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ORIGINAL ARTICLE

Exploring the *ex vivo* effects of *Naja mossambica* venom on the ultrastructure and viscoelastic properties of human blood

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Abstract

Background: Venomous snake bites have been listed as a neglected tropical disease by the World Health Organization. The Mozambique spitting cobra (*Naja mossambica)* is found in Sub-Saharan African countries, and its venom has been identified to predominantly result in cytotoxic effects. However, there is limited evidence on the possible hemotoxic effects of this venom on human blood.

Objectives: In this cross-sectional study, we investigated how Mozambique spitting cobra venom affects blood clot formation.

Methods: Cell morphology and clot architecture were studied by using microscopy techniques. We also studied the effects of the venom on platelets by measuring platelet activity with the global thrombosis test, followed by analyzing the viscoelasticity with thromboelastography using a 0.025 ng/μL venom concentration.

Results: The most prominent findings indicated that the viscoelastic profile in the venom-treated blood samples formed an unstable and elastic clot. The clot architecture seen with the scanning electron microscopy analysis showed an altered fibrin network and red blood cells, confirmed by the increased axial ratios, and aggregated platelets with spreading.

Conclusion: These findings may offer insights into the species-specific effects of snake venom on human blood and add value to the clinical workup in confirming envenomation. Further research is needed to correlate the 20 minute whole blood clotting test with measurable values from the thromboelastography within the context of snake envenomation. This may offer a bridge between cost, early diagnosis, and treatment of snake envenomation in resource-constrained countries.

KEYWORDS
blood platelets, red blood cells, snake bites, snake venom, thromboelastography

Essentials

- There is limited evidence on the hemotoxic effects of the *Naja mossambica* venom.
- The viscoelastic profile in the venom-treated blood samples formed an unstable and elastic clot.
- The clot architecture showed an altered fibrin network and red blood cells.
- These findings offer insights into the species specific effects of *N mossambica* venom on human blood.

1 | INTRODUCTION

Venomous snake bites in Southern Africa carry a huge burden on several levels of society and were listed as a neglected tropical disease as recognized and categorized by the World Health Organization [1,2]. Venomous snake bites and risks continue to prove a hazard to human health, predominantly in tropical and subtropical regions such as Sub-Sahara Africa, South Asia, and Southeast Asia, mostly affecting tropical rural and remote areas [1,3–5].

Snake venoms are classified as neurotoxic, cytotoxic, or hemostatic toxins based on their pharmacologic effects, symptoms, and biochemical outcomes produced after envenomation, which may sometimes produce overlapping signs and symptoms [6]. In addition, the type of venom also depends on the composition, 80% to 100% of which is composed of proteins and nonprotein type of substances such as carbohydrates, lipids, inorganic salts, and amines [1,7]. In the family *Elapidae*, venoms are mainly comprised of 2 protein families: three-finger toxins and phospholipase A_2s [1,7]. It is interesting to note that all venomous snakebites may affect blood coagulation to some degree, implicating a hemotoxic profile for all venomous snakes at various grades of intensities [6,8]. This gives us the opportunity for a more in-depth investigation of this common ground phenomenon, which may give us better insights into possible species-specific differences by assessing their effects on human blood, as previously described for puff adder (*Bitis arietans*) venom [9]. This has the potential to unlock the development of novel methods of snake identification on a biochemical level. Snake venom may produce either hemorrhagic (anticoagulative) or coagulative effects based on the biochemical properties present in the venom. Venom that is able to produce anticoagulative effects inhibits the blood coagulation cascade, inhibiting the main enzymes responsible for blood clotting, which are thrombin and activated factor X [10,11]. In addition, Bittenbinder et al. [10] studied the coagulotoxic effects of elapid snake venom, which revealed that it has the ability to inhibit thrombin function and activated factor X from forming the prothrombinase complex. In our study, we focused on a specific member of the family *Elapidae*, the Mozambique spitting cobra (*Naja mossambica*), where limited information is currently available on the coagulotoxic properties of its venom.

The Mozambique spitting cobra is found in Sub-Saharan Africa in countries such as Botswana, Angola, Zimbabwe, Zambia, Tanzania, Namibia, Swaziland, Malawi, Mozambique, and South Africa and commonly found in low-lying areas and around constant water bodies [12–14]. The Mozambique spitting cobra has been described as the "deadly bed partner" since victims may be attacked when asleep and usually sustain 1 or multiple bites as a result of individuals rolling over these snakes in an unintentionally provocative manner [13]. The Mozambique spitting cobra venom has been identified to predominantly result in cytotoxic effects [15].

In this study, we aimed to investigate how Mozambique spitting cobra venom affects blood cell morphology and clot architecture using microscopy techniques. The effects of Mozambique spitting cobra venom on platelets were also determined by measuring platelet activity with the global thrombosis test (GTT), followed by measuring the viscoelastic properties of clot formation using thromboelastography (TEG).

2 | METHODS

2.1 | Study design and study population

This was a cross-sectional analytical study. Healthy members of the general public were invited to participate in the study by means of convenience sampling. The inclusion criteria included healthy individuals, any biological sex (being male or female) [16] or race, and ages between 18 and 60 years. The exclusion criteria were set to limit any confounding factors such as smokers, use of chronic medication, and any known acute or chronic disease. Blood was collected from 38 participants with ages ranging from 20 to 59 years. Only complete data for each variable were used; therefore, the number of individuals analyzed per variable may differ. If there were data sets with incomplete data, they were removed by pairwise deletion from the data set. Reasons for incomplete data included inadequate amount of blood collected to be analyzed, no visible sample on the microscopy slides, and equipment errors. There were 2 groups in this study: an untreated group (naïve samples) and a treated group (exposed to *N mossambica* snake venom). Blood collection was done by a medically trained professional. Blood was collected in two 4 mL citrate blood tubes (1:9, 3.2% sodium citrate anticoagulation) and one 10 mL syringe. Treated samples consisted of whole blood exposed to 0.025 ng/μL lipolyzed *N mossambica* snake venom (donated by the African Snakebite Institute) and incubated for 10 minutes before sample analysis. Whole blood samples from the citrate tubes were used for light microscopy, scanning electron microscopy (SEM), and TEG analyses. The residual untreated and treated citrated whole blood samples were centrifuged at 2000 *g* for 20 minutes at room temperature. The platelet-poor plasma was obtained by removing the supernatant. The platelet-poor plasma was used to create fibrin fiber smears to measure fibrin thickness and branching. Whole blood collected in the syringes was used for GTT analyses.

This study took place in the Basic Medical Sciences building at the University of Pretoria, South Africa.

2.2 | Light microscopy and axial ratios

Thin whole blood (citrated) smears were made following a standard method for light microscopy, as described by Bester et al. [17]. Light microscopy micrographs were obtained on a Zeiss Axio Imager M2/A1m (Carl Zeiss Microscopy) microscope, and 5 micrographs per participant were obtained. Axial ratios of red blood cells from the untreated and treated samples were measured using ImageJ (ImageJ is a public domain, Java-based image processing program developed at the National Institutes of Health; http://rsbweb.nih.gov/ij/). Axial ratios were calculated by using the largest diameter overall as the

numerator and the length at 90° to the line used to provide the numerator as the denominator. On average, 50 red blood cells were assessed for each participant.

2.3 | TEG of whole blood

Clot kinetics was analyzed by using viscoelastic assessment performed by TEG. Citrated whole blood was used, and 340 μL of the sample was added to 20 μL of 0.2 M $CaCl_2$ in a disposable TEG cup. The samples were then placed in the computer-controlled Thromboelastograph 5000 Hemostasis Analyzer System (Haemonetics Inc) for analysis at 37 °C. The TEG measured 8 parameters, which included reaction time (R time), kinetics time, alpha angle, maximal amplitude (MA), G-value, maximum rate of thrombin generated, time maximum rate of thrombin generated, and total thrombin generated [18]. The units and a description of each of these parameters are shown in Table 1 [18]. For this study, the test was terminated after MA was achieved as clot formation was mainly studied [18–20].

2.4 | GTT

Naïve, uncitrated whole blood (4 mL) was collected with a 10 mL syringe from participants. The untreated samples were left to stand for 30 seconds and placed into GTT-specific cuvettes (Thromboquest Ltd). The treated samples were first incubated with 0.025 ng/μL lipolyzed *N mossambica* snake venom, left to incubate for 30 seconds, and placed into GTT-specific cuvettes (Thromboquest Ltd). After the blood sample was added to the cuvettes, the analysis was initiated. The treated and untreated samples were analyzed with the GTT-2 model (Thromboquest Ltd). The occlusion time (OT) time was recorded as soon as the blood flow stopped due to platelet-rich thrombus formation. Due to endogenous thrombolysis, the platelet-rich clot lysed, partially restoring the flow, and the first drop that was detected was recorded as the lysis time (LT), indicating the time it took for the platelet-rich clot to lyse.

Platelet activity was measured by the OT and LT from the GTT, and the interpretation of the results is described in Table 2.

2.5 | SEM of whole blood

Citrated whole blood and platelet-poor plasma smears were prepared for SEM analysis. Blood smears were made by placing 10 μL of whole blood on glass coverslips (with and without the addition of 5 μL thrombin at a final concentration of 3.33 IU/mL). The platelet-poor plasma smears were prepared by placing 10 μL of platelet-poor plasma on glass coverslips with the addition of 5 μL thrombin. All the smears were prepared for SEM using a standardized protocol described by Bester et al. [21]. Micrographs of the smears were taken using a high-resolution Zeiss Crossbeam 540 field emission gun scanning electron microscope (Carl Zeiss Microscopy) at the Unit for Microscopy and Microanalysis at the University of Pretoria to study the ultrastructure of red blood cells, platelets, and fibrin fibers. Micrographs were obtained at 2 kV with the InLens detector.

Fibrin fiber thickness was measured by using the platelet-poor plasma SEM micrographs. The micrographs were imported into ImageJ, and 50 fibers per participant were measured using 4 images per participant. Fractal dimension analysis was performed to measure fibrin branching by using the Fractal Analysis system supplied by the National Agricultural and Food Research Organization, Japan, as previously described [22,23]. For each participant, 4 images were analyzed.

2.6 | Statistical analysis

Descriptive statistics are given using tables, and continuous data were checked for normality using the Shapiro–Wilk test. Measurements of the TEG and other outcome variables were compared before and after treatment using Wilcoxon signed-rank tests. A 5% CI was used to determine statistical significance. GraphPad Prism 9.5.1 was used to analyze the data.

2.7 | Sample size

In order to detect a mean difference of 3 seconds in R time between untreated and treated samples with 90% power, with an SD of 3 seconds in the untreated samples and 6 seconds in the treated samples and a correlation of 0.5 between pretreatment and post-treatment samples, a minimum of 28 samples are required.

3 | RESULTS

All the TEG parameters from the treated group were significantly different from those in the untreated group. The R time, kinetics, and time maximum rate of thrombin generated were decreased, while the angle, MA, G-value, maximum rate of thrombin generated, and total thrombin generated were increased for the treated group. There was no significant difference between the control and treated groups for the OT, LT, fibrin fiber thickness, and fibrin branching. The axial ratios for the treated group were significantly increased. The results for the TEG, GTT, fibrin fiber measurements, fibrin branching, and axial ratios are shown in Table 1.

The results from the SEM for the untreated group showed normal red blood cell morphology, limited presence of platelets with slight pseudopodia, and spreading, shown in Figure 1A, B. Also visible in these micrographs was limited interaction between the red blood cells and platelets. The clot structure was normal, with a uniform network of fibers entrapping discoid red blood cells, as shown in Figure 1C. The treated group had abnormally shaped red blood cells with an increased presence of platelets with extensive pseudopodia formation and spreading, which led to an increase in

TABLE 1 Results from thromboelastography, global thrombosis test, fibrin fiber measurements, and axial ratios.

Parameter	Description	Reference range and unit	Untreated, median (min, max)	Venom-treated, median (min, max)
Participant demographics (n = 38)				
Age		Years	25 (20, 59)	
Sex (24/14)	Male/female			
TEG n = 30				
Reaction time	Clot initiation	9-27 min	14.70 (11.75, 18.53)	22.25 (15.63, 28.05)
	P value		**<.001**	
Kinetics	Amplification	2-9 min	5.15 (2.80, 7.13)	9.30 (5.78, 13.93)
	P value		**<.001**	
Angle	Thrombin burst	22-58 degrees	47.65 (41.33, 61.20)	38.30 (36.50, 42.70)
	P value		**<.001**	
MA	Overall clot stability	44-64 mm	58.25 (50.25, 63.00)	49.55 (42.58, 56.25)
	P value		**<.001**	
G-value	Shear elastic modulus strength	3.5-8.6 dynes/cm^2	7.00 (5.05, 8.50)	4.90 (3.73, 6.43)
	P value		**<.001**	
MRTG	Maximum rate of thrombus generation	0-10 dynes/cm^2/s	3.87 (2.22, 4.83)	1.67 (0.93, 2.43)
	P value		**<.001**	
TMRTG	Time to maximum rate of thrombus generation	5-23 min	18.67 (16.12, 26.44)	31.67 (20.02, 38.79)
	P value		**<.001**	
TTG	Total thrombus generation	251-1014 dynes/cm^2	705.4 (491.30, 855.20)	483.7 (377.50, 646.10)
	P value		**<.001**	
GTT n = 26				
Occlusion time	Time to create an occlusive thrombus	300-500 s	343.2 (220.10, 456.50)	253.0 (73.78, 479.80)
	P value		.40	
Lysis time	Time taken to lyse the thrombus	<2000 s	2719 (2129, 4809)	3373 (1963, 6000)
	P value		.81	
Fibrin measurements n = 26				
Branching	Fibrin branching		2.55 (2.47, 2.61)	2.51 (2.47, 2.56)
	P value		.35	
Fiber thickness	Fibrin fiber thickness	nm	168.20 (143.30, 184.10)	160.40 (141.60, 200.60)
	P value		.89	
Red blood cell axial ratio n = 35				
Axial ratio	Red blood cell deformability		1.10 (1.08, 1.12)	1.12 (1.09, 1.15)
	P value		**.03**	

Significant P values are shown in bold.

GTT, global thrombosis test; MA, maximal amplitude; MRTG, maximum rate of thrombin generated; TEG, thromboelastography; TMRTG, time maximum rate of thrombin generated; TTG, total thrombin generated.

TABLE 2 Interpretation of global thrombosis test results.

Parameter result	Result interpretation
OT: 300-500 s	Range of normal hemostatic activity
OT: 400-500 s	Effective antiplatelet/anticoagulant medication
OT: >900 s	Possible bleeding risk
OT: <300 s	Platelet hyperactivity: antiplatelet medication is indicated
LT: <2000 s	Normal spontaneous thrombolytic activity
LT: 2000-4000 s	Low thrombolytic activity
LT: >6000 s	Lack of thrombolytic activity
If OT is >900 s	No occlusion, LT cannot be measured

LT, lysis time; OT, occlusion time.

interaction between the cells, which can be seen in Figure 1D, E. The whole blood clot structure had deformed red blood cells with a fibrin network that appeared to have an increased presence of fibers, as shown in Figure 1F.

4 | DISCUSSION

Mozambique spitting cobra venom contains three-finger toxins and phospholipase A_2, which have been shown to be hemotoxic. In this study, we have shown that Mozambique spitting cobra venom has a hypocoagulable effect on human blood at a concentration of 0.025 ng/μL. The most prominent findings indicated that the viscoelastic profile in the treated group had a prolonged initiation time and increased amplification and ultimately formed an unstable and elastic clot. The clot structure seen with the scanning electron microscope (Figure 1F) showed an increased presence of fibrin fibers, which could have contributed to the change in the elasticity of the clot detected by TEG. Even though there were no significant differences between the treated and untreated groups for the GTT, the median of the treated group was below 300 seconds, which could indicate that platelet hyperactivity was present to some extent, as seen in the SEM analyses (Figure 1E). The axial ratios revealed that there was increased red blood cell deformability in the treated group, which was also visible with SEM (Figure 1D).

From a clinical perspective, the results demonstrate the importance of evaluating the hemodynamic status of a patient with a snakebite. Patient care outcomes may be directly influenced by the delay between envenomation and antivenom administration for susceptible snakebite envenomation cases. As most snakebites are concentrated in rural areas of low-resource settings, investigations may be limited to basic tests, such as full blood counts, 20-minute whole blood clotting test, urine examinations, and serum renal function tests [24]. The increased platelet hyperactivity may result in thrombocytopenia, as demonstrated in *B arietans* envenomation [9]. TEG may add value in the clinical workup of the snakebite victim to confirm envenomation. Its improved sensitivity compared to that of a 20-minute whole blood clotting test may detect and quantify earlier stages of envenomation and promote confidence in clinical decision making on whether to start antivenom administration sooner rather than later.

4.1 | Limitations

More males than females were included in the study; therefore, the results might be biased toward males. However, we made use of

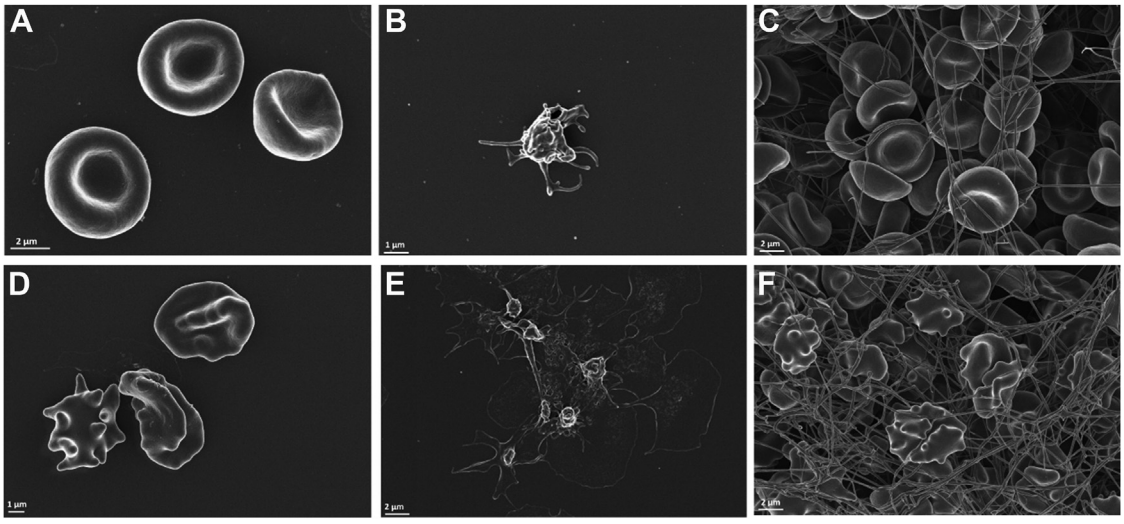

FIGURE 1 Whole blood smears for the untreated and treated samples. Micrographs A to C represent the untreated group. (A) Normal-shaped red blood cells with limited interaction. (B) The untreated group showed some platelet presence with limited spreading and pseudopodia formation. (C) A whole blood clot with normal red blood cell morphology and a uniform fibrin network. Micrographs D to F represent the treated group. (D) Abnormally shaped red blood cells with some degree of interaction between the cells. (E) Increased presence of aggregated platelets with spreading and pseudopodia. (F) Whole blood clot with abnormally shaped red blood cells with a denser fibrin network.

convenience sampling that limited specific selection of the participants. Also, some of the variables had missing data, and we applied the pairwise deletion approach to the data sets. No standard clinical coagulation analyses were included in this study. These analyses will be included in future studies.

5 | CONCLUSION

In conclusion, bleeding syndromes are a known and expected complication in snakebite victims. The *ex vivo* effects of Mozambique spitting cobra venom on blood cell ultrastructure and coagulation have been described and should be investigated further in a clinical or animal study. Coagulation parameters and blood cell ultrastructure may assist the clinician in confirming envenomation that may promote clinical decision making. Due to the burden of snakebites in rural communities, funding and availability of more sophisticated laboratory investigations remain a challenge. Further research is needed to correlate the 20-minute whole blood clotting test with measurable values from TEG within the context of snake envenomation. This may offer a bridge connecting cost, early diagnosis, and treatment of snake envenomation in resource-constrained countries.

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ETHICS STATEMENT

This study was approved by the Research Ethics Committee and Animal Ethics Committee of the Faculty of Health Sciences at the University of Pretoria, South Africa (ethics clearance number: 344/2021). A written form of informed consent was obtained from all participants before sample collection took place. We adhered strictly to the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

T.C.: sample collection, execution of experiments, data analysis, and manuscript editing. J.L.: sample collection, execution of experiments, data analysis, and manuscript editing. M.A.S.: concept development, data interpretation, and manuscript writing. J.B.: concept development, project management, data interpretation, and manuscript writing.

RELATIONSHIP DISCLOSURE

T.C. reports National Research Foundation (NRF) scholarship funding. M.A.S. and J.B. are responsible for the equipment and materials used during the course of the research. M.A.S. reports support via Thuthuka NRF Grant TTK2204072413 and University of Pretoria Research Developmental Grant for this study. J.B. reports funding to conduct research from National Research foundation. J.L. has no conflict of interest to declare.

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