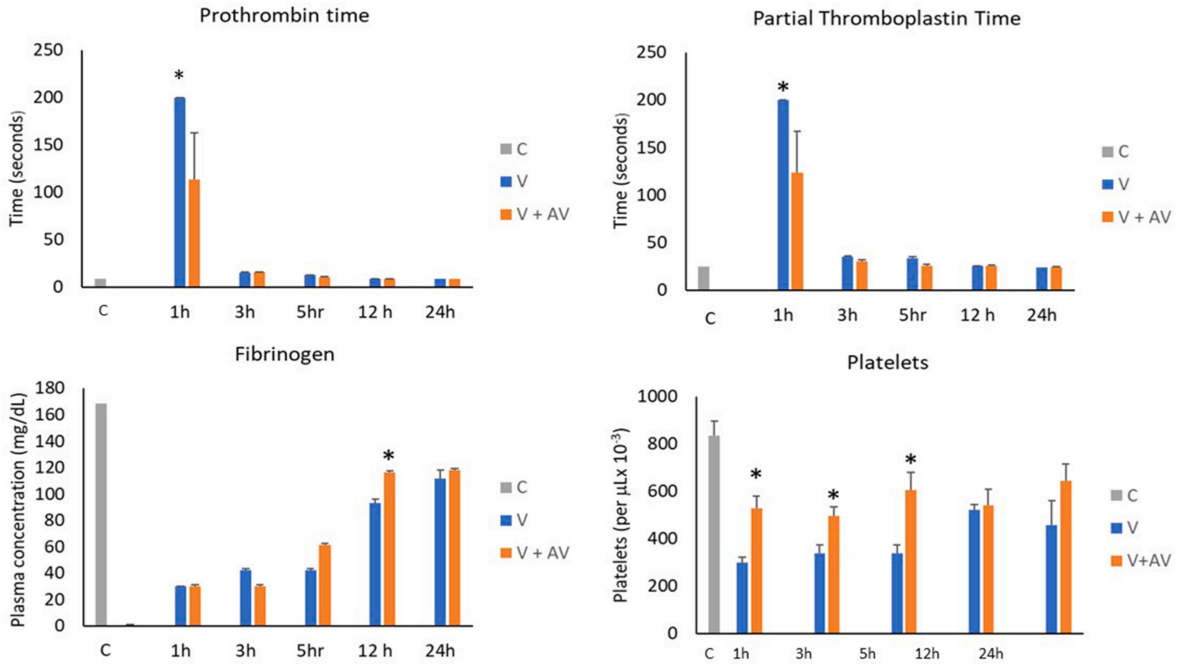

Fig. 4. Recovery from coagulopathy and thrombocytopenia in mice injected i.v. with 7.5 μg B. *asper* venom. After 20 min, one group of mice received an i.v. administration of 100 μL of antivenom diluted 1:2 with PBS (V + AV) whereas another group did not receive antivenom (V) (see materials and methods for details). At various time intervals mice were bled under isofluorane anesthesia and blood was added to citrate anticoagulant for determination of clotting parameters and platelet counts. Controls (C) received an i.v. injection of PBS and were bled 1 h later. Results are presented as mean ± SEM (n = 4). *p < 0.05 when comparing envenomed mice receiving and not receiving antivenom.

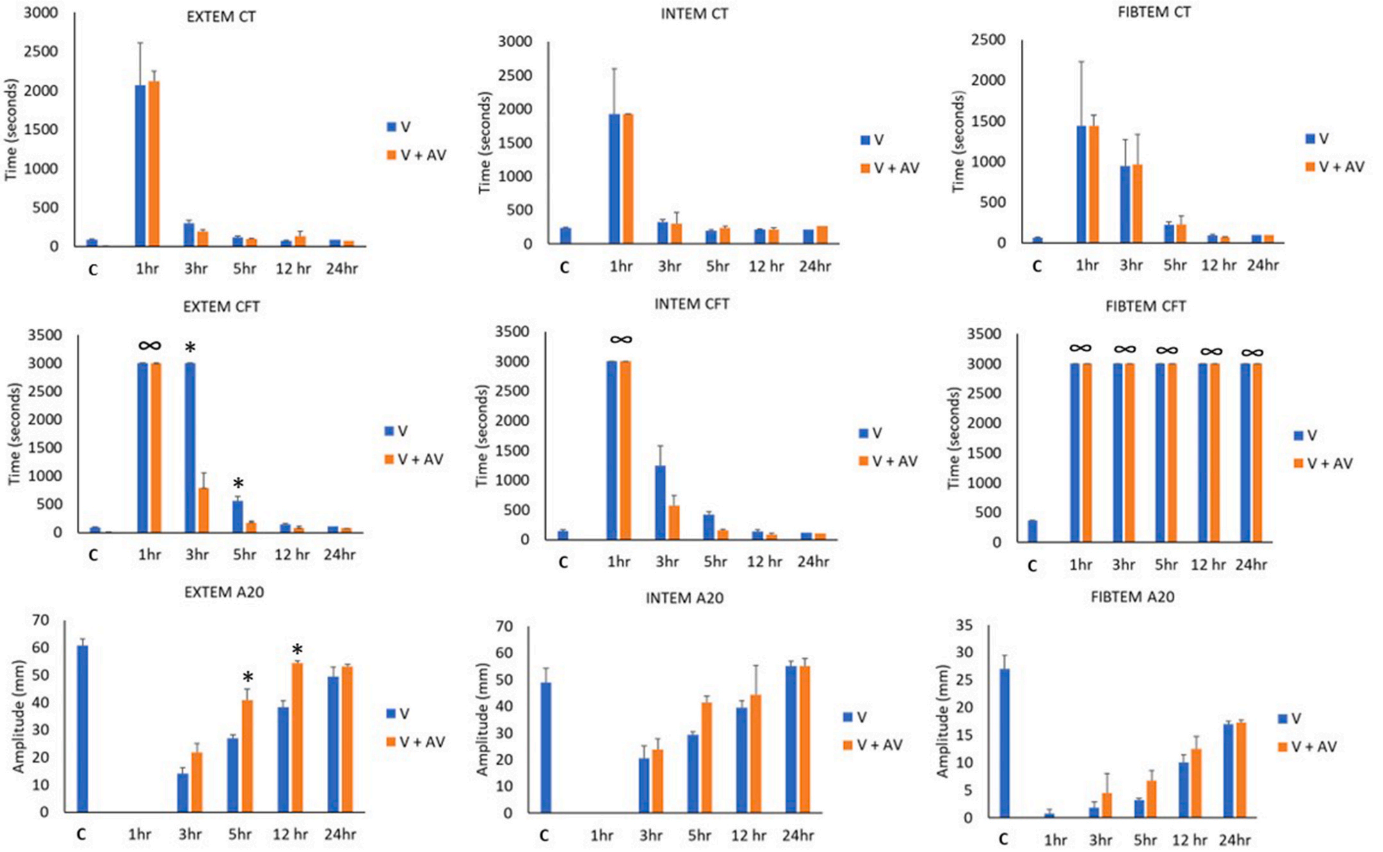

Fig. 5. Recovery from coagulopathy, as judged by rotational thromboelastometry parameters, in mice injected i.v. with 7.5 μg B. *asper* venom. After 20 min, one group of mice received an i.v. administration of 100 μL of antivenom diluted 1:2 in PBS (V + AV) whereas another group did not receive antivenom (V) (see materials and methods for details). At various time intervals mice were bled under isofluorane anesthesia and blood was added to citrate anticoagulant for determination of Extem, Intem and Fibtem parameters (clotting time (CT), clot formation time (CFT) and A20). Controls (C) received an i.v. injection of PBS and were bled 1 h later. Results are presented as mean ± SEM (n = 4). $^*p < 0.05$ when comparing envenomed mice receiving and not receiving antivenom.

analysis of clotting alterations, as they are based on the viscoelastic properties of clot formation, allowing not only the determination of clotting times but also assessing the kinetics of clot formation, the role of platelets and the clot strength (Abdelfattah and Cripps, 2016; Cannata et al., 2021; Luddington, 2005).

In vivo preclinical tests of antivenom efficacy have been traditionally divided into 'preincubation assays', in which venom and antivenom are incubated prior to injection, and 'independent injection' or 'rescue-type' tests, in which antivenom is administered at various time intervals after venom injection (Gutiérrez et al., 2013; Knudsen et al., 2020; León et al., 1999). The first method establishes whether an antivenom contains antibodies able to neutralize the relevant toxins, while the latter takes into consideration the toxicokinetics of venom toxins and the pharmacokinetics of antibodies, thus better modeling a real scenario situation. Our observations indicate that the polyvalent antivenom tested effectively neutralizes toxins responsible for venom-induced coagulopathy and thrombocytopenia when incubated with venom prior to injection since all parameters affected by the venom were corrected. These findings agree with and expand previous studies using a variety of methods, which have demonstrated neutralization of *in vitro* coagulant and *in vivo* defibrinogenating activities of *B. asper* venom by this antivenom (Alsolaiss et al., 2023; Bourke et al., 2021; Gené et al., 1989; Mora-Obando et al., 2021; Saravia et al., 2001; Segura et al., 2010, 2012).

When tests involved the independent injection of venom and antivenom, an almost complete neutralization was observed when antivenom was administered immediately after venom, indicating that antivenom could bind and neutralize toxins already present in the circulation. The only exceptions were Fibtem CFT, which was not neutralized, and Fibtem A20, which was neutralized only partially. Since Fibtem parameters are closely related to the status of fibrinogen, these observations highlight a rapid effect of venom on fibrinogen upon injection before antivenom is administered.

Since a profound coagulopathy developed in our model as soon as 20 min after venom injection, we studied whether the administration of antivenom at that time results in a more rapid recovery of clotting parameters compared to untreated envenomed mice. While there was a higher recovery of some parameters in antivenom-treated mice, no significant differences were observed between the two groups in other parameters analyzed. This is likely to depend on the fact that mice receiving an i.v. dose of 7.5 μg of *B. asper* venom rapidly develop coagulopathy, but there is recovery of clotting parameters as soon as 3 h after envenoming, except for fibrinogen concentration, and Fibtem CFT and A20, which largely depend on fibrinogen levels (Rucavado et al., 2022; this study). Thus, the rapid spontaneous recovery of most clotting parameters after the onset of coagulopathy following an i.v. bolus venom injection largely precludes the assessment of the ability of antivenom to accelerate such recovery.

These observations are likely to depend on the rapid elimination of procoagulant toxins from the circulation, similar to a model describing a short half-life of procoagulant toxins of taipan (*Oxyuranus scutellatus*) venom (Tanos et al., 2008). Since our model is based on the i.v. injection of venom, it is likely that coagulopathic toxins are rapidly eliminated from the bloodstream, and the synthesis of clotting factors by the liver rapidly enables the recovery of coagulation tests, as previously described for *B. asper* venom (Rucavado et al., 2022). Quantification of venom concentration in plasma corroborated this hypothesis, as it drops

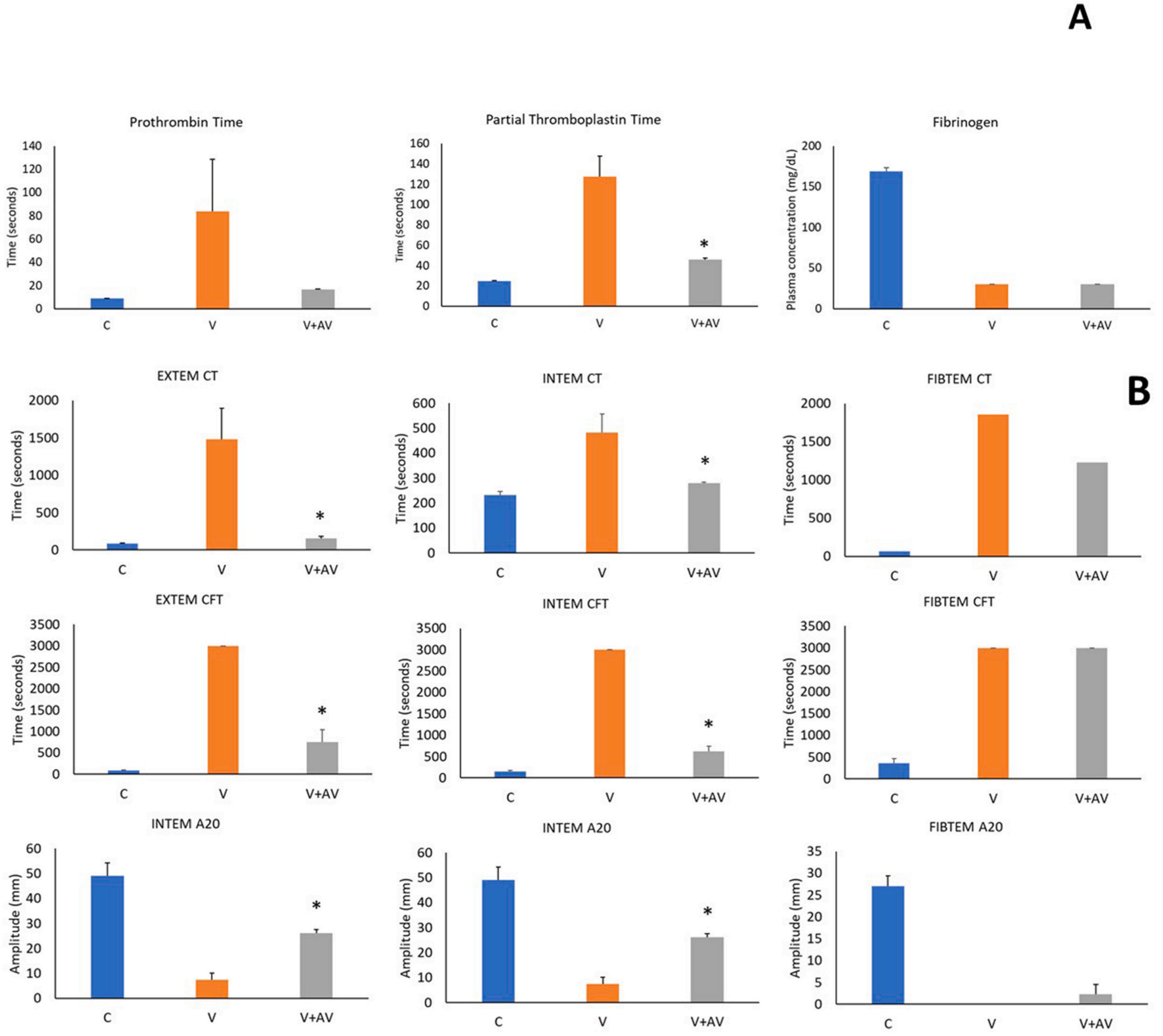

Fig. 6. Recovery from coagulopathy in mice injected i.v. with 15 μg B. *asper* venom. After 20 min, one group of mice received an i.v. administration of 100 μL of undiluted antivenom (V + AV) whereas another group did not receive antivenom (V) (see materials and methods for details). Three hr after venom injection mice were bled under isofluorane anesthesia and blood was added to citrate anticoagulant for determination of classical clotting (A) and rotational thromboelastometry (B) parameters. Controls (C) received an i.v. injection of PBS and were bled 1 h later. Results are presented as mean ± SEM (n = 4). *p < 0.05 when comparing envenomed mice receiving and not receiving antivenom.

to low levels as early as 20 min after injection. We then attempted to adapt our model to extend the coagulopathy duration, as occurs in real snakebite envenomings. The i.m. and i.p. routes of injection were used to simulate a slow absorption of venom toxins into the vasculature, resembling a real case scenario. However, mice only developed incoagulability 3 h after i.p. venom injection, but not at the other time intervals by this route nor at any time interval after i.m. injection at the venom doses tested.

Then, we increased the i.v. dose of venom to 15 μg in order to prolong the presence of procoagulant toxins in the blood. In this case, coagulopathy developed beyond the 1 h interval since clotting tests were also altered 3 h after injection. This allowed us to assess whether antivenom administration at 20 min, when coagulopathy was well established, would speed up the recovery of coagulation by 3 h. This was indeed the case for some laboratory tests, particularly those related to Extem and Intem, thus indicating that, by neutralizing procoagulant toxins *in vivo* after the onset of coagulopathy, antivenom is effective in accelerating the recovery of some clotting tests. However, in the case of other parameters, such as those of Fibtem, no significant differences were observed between groups receiving and not receiving antivenom, probably because venom concentration in plasma drops significantly by 20 min even when using this higher dose. Thus, when working with the venom of *B. asper*, to better reproduce in animal models the more prolonged coagulopathy observed in the clinical setting, it is necessary to search for alternative experimental protocols. It is suggested that a device allowing a slow i.v. delivery of venom, e.g., by using a controlled infusion system, would better reproduce the kinetics of venom absorption observed in clinical cases, as in a model developed to study venom-induced shock in rats (Carlson et al., 1975). Previous clinical observations have demonstrated that patients receiving antivenom show a more

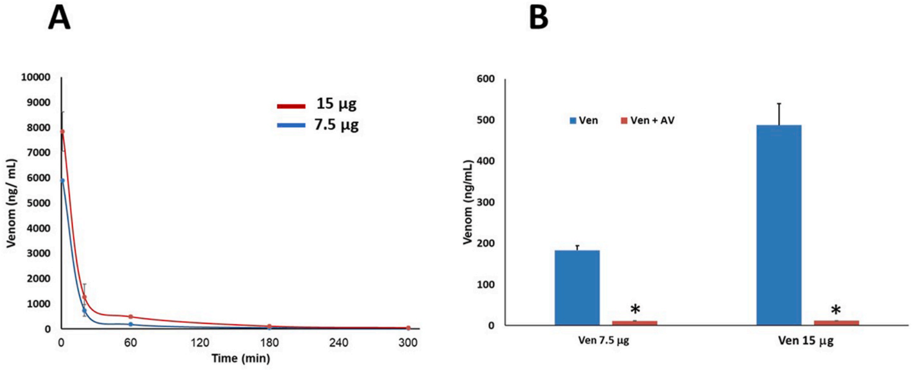

Fig. 7. (A) Time-course of changes in venom concentration in plasma of mice receiving i.v. injections of either 7.5 μg or 15 μg B. *asper* venom. Venom concentration was estimated by immunoassay (see materials and methods for details). (B) Effect of antivenom administration on the plasma concentration of *B. asper* venom. Mice received either 7.5 μg or 15 μg of *B. asper* venom by the i.v. route, followed, at 20 min, by either PBS (Ven) or antivenom (Ven + AV,see materials and methods for details) by the same route, as described in the legends of previous figures. Mice were bled by cardiac puncture 1 h after venom injection and added to citrate anticoagulant. After centrifugation, plasma was separated, and venom concentration estimated as described in materials and methods. Results are expressed as mean ± S.D. (n = 3). *$p < 0.05$ when comparing envenomed mice receiving and not receiving antivenom.

rapid recovery from coagulopathy as compared to those that did not receive antivenom in envenomings by the viperids *Bothrops atrox* (Resiere et al., 2020), *Echis ocellatus* (Mion et al., 2013), and *Daboia russelii* (Silva et al., 2022).

We also assessed the ability of the polyvalent antivenom to neutralize the toxins responsible for thrombocytopenia in the venom of *B. asper*. Drops in platelet counts have been reported in 15–30% of cases by *B. asper* (Otero-Patiño, 2009) and constitute a risk factor for the development of systemic bleeding in *Bothrops* sp envenomings (de Oliveira et al., 2020; Santoro et al., 2008). In the case of *B. asper* venom, this effect is due to aspercetin, a C-type lectin-like component that induces platelet agglutination/aggregation (Rucavado et al., 2001, 2005). Our data indicate that polyvalent antivenom is effective at abrogating the thrombocytopenic effect when incubated with venom before injection and when administered immediately after venom. Moreover, when antivenom administration was delayed 20 min, platelet numbers in antivenom-treated mice were higher than in control envenomed mice at 1, 3, and 5 h, thus indicating that antivenom accelerates the recovery of thrombocytopenia in this model.

In conclusion, we have developed an *in vivo* model to assess the efficacy of antivenoms to neutralize *B. asper* venom toxins responsible for consumption coagulopathy and thrombocytopenia. Combining classical clotting tests with rotational thromboelastometry and platelet counts allows for a detailed assessment of the various aspects of hemostasis. Experiments involving preincubation of polyvalent antivenom and *B. asper* venom prior to injection demonstrated that antivenom neutralized coagulopathic and thrombocytopenic toxins. This experimental platform could be applied to assess the efficacy of different antivenoms to neutralize coagulopathy induced by various snake venoms. When experiments involved the independent injection of venom and antivenom (rescue-type protocol), antivenom was effective when administered immediately after venom. On the other hand, in order to implement experimental models to assess the effect of antivenoms on the recovery of clotting parameters once the coagulopathy has developed, bolus i.v. injection of venoms does not seem to be the best option, since coagulopathic toxins are rapidly eliminated from the circulation and clotting parameters recover spontaneously. Thus, it is necessary to develop slow venom absorption models which ensure that the coagulopathic state remains for several hours, thus resembling the actual circumstances of envenomings.

Credit author statement

Conceptualization: EC, AR, GG, TE, JMG-Formal analysis: EC, GV, KV, AR, TE, IA, MC, GG, MLM, JMG, -Investigation: EC, GV, KV, AR, TE, IA, MV, AS, MC, JMG, -Methodology: EC, AR, TE, JMG-Writing-original draft preparation: EC, JMG-Writing-review and editing: EC, GV, KV, AR, TE, MV, AS, IA, MC, GG, MLM, TEM, JMG All authors agree with the final content of this manuscript.

Ethical statement

The experimental protocols were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUA) of the University of Costa Rica (approval number CICUA-062-2021).

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marilla Lamela Méndez works for Capris S.A., which distributes equipment and reagents for rotational thromboelastometry analysis.Erika Camacho, Alexandra Rucavado, Teresa Escalante, Mariángela Vargas, Álvaro Segura and José María Gutiérrez work at Instituto Clodomiro Picado, Universidad de Costa Rica, where the antivenom used in this study is manufactured.

Data availability

Data will be made available on request.

Acknowledgements

The authors thank Adriana Sánchez, Gabriela Solano, and Daniela Solano for their support in the experimental work, Kalana Maduwage for valuable discussions on this topic, and Capris S.A. for providing the reagents for ROTEM assays. This study was supported by Instituto Clodomiro Picado. Thanks are due to Hospital Nacional de Niños "Dr Carlos Sáenz Herrera" for the support in various aspects of this study.

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