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ORIGINAL ARTICLE

Effect of Vipoxin (*V. amm. meridionalis*) and its components on neuromuscular transmission

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ABSTRACT
Vipoxin is the main toxic component isolated from the venom of long-nosed viper *Vipera ammodytes meridionalis,* which population is widely distributed on the Bulgarian area. The neurotoxin consists of two subunits – unstable and toxic phospholipase A_2 (PLA_2) and non-toxic acidic component without enzymatic activity (Vipoxin's acidic components, VAC). In the current study, the action of neurotoxic complex was examined by neurophysiological *in vivo* experimental model on anaesthetized rats. Vipoxin produces neuromuscular blockade in a dose-dependent manner with non-depolarizing post-junctional site of action. It was found that isolated PLA_2 displays significantly lower blocking activity in comparison with Vipoxin when both have been applied in equimolar concentration. That implies the stabilizing role of VAC and its significance for the toxicity of the whole complex. The Vipoxin-induced neuromuscular blockade is completely reversed when antivenom is administrated.

KEY WORDS: snake venom; Vipoxin; *in vivo* neurotoxicity; neuromuscular transmission

Introduction

Snake venoms are a cocktail of different compounds such as proteins with enzymatic and non-enzymatic activity, polypeptides and inorganic components that have evolved to assist in the capture and digestion of prey, as well as for use in defense against potential enemy. Each of these compounds may possess one or more different function as anticoagulant, hemolytic and cytolytic activities, necro-, myo-, nephro-, cardio- or neurotoxicity that could not be directly correlated with the enzymatic activity (Kini & Evans, 1989, Joseph *et al*., 2011).

Skeletal neuromuscular junction is one of the major targets of snake venom in the somatic nervous system (Utkin & Krivoshein, 2016). Particularly, attention needs to be paid to neurotoxins, which can be classified according to their site of action as pre- or post-synaptic (Gawade, 2004). Pre-synaptic neurotoxins with varying phospholipase A_2 activities were identified in the venoms of the four major families of venomous snakes – *Crotalidae, Elapidae, Hydrophiidae* and *Viperidae* (Harris, 1997, Kuruppu *et al*., 2008). A triphasic effect on acetylcholine release could be seen when the blockade of transmission is produced by these toxins (Su & Chang, 1984). Post-synaptic neurotoxins are known to be antagonists of the nicotinic receptor in the skeletal muscle (Gong *et al*., 1999). Depending on their sequence, post-synaptic toxins are subdivided into short- and long-chain toxins (Phui Yee *et al*., 2004). These toxins display different binding kinetics and different affinity for subtypes of nicotinic receptors. Post-synaptic neurotoxins are identified only in venoms of snakes from the families *Elapidae* and *Hydrophiidae*. (Hodgson & Wickramaratna, 2002). Effects of neurotoxins are manifested as interference of neuromuscular signal transmission and can vary from subtle alteration of neurotransmitter release to complete neuromuscular block. The activity of neurotoxins can be exerted at the pre-synaptic elements, post-synaptic or both (Harris, 1984). There are different kinds of venom, which contain both pre- and post-synaptically active toxins.

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For example, a post-synaptic toxin Daboia Neurotoxin isolated from the venom of Russell's viper *Daboia russelii* contains phospholipase A_2 with pre-synaptic site of action. Venom of kraits (*Bungarus* spp.) consists of several different types of neurotoxins (Kini, 2003), in addition to the alpha-bungarotoxin (post-synaptic) and beta-bungarotoxin (pre-synaptic) already described (Lee *et al*., 1977, Rugulo *et al*, 1986). Venom of kraits also contains kappa-bungarotoxin that binds to the neuronal nicotinic acetylcholine receptors at the post-synaptic level in central cholinergic synapses in autonomic ganglia (Ranawaka *et al*., 2013). The main toxic component of the South American rattlesnake – the β-neurotoxin crotoxin (*Crotalus durissus terrificus*) may cause blockade via a post-synaptic action involving stabilization of the desensitized form of the nicotinic acetylcholine receptor (Sampaio *et al.,* 2010).

The family *Viperidae* is the most diverse family of venomous snakes (Tasoulis & Isbister, 2017). Most vipers have numerous heavily keeled scales and possess a large, flattened triangular head. The venom fangs are large, which permit deep penetration during envenomation of prey. They are found throughout Europe, Africa, Asia and America (Lewis & Gutmann, 2004).

Vipoxin is the main toxic component isolated from the venom of long-nosed Bulgarian viper – *Vipera ammodytes meridionalis* (Tomovic, 2006). It is a complex of two subunits – a basic and toxic phospholipase A_2 enzyme (EC 3.1.1.4, $sPLA_2$) and an acidic non-enzymatic component (Vipoxin's acidic components, VAC) with 62% amino-acid sequence identity (Mancheva *et al.,* 1987). This toxin is the first reported example of a high structural similarity between an enzymatic and non-enzymatic subunit and it is a unique example of regulation of toxic function generated by molecular evolution that is of great pharmacological interest. The complex formation between the two subunits is necessary for the physiological function of Vipoxin (Noetzel *et al*., 2002). The function of VAC is to stabilize the neurotoxin's quaternary structure, required for Vipoxin's toxic and enzymatic activities, similarly to the role of the acidic component of crotoxin (Atanasov *et al*. 2012). According to the published preliminary data, the neurotoxic complex of Vipoxin appeared able to block the post-synaptic membrane, whereas phospholipase A_2 manifested a pre-synaptic action that was possibly due to its enzymatic activity (Tchorbanov *et al*., 1978). In the present study, we are trying to investigate the mechanism of neuromuscular blockage *in vivo* as well as to answer the question about the difference in the toxic action of Vipoxin and its PLA_2.

Material and methods

Animals and surgical procedures, *in vivo* experiments

Experiments were carried out on 18 male albino "Wistar" rats (180–220 g) obtained from the Laboratory Animal Farm of Military Medical Academy (Sofia, Bulgaria). Prior to the experiments they have been housed with 6 animals per cage. Temperature was kept at 18–22 °C, humidity was maintained at 50–65% and 12 h light-dark cycle was available. Rats were allowed to standard rodent food and tap water *ad libitum.* The animals were anaesthetized by intraperitoneal injection of 1.5 g/kg 20% urethane applied 45 min before the beginning of experiment to minimize any pain and discomfort caused.

Experimental surgical protocol for cannulation of femoral vein and intravenous administration of the testing compounds was used (Goranova *et al*., 2018). This provides prolonged monitoring within the framework of 60–120 min and toxicological evaluation of the testing compounds and observation. The trachea was cannulated with an endotracheal tube connected to rodent ventilator with artificial ventilation. Tetanic responses of the *m. extensor digitorum longus* (EDL) evoked by *n. ischiadicus* stimulation.

Experiments were carried out in accordance with the Regulation № 20/2012 "Minimal requirements for protection and humanely attitude to the experimental animals, Bulgarian Food Safety Agency, Animal Health and Welfare and Feed Control Directorate, Animal Welfare Unit (Member State authorities for Directive 2010/63/EU).

The scientific examination *in vivo* of experimental animals, has been conducted in accordance with the principles of ICLAS/FELASA and the Ordinance for a humanely attitude towards the experimental animals, with regard to which Military Medical Academy (Sofia, Bulgaria) has received the necessary license-permit received by the National Commission. /License-permit from the National Commission 13/06.02.2013/ Standpoint № 6/ expiration date: 06.02.2018/

Toxins

Vipoxin was isolated from crude air-dried venom from Bulgarian long-nosed viper *Vipera ammodytes meridionalis* (purchased from the Thracian Herpetological Society, Stara Zagora, Bulgaria) by ion-exchange chromatography on SP-Sephades C-50 (Pharmacia, Sweden) using linear gradient from 0.05 M up to 0.4 M Tris-citrate buffer, pH 7.3 (Tchorbanov & Aleksiev, 1981; Atanasov *et al*., 2009). It was dialyzed and lyophilized before separation of its component using charged chromatography with carboxymethylcellulose. The homogeneity of the basic and acidic subunits was verified by SDS-PAGE (Laemmli, 1970). Total protein content was determined according to Smith *et al.* (1985). Enzymatic activity was assayed as described by Cho *et al.* (1988) and Holzer & Mackessy (1996).

Doses, *in vivo* experiments

Vipoxin was administered intravenously in three different doses – 1/5, 1/10, and 1/20 of LD_{50} (7.8 mg/kg b.w.). LD_{50} (rats) of the neurotoxin was calculated using conversion factor between animals (Nair & Jacob, 2016) based on previously published LD_{50} (mice) (Atanasov *et al*., 2012). PLA_2 and VAC were also applied intravenously in the dose equimolar to 1/5 LD_{50} of Vipoxin and 1/10 LD_{50} (rats) of Vipoxin, respectively (the number of repetitions is 3 for each dose administered). To reconstitute the

Vipoxin complex, the PLA_2 subunit was incubated with the same molar concentration of VAC (1:1 molar ratio as in the native Vipoxin) for 30 min at 4 °C (Atanasov *et al*., 2012). To analyze the effect of antivenom (100AE, 1:100, Bul Bio, Bulgaria), it was injected intravenously without further dilution.

Devices

Measuring system for investigation of neuromuscular conduction and contractile function on rat skeletal muscle was used (Experimetria, Ltd., Hungary). The system is designed to study on neuromuscular connection in *in vivo* condition on small laboratory animals (rats and guinea pigs). It mainly consists of microprocessor controlling modular square wave stimulator. SPEL Advanced Haemosys Software is an integral part of the compact research system and has been developed to monitor, save and analyze all measuring online and offline data. The contractile response is measured following either direct muscle stimulation, or by nerve stimulation. Parameters of the electrical stimulus were: PP (pulse period) – 45 s, PW (pulse width) – 1 ms, TD (train delay) – 0, TN (train numbers) – 0, DE (delay) – 5 ms, pressure – 3 V. Tetanic responses were elicited by 50 Hz trains of stimuli for five seconds.

Red blood cells (RBC) morphology and complete blood count

The effect of Vipoxin and its components to RBC morphology was assessed by examination of stained blood smears. To make a visual evaluation of RBC, 50 µl whole blood (K_2EDTA) was collected from the treated animal in the end of the neuromuscular transmission experiment. The staining of blood smears was accomplished according to the Pappenheim procedure using Giemsa and May-Grünwald dyes (Penev & Dukova-Peneva, 2007). Visualization of RBC morphology was performed using light microscope Motik 1820 (Motik, China, 1000×, oil immersion). Complete blood count was measured using analyzer "Erma Inc".

Results and discussion

Neuromuscular transmission

Neuromuscular synaptic transmission involves sequence of events, in which every single step is a possible target for neurotoxic activity. Acetylcholine is neurotransmitter synthesized from inactive precursors within the pre-synaptic nerve terminal (Bowman, 1995). Arrival of action potential in pre-synaptic motor axon terminals cause to open of voltage gated Ca^{2+} channels and Ca^{2+} moves down its electrochemical gradient into the nerve terminal. The elevation of unbound Ca^{2+} leads to fusion of the vesicles with the pre-synaptic membrane. Acetylcholine is released into the synaptic cleft as a response to an action potential and bounds to the acetylcholine receptors on the post-synaptic membrane (Sakmann, 1992). This opens ion channels in the post-synaptic membrane that allow the flow of positive-charged ions down the concentration gradient and consequently depolarization of the post-synaptic membrane occurs. If the response reaches threshold, a regenerative action potential is generated on the post-synaptic membrane, which ultimately results in muscle contraction. The action of acetylcholine is terminated rapidly by the enzyme acetylcholinesterase and the process repeats (Lewis & Gutmann, 2004).

Snake venoms contain potent toxins that are capable of inhibiting neuromuscular transmissions on pre-synaptic (β-neurotoxins) and post-synaptic (α-neurotoxins) sites. The pre-synaptic neurotoxicity is associated with PLA_2 activity found in the venoms. Finding that after a relatively short time period (*i.e.* approximately 30 min) the inhibitory effects of the β-neurotoxins cannot be reversed by the addition of antivenom is of clinical interest (Hodgson & Wickramaratna, 2002). It is known that some β-neurotoxins (taipoxin, notexin) cause irreversible pre-synaptic damage and depletion of pre-synaptic vesicles one hour after injection of venoms (Bowman, 2006, Cull-Candy *et al*., 1976).

Vipoxin is one of the few known multichain neurotoxins with post-synaptic toxicity. The results obtained in previously *in vitro* experiment in 1978 showed that the toxin isolated from *Vipera ammodytes meridionalis* is an α-neurotoxin that acts on the postsynaptic membranes preventing the binding of acetylcholine to its receptor and blocking the neuromuscular transmission of skeletal muscles (Tchorbanov *et al*., 1978). In this way, the toxin exerts its lethal action similar to another multimeric post-synaptic snake venom α-bungarotoxin. When isolated from the complex, the vipoxin's PLA_2 manifests a presynaptic action. Evidently, the complex formation changes the target of the pharmacological action of the neurotoxin and Vipoxin is "targeted" to the post-synaptic site (Banumathi *et al*., 2001).

In the current study, we assessed *in vivo* effects on the neuromuscular transmission of native Vipoxin in rats, isolated from the venom of Bulgarian long-nosed viper *Vipera ammodytes meridionalis*, and its subunits – PLA_2 and VAC, each applied alone as well as reconstituted complex Vipoxin (a mixture of preliminary separated PLA_2 and VAC in 1:1 molar ratio, incubated for 30 min at 4 °C).

In blank experiment (control), the tetanic responses of EDL after nerve stimulation were 95±5% of control group (n=3) after 60 min of *n. ischiadicus* stimulation were induced.

In our experiments Vipoxin produces a dose-depended blockade of the tetanic responses of the EDL to nerve stimulation. 75% inhibition of neuromuscular transmission was recorded at higher dose of Vipoxin 1/5 LD_{50} (rats) (Figures 1A, B). In contrast, when PLA_2 was applied in equimolar dose of 1/5 LD_{50} (rats) Vipoxin, causes only 15% depression of neuromuscular transmission (Figures 1A, B). The reconstituted Vipoxin demonstrates 40% depression of neuromuscular transmission, that is practically the same effect as native Vipoxin in the same dose used, which means that the Vipoxin complex is completely restored (Figure 1A). These findings support the presumption that the integrity of the neurotoxin is

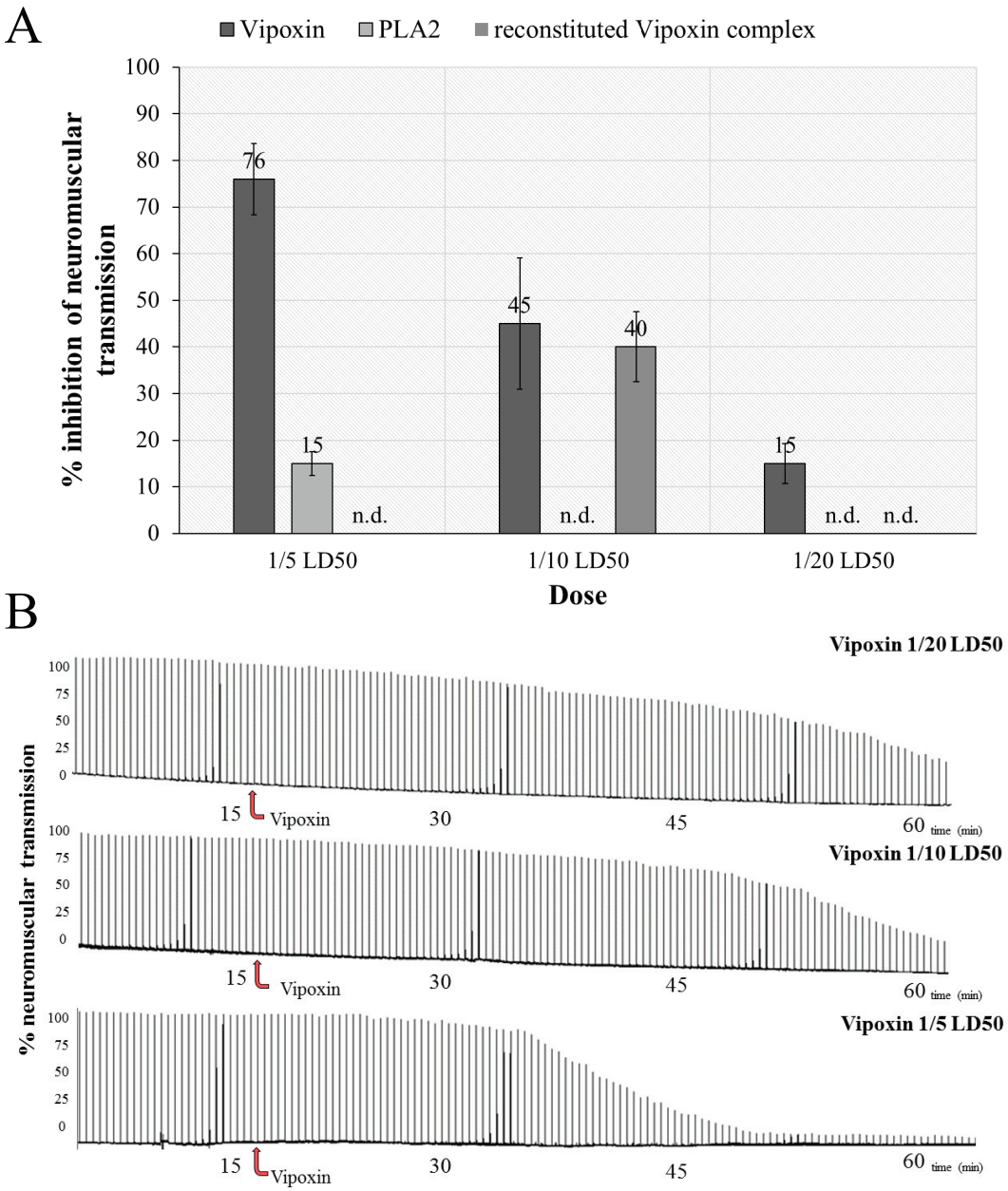

Figure 1. A) Evaluation of inhibitory effect of Vipoxin, PLA_2 and reconstituted Vipoxin complex (PLA_2 and VAC in ratio 1:1) on neuromuscular transmission at 60 min. All compounds tested were used at doses equimolar to 1/5, 1/10 and 1/20 LD_{50} Vipoxin. Each experiment was performed in triplicate (three independent analysis). B) Representative mechanograms of neuromuscular transmission following application of neurotoxin Vipoxin at doses to 1/5, 1/10 and 1/20 LD_{50}. Administration of compound tested was labeled and indicated by a bent arrow.

important for its toxic activity and raise questions about the VAC role on the whole complex.

The onset of neuromuscular block was gradual in all of the doses and testing compounds used. The depressions in the tetanic responses became detectable approximately 14 min (8–20 min) after intravenous injection of the testing compounds. The presence of a fade response during the blockade of nerve-evoked tetanic responses of the EDL has been observed for all Vipoxin and reconstituted complex doses applied.

The muscle response to tetanic stimulation is sustained during normal neuromuscular transmission and a pure depolarizing block, whereas during a non-depolarizing block fade occurs and the response is not sustained. A possible explanation for the fade response is that under tetanic stimulation the acetylcholine, available in pre-synaptic stores, bursts into synaptic cleft and starts to stimulate post-synaptic cholinergic receptors. The process continues until a depletion of stores occurs followed by a period of equilibrium, meanwhile mobilization and synthesis of acetylcholine is achieved. However, even in condition of this equilibrium a tetanic stimulation of the nerve (50 Hz) maintain muscle response due to incongruity between the release of acetylcholine (much higher) and

its amount necessary to evoke a response. When a non-depolarizing neuromuscular blocking agent provokes reduction of the "margin of safety" of the post-synaptic membrane the decrease in release of acetylcholine during tetanic stimulation produces fade (Miller, 2010).

The results obtained from this study well correlate to the data about acute toxicity of Vipoxin and its PLA_2 confirming that native Vipoxin is more toxic in comparison to PLA_2 and the VAC is of importance for toxicity of the whole complex (Atanasov *et al*., 2012). It can be concluded that Vipoxin is the carrier of the toxic effects on the skeletal muscles and neuromuscular transmission particularly.

There is a dose-response relationship between the amount of Vipoxin and the reducing of the neuromuscular transmission. This finding explains the clinical signs followed envenoming by Bulgarian viper and the corresponding effects on the skeletal muscles.

Here, for the first time, we have tested the effect of the antivenom (commercial product; fraction from horse hyperimmune plasma) on the neuromuscular transmission after Vipoxin-induced inhibition. It was found that the neuromuscular activity is successfully recovered when the antivenom have been applied (Figure 2). In our experimental protocol, the specific treatment started immediately when the neuromuscular block was registered. In the future project, the antidote effects of the same antivenom will be assessed after postpone application.

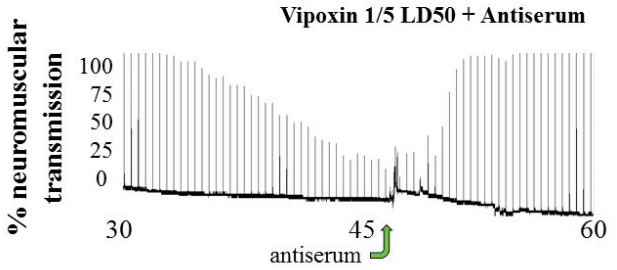

Figure 2. Representative mechanogram of effect of antivenom (applicated 30 min after neurotoxin) on neuromuscular blockade caused by 1/5LD50 Vipoxin (administered at 15 min).

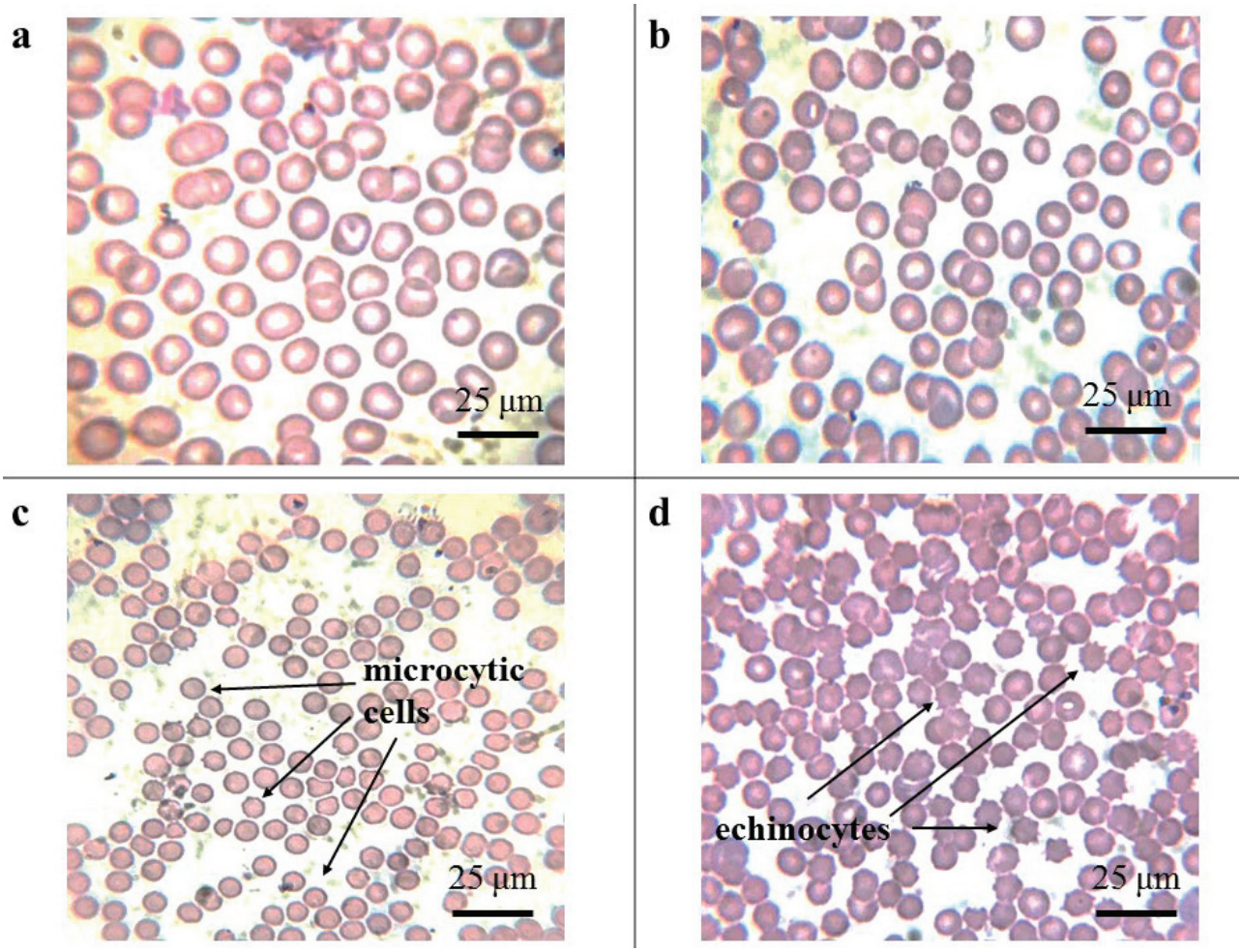

Figure 3. Representative images of effect of Vipoxin at doses of 1/5, 1/10 and 1/20 LD_{50} on morphology of RBC (blood smears): **(a)** normal erythrocytes control; (b) RBC treated with Vipoxin at dose 1/20 LD_{50} where no morphology deviation was observed; (c) RBC treated with 1/10 LD_{50} Vipoxin – an increased number of microcytic cells suggesting initial membrane lysis can be observed; (d) RBC treated with Vipoxin at dose 1/5 LD_{50} – registration of numerous echinocyte formation.

Red blood cells morphology and complete blood count

Normocytic RBCs are biconcave, disc-shaped, anuclear cells. Their morphology has five important parameters: shape, size, color, inclusions and arrangement. The abnormalities of RBC morphology are source of the key information in establishing a differential diagnosis. In our experiment, no deviation in RBC morphology was observed in the control sample (Figure 3a) and at the lowest dose of Vipoxin 1/20 LD_{50} (rats) used (Figure 3b). When the dose of 1/10 LD_{50} (rats) Vipoxin was applied (Figure 3c) an increased number of macrocytic cells was observed, whereas when the highest dose of 1/5 LD_{50} (rats) Vipoxin was applied numerous echinocyte formations were found (Figure 3d). It should be mentioned that macrocytosis supposes an initial membrane lysis. The presence of echinocytes in this case may be used as a diagnostic and hemolytic prognostic factor. The effect on RBC morphology due to Vipoxin's PLA_2 subunit have been studied (Stoykova *et al*., 2013) and apparently, it was found that echinocytes formation is a main result of PLA_2 enzymatic activity.

Conclusion

The effect of Vipoxin (the main toxin isolated from the long-nosed Bulgarian viper – *Vipera ammodytes meridionalis*) on the neuromuscular transmission and its ability to cause disturbance in physiological functions of the neuromuscular synapse was studied for the first time using neurophysiological *in vivo* experiment. A relation between the doses of the neurotoxin Vipoxin and the extent of reduction of neuromuscular transmission was registered. The comparison between the effects of PLA_2 and recombinant Vipoxin (complex of PLA_2 and VAC) on the neuromuscular transmission showed that recombinant Vipoxin possesses the same potential as the native Vipoxin. Vipoxin induces neuromuscular block with non-depolarizing post-junctional site of action. Applying of antivenom reverses the neuromuscular transmission when the Vipoxin-induced blockade is realized. The dose-responded changes on RBC's morphology was observed during the experiments displaying *in vivo* hemolysis of the cells.

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