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ORIGINAL ARTICLE

Exploring the *ex vivo* effects of *Bitis arietans* snake venom on the coagulation, ultrastructure, and viscoelastic properties of human blood

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Abstract

Background: Snakebite envenoming represents a significant and frequently overlooked public health challenge affecting tropical and subtropical regions. *Bitis arietans* venom toxins have cytotoxic effects and result in coagulopathy. However, there is limited literature on coagulopathies associated with *B arietans* envenomation or comparing traditional diagnostic tests with point-of-care (POC) methods.

Objectives: This study investigated the effects of *B arietans* venom on the coagulation of human blood, with a focus on comparing the 20-minute whole blood clotting test (20-WBCT) to other POC coagulation tests.

Methods: This study exposed human blood to 2 ng/μL *B arietans* venom *ex vivo*. Clot formation was studied using the 20-WBCT. Prothrombin time, activated partial thromboplastin time, thrombin time, and fibrinogen levels were measured to obtain hematological clotting profiles of each participant. Viscoelastic properties of whole blood clot kinetics were quantified using thromboelastography (TEG). Red blood cell morphology and clot architecture were analyzed using scanning electron microscopy.

Results: *Bitis arietans* venom had significant effects on red blood cell morphology and clot structure. Both the 20-WBCT and clinical coagulation assays revealed notable differences in the results of venom-exposed samples; however, they were still in the normal range. TEG indicated hypocoagulation and decreased clot stability. Morphological studies of venom-exposed samples revealed echinocytes with varying degrees of morphological abnormalities and membrane blebbing. In addition, venom-exposed blood clots had sparse, disorganized fibrin networks and limited crosslinking.

Conclusion: *Bitis arietans* venom contains various hemotoxins that disrupt normal clot formation and affect TEG parameters. These insights provide a necessary link between clinical and laboratory analysis of *B arietans* venom. The study demonstrates the value of TEG as a POC test in snakebite management as it could provide a better indication of coagulopathy associated with envenomation.

KEYWORDS
Bitis arietans, snake venom, scanning electron microscopy, thromboelastography

Essentials

- Current research focusing on the hemotoxic effects of *Bitis arietans* venom is limited.
- Small, unstable clot formation with disrupted fibrin networks were observed in the venom-exposed samples.
- Venom-exposed samples also displayed changes in red blood cell morphology and ultrastructure.
- The study findings expand the understanding of the effects of *B arietans* venom on human blood coagulation.

1 | INTRODUCTION

Venomous snakes bite approximately 5.4 million individuals every year [1–3]. It is estimated that these bites cause between 1.8 and 2.7 million cases of envenoming and lead to over 80,000 mortalities [1,3]. Snake venom disrupts hemostasis through various toxic compounds [4]. Envenomation by cytotoxic snakes is a critical health concern worldwide [4]. Coagulopathy-induced hemorrhage is a major complication of Viperidae envenomation. Viper envenomation, specifically from *Bitis arietans*, is associated with tissue necrosis, hypotension, and severe coagulopathy characterized by spontaneous bleeding and thrombocytopenia [5].

The snake is commonly found in the sub-Saharan African savannah regions and inhabits a wide range of habitats [6]. This extensive species distribution is widely believed to be an important factor contributing to the estimated 43,000 deaths from *B arietans* bites reported in Africa annually [6]. The venom of *B arietans* is considered one of the most toxic/potent of any viper species (murine median lethal dose [LD_{50}] values of 9–13 μg/mouse) and leads to a number of cytotoxic crises [6,7]. In addition, *B arietans* venom has proven to inhibit the activity of factors vital in blood coagulation producing hypocoagulative effects [4].

Early diagnosis and treatment are essential in the management of snakebite patients, in terms of limiting the ability of toxic venom proteins to cause morbidity and mortality. The 20-minute whole blood clotting test (20-WBCT) is a simple, well-established point-of-care method, used to detect coagulopathy and confirm systemic envenomation [8]. This test is particularly useful in emergency departments in resource-limited settings [8]. The test provides the clinician with valuable information during the initial patient assessment and ongoing management of snakebite patients [8,9]. Furthermore, it is the only point-of-care clotting test that has been scientifically validated to detect coagulopathy following envenomation [10]. Unfortunately, the test lacks standardization in key aspects, including test duration and the specific materials used during the method [11]. Additionally, the simplicity of the test limits its ability to account for the changes that occur throughout the 20-minute duration and overlooks the possibility for earlier diagnostics without the risk of missing critical diagnostic information [12]. Traditionally, the detection and management of clotting abnormalities are achieved by using standardized conventional clotting tests like prothrombin time (PT), activated partial thromboplastin time (aPTT), and thrombin time (TT) [3]. In addition, clinicians rely on these clotting assays to assist in determining whether systemic envenoming has occurred and to monitor antivenom treatment regimens [8]. Unfortunately, many envenoming cases are managed in rural health care settings that lack the resources to perform automated laboratory tests for diagnosis or management [13]. Furthermore, conventional clotting tests are time consuming in a time-sensitive situation as they require specialized equipment and personnel to perform. Despite such concerns, conventional clotting tests are routinely performed in this patient group as these tests supplement clinical features of hypocoagulation as evidence of envenomation and thus in the decision to administer antivenom.

A potentially more physiologically accurate evaluation of what occurs during snake bite envenoming may be provided by thromboelastography (TEG). Unlike the 20-WBCT and conventional clotting tests, TEG uses the principle of viscoelasticity to measure coagulation parameters and provide a more comprehensive assessment of hemostatic function as it considers whole blood (WB) and not only platelet-poor plasma. TEG is a rapid, point-of-care test and is being increasingly used in cardiac surgery, trauma, and massive transfusions [14]. In addition, TEG is currently used in wide variety of (pit)viper envenomations [15–17].

A major issue associated with snake bites is the difficulty in identifying the snake responsible for the envenomation and to determine the level of venom exposure, as both factors are crucial for effective antivenom administration [10]. The responsible snake is often misidentified, resulting in poor patient management and treatment. This highlights the need for the development of improved diagnostic methods to enhance accuracy and improve subsequent patient treatment. Studies have previously demonstrated that in venom-exposed samples, TEG shows delayed coagulation initiation, weaker clot strength, and impaired fibrinogen function [5]. However, the relationship between TEG, 20-WBCT, and conventional clotting tests in this population remains unknown. Developing fast, reliable diagnostic methods require a better understanding of the underlying pathophysiology of *B arietans* envenomation. The effects of South African snake venoms on traditional coagulation tests, viscoelastic testing and imaging also needs to be explored. Accordingly, we

compared the 20-WBCT, conventional coagulation tests and laboratory tests, including TEG and scanning electron microscopy (SEM), before and after the *ex vivo* addition of *B arietans* venom. The combined information will highlight possible diagnostic limitations of the 20-WBCT in the 20-minute test window when other clinical tests are unavailable.

2 | METHODS

2.1 | Study design, sampling criteria, and setting

This analytical, laboratory-based experimental study took place at the University of Pretoria and the blood coagulation tests were performed at the Haematology Core Laboratory of the National Health Laboratory Service in the Steve Biko Academic Hospital. Sample viewing took place at the unit of Microscopy and Microanalysis on Hatfield campus, University of Pretoria.

Blood samples were collected from 28 healthy members of the general public. The inclusion criteria included healthy individuals of any sex, between the ages of 18 and 50 years, without any scheduled drug use, tobacco or tobacco-related product use, any acute or chronic illness, and pregnancy in females. Participants were recruited using convenience sampling to avoid any bias and only participants with complete data were included in the study. The study consisted of 2 groups, namely, a naive group (control samples) and a venom-exposed group (*B arietans* snake venom).

Blood was collected in four 4.5-mL evacuated citrate blood tubes (1:9 3.2% sodium citrate anticoagulation) and two 2.5-mL evacuated nonadditive tubes. Exposed WB samples were exposed to a final concentration 2.0 ng/μL *B. arietans* snake venom (donated by African Snakebite Institute). The use of this venom concentration was initially based on previous studies conducted on *B. arietans* snake venom by Strydom et al. [5]. In addition, this study concentration was optimized to just before the TEG flatlined. The venom was received as a lyophilized powder, stored at room temperature, and reconstituted when required for experimentation.

The 20-WBCT was conducted in nonadditive tubes immediately after sample collection. In addition, WB in citrate was incubated for 10 minutes at room temperature (18-25°C) before running the clinical coagulation assays and SEM and TEG analyses.

2.2 | Clinical techniques

2.2.1 | 20-minute WB clotting test

The method as described by Benjamin et al. [9] was applied in this study. The 20-WBCT was conducted immediately after sample collection, where 2 mL of venous blood was collected and tested in two zero-additive tubes. One tube was used as a control (naive sample) and the other, exposed to *B arietans* snake venom. The tubes were left upright, open and undisturbed for a period of 20 minutes. During the allocated period, the tubes were observed and time was recorded when a solid clot was formed. After 20 minutes, the tubes were inverted to confirm that the solid clot maintained its shape and normal coagulation status.

2.2.2 | Conventional coagulation testing of citrated WB

One of the collected citrate tubes of each participant was exposed to *B arietans* venom and left to stand for 10 minutes before beginning the test. Two tubes, one exposed and one control, were centrifuged for 15 minutes at 1500 *g* to obtain platelet-poor plasma for analysis. The PT (international normalized ratio), aPTT, TT, and fibrinogen level were all performed as 1-stage clotting assays on citrated plasma using the Sysmex CS-2500 System (Siemens Healthcare Diagnostics), according to the manufacturer's recommendations. The results were analyzed and interpreted according to laboratory-established reference ranges for adults.

2.2.3 | TEG of citrated WB

A volume of 340 μL WB was pipetted into the TEG cup, with 20 μL 0.2 M calcium chloride ($CaCl_2$). The samples were placed in the computer-controlled Thromboelastograph 5000 Haemostasis Analyzer System (Haemonetics) for analysis at 37 °C. The viscoelastic parameters measured included reaction time (R time), clot kinetics (K), α angle, maximal amplitude (MA), G-value, maximum rate of thrombin generated (MRTG), time maximum rate of thrombin generated and total thrombin generated (TTG) and coagulation index (CI). For this study, the test was terminated after MA was achieved as only clot formation was studied.

2.3 | SEM

The method described by Strydom et al. [5] was used in the study. The method included WB, WB clots, and 20WBCT smears for both naïve and venom-exposed samples. WB smears were made by pipetting either 10 μL of WB or adding a piece of clot onto glass cover slips. In addition, for WB clot analysis, 10 μL of WB was combined with 5 μL of thrombin (donated by South African National Blood Service) and smeared onto the coverslip. The sample smears/clots were fixed (4% formaldehyde and 1% Osmium Tetra-oxide), dehydrated using graded dehydration in 30%, 50%, 70%, 90%, and 100% ethanol and dried using hexamethyldisilane (Sigma-Aldrich). Blood cell morphology and clot structure micrographs were captured and analyzed using a high-resolution Zeiss Ultra Plus Zeiss Crossbeam 540 field emission gun scanning electron microscope (Carl Zeiss Microscopy).

TABLE Summarized statistics of the 20-WBCT, clinical coagulation assays, and TEG data.

Parameter	Description	Reference range	Naïve, median (minimum, maximum)	Venom exposed, median (minimum, maximum)
Age (y), *n* = 28			24 (20, 49)	
Sex	Male/female		14/14	
20-min whole blood clot test, *n* = 26				
All tests observed as normal coagulation/clot formation				
Time (min:s)	Observed time to solid clot formation		5:25 (3:09, 11:30)	15:14 (8:08, 19:38)
Conventional coagulation assays, *n* = 26				
PT (s)	Measure of clotting time (extrinsic and common pathways)	10-14	11.10 (10.10, 12.10)	11.70 (10.70, 13.20)
aPTT (s)	Measure of clotting time (intrinsic and common pathways)	30-40	26.65 (23.00, 34.90)	30.75 (23.10, 41.40)
Fibrinogen (g/L)	Measure of fibrinogen levels	1.8-3.5	2.54 (1.84, 4.53)	2.15 (0.90, 4.32)
TT (s)	Analyze conversion of fibrinogen to fibrin	14-21	19 (17.30, 21.30)	20.70 (18.30, 23.60)
INR	Ratio of time taken for clot to form	0.8-1.1	0.97 (0.88, 1.06)	1.02 (0.93, 1.16)
TEG, *n* = 28				
Reaction time (min)	Clot initiation	9-27	12.80 (5.50, 23.10)	31.5 (11.50, 72.60)
P			<.001	
Kinetics (min)	Amplification	2-9	4.80 (1.80, 21.80)	13.60 (7.30, 32.30)[a]
P			<.001	
Angle (°)	Thrombin burst	22-58	36.75 (10.50, 65.00)	13.2 (1.70, 29.10)
P			<.001	
MA (mm)	Overall clot stability	44-64	56.40 (31.00, 73.50)	34.40 (2.50, 57.40)
P			<.001	
G-value (dynes/cm^2)	Shear elastic modulus strength	3.5-8.6	6.45 (2.20, 13.90)	2.60 (0.10, 6.70)
P			<.001	
MRTG (dynes/cm^2/s)	Maximum rate of thrombus generation	0-10	3.02 (0.54, 7.06)	0.83 (0.09, 2.60)
P			<.001	
TMRTG (min)	Time to maximum rate of thrombus generation	5-23	18.71 (7.75, 39.67)	43.42 (19.17, 92.33)
P			<.001	
TTG (dynes/cm^2)	Total thrombus generation	251-1014	688.55 (225.37, 1391.91)	262.63 (13.21, 677.86)
P			<.001	
Run time (min)	Time to test completion	–	44 (30, 69)	70 (32, 99)
P			<.001	
CI	Clotting index	+3 > CI < -3	0.19 (-5.41, 3.05)	-5.92 (-21.89, -0.64)[a]
P			<.001	

aPTT, activated partial thromboplastin time; INR, international normalized ratio; MA, maximum amplitude; MRTG, maximum rate of thrombus generation; PT, prothrombin time; TEG, thromboelastography; TGG, total thrombus generation; TT, thrombin time.
[a]Calculated as *n* = 20 due to missing K values.

2.4 | Sample size and statistical analysis

The sample size of the study was based on the detection of a mean difference of 3 seconds in reaction (R) time between naïve and venom-exposed samples with 90% statistical power. Normal distribution of R time and an SD of 3 and 6 seconds for naïve and venom-exposed samples, respectively, were assumed. In addition, a correlation coefficient of 0.5 between preexposed and postexposed

samples was used. The calculation was based on prior sample size determination that made use of TEG experimental data conducted by Strydom et al. [5] and Chamboko et al. [18].

Basic descriptive statistics for numerical data were used in the data analyses. Normality was established using the Shapiro–Wilk test, and based on this result, the median and minimum and maximum was used for nonparametric data. A Wilcoxon matched-pairs signed-rank test was used to compare the results between the two groups. A 95% CI was used, with $P < .05$. Statistical analysis was conducted using GraphPad10 prism 10.4.1 software.

3 | RESULTS

The 20WBCT revealed a difference in clot formation time between the naïve and venom-exposed groups, with the venom-exposed samples having a prolonged clotting time. There were differences in all conventional coagulation values between venom-exposed and naïve samples. The venom-exposed group demonstrated longer PT, aPTT, and TT values. In addition, venom-exposed samples also showed a decrease in fibrinogen levels. However, all venom-exposed test values were still within accepted normal laboratory ranges. All analyzed viscoelastic parameters in the naive group were significantly different from those of venom-exposed group. In addition, the venom-exposed group indicated a hypocoagulable state. The venom-exposed group demonstrated significantly increased runtime, R-time, K-value, and MRTG values ($P < .0001$). In addition, there was a significant decrease in the angle, kinetics, MA, and time MRTG of the venom-exposed group ($P < .0001$).

In addition to Table, TEG trace graphs in Figure 1 showcases how the typical tracings of the venom-exposed samples compare with a normal tracing. Observations of the morphological studies indicated clear differences between naïve and venom-exposed samples. SEM micrographs of red blood cells (RBCs) extracted from the naïve clot formed during the 20-WBCT depict smooth, biconcave cells indicative of healthy cells with membrane normal integrity (Figure 2A). The slight membrane deformation in Figure 2B is characteristic of normal cell interaction during clot formation. In comparison, RBCs extracted from the venom-exposed 20-WBCT clot showed a significantly high frequency of abnormal cell characteristics. Figure 2C shows bulging blood cells with irregular protrusions on the cell membranes with the loss of cell biconcavity. There was also extensive aggregation of RBCs (Figure 2D).

Figure 3 depicts SEM micrographs taken at a high magnification to analyze cell morphology. Figure 3A, B is representative of normal naïve sample cell morphologies. Red blood cells have a biconcave, discoid shape, and membranes display a slight granularity. In contrast, venom-exposed samples consisted mainly of echinocytes with varying degrees of morphological abnormality (Figure 3C). Cell membranes appear rough and show abnormal characteristics of "pin pricks" (Label 1) and membrane blebbing (Figure 3D).

Figure 4A, B demonstrates the clot network of naïve samples. The dense clot network was composed of organized, branching fibrin fibers and healthy RBCs. Cell membranes maintain their shape during clot formation and interaction with fibrin fibers (Figure 4A). Venom-exposed samples clearly demonstrated a sparse clot network composed of disorganized fibrin fibers interacting with abnormal RBCs. There are visible morphological changes to RBCs and fewer interactions between the cells and fibrin fibers within the clot (Figure 4C, D). The micrographs also show fiber fusion and tangling (Label 2) and a dispersed fibrin fiber network with limited areas of adhesion. In comparison, Figure 4A, B show overall thinner, single fibrin strands. It is not uncommon to see some degree of 'deformation' in control samples. However, when comparing micrograph (Figure 4C, D) with the fibrin tangling (Figure 4A, B) is more pronounced and visible throughout the venom-exposed samples. However, it is also important to note that the venom-exposed fibers are sparser and most that are present have abnormal characteristics.

Figure 5A, B depicts the WB TEG clot network of the naïve sample group. The RBCs of the well-formed clot had minimal deformation and maintained their discoid shape. There was normal interaction between a dense fibrin fiber network and healthy RBCs (Label 3) with evidence of fiber fusion in Figure 5B. In contrast, venom-exposed samples depict abnormal WB TEG clot networks where the majority of RBCs are round and small with irregular protrusions on the cell membranes (Figure 5C). The fibrin fiber network appears broken with sparse, thin fiber strands (Label 4) with cleaved ends. Although the clot is dense, the fibrin fiber network showed decreased fiber crosslinking and aggregation of abnormal cells.

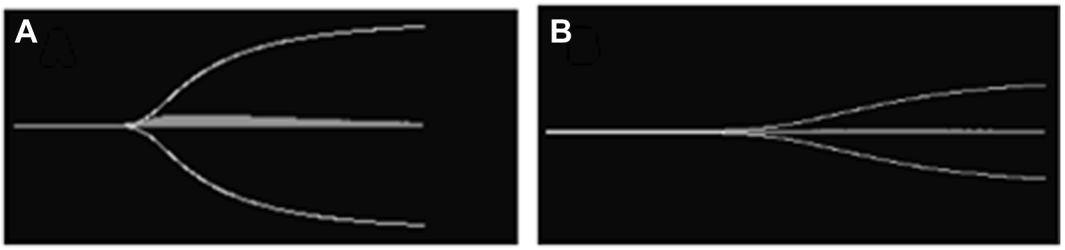

FIGURE 1 Typical thromboelastography trace graphs of (A) naïve sample groups and (B) *Bitis arietans* venom–exposed group.

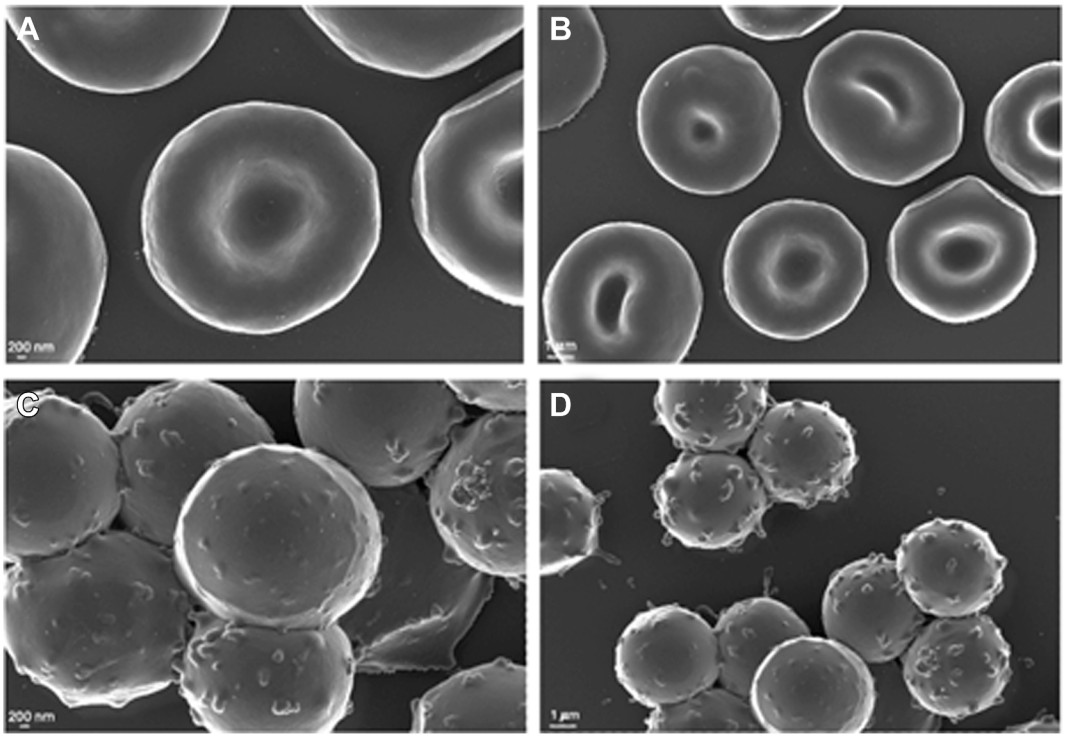

FIGURE 2 Scanning electron microscopy micrographs depicting red blood cells (RBCs) extracted from the clots formed during the 20-minute whole blood clotting test, before and after exposure to *Bitis arietans* venom. (A) Healthy RBCs with smooth cell membranes with only deformation caused by normal cell interaction during clot formation (B). (C) Overexposed, bulging RBCs with irregular protrusions on the cell membranes. (D) More aggregation of cells in venom-exposed group.

4 | DISCUSSION

This study aimed to expand on the understanding of the effects of *B arietans* venom on coagulation with a focus on comparing the 20-WBCT, point-of-care coagulation tests, and current clinical laboratory tests when assessing envenomation. This was achieved by exploring clot formation and kinetics, coagulation parameters, RBC morphology, and formed clots in venom-exposed samples. The toxic protein components found in *B arietans* that are known to be responsible for causing physiological and biochemical harm include snake venom serine proteases (SVSPs), snake venom metalloproteases (SVMPs), C-type lectin–like proteins (CTLs) proteins, phospholipases (PL)A_2, L-amino acid oxidases, and disintegrins (DISs) [5,6].

The prolonged clotting time seen in the 20-WBCT of exposed samples compared with naïve samples indicates the presence of toxins to prevent/disrupt coagulation. SVMPs and SVSPs are present in *B. arietans* venom in large concentrations, accounting for approximately 50% of dried venom weight and could contribute to coagulopathy [19]. SVMPs are Zn^{2+}-dependent, endoproteolytic enzymes that aid in the digestion of prey and affect various physiological aspects of cell function [20]. The enzymes selectively cause fibrin degradation and fibrinolysis, contributing to coagulopathy and more specifically, clot instability [20]. Additionally, SVSPs are enzymes that have been observed to exhibit fibrinogenolytic activities analogous to thrombin [21,22]. The toxins are the second most abundant compound in *B. arietans* venom and act by cleaving both α and β chains of fibrinogen, preventing fibrin formation and thus solid clot formation [19]. These proteases reduce fibrin fiber production and disrupt fibrin crosslinking, prolonging clot formation and decreasing overall clot strength.

In addition, fibrinogenolytic enzymes, like metalloproteinases in *B. arietans* venom, do not completely destroy fibrinogen but instead degrade it so that kinetics are compromised via poor preliminary polymerization and incomplete fiber crosslinking. The SEM micrographs of the venom-exposed samples indicate this phenomenon very well. This is in contrast to the effects of the Malayan Pit Viper and Eastern diamondback rattlesnake venoms, which are composed of well-characterized serine proteases that polymerize and remove fibrinogen from blood plasma [23,24].

Clot formation is dependent on the activation of the coagulation cascade to ultimately form a fibrin-rich platelet plug that prevents blood flow [25]. The longer clotting time seen in the PT and aPTT of exposed samples compared with naïve samples indicates the presence of toxins that disrupt normal coagulation. Kitchens and Van Mierop [26] conducted a retrospective chart review of patients admitted to a U.S. hospital for rattlesnake envenomation. Envenomation by North American rattlesnakes is known to result in fibrinogenolysis and thrombocytopenia, prolonging clinical coagulation tests such as PT and aPTT [26]. Since

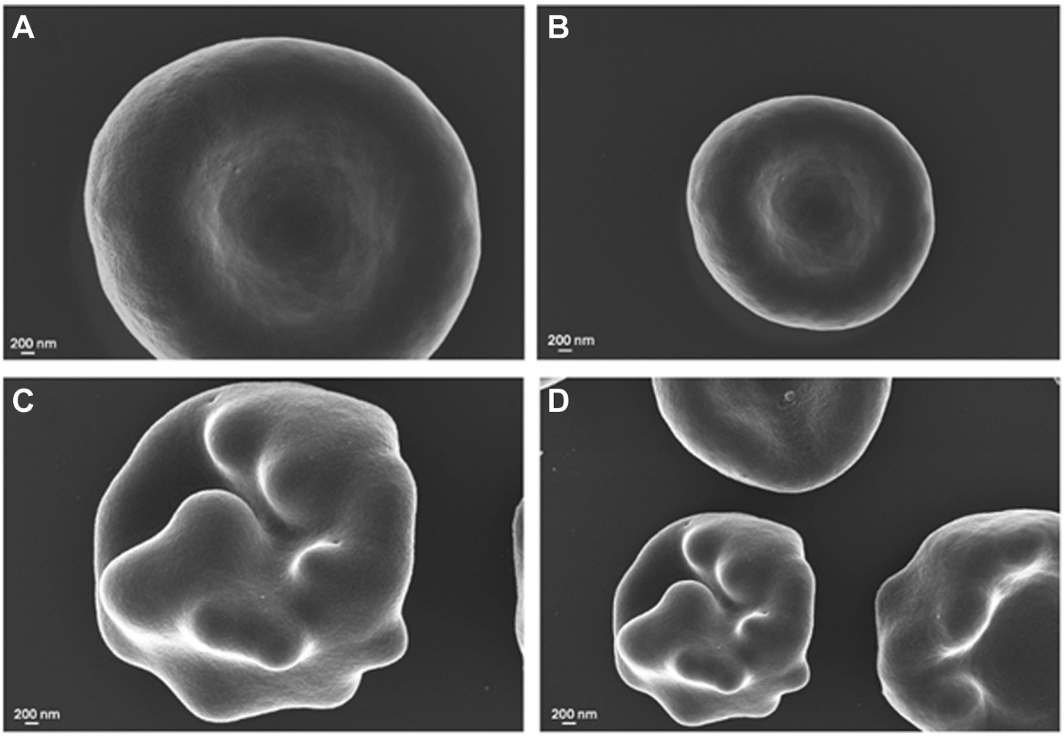

FIGURE 3 Scanning electron microscopy micrographs depicting whole blood before and after exposure to *Bitis arietans* venom showing cell membrane damage. (A, B) Healthy, round red blood cell with a central indentation and smooth membrane. (C, D) Venom-exposed echinocytes with varying degrees of morphological abnormality. Cell membranes show abnormal characteristics of pin pricks (Label 1) and membrane blebbing.

rattlesnakes form part of the Viperidae family, their venom composition consists of toxic proteins, which are similar to *B. arietans* venom, like CTLs and DIS [27]. CTLs have been described that exhibit a number of functional activities relating to haemostasis, including binding to factor IX and X to inhibit blood coagulation and inhibiting the binding of thrombin to fibrinogen [7]. Lastly, DIS are strong inhibitors of platelet aggregation by binding to glycoprotein IIb/IIIa platelet fibrinogen receptor, thereby preventing fibrinogen binding to platelets [28].

According to World Health Organization snakebite management and treatment guidelines, the 20-WBCT is considered to be an inexpensive and reliable method in detecting early systemic envenomation, together with PT, aPTT, and INR in cases of hematotoxic snake bites [3].

In this study, TEG provided insights into the effects of *B. arietans* venom on clot kinetics. Analysis of the venom-exposed TEG results revealed overall hypocoagulation, characterized by delayed clot initiation, reduced clot strength, decreased clot size, and impaired clot stability. In a study by Kang and Fisher [29] exploring the fibrinogenolytic and thrombocytopenic effects of North American rattlesnake envenomation, the TEG data indicated impaired hemostasis, with low clot stability (MA) and decreased rates of clot formation. The most obvious features observed in venom-exposed TEG clot micrographs were deeper clot concavities with a clear decrease in clot thickness and the presence of many cleaved fiber ends. This pattern of degradation is consistent with investigations conducted by Veklich et al. [30] on fibrin clots incubated with plasmin, a key fibrinolytic enzyme.

In the venom-exposed samples, echinocytes (Figure 3C) and membrane blebbing (Figure 3D) were the most prevalent morphological abnormalities observed. These findings were consistent with that of Strydom et al. [5], whose study investigated the effects of *B. arietans* venom on RBC ultrastructure. Snake venom PLA_2 are cytotoxic compounds that are known to trigger eryptosis [31]. The toxins cause RBC membrane destabilization by hydrolyzing and destroying membrane phospholipids [31,32]. The more extreme RBC abnormalities observed by Strydom et al. [5] compared with this study may be a result of the use of venom in its natural raw state (at a higher concentration) instead of a lyophilized form. Additionally, Pretorius et al. [33] investigated the effects of heavy metals on RBCs and fibrin networks of WB. SEM micrographs revealed echinocyte formation and membrane instability due to oxidative stress, generated by heavy metal exposure [33]. The predominant altered RBC shape observed was echinocytes with the formation of nodule-like projections [33]. The morphological observations that resemble Pretorius et al. [31] findings in this study may be a result of the presence of L-amino acid oxidase. Although the mechanism of toxicity is poorly understood, LAAOs are presumed to mediate cytotoxicity by causing direct deterioration of cell membranes due to reactive oxygen species and hydrogen peroxide (H_2O_2)

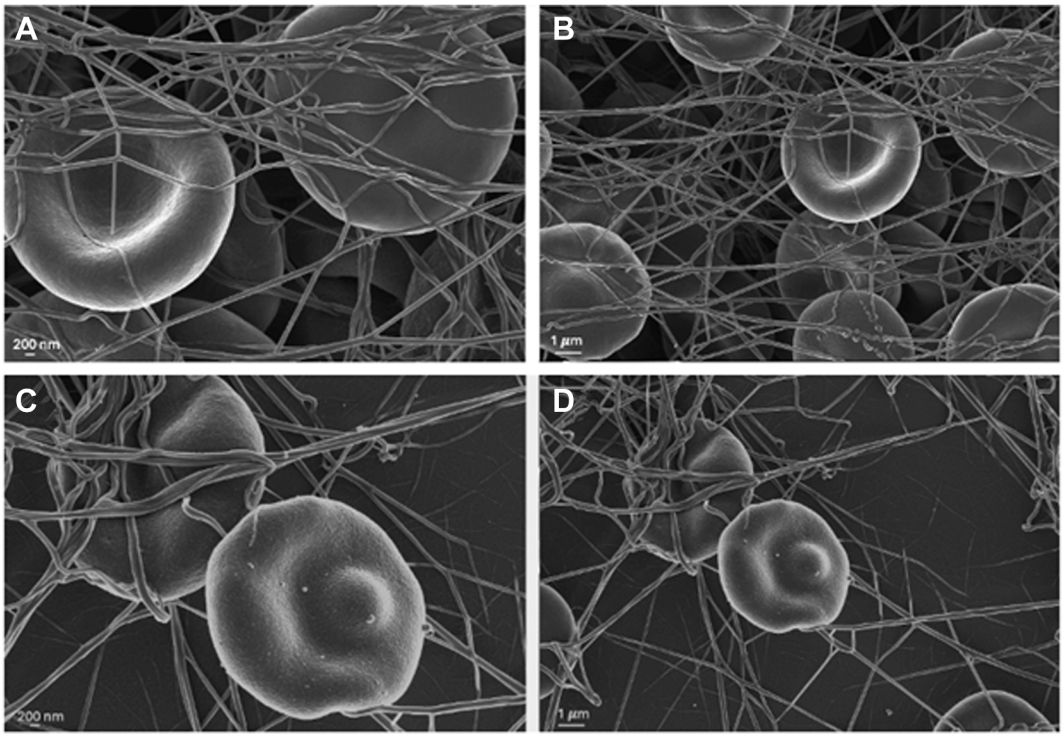

FIGURE 4 Scanning electron microscopy micrographs depicting whole blood, before and after exposure to *Bitis arietans* venom, with the addition of thrombin in order to demonstrate interactions between red blood cells (RBCs) and fibrin networks within the blood clot. Micrographs (A) and the clot network (B) composed of dense fibrin fibers and healthy RBCs of the naive group. Cell membranes maintain their shape during clot formation and interaction with fibrin fibers. (C) Abnormal cell shape and membranes of venom-exposed RBCs. (D) Dispersed fibrin fiber network with fiber fusion and tangling (Label 2).

accumulation [31]. This oxidative stress results in cell membrane instability and RBC eryptosis [31].

An important finding to note is that although the 20-WBCT assay revealed a prolonged clotting time in venom-exposed samples, all samples produced a solid clot (that did not break apart after inversion) within the accepted 20-minute period and would be considered normal in a clinical setting. In addition, PT and aPTT assays also revealed prolonged clotting times in venom-exposed samples; however, data values were within accepted normal laboratory ranges. Standard coagulation assays used clinically did not show deviation from the reference intervals during venom exposure. In contrast, the SEM morphology and some TEG parameters were able to indicate the changes the venom introduced on the blood. These findings were similar to that of Nag et al. [14] when monitoring the clinical management of a viper snakebite patient in rural India. Upon admission, the patient's 20-WBCT was considered normal and initial laboratory coagulation status (PT and aPTT) was also within accepted ranges [14]. After 12 hours, the patient's TEG graph showed severe hypocoagulability, whereas PT and aPTT were prolonged but still within the normal range [14]. The patient was admitted to a critical care unit and received antivenom treatment, highlighting that although the gold standard envenomation tests indicated normal coagulation, lifesaving intervention was necessary. In this study, conventional coagulation assays indicated similar results (within normal range) to the 20-WBCT; however, inconsistent with the TEG. The TEG results and accompanying SEM micrographs show detailed and informative abnormalities after 10 minutes of venom exposure. Incorporating and considering laboratory parameters, including PT, aPTT, and fibrinogen, in the study, is still appropriate since they are recommended as standard measures for evaluating patients with snakebite [34,35]. These biomarkers are also incorporated into snakebite severity scoring systems [36].

In contrast to this study, Costa et al. [8] and Lamb et al. [37] concluded the 20-WBCT was a highly specific and a fairly sensitive point-of care clotting test. This inconsistency with this study's results could be due to the sample incubation time with the venom or that this study was conducted *ex vivo*. However, both SEM and TEG results of the venom-exposed samples in this study indicate RBC membrane abnormalities, fibrin network disruptions and hemostatic disturbances associated with coagulopathy. In addition, SEM micrographs of the 20-WBCT imply that after 20 minutes, there are already significant changes in RBC structure and morphology despite normal clot formation before the 20-minute period has lapsed. Additionally, it highlights the need for developing alternative diagnostic tools that provide more accurate, reliable results in a short period as well as the need for further investigation into 20-WBCT efficacy and whether adjustments need to be made to the test parameters.

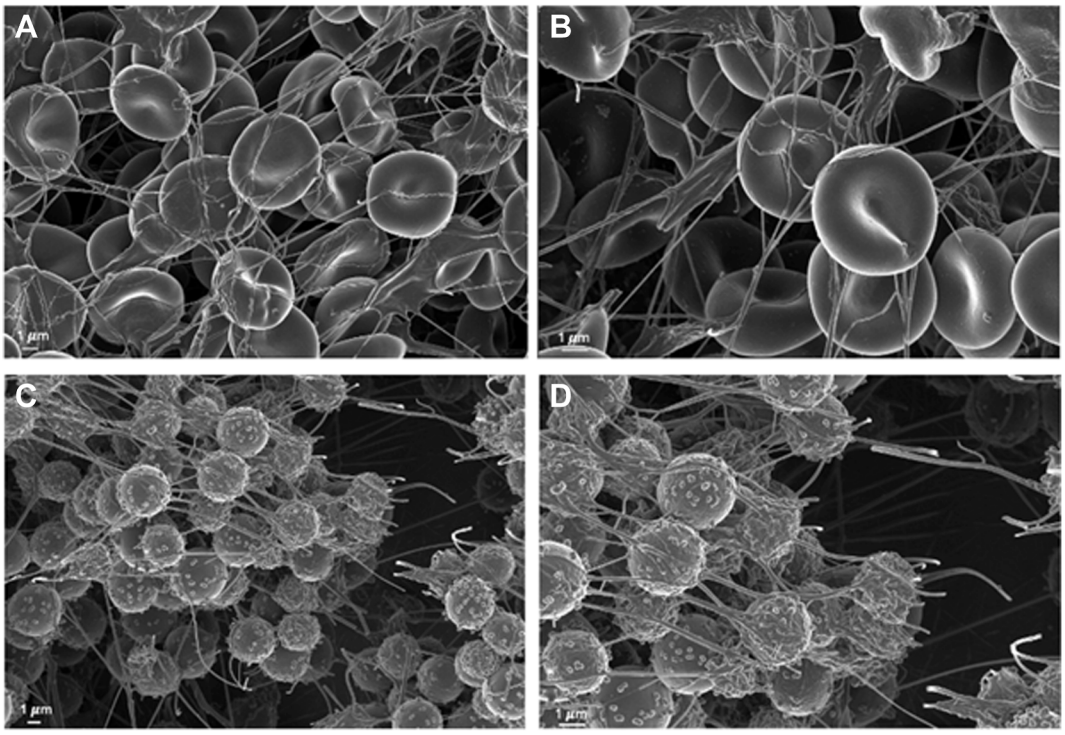

FIGURE 5 Scanning electron microscopy micrographs depicting whole blood thromboelastography (TEG) clots, before and after exposure to *Bitis arietans* venom. (A, B) Micrographs show the well-formed TEG clot of the naïve group, consisting of normally shaped RBCs interacting with fibrin fibers. There is some evidence of RBC membrane deformation and fibrin fiber fusion (Figure 5B). Label 3 indicates normal interaction between fibrin fiber network and RBCs. (C, D) Micrographs show the abnormal TEG clot of the venom-exposed group, consisting of abnormal RBCs interacting with partially formed fibrin fibers. (D) Fibrin fiber network appears broken with thin fiber strands (Label 4).

5 | CONCLUSION

This study emphasizes the hypocoagulable effects of *B. arietans* venom, as demonstrated by the clinical and laboratory-based results and imaging. These changes may be associated with the venom's components. Prolonged clotting time/initiation, observed in the 20-WBCT and conventional coagulation tests, could be influenced by SVSPs and SVMPs. Additionally, disruption of stable clot formation, observed in TEG results, could be influenced by CTL proteins and DIS. The proeryptotic mechanisms of PLA_2, L-amino acid oxidases could result in the presence of echinocytes observed in SEM micrographs. The venom exposure time of the SEM micrographs was the same as the coagulation tests (10 minutes) and was less than the 20-WBCT (20 minutes). SEM micrographs indicated altered clot structure and changes in RBC morphology and membrane ultrastructure. This disconnect highlights that although the 20-WBCT is considered effective, it loses efficacy at lower venom concentrations and information that could provide clinicians with life-saving information in time-sensitive situations. The TEG data, which were consistent with the SEM results, show this diagnostic limitation and emphasize the disadvantages in resource-constrained facilities.

This study provides a necessary comparison between clinical and laboratory analysis of *B arietans* venom and provide insight into the diagnostic limitations of the 20WBCT, 20-minute test window when other clinical tests are unavailable. Further research could focus on studying the effect of different exposure concentrations on the clotting times and the development of a more robust point-of-care snakebite test to aid in the management of this neglected tropical disease.

5.2 | Limitations

Although participants were recruited at random, the median age of the study participants was 24 years. Future studies could include focusing on an older or more specific age range. In addition, several datasets within the study contained missing data. This was addressed by applying pairwise deletion to maintain the analytical consistency throughout the study. For this study, the TEG was terminated after MA was achieved, instead of after fibrinolysis, meaning only clot formation was studied. *B. arietans* snake venom is composed of various fibrinolytic toxins, and analyzing these effects could be helpful in creating venom profiles. Lastly, additional experiments using platelet-inhibited plasma or isolated plasma components would be useful in providing further insight into the specific effects on fibrinogen polymerization and platelet function.

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ETHICS STATEMENT

This study was approved by the Research Ethical Committee and the Animal Ethics Committee of the University of Pretoria (ethics clearance number: 301/2024). Permission to do research in terms of Section 20 of the Animal Diseases Act, 1984 (Act no 35 of 1984) was obtained. Written informed consent was obtained from all healthy participants (available upon request). All blood collection and methods were carried out in accordance with the approved guidelines of the ethics committee.

AUTHOR CONTRIBUTIONS

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

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

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