Reversible and transient thrombocytopenia of functional platelets induced by nose-horned viper venom

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Letter to the Editors-in-Chief

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In Slovenia *Vipera b. berus* (*Vbb*) and *Vipera a. ammodytes* (*Vaa*) are the only medically important venomous snakes. The most common laboratory findings in *Vaa* envenomation are mild leukocytosis and severe thrombocytopenia [1]. The latter can be rapidly reversed with an antivenom raised against the whole *Vaa* venom. The absence of clinically significant bleeding in *Vaa*-envenomed patients suggests that platelet function is preserved despite transient and severe thrombocytopenia [2].

Overall platelet function can be assessed by rotational thromboelastometry, as only normal platelet count and function lead to normal values, and by the aggregometry assay, which detects platelet function triggered *via* the thrombin receptor without triggering fibrin formation [3].

Only platelets with preserved function are able to activate, which is a pivotal event in haemostasis. The process of platelet activation and degranulation is irreversible and is accompanied by distinct morphological changes [4]. Activated platelets can be recognised by flow cytometry by the expression of P-selectin (CD62P) on the surface of their plasma membrane, as it is rapidly translocated from the α-granules of platelets to the cell surface upon stimulation and activation [5].

According to PubMed search, there is no clinical study that has investigated platelet function in *Vaa*-envenomed patients with thrombocytopenia. The aim of this work was to evaluate platelet function in *Vaa*-envenomed patients with thrombocytopenia using thromboelastometry and aggregometry assays as well as by flow cytometry.

This was a prospective study of consecutive *Vaa*-envenomed patients with thrombocytopenia and treated with Viperfav ($F(ab')_2$ fragments) at the government University Medical Centre Ljubljana. Cases were included only if they had thrombocytopenia ($< 150 \times 10^9$/L) and *Vaa* envenomation was confirmed by quantification of *Vaa* venom in the patient's serum by ELISA assay [2].

The clinical picture and laboratory results (myoglobin, creatine kinase, creatine, troponin I, liver tests, prothrombin time (international normalized ratio), activated partial thromboplastin time, D-dimer, fibrinogen, platelet count, leukocyte count, lactate), rotational thromboelastometry, aggregometry assays and flow cytometric analysis were evaluated at admission before antivenom therapy and again after antivenom application. Patients received a dose of 4 mL of Viperfav ($F(ab')_2$ fragments at a concentration of 99–117 mg/mL intravenously over 60 min.

The citrated whole blood samples were used for rotational thromboelastometry with the ROTEM® (Pentapharm, Munich, Germany). Multiple electrode aggregometry was performed with the Multiplate® analyzer (Roche Diagnostics, Mannheim, Germany). The agonists TRAP-6 (thrombin receptor activating peptide, TRAPtest) was used for quantitative *ex vivo* determination of platelet function after antivenom therapy.

Changes in platelet activation were assessed by flow cytometry (Navios, Beckman Coulter, USA). The fresh citrated whole blood samples were centrifuged to obtain platelet rich plasma. Platelet concentration was set to 20×10^9/L using PBS. Platelets were incubated with monoclonal antibody, *i.e.* FITC-labelled CD62P (P-selectin) (Beckman Coulter, Brea, California, USA) as a marker for platelet activation. Human P-selectin (granule membrane protein 140) is found in intracellular granules and is released after platelet activation. It can be used as a marker for platelet activation in general and platelet secretion in particular. The percentage of expression of the antigen was determined using an appropriate isotype control. The percentage of platelets expressing P-selectin can be up to 10 % in healthy volunteers [6,7].

Data are reported as median and interquartile range (IQR) for continuous variables and frequency (percentage) for categorical variables. The Wilcoxon test was used to analyze differences. A p value of <0.05 was considered significant.

Nine *Vaa*-bitten patients with severe thrombocytopenia were included. *Vaa* envenomation was confirmed by determination of *Vaa* venom in serum (median value: 54.3 ng/mL; range 2.6–154 ng/mL). Their general characteristics are listed in Table 1.

On admission, a profound thrombocytopenia (37×10^9; 18–90 $\times$ 10^9/L) was observed in all patients. Only two patients had laboratory signs of coagulopathy with a slightly decreased prothrombin time (PT) (0.47 and 0.48 s) and decreased activated partial thromboplastin time (aPTT) (22.5 s) (Table 2). Fibrinogen levels were normal in all patients (Table 2). Mild rhabdomyolysis was present in two patients whose serum myoglobin levels were only three times the upper normal value (Table 1).

Rotational thromboelastometry assays were available for all patients. On admission, the clot formation time (CFT) was abnormally prolonged for EXTEM and INTEM (289 s (165–413 s) and 308 s

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Table 1
General characteristics of nine *Vaa*-envenomed patients with severe thrombocytopenia on admission at the Emergency Department ($n = 9$ patients).

Male	7/9 (78 %)
Age (median, IQR) (year)	67 (27.5–74.5)
Bite location	
Arm	7/9 (78 %)
Leg	2/9 (22 %)
Venom concentration (median, IQR) (ng/mL)	54.3 (35.2–107.3)
Local pain	9/9 (100 %)
Local oedema	9/9 (100 %)
Ecchymosis	6/9 (67 %)
Nausea	6/9 (67 %)
Vomiting	5/9 (56 %)
Dizziness	3/9 (33 %)
Syncope	0/9 (0 %)
Cranial nerve palsies	0/9 (0 %)
Tachycardia	1/9 (11 %)
Hypotension	3/9 (33 %)
Shock	0/9 (0 %)
Rhabdomyolysis	2/9 (22 %)
Acute renal failure	1/9 (11 %)
Acute respiratory failure	0/9 (0 %)
Acute myocardial injury	0/9 (0 %)
Death	0/9 (0 %)

Table 2
Platelet count, rotational thromboelastometry, aggregometry assays, flow cytometric analysis of platelets and coagulation parameters before and after administration of the antivenom in *Vaa*-envenomed patients. Analyses were performed at a median of 1.0 h (1.0–4.5 h) after antivenom administration.

Parameter (units; reference range)	On admission	After antivenom	p
Platelet count ($\times 10^9$/L) (150–410)	37 (18–90)	158 (145–169)	0.01
Rotational thromboelastometry			
Extem			
CT (s; 35–80)	94 (78–142)	61 (55–72)	0.01
CFT (s; 35–160)	289 (165–413)	103 (82–113)	0.01
MCF (mm; 53–72)	39 (36–49)	57 (56–61)	0.01
Intem			
CT (s; 100–240)	159 (132–183)	145 (123–161)	0.37
CFT (s; 35–110)	308 (158–450)	90 (73–107)	0.01
MCF (mm; 53–72)	36 (34–49)	54 (53–60)	0.01
Aggregometry			
TRAPtest (U; 86–159)	NA	128 (114–132)	NA
Flow cytometric analysis of platelets			
Platelets with expressed P-selectin (%)	0.5	0.5–1.3	NA
Coagulation parameters			
Prothrombin time (s; 0.7–1.3)	0.76 (0.59–0.85)	0.81 (0.62–0.86)	0.11
Activated partial thromboplastin time (s; 25.9–36.6)	27.9 (26.0–29.8)	30.1 (25.1–32.0)	0.51
Fibrinogen (g/L; 1.8–3.5)	2.8 (2.3–3.5)	2.8 (2.3–3.3)	0.68
D-dimer (μg/L; <500)	2615 (775–8526)	2653 (436–17,215)	0.09

Legend: CT - clotting time, CFT- clot formation time, MCF - maximum clot firmness, TRAP - thrombin receptor activating peptide.

(158–450 s), respectively), and maximum clot firmness (MCF) was abnormally low for EXTEM and INTEM (39 mm (36–49 mm) and 36 mm (34–49 mm), respectively) (Table 2). The initial INTEM and EXTEM CFT and MCF abnormalities indicated poor clot formation and weak clot firmness due to thrombocytopenia and possible platelet dysfunction in the absence of fibrinogen deficiency.

Patients were treated with one intravenous dose of $F(ab')_2$ fragments raised against the whole viper venom. Thrombocytopenia was reversed in all patients (158×10^9; 145–169 $\times 10^9$/L) within a median time of 1 h after one dose of $F(ab')_2$ fragments. Rotational thromboelastometry, including CFT for EXTEM and INTEM (103 s (82–113 s) and 90 s (73–107 s), respectively), and MCF for EXTEM and INTEM (57 mm (56–61 mm) and 54 mm (53–60 mm), respectively), returned to within normal limits 1 h after $F(ab')_2$ fragments administration, indicating preserved platelet function after profound and transient/reversed thrombocytopenia (Table 2).

The aggregometry TRAPtest was within normal limits (128 U; 114–132 U) after administration of the antivenom and reversal of thrombocytopenia and also showed preserved platelet function (Table 2).

Flow cytometry of the patients' platelet-rich plasma samples was performed in only three *Vaa*-envenomed patients. The other 6 snakebites occurred in the afternoon and on weekends when flow cytometry was not available. In the first patient with thrombocytopenia, flow cytometry of platelets was performed only on admission, revealing 0.5 % of platelets expressing P-selectin. In the second patient with thrombocytopenia, flow cytometry of platelets was conducted on admission and after reversal of thrombocytopenia with antivenom and showed 0.5 % and 1.3 % of platelets expressing P-selectin, respectively. In the third patient with thrombocytopenia, flow cytometry of platelets was performed only after reversal of thrombocytopenia with antivenom and revealed 0.6 % of platelets expressing P-selectin (Table 2).

In this study, we have shown that *Vaa* envenomation causes profound and transient thrombocytopenia with functional platelets after reversal of thrombocytopenia. Platelet function was not impaired after *Vaa* venom-induced thrombocytopenia, as thromboelastometry, which provides information on the overall kinetics of haemostasis (clot formation and clot stability), was within normal limits after reversal of *Vaa* venom-induced thrombocytopenia. The preserved platelet function was also confirmed by aggregometry, which was normal after reversal of *Vaa* venom-induced thrombocytopenia, as the aggregometry results depend on platelet function only when the platelet count is within the normal range [3]. Furthermore, only 0.5–1.3 % of platelets had exposed P-selectin on their surface, a widely used marker of platelet activation, despite the observed thrombocytopenia with a five-fold drop in platelet count. The results of flow cytometry indicate that platelets were not activated and were most likely functional even during thrombocytopenia prior to antivenom administration. Although reversible inhibition of platelet activation during thrombocytopenia by unknown venom components could not be completely ruled out in this study due to limitations in assessing platelet function by aggregometry, the absence of identified inhibitors of platelet activation by the *Vaa* venom proteome studies provides additional support for platelet functionality during thrombocytopenia [8].

The mechanism of thrombocytopenia in *Vaa* envenomation remains unclear. Pseudo-thrombocytopenia or analytical error due to possible *in vitro* formation of aggregates within a tube was excluded [2]. The possibility of an immunological mechanism was rejected by performing anti-platelet antibody tests [2]. Other possible causes of thrombocytopenia, such as significant local tissue damage with coagulopathy or sequestration of platelets at the site of envenomation onto the exposed and damaged endothelium, are unlikely, as no consumption of clotting factors and no mechanical damage to the red blood cells due to microangiopathic changes leading to the formation of schistocytes were detected [2]. This is consistent with the results of this study, as only two out of nine patients with thrombocytopenia had mild local rhabdomyolysis. Venom-induced consumptive coagulopathy (VICC) or even disseminated intravascular coagulation (DIC) with thrombocytopenia were excluded by normal thromboelastometry and aggregometry after reversal of thrombocytopenia, despite elevated D-dimer and slightly decreased PT and aPTT in two patients. Furthermore, in this study, only 1 % of platelets with expressed P-selectin excluded VICC and DIC. In sepsis, up to 40 % of platelets had an increased level of surface P-selectin and 18 % of platelets were P-selectin positive in septic patients with thrombocytopenia [6,7]. However, the different results of CT between the INTEM assay (normal CT) and the EXTEM assay (prolonged CT)

during thrombocytopenia suggest involvement of the extrinsic coagulation pathway in *Vaa*-envenomation.

The most probable mechanism of thrombocytopenia induced by *Vaa* venom appears to be platelet agglutination. Several studies on *Viperidae* venom have already pointed to platelet agglutination as a possible cause of thrombocytopenia and to snaclecs as the most likely venom components responsible for this effect [9,10]. Platelet aggregation is less likely after *Vaa* envenomation, since aggregation follows platelet activation, which was not demonstrated in this study. Furthermore, *Vaa* venom does not contain any known agonists of platelet aggregation [8].

On the other hand, it was recently shown in a proteomic study that the amount of snaclecs in *Vaa* venom is high [8]. It was also shown that thrombocytopenia caused by *Vaa* venom could be rapidly and completely reversed by an antivenom raised against whole *Vaa* venom, but not by an antivenom raised against *Vbb* venom, which contains only a small amount of snaclecs [1,8]. Therefore, *Vaa* snaclecs-induced agglutination with thrombocytopenia is very likely and should be further investigated. The antivenom raised against whole *Vaa* venom should also effectively neutralize any potential inhibitors of platelet activation that might be present during thrombocytopenia in *Vaa* envenomation, as platelets were functional after reversal of thrombocytopenia with the antivenom.

The finding of transient and rapidly reversible thrombocytopenia without clinically significant platelet activation in this study may stimulate the exploration and isolation of antiplatelet compounds from *Vaa* venom. Novel antiplatelet agents may be useful in procedures where only a transient reduction in platelet count is required without clinically significant platelet activation and aggregation.

In conclusion, *Vaa* envenomation causes profound and transient thrombocytopenia with functional platelets after reversal of thrombocytopenia, probably due to their agglutination by the action of *Vaa* venom components.

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Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Slovenian National Medical Ethics Committee (No. 87/07/15 and No. 0120-546/2017/5).

CRediT authorship contribution statement

MDB, MB and IK conceived and designed the study; MDB, MB and DG treated the patients; TK and BH measured venom in blood samples; RK and HP performed flow cytometry; MDB, AL and MB analyzed the data; IK, HP and BH contributed reagents/materials/analysis tools; MDB, IK and MB wrote the paper. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known conflict of interests or personal relationships that could have appeared to influence the work reported in this paper.

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