Blistering in *Bothrops atrox* envenomings: Evidence of antivenom and inflammatory factors in the bite site

Sarah N. C. Gimenes[1]; Jacqueline de A. G. Sachett[2]; Mônica Colombini[1]; Luciana A. Freitas-de-Sousa[1]; Hiochelson N. dos S. Ibiapina[2]; Allyson G. da Costa[2]; Monique F. Santana[2], Jeong-Jin Park[4]; Nicholas E. Sherman[4]; Luiz C. de L. Ferreira[3], Fan H. Wen[1]; Wuelton M. Monteiro[2]; Ana M. Moura da Silva[1]; Jay W. Fox[4]

[1]Laboratório de Imunopatologia, Instituto Butantan, São Paulo, SP, Brazil

[2] Escola Superior de Ciências da Saúde, Universidade do Estado do Amazonas, Manaus, AM, Brazil

[3] Departamento de Ensino e Pesquisa, Fundação de Dermatologia Alfredo da Matta, Manaus, AM, Brazil

[4] University of Virginia, Charlottesville, VA, USA

Introduction

In Brazil, the northern states present the highest prevalence of snakebite. *Bothrops atrox* is the species responsible for the majority of snakebites, which consists in an economic impact. The local symptoms (edema, necrosis, inflammation, tissue damage and blistering) are partially responsible for this impact. Inflammatory reactions play an important role in the onset of local tissue damage; and one of the major components of *B. atrox* venom responsible for blister formation are the snake venom metalloproteinases (SVMPs). SVMPs are responsible for hemorrhagic effect and is also correlated with ability to degrade ECM components. These fragments may potentially act as immunomodulator and Damage-Associated Molecular Patterns (DAMPs), increasing the inflammatory response. Currently, the use of antivenom is recognized as the best approach to systemic effects. However, is not particularly effective in preventing the initial damage trigged by the venom toxins, and consequently we observed the worsening of tissue damage at the site of bite.

Aims

The examination of blister fluids as a window, which can provide important information about the pathophysiological origin of blister formation, considering its biochemical composition. Moreover, to investigate the possible reasons of limited protective efficiency of antivenom to be able to treat the local damage in *B. atrox* envenoming.

Results

Five patients were attended at Tropical Medicine Hospital, Manaus, Brazil (CAAE 53192516.8.0000.0005). They were classified according to the clinical data: moderate (patients 1, 2 and 3) and severe (patients 4 and 5) accident. The clinical data provide insights into the local symptoms experienced in this patient cohort. The presence of moderate to severe edema and elevated levels of LDH (>190UI/I), which is a marker indicative of tissue damage, was observed during the whole time of hospitalization (**Table 1**). The histological examination of tissue at the local site corroborated the clinical data, showing an acute inflammatory and hemorrhage in the area around the snakebite (**Figure 1**).

Table 1: Clinical data of patient whose blister fluid.

Patient/Clinical data		Patient 1 (Moderate)	Patient 2 (Moderate)	Patient 3 (Moderate)	Patient 4 (Severe)	Patient 5 (Severe)
Gender		Male	Female	Male	Male	Male
Bite site		Foot	Foot	Foot	Leg	Foot
Time from envenomation until hospital admission		5 hrs.	2 hrs.	6 hrs.	11 hrs.	4 hrs.
Time of blister after hospital admission		58 hrs.	135 hrs.	77 hrs.	111 hrs.	80 hrs.
Tourniquet Applied		No	No	No	Yes	No
* Pain (0-10)	Time 0	10	8	7	10	10
	Time 24	4	7	5	0	5
	Time 48	0	5	4	0	0
	Time 72	0	5	3	0	7
	Time 144	0	4	2	8	0
Ecchymosis		No	After 48 hrs. of hospital admission	No	No	No
Edema	Time 0	Moderate	Moderate	Mild	Moderate	Moderate
	Time 24	Moderate	Moderate	Moderate	Moderate	Severe
	Time 48	Moderate	Moderate	Moderate	Moderate	Severe
	Time 72	Moderate	Moderate	Moderate	Moderate	Moderate
	Time 144	Moderate	Mild	Mild	Moderate	Moderate
** Lactic Dehydrogenase	Time 0	464	234	715	935	264
	Time 24	335	221	501	305	255
	Time 48	308	115	281	286	344
	Time 72	313	283	302	328	255
	Time 144	285	263	289	230	289
*** C-reactive protein	Time 0	6,5	6,5	48	96	6,5
	Time 24	96	96	48	96	48
	Time 48	96	96	192	24	48
	Time 72	24	96	96	48	96
	Time 144	48	96	96	12	48

* 0 means less pain and 10 means intense pain. ** Lactic Dehydrogenase normal value 190 UI/l. *** C-reactive protein normal value 0.8 mg/dL.

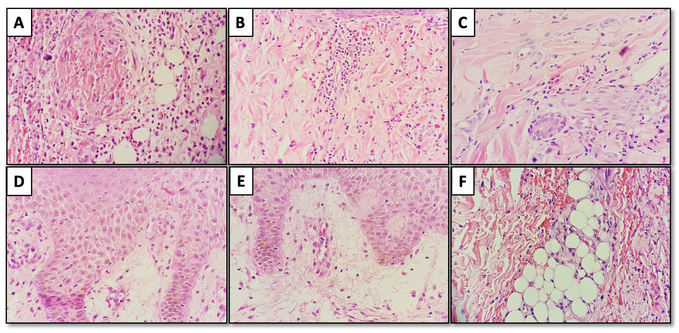

Figure 1: Illustrative figure of local damage. **A, B and C)** vascular thrombosis associated with vessel disruption and inflammatory infiltrate of mainly eosinophils and neutrophils. **D, E and F)** exocytosis of neutrophils in the epidermal layer associated with the presence of edema and papillary edema, and intense erythrocyte extravasation in the deep dermis layer, associated with mononuclear inflammatory infiltrate.

The proteomic data of the blister fluids correlated with previous blister fluid studies showing the presence of ECM and coagulation's proteins (**Figure 2A and B**), as well as DAMPs and immunomodulators (**Figure 2C and D**).

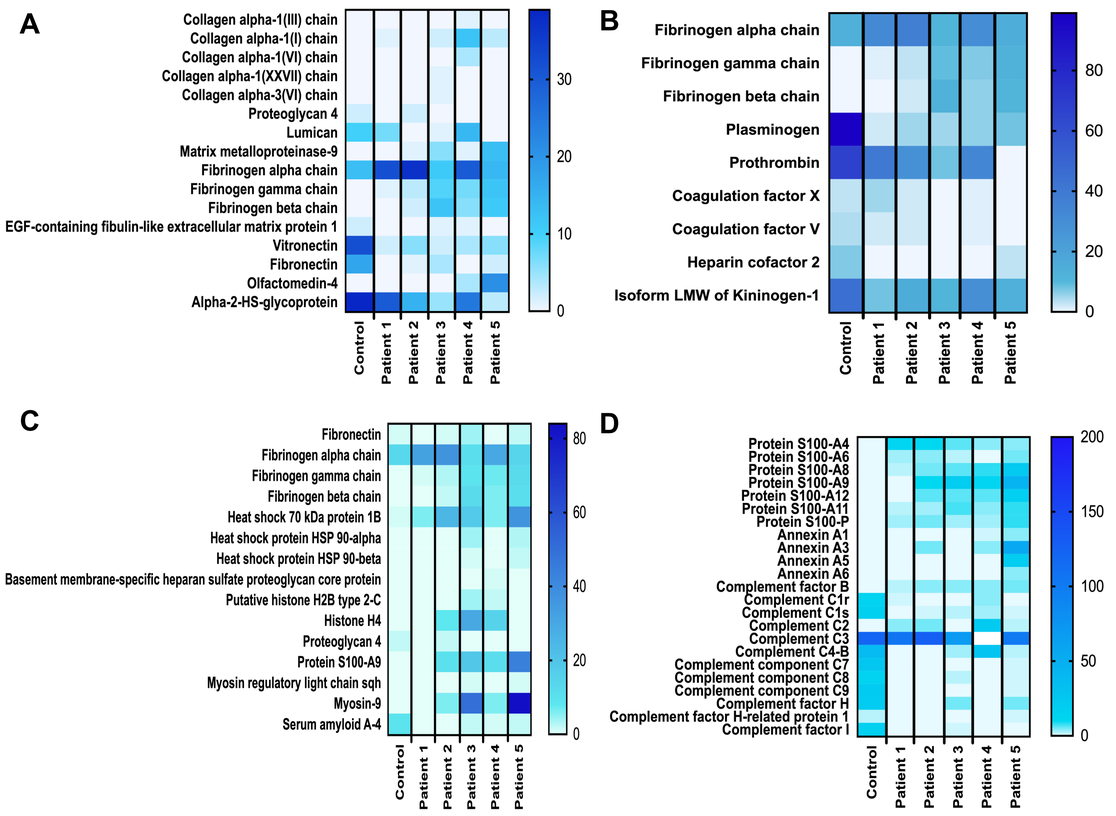

Figure 2: Proteomic data from blister fluids. **A)** Proteins present in blister fluid related to extracellular matrix; **B)** Proteins present in blister fluid related with coagulation; **C)** DAMPs and **D)** Immunomodulators. Proteins were identified from the raw data using the Sequest search algorithm Proteome Discovery against human basic database. The bar indicates the value of normalized total spectra count.

The blister composition was observed to be similar among the patients regardless of the clinical severity of envenomation. An unprecedented additional finding was that we identified venom (Figure 3A) and antivenom proteins (**Figure 3B**) in the bite site by ELISA. The venom was quantified in the fluid a significant time after envenommation (up to135 hours), suggesting a slow clearance of venom at the site of bite, which might have influence on local tissue well after the time of envenomation (**Figure 3A**).

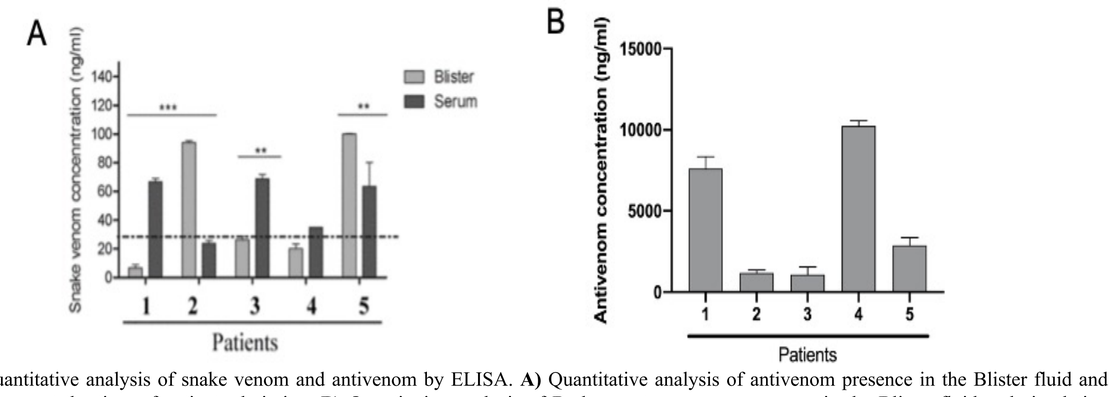

Figure 3: Quantitative analysis of snake venom and antivenom by ELISA. **A)** Quantitative analysis of antivenom presence in the Blister fluid and circulating serum at the time of patient admission. **B)** Quantitative analysis of Bothrops atrox venom presence in the Blister fluid and circulating serum at the time of patient admission.

Antibodies from the administered antivenom identified in the blister fluids were shown capable of binding venom proteins by Western blotting (**Figure 4**). Thus, blister fluid antibodies should be capable of neutralizing the any venom components in the fluid. However, taken together, these findings suggest that although blistering is a delayed phenomenon of envenomation, its likely pathophysiological origins occur in advance of antivenom administration and venom neutralization at the site of envenomation and continues despite the eventual neutralization of venom.

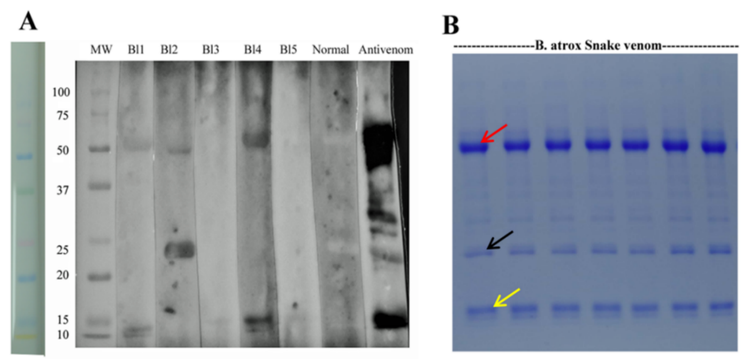

Figure 4: Analysis of the antivenom in Blister by Western Blotting. **A)** Western Blotting demonstrating the recognition of *B. atrox* venom proteins by the blister content. MW: Molecular Weight standard; Bl1: Blister Patient 1; Bl2: Blister Patient 2; Bl3: Blister Patient 3; Bl4: Blister Patient 4; Bl5: Blister Patient 5; Normal: serum from volunteer without antivenom exposure; Antivenom: antivenom produced by Butantan Institute. B) SDS-PAGE with *B. atrox* venom used for protein transferred in western blotting assay. Red arrow represents range of SVMP P-III; Black arrow represents range of SVMP P-I; Yellow arrow represents range of Phospholipases A_2.

Conclusion

We demonstrates that antivenom and venom reach and stay even after long time on the local lesions in *B. atrox* snakebite. However, the presence of antivenom in the blister did not prevent the severity of tissue damage or blister formation. It suggest that the blister is an independent process, and the pathophysiology of blister formation is more related to the presence of proinflammatory molecules released by the toxins action since the first moments after venom inoculation (**Figure 4**). Our studies underscore the need to develop rapid, in situ therapeutics to eliminate or at least attenuate the local effects of envenomation.

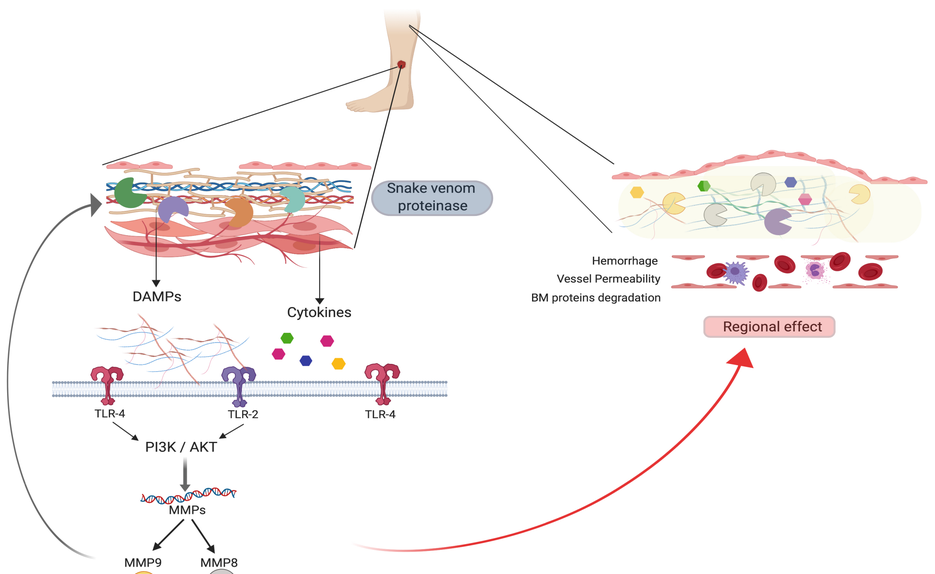

Figure 4: Representation of hypothetical pathway of blister formation triggered by snake venom proteinase from *Bothrops atrox* envenoming. The BioRender Standard Academic software was used.