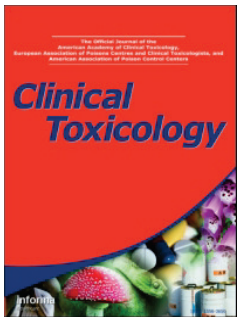

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CLINICAL RESEARCH

Vipera ammodytes bites treated with antivenom ViperaTAb: a case series with pharmacokinetic evaluation

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ABSTRACT

Context: In clinical practice it is difficult to differentiate between *V. berus* and *V. ammodytes* venomous bites. In the past this was not a concern, but due to the current shortage in Viperfav™ and European viper venom antiserum availability, *V. a. ammodytes* venomous bites have recently been treated with ViperaTAb®, which is a pharmaceutical formulation containing a monospecific ovine Fab fragments against the venom of *V. berus*.

Objective: To evaluate ViperaTAb® in *V. a. ammodytes* envenomations.

Materials and methods: This is a prospective case series of three consecutive patients envenomed by *V. a. ammodytes* snakebite treated with ViperaTAb®. *V. ammodytes* venom, neurotoxic ammodytoxins, and Fab fragment levels were determined in serum samples and a pharmacokinetic analysis of the antivenom Fab fragments was carried out.

Results: Three patients bitten by *V. a. ammodytes* with extensive local swelling, neurological symptoms and recurrent thrombocytopenia were treated with ViperaTAb®. *V. ammodytes* venom was detected in serum of all three patients. Ammodytoxins were detected in the serum of only the most severely envenomed patient who developed neurological symptoms. In the presented moderate cases, a dose of 8 mL of ViperaTAb® reduced swelling and improved systemic effects, such as thrombocytopenia. However, this dose of ViperaTAb® was not effective in the most severely envenomed patient with the highest serum values of *V. ammodytes* venom. In this case ViperaTAb® did not stop local swelling and it had no effect on neurological signs. ViperaTAb®'s systemic clearance, distribution and elimination half-lives were 4.3–13.4 mL/h/kg, 1.2–3.2 h and 14.1–55.4 h, respectively.

Conclusions: In patients envenomed by *V. a. ammodytes* venom, ViperaTAb® reduces moderate swelling and temporarily improves systemic effects, except neurological symptoms. ViperaTAb® application induces a decrement of *V. ammodytes* venom level in the blood, but did not affect serum concentration of neurotoxic ammodytoxins in the one patient with measurable concentrations.

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V. a. ammodytes; nose-horned viper; ViperaTAb; Fab fragments; pharmacokinetics

Introduction

In Slovenia *Vipera b. berus* (*Vbb*) and *Vipera a. ammodytes* (*Vaa*) are the only medically important venomous snakes [1–4]. In clinical practice it is difficult to differentiate between *Vbb* and *Vaa* venomous bites. However, in the 15% of cases with neurological signs, a clinical diagnosis of *Vaa* bite can be made since ammodytoxins (Atxs), the main neurotoxins of *Vaa* venom, are not present in *Vbb* venom [4]. Local and systemic symptoms are usually more pronounced in the cases of *Vaa* envenomation; however, the clinical picture may depend on the patient's age, body mass, co-morbidities, site of the bite, and amount of injected venom. Accordingly, the differentiation between *Vbb* and *Vaa* venom envenoming based on clinical presentation is very difficult and unreliable.

In the past this was not a concern, since *Vbb* and *Vaa* snakebites were successfully treated with Viperfav™ (Aventis Pasteur, MSD, Lyon, France), a formulation containing polyvalent equine $F(ab')_2$ fragments as an active principle against venoms of *V. aspis*, *V. berus* and *V. ammodytes* [5], or European viper venom antiserum (Zagreb antivenom) (Institute of Immunology Inc., Zagreb, Croatia), a formulation containing monospecific equine $F(ab')_2$ against *V. ammodytes* venom [2,3]. European viper venom antiserum was clinically effective also in paraspecific neutralisation of several venoms from viperid snakes closely related to *Vaa* – *V. aspis, V. berus, V. xanthina* and *V. lebetina*. However, due to a current shortage in Viperfav™ and European viper venom antiserum availability, *Vbb* and *Vaa* venomous bites have recently been treated with ViperaTAb® (MicroPharm Limited, Newcastle Emlyn, UK), which is a pharmaceutical formulation containing monospecific ovine Fab fragments against the venom of *V. berus* [6].

The use of monospecific *V. berus* antivenom in *Vaa* bites might be insufficient since it was recently shown that the proteome of *Vbb* venom is much less complex than the

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venom of *Vaa*. In particular, it contains lower levels of snake c-type lectin-like proteins (snaclecs) and no Atxs, neurotoxic-secreted phospholipases A_2 ($sPLA_2$s) [4]. Atxs are responsible for the most characteristic feature of *Vaa* venom envenoming – induction of neurotoxic signs in patients, while snaclecs are probably responsible for thrombocytopenia [4].

However, *in vitro* immunological experiments revealed that the anti-*V. berus* antivenom ViperaTAb® exhibits substantial cross-reactivity with the venom of other *Vipera* snake species, including *V. ammodytes* [6]. In addition, *in vivo* preclinical efficacy studies demonstrated that ViperaTAb® effectively neutralises lethality induced by *V. berus* and *V. ammodytes* and at much higher levels than those outlined by regulatory pharmacopoeial guidelines and by the other antivenoms [6]. However, there is no clinical report of monospecific *V. berus* antivenom usage in the patient bitten by *Vaa*.

In this case series we describe patients envenomed by *Vaa* snakebite treated with ViperaTAb® and pharmacokinetics of the antivenom Fab fragments.

Materials and methods

Patients

This is a prospective case series of consecutive patients envenomed by *Vaa* snakebite and treated with ViperaTAb® at the Emergency Department and Centre for Clinical Toxicology and Pharmacology at the government University Medical Centre Ljubljana in 2015. The hospital is a tertiary referral center in the Slovenian capital city of Ljubljana, serving a local population of 400,000 inhabitants and a national population of two million.

The study was approved by the Slovenian National Medical Ethics Committee (No. 87/07/15).

Reagents and chemicals

Horseradish peroxidase-conjugated rabbit anti-guinea pig IgG (HRP-anti-guinea pig IgG), horseradish peroxidase-conjugated rabbit anti-equine IgG (HRP-anti-equine IgG) and horseradish peroxidase-conjugated rabbit anti-equine $F(ab')_2$ (HRP-anti-equine $F(ab')_2$) were from Bio-Rad Laboratories, USA. Horseradish peroxidase-conjugated rabbit anti-ovine IgG (HRP-anti-ovine IgG) was from antibodies-online.com, USA. Bovine serum albumin (BSA), Tween 20 and *o*-phenylendiamine dihydrochloride (OPD) were from Sigma-Aldrich, USA. Chemicals for buffers and solutions were from Kemika, Croatia.

Snake venoms and antivenoms

Venoms were collected by manual method of milking snakes. Snakes (*V. ammodytes ammodytes*) were collected in different geographical regions of Croatia and kept in serpentarium (Institute of Immunology, Inc., Zagreb, Croatia) for several years according to good manufacturing and veterinary practice. Approximately, 100 snakes, both male and female, over 3-year-old, per one venom batch were used. Immediately after milking, venom batch was dried in vacuum desiccator with a silica gel at room temperature. After venom crystallisation, obtained venom was kept in dark bottle and stored in the dark at 2–8 °C until used. All venom batches were tested for biochemical properties (protein content and analysis by SDS-PAGE and HPLC) and biological activity (lethal dose (median lethal dose, LD_{50}), haemorrhagic (minimum haemorrhagic dose – MHD) and necrotic (minimum necrotic dose – MND) activity) [7–9]. Recombinant AtxA used as standard in ELISA assay was produced as described [10]. ViperaTAb® was supplied by MicroPharm Ltd., Newcastle Emlyn, UK. European viper venom antiserum was from Institute of Immunology Inc., Croatia.

Determination of Vaa venom in sera samples

Microtiter plate was coated with in-house rabbit anti-*Vaa* venom IgG (1 μg/mL) in 0.05 M carbonate buffer, pH 9.6 (100 μL/well) and left overnight at room temperature (RT). After washing and blocking with 2% (*w/v*) BSA in PBS buffer comprising 0.05% (*v/v*) Tween 20 (200 μL/well) for 2 h at 37 °C, the investigated sera (2 or 10-fold diluted, depending on the patient) were added in duplicates and incubated overnight at RT.

The whole venom solution (100 ng/mL) used as a standard was prepared in the respective matrix and added in eight serial 2-fold dilutions in duplicates (100 μL/well). Pool of sera from non-bitten individuals (2 or 10-fold diluted) was used as negative control. The plate was extensively washed and incubated first with in-house equine anti-*Vaa* venom IgG (100 μL/well of 5.7 μg/mL) and then with HRP-anti-equine IgG (100 μL/well of 4000-fold dilution). Finally, after washing, OPD solution (5.5 mM in 0.15 M citrate-phosphate buffer, pH 5.0) with 30% H_2O_2 (0.5 μL/mL of OPD solution) was added and incubated for half an hour at RT in the dark. The enzymatic reaction was stopped with 1 M H_2SO_4 (50 μL/well) and absorbance at 492 nm was measured. Venom content was determined by multiplying each concentration, which was obtained from the standard curve, by the corresponding dilution factor. The assay was performed independently minimally two times and the results are given as mean ±95% confidence interval (CI). Pharmacokinetic parameters were determined as described [5].

Determination of Atxs in sera samples

ELISA for determination of Atxs was performed in a similar manner with few exceptions. Coating was done with in-house rabbit anti-Atx IgG (1 μg/mL, 100 μL/well). The investigated sera (2 or 4-fold diluted) and pure Atx solution as standard (eight serial 2-fold dilutions starting from 10 ng/mL) were added after washing and blocking. After washing, plates were incubated first with in-house guinea pig anti-Atx IgG (100 μL/well of 0.3 μg/mL) and then with HRP-anti-guinea pig IgG (100 μL/well of 10,000-fold dilution). The final steps were performed as already described in previous section.

Quantification of antivenom in sera samples

ELISA for determination of antivenom IgG fragments – Fab or $F(ab')_2$ was performed as follows. Microtiter plate was coated with *Vaa* venom (1 μg/mL) in 0.05 M carbonate buffer, pH 9.6 (100 μL/well) and left overnight at RT. After washing and blocking with 2% (*w/v*) BSA in PBS buffer comprising 0.05% (*v/v*) Tween 20 (200 μL/well) for 2 h at 37 °C, the investigated sera were added in a few suitable dilutions in duplicates (100 μL/well). ViperaTAb® (100 ng/mL) or European viper venom antiserum (125 ng/mL) used as standards for Fab and $F(ab')_2$ fragments quantification, respectively, were added in eight serial 2-fold dilutions (100 μL/well), also in duplicates. Incubation was performed overnight at RT. The plate was extensively washed and incubated with HRP-anti-ovine IgG (100 μL/well of 5000-fold dilution) for Fab or HRP-anti-equine $F(ab')_2$ (100 μL/well of 25,000-fold dilution) for $F(ab')_2$ fragments quantification. The final steps were performed as already described in previous section. The assay was performed independently three times and the results are given as mean ±95% CI.

Pharmacokinetic analysis

Pharmacokinetic analysis of the measured concentrations was performed using PKSolver add-in software (version 2.0, China Pharmaceutical University, Nanjing, China) for Microsoft Excel [11]. Concentration-time data was fitted either to one-, two- or three-compartment model. Akaike information criterion (AIC) and Schwarz criteria (SC) were used for comparison of goodness of their fit [12].

Results

Case series

Case no. 1

A 60-year-old man with arterial hypertension without therapy and with a history of alcohol abuse was working in a remote vineyard in the southern region of Slovenia when he was bitten in the thenar of the right hand by an approximately 50 cm nose-horned viper (*Vaa*). Immediately after the bite he felt pain and noticed two puncture wounds. Within 10 min his right hand started to swell. Upon arrival to the local physician, approximately one hour after the viper bite, the patient was faint, dizzy, confused, tachycardic (100/min) and hypotensive (85/50 mmHg), with oedema of the affected hand, extending up to a fifth of his forearm toward the elbow. He was vomiting and had syncope. He was given antiemetic thiethylperazine and infusion of 0.9% NaCl (1000 mL).

The patient was immediately transferred to the University Medical Centre Ljubljana (UMCL) due to a shortage of antivenom. Upon arrival to the Emergency Department (ED) of UMCL, 3 h after the viper bite, the patient was somnolent, tachycardic (100/min), normotensive (110/80 mmHg) and tachypneic (30/min). He felt a strong and intense pain at the envenomation site and the local oedema with erythema extended up to a half of his forearm. However, the remainder of his physical examination was unremarkable and the patient had no neurological symptoms. The initial laboratory tests 3 h after the bite showed leucocytosis (22×10^9/L), thrombocytopenia (26×10^9/L), coagulopathy with prolongation of prothrombin time (0.41), increased D-dimer (10,469 μg/L) and normal fibrinogen level (3.14 g/L). The patient had rhabdomyolysis (myoglobin 1101 μg/L and creatine kinase 7.9 μkat/L) and acute renal failure (creatine 113 mmol/L). Treatment was continued with isotonic electrolyte composition infusion at the rate 200 mL/h, and 4.5 h after the bite the patient was given 8 mL of ViperaTAb® diluted in 100 mL of 0.9% NaCl within 30 min as the envenomation was graded as 2b according to a modified Audebert's clinical severity grading [13].

Follow-up studies, 7 h after the bite, revealed significant increase of platelet count (84×10^9/L) and a slight improvement of coagulopathy (prothrombin time 0.48) and rhabdomyolysis (myoglobin 677.6 μg/L). Renal function normalised due to hydration. These transient improvements were followed by profound second thrombocytopenia (18×10^9/L), coagulopathy (prothrombin time 0.24) and extension of local pain, oedema, erythema, lymphangitis, petechiae and ecchymosis up to the upper arm 12 h after the bite. Pseudothrombocytopenia or analytical error due to possible *in vitro* formation of aggregates within a tube of the first blood sample was excluded by microscopic examination of blood smear and use of different buffers. Direct and indirect anti-platelet antibody tests were negative, as were direct and indirect Coombs tests.

Fourteen hours after the bite, the patient was given a second dose of 8 mL of ViperaTAb® diluted in 100 mL of 0.9% NaCl within 30 min. Platelet number normalised within 2 h (177×10^9/L) again and remained normal afterwards, while coagulopathy significantly improved with prothrombin time reaching 0.53. However, local oedema and erythema extended further to the shoulder and the patient developed neurological signs (bilateral ptosis, ophthalmoplegia and dysphagia) despite treatment with ViperaTAb®.

Eighteen hours after the bite the patient aspirated gastric content due to dysphagia and developed pneumonitis with acute respiratory failure. The patient was sedated with propofol and intubated, and assisted mechanical ventilation and antibiotics were started. Bronchoscopy with pulmonary toilet was done, but immediately afterwards the patient developed extreme arterial hypertension (300/160 mmHg) and treatment with glyceryl trinitrate infusion was started. Blood pressure dropped to 120/60 mmHg within an hour and immediate brain CT scan was normal. This episode with aspiration was followed by a dose of 4 mL of expired Viperfav™ diluted in 100 mL of 0.9% NaCl within 60 min as envenomation was deemed to be grade 3, 21 h after the bite. Viperfav™ effectively reduced local swelling extension. The patient was extubated 72 h after the bite and had no neurological signs, except persistent dysphagia that slowly improved during subsequent days. Brain magnetic resonance imaging and magnetic resonance angiography revealed moderate brain atrophy with chronic microangiopathy and leukoaraiosis of grade 2 according to the Fazekas scale for white matter lesions, but no signs of acute bleeding were present. The patient was ultimately discharged in good condition.

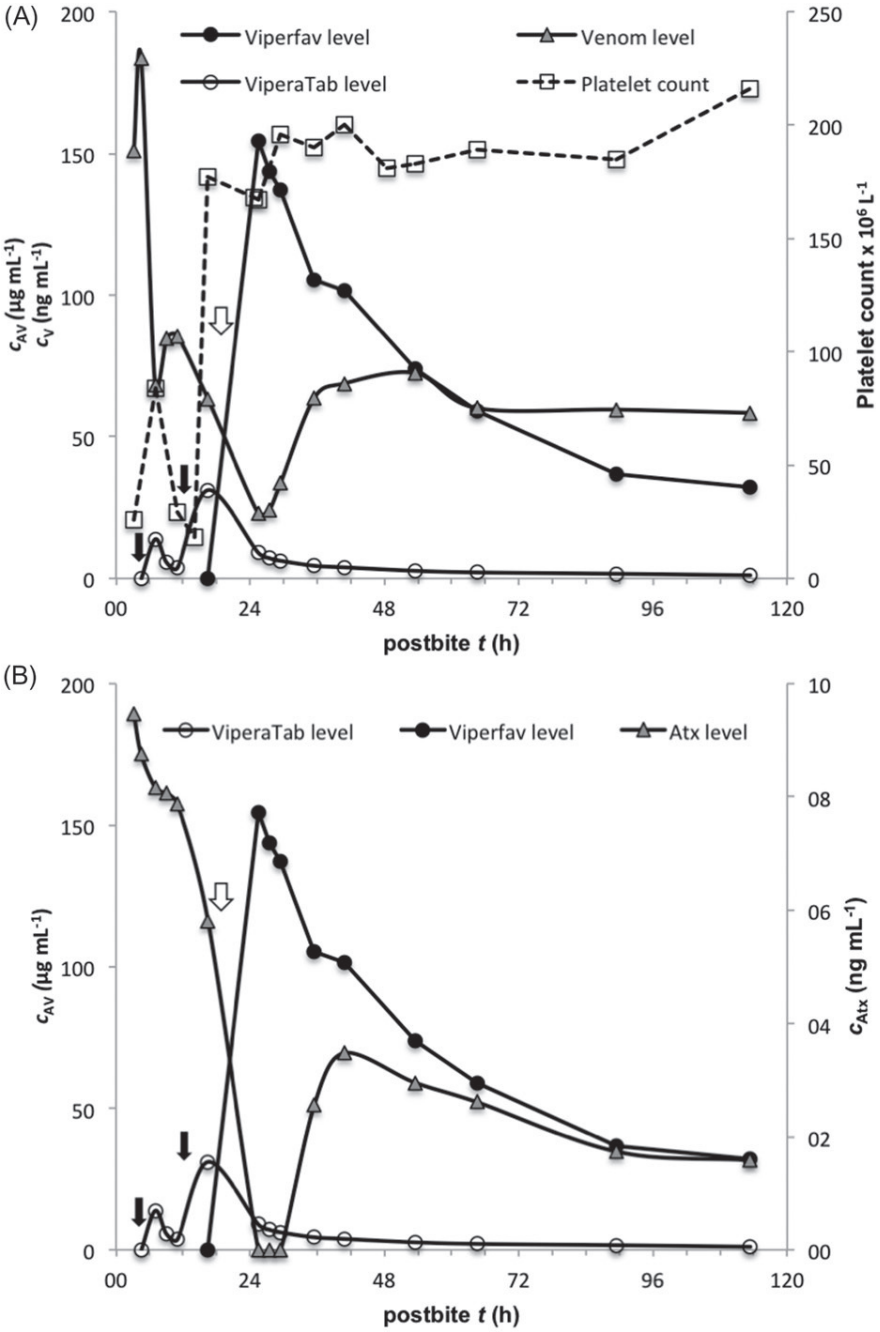

Figure 1. Analysis of the serum of the patient #1 bitten by *Vaa* and treated with ViperaTAb® and Viperfav™. Panel (A) displays the concentration of *Vaa* venom in the serum (c_V), platelets count and serum concentrations of ViperaTAb® (c_{AV}) and Viperfav™ (c_{AV}). Panel (B) shows serum concentrations of Atxs (c_{Atxs}), ViperaTAb® (c_{AV}) and Viperfav™ (c_{AV}). Legend: black arrow – ViperaTAb application; white arrow – Viperfav application.

Subsequent ELISA analysis of serum samples taken 3 h after the bite revealed the *Vaa* venom level of 160 ng/mL and the Atxs level 9.5 ng/mL. These and subsequent serum concentrations of *Vaa* venom and Atxs are presented in Figure 1. Antivenoms ViperaTAb® and Viperfav™ serum concentrations measured by ELISA are presented in Figure 1, while ViperaTAb® pharmacokinetic data is presented in Table 1.

Table 1. Pharmacokinetic parameters of monospecific ovine Fab fragments (ViperaTAb®) in the patients envenomed by *Vaa* after *I.V.* administration.

	Case #1 (2nd dose)	Case #2 (1st dose)	Case #2 (2nd dose)	Case #3 (1st dose)
$t_{1/2\alpha}$ (h)	3.2	1.6	1.2	1.2
$t_{1/2\beta}$ (h)	55.9	22.2	14.1	45.3
V_{ss} (mL/kg)	252.9	154.5	118.3	524.2
MRT (h)	59.0	19.6	13.0	39.1
AUC_∞ (µg h/mL)	666.8	373.1	323.5	198.7
AUMC (µg h^2/mL)	39,347.1	7311.0	4207.5	7760.0
CL (mL/h/kg)	4.3	7.9	9.1	13.4

$t_{1/2\alpha}$: distribution half-life; $t_{1/2\beta}$: elimination half-life; *V*ss: steady-state volume of distribution; MRT: mean residence time; AUC_∞: area under the curve at $t = \infty$; AUMC: area under the first(-order) moment curve; CL: systemic clearance. We were not able to calculate pharmacokinetic parameters for the first dose of the first case due to lack of data.

Case no. 2

An 83-year old man with arterial hypertension was bitten in the dorsal side of the right foot in the central part of

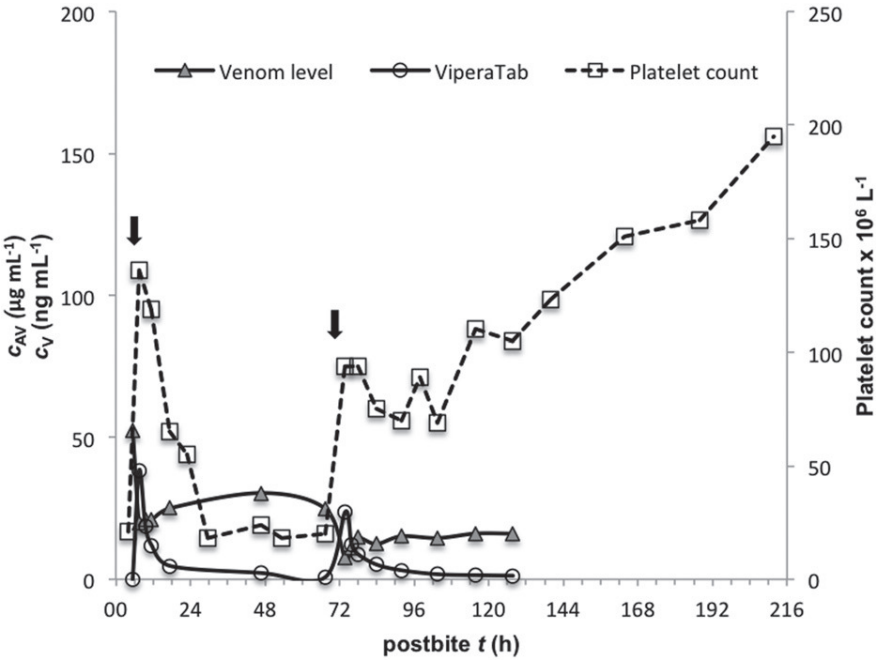

Figure 2. Analysis of the serum of the patient #2 bitten by *Vaa* and treated with ViperaTAb®. Serum concentrations of *Vaa* venom (c_V) and ViperaTAb® (c_{AV}) were determined using ELISA. Platelet count was also monitored in the samples of serum. Legend: black arrow: ViperaTAb application.

Slovenia by a nose-horned viper (*Vaa*). Immediately after the bite he felt pain and within a few minutes the right foot started to swell. Upon arrival at the ED of the UMCL four hours after the viper bite the patient was in extreme pain, confused and hypertensive (200/100 mmHg). Oedema, lymphangitis and haematoma were extending up to a third of the lower leg. The remainder of his physical examination was unremarkable and the patient had no neurological symptoms. The initial laboratory tests 3 h after the bite showed leucocytosis (21×10^9/L), thrombocytopenia (21×10^9/L), slight rhabdomyolysis (myoglobin 97.6 µg/L) and coagulopathy with increased D-dimer (5428 µg/L). Prothrombin time was at lower normal value (0.70) and fibrinogen level was normal (3.29 g/L). 5.5 h after the bite the patient was given 8 mL of ViperaTAb® diluted in 100 mL of 0.9% NaCl within 30 min as the envenomation was graded as grade 2b. The extension of local signs stopped and follow-up studies 7.5 h after the bite revealed an increase of platelet count (136×10^9/L). Afterwards, profound thrombocytopenia reappeared (18×10^9/L) with nadir 30–70 h after bite. 72 h after the bite the patient was given a second dose of 8 mL of ViperaTAb® diluted in 100 mL of 0.9% NaCl within 30 min. Platelet number increased to 94×10^9/L within next 2 h, but 12 h after the second dose it decreased to 70×10^9/L again and remained between 70 and 140×10^9/L until the seventh day, when platelets count finally normalised. The patient was ultimately discharged in good condition.

Subsequent ELISA analysis of serum samples taken 4 h after the bite revealed *Vaa* venom level of 50 ng/mL. The serum concentrations of the *Vaa* venom are presented in Figure 2. None of the analysed sera samples for Atxs in this patient gave absorbance values higher than those obtained for the simultaneously assayed negative control. Antivenom ViperaTAb® serum concentrations measured by ELISA are presented in Figure 2, while ViperaTAb® pharmacokinetic data is presented in Table 1.

Case no. 3

A 73-year old man was bitten in the left thumb in the southern part of Slovenia by a nose-horned viper (*Vaa*). The patient killed the snake and took a photo of it. Immediately afterwards he felt pain and his right arm started to swell and become erythematosus. After 6 h local pain, oedema, erythema and lymphangitis extended up to the elbow and the patient was admitted to the ED of the UMCL. On admission the patient was tachycardic (124/min) and normotensive (150/90 mmHg). The initial laboratory tests 7 h after the bite showed slight thrombocytopenia (133×10^9/L) and increased D-dimer (3639 µg/L). All other laboratory tests were normal. 14 h after the bite the patient was given 8 mL of ViperaTAb® diluted in 100 mL of 0.9% NaCl within 30 min as the envenomation was graded as 2a due to the extension of local signs above the elbow and platelet number further decreased (113×10^9/L). After the ViperaTAb® application the extension of local signs stopped and the follow-up studies revealed an increase of platelet count (147×10^9/L). The patient developed no neurological symptoms and was discharged in good condition on the third day.

ELISA analysis of serum samples taken 7 h after the bite revealed the *Vaa* venom level of 40 ng/mL. The serum concentrations of *Vaa* venom are presented in Figure 3. Measurable Atxs quantity was not detected in the patient's serum. Antivenom ViperaTAb® serum concentrations are

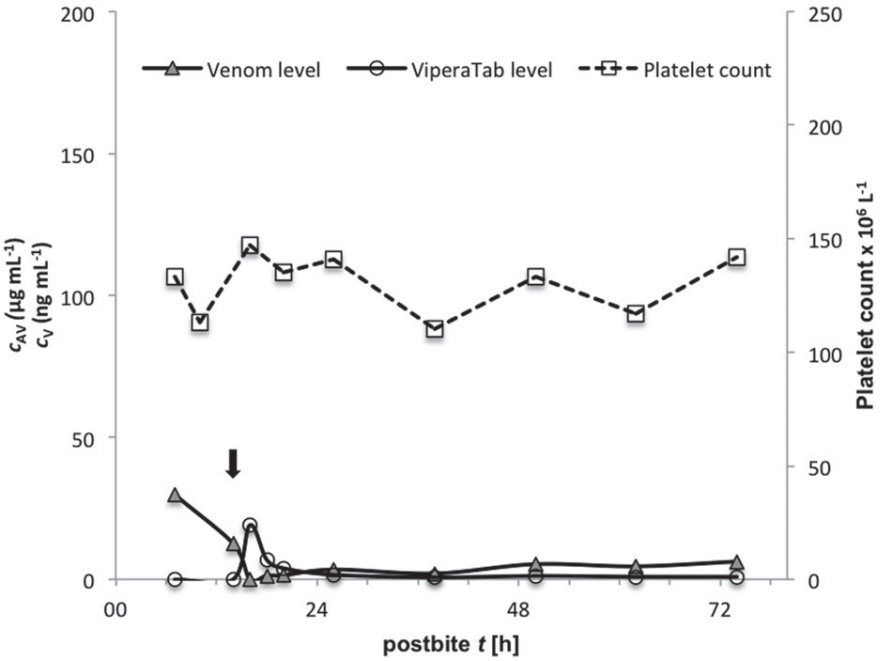

Figure 3. Analysis of the serum of the patient #3 bitten by *Vaa* and treated with ViperaTAb®. Using ELISA, concentrations of *Vaa* venom (c_V) and ViperaTAb® (c_{AV}) were followed in the serum. Platelets were counted in the serum samples. Legend: ViperaTAb application.

presented in Figure 3 and ViperaTAb® pharmacokinetic data in Table 1.

Pharmacokinetics of antivenom level decrement

Pharmacokinetic parameters were determined as already described [5]. Pharmacokinetic parameters of ViperaTAb® obtained from each of three antivenom treated patients are summarised in Table 1.

Discussion

ViperaTAb®, which is a pharmaceutical formulation containing a monospecific ovine Fab fragments against the venom of *V. berus* [6], was used in patients envenomed by *Vaa* for the first time according to the PubMed search. The main reason for this was the current shortage of antivenom against *Vaa*, such as Viperfav™ and European viper venom antiserum that were successfully used in *Vaa* venomous bites in the past [2,5,14].

Vaa venom was detected in all three cases in the patients' serum and its concentration correlated with the severity of envenomation. Atxs, the most toxic components of *Vaa* venom purified and characterised so far [15], were detected in only the most severely envenomed patient who developed neurological symptoms. In this case, serum venom level was 160 ng/mL, while Atxs concentration was 9.5 ng/mL, what is 17-times less, since Atxs represents only a small part of *Vaa* venom [16,17]. Accordingly, Atxs were not detected in the other two moderately envenomed patients with serum venom levels of only 50 and 40 ng/mL, since the detection limit for Atxs by ELISA test was about 2 ng/mL. Nevertheless, in all three cases the snakes were identified as *Vaa* by the patients or based on the picture of the dead snake.

It seems that ViperaTAb® is effective in moderate *Vaa* bites, graded as 2a and 2b according to modified Audebert's clinical severity grading [13]. In the presented moderate cases ViperaTAb® reduced swelling and improved systemic effects, such as thrombocytopenia. However, ViperaTAb® was not effective in the case of the most severe envenoming with the highest serum values of the *Vaa* venom (grade 3). In this case ViperaTAb® did not stop the extension of local swelling and it had no effect on neurological signs. We hypothesise that this is due to neurotoxic Atxs, since they are not present in the *V. berus* venom used in ViperaTAb® production [4]. This is important information since neurotoxic effects in *Vaa* bites constitute a medical emergency as they can progress from ptosis to intense muscular weakness [2,3]. In this case prolonged dysphagia due to neurotoxicity of *Vaa* venom may result in life-threatening aspiration pneumonitis. Nevertheless, cranial nerve palsies such as dysphagia with aspiration and acute respiratory failure due to neurotoxicity of *Vaa* venom could be treated symptomatically with airway protection, pulmonary toilet and assisted mechanical ventilation.

Toxicokinetic study revealed that ViperaTAb® application induced immediate *Vaa* venom level decrement, although only temporarily. Namely, in two envenomed patients the venom antigen concentration increased once again, remaining measurable for more than 4 days after the snakebite. On the other hand, Atxs serum concentrations underwent only minor change in the most severely envenomed patient, decreasing from 9.5 to 7.9 ng/mL, irrespective of the consecutive administration of two ViperaTAb® doses. In contrast, Viperfav™ application resulted in a prompt drop of Atxs concentration to a level under the detectable level, but this was followed by Atxs re-appearance in low concentration during the remaining time-course period as well. This support our

clinical observation and hypothesis that neurological symptoms in severely envenomed patient bitten by *Vaa* cannot be reversed by ViperaTAb®, but this can be done only with antivenom against *Vaa* venom containing anti-ammodytoxins fragments, such as European viper venom antiserum and Viperfav™ [2,14]. However, in this case we were unable to evaluate Viperfav™ effectiveness on neurological symptoms since the patient was sedated due assisted mechanical ventilation at the time of Viperfav™ application. Prolonged dysphagia in the most severely envenomed patient was probably due to Atxs re-appearance from tissue depot of unneutralised venom at the site of bite. Dysphagia due to ischemic or haemorrhagic cerebrovascular event was excluded by brain CT scan and magnetic resonance imaging.

On the other hand, thrombocytopenia established in all presented patients bitten by *Vaa* was successively reversed by ViperaTAb®, since a decrease of platelet count in *Vaa* envenomations is probably due to component(s) present in both *Vbb* and *Vaa* venoms [4]. Since no agonists of platelet aggregation have been detected in the venoms, snaclecs may have been responsible for the occurrence of thrombocytopenia *in vivo* by activating platelets through the interaction with platelet GPIb receptor or mediating platelet adhesion to blood vessel walls [4]. Accordingly, the significant and prompt post-treatment increase in the platelets count observed within a few hours after ViperaTAb® antivenom administration in these patients could be secondary to deadhesion or disaggregation of venom-aggregated platelets. Similar effectiveness in reversing thrombocytopenia has been reported for crotaline-specific antivenom, where the Fab binding to venom proteins responsible for aggregation and rapid subsequent release of platelets has been reasonably supposed as the most probable scenario creditable for normalisation of their count [18,19].

The increase of the platelet count after ViperaTAb® application was however only transient and it correlated with a temporary decrease in serum venom level due to ViperaTAb® application (Figures 1–3). This might be due to low level of anti-snaclecs Fab in ViperaTAb®, since ViperaTAb® is produced by using *V. berus* venom, where snaclecs are only scarcely present, while they are abundant in the *Vaa* venom [4]. Furthermore, recurrent thrombocytopenia within a few hours after ViperaTAb® application correlated well with *Vaa* venom reappearance in serum. Similar recrudescence of thrombocytopenia was shown due to renewed venom antigenemia after clearance of Fab antivenom (CroTAb) in a woman bitten by a Western diamondback rattlesnake [16]. According to the presented cases thrombocytopenia in *Vaa* bitten patients correlates with serum venom level that depends on ViperaTAb®'s pharmacokinetic parameters such as systemic clearance of 4.3–13.4 mL/h/kg and distribution and elimination half-lives 1.2–3.2 h and 14.1–55.4 h, respectively (Table 1). As expected, ViperaTAb®'s pharmacokinetic parameters are less favourable compared to equine $F(ab')_2$ fragments (Viperfav™) that have systemic clearance of only 1.64 mL/h/kg and distribution and elimination half-lives 7 h and 4 days, respectively [5]. This heterogeneity in pharmacokinetics of different types of neutralising molecules has evident potential pharmacodynamic implications since high tissue depot of unneutralised venom, which continuously keeps being absorbed from the inoculation site into circulation, creates conditions for recurrence phenomena, mostly local or coagulopathic, if free plasma antivenom is exhausted prematurely [20].

In this study heterogeneity in ViperaTAb® pharmacokinetics between different subjects was observed as well, since it depends on several factors, such as patient's age, genetic makeup and co-morbidities, such as alcohol abuse. Additional limitation of this study is a small number of included patients and infrequent blood sampling during the first hours after antivenom application. However, we should be aware that this study was the result of *V. ammodytes* antivenom shortage and it emphasises the importance of the specific *V. ammodytes* antivenom availability.

Conclusions

In *V. a. ammodytes* bitten patients ViperaTAb®, a monospecific *V. berus* antivenom with good *in vitro* and preclinical cross-reactivity with *V. ammodytes* venom, reduces moderate swelling extension and temporarily improves systemic effects, except neurological symptoms. ViperaTAb® application induces *V. ammodytes* venom level decrement, but it does not affect serum concentration of neurotoxic Atxs. ViperaTAb® doses in *V. a. ammodytes* bites should be higher and given repeatedly despite its maximum 55-h long elimination half-life. No adverse effects of ViperaTAb® were noted in *V. a. ammodytes* bitten patients. In future, specific antivenom against *V. ammodytes* should be produced, since full protection in *V. a. ammodytes* bitten patients by a monospecific *V. berus* antivenom could not be always expected.

Disclosure statement

The authors declare no conflict of interest.

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