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EXPERIMENTAL STUDIES

THE EFFECT OF THE SECRETORY PHOSPHOLIPASES A_2 FROM *GLOYDIUS BLOMHOFFII* AND *GLOYDIUS HALYS* VENOMS ON HEMOSTASIS COMPONENTS *IN VITRO*

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The aim of this study was to investigate the effects of *Gloydius blomhoffii* and *Gloydius halys* venoms and their fractions on platelet and plasma hemostasis, and to evaluate the ability of the secretory phospholipase A_2 (sPLA_2) inhibitor Varespladib to neutralize the coagulopathic activity mediated by sPLA_2 present in these venoms. The proaggregatory effect of whole *G. halys* and *G. blomhoffii* venoms on platelet hemostasis was associated with multidirectional effects of their multiple protein components, while sPLA_2-containing fractions exhibited an antiaggregatory effect. Whole *G. halys* and *G. blomhoffii* venoms and Varespladib added to them did not affect the extrinsic pathway of hemocoagulation, but inhibited the intrinsic pathway of human blood coagulation. The paradoxical effect of further enhancing the anticoagulant activity of *G. halys* and *G. blomhoffii* venoms with the sPLA_2 inhibitor Varespladib could be determined by a stronger sPLA_2 interaction with blood coagulation factor Xa after formation of the enzyme-inhibitor complex.

Keywords: secretory phospholipase A_2; hemostasis; Varespladib; *Gloydius blomhoffii*; *Gloydius halys*

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INTRODUCTION

Venomous snakes have evolved the ability to exert a generalized effect on their prey. Secretory phospholipase A_2 (sPLA_2), which has anticoagulant activity, plays a significant role in the spread of venom throughout the body [1, 2].

Oral administration of the low-molecular-weight sPLA_2 inhibitor Varespladib-methyl (Var-m; methyl 2-((3-(2-amino-2-oxoacetyl)-1-benzyl-2-ethyl-1H-indol-4-yl)oxy)acetate) is considered as an adjunctive treatment for snakebites (along with antiserum administration) [3, 4]. Certain evidence exists that this drug is capable of neutralizing the anticoagulant effect caused by sPLA_2 in the venom of some hemotoxic snakes [5, 6].

Like the venoms of other vipers, the venoms of the copperhead snakes *Gloydius blomhoffii* (eastern copperhead snake) and *Gloydius halys* (common copperhead snake) contain significant amounts of group IIA phospholipase A_2, classified as a secretory phospholipase (sPLA2). The venoms of *G. blomhoffii* and *G. halys* contain 62.5% [7] and 14–27% [8] sPLA_2, respectively.

The aim of our study was to investigate the effects of *G. blomhoffii* and *G. halys* venoms and their fractions on platelet and plasma hemostasis, as well as to assess the possibility of neutralizing the coagulopathic activity caused by sPLA_2 in these venoms.

MATERIALS AND METHODS

Reagents

The following reagents have been used in this study: lyophilized samples of *G. blomhoffii* and *G. halys* venoms provided by the Siberian Serpentarium (Russia) under a scientific cooperation agreement; phospholipids (Lecithin from egg BioChemica, AppliChem, Germany); reagent kits for determining ADP-induced platelet aggregation (ADP), activated partial thromboplastin time (APTT-TEST), and prothrombin time (Techplastin test) (Technologiya-Standard, Russia). The venom was dissolved in phosphate-buffered saline (diaGene, Russia), pH 7.4, to 10 mg/ml; Varespladib (Var; LY315920; Sigma-Aldrich, USA) was dissolved in 10% DMSO (dimethyl sulfoxide) (PanEco, Russia), the initial concentration was 13.1 mM.

Snake Venom Fractionation

Prior to fractionation, the venoms were pre-dissolved in 0.1% aqueous trifluoroacetic acid (TFA) (Sigma-Aldrich) and analyzed by analytical reversed-phase HPLC (RP-HPLC) with a 25–40% acetonitrile gradient (Panreac, Spain) using a XBRIDGE C18 BEH 130 Ang 5 μm column (Waters, Ireland). An Agilent 1200 Series system (Agilent Technologies, USA) was used for analytical RP-HPLC, and a Gilson 302/303 PUMP system (Gilson, France) was used for semi-preparative analysis. UV detection (at 214 nm) was used for analytical RP-HPLC, and at 280 nm for semi-preparative analysis. In the first case, the load was 1.0–1.5 mg, in the second it was 3.0–4.0 mg. The resulting fractions were evaporated (SpeedVac vacuum concentrator, Labconco, USA), lyophilized (FreeZone freeze dryer, Labconco), and stored at -80°C until use.

Determination of the Primary Structure of Proteins from G. blomhoffii and G. halys Venoms

The primary structure of the venom proteins was determined by automated Edman stepwise degradation using a PPSQ-33A protein and peptide sequencer (Shimadzu Corp., Japan) according to the manufacturer's protocol. For analysis, 800 pmol of protein, purified by analytical RP-HPLC, was dissolved in 30 μl of 50% aqueous acetonitrile supplemented with 0.1% TFA. Identification of phenylthiohydantoin derivatives of amino acids was performed using LabSolution software (Shimadzu Corp.).

Phospholipase A_2 Activity Assay

PLA_2 activity was assayed by proton release for 2 min at 37°C in a medium containing phenol red (ΔA_{557}) as a pH indicator. The reaction was started by adding 0.5 μg of snake venom to the sample. The sample volume was 1.8 ml. The activity assay medium contained the following components (final concentrations): 5 mM HEPES (Servicebio, China), pH 7.8, 88 μM phenol red (Vekton, Russia), 150 mM NaCl (Grotex, Russia), 9 mM $CaCl_2$ (CDH, India), 3.1 mM egg yolk phosphatidylcholine (Lecithin from egg BioChemica, AppliChem), 6.14 mM Triton X-100 (CDH). Phospholipase A_2 activity was determined on a BioRadxMarx spectrophotometer (Bio-Rad Laboratories, USA). The studies were performed in 5 replicates.

The Study of the Effect of Whole Venom and Individual Fractions on Platelet and Plasma Hemostasis

Sample Preparation. Blood for the study of the parameters determined in this study was obtained from the Blood Transfusion Department of the Pavlov First Saint Petersburg State Medical University. To prevent platelet activation, blood was collected in vacutainers containing 3.8% sodium citrate as a stabilizer at a sodium citrate:blood ratio of 1:9. Stabilized blood was centrifuged for 7 min at 150 *g* and room temperature to obtain platelet-rich plasma (PRP). A portion of the PRP was collected in a plastic tube in the amount required for analysis. Platelet-poor plasma was obtained from the remaining blood by centrifugation for 30 min at 2500 *g* to calibrate the aggregometer's optical density scale and to study the effect of venoms on plasma hemostasis.

Platelet aggregation. Platelet aggregation in PRP was studied using a Solar AP 2110 aggregometer (Solar, Belarus) at 37°C and a magnetic stirrer at 1200 rpm. The effect of the venom and individual fractions on platelet aggregation was assessed after addition of 10 μM ADP as an inducer to samples. The venom and lyophilized fractions were pre-dissolved in saline (1 μg in 1 ml). The study procedure: 270 μl of PRP and 30 μl of the corresponding solutions were sequentially mixed in cuvettes, the mixture was incubated for 5 min at 37°C and then 10 μl of ADP was added. In the control, saline (30 μl) was added to 270 μl of PRP. Aggregation was recorded until the curve reached a plateau.

Plasma hemostasis. The effect of the venom and its fractions on plasma hemostasis was assessed by their ability to prolong or shorten the clotting time of platelet-poor plasma (compared to the control) in activated partial thromboplastin time (APTT) and prothrombin time (PT) tests. The studies were conducted using an APG2-02-P coagulometer (ECMO, Russia). The study procedure included mixing 50 μl of plasma, 40 μl of venom and fraction solutions, and 10 μl of saline. The mixture was then incubated at 37°C for 60 s, and the coagulation time was determined using the analyzer according to the study protocol. For the control, 50 μl of saline was added to the plasma samples. To assess the effect of Var on the blood coagulation system *in vitro*, 40 μl of saline, 10 μM Var, and 50 μl of plasma were mixed. To determine the contribution of $sPLA_2$ enzymatic activity in the whole venom of *G. blomhoffii* and *G. halys*, 40 μl of venom and 10 μM Var were mixed, incubated for 5 min at room temperature, and then 50 μl-aliquots of plasma were added. Measurements were performed in seven replicates.

Statistical Analysis

Statistical analysis of the obtained results was performed using GraphPad Prism software (USA). Data are presented as mean ± standard error of the mean (SEM). Statistical evaluation of the results was performed by comparing the parameters in the experimental and control groups. Comparisons between groups were performed using the Mann-Whitney U test. The critical significance level of differences was set at 5% ($p < 0.05$).

RESULTS AND DISCUSSION

Determination of $sPLA_2$ Activity

The enzymatic $sPLA_2$ activity of the venoms of *G. blomhoffii* and *G. halys* was quite high, 26.5±2.64 U/mg venom and 30.6±3.98 U/mg venom, respectively. No statistically significant differences in $sPLA_2$ activity were found between the two studied copperhead species.

Snake Venom Fractionation

Fractionation of the venoms using RP-HPLC yielded 23 fractions of the *G. blomhoffii* venom and 26 fractions of the *G. halys* venom.

The molecular mass range of the studied fractions after separation of whole venoms of the copperhead snakes *G. blomhoffii* and *G. halys* by analytical reversed-phase HPLC, according to MALDI-TOF MS, was 2.8–13.6 kDa. The lowest molecular weight peptides are neurotoxins, while the highest molecular weight peptides are various PLA_2 isoforms.

Determination of the Primary Structure of Proteins from G. blomhoffii and G. halys Venoms

The protein fractions of *G. blomhoffii* and *G. halys* venoms (fraction **16** of *G. blomhoffii* and fraction **22** of *G. halys*) were analyzed using the Edman automated degradation method. A BLASTP search for primary structure homology identified both of these protein components as acidic phospholipase A_2 (PA2-II) or phosphatidylcholine 2-acylhydrolase (UniProt ID: P20249.1).

Platelet aggregation. Table 1 presents our data on the effects of *G. blomhoffii* and *G. halys* venoms, as well as their fractions, on platelet hemostasis in the ADP-induced aggregation test.

As can be seen from the data presented in this table, whole *G. halys* and *G. blomhoffii* venoms exhibited proaggregatory properties. Enhancement of platelet aggregation was observed with ten fractions of *G. halys* venom, with the most active fractions **6**, **7**, and **9**, and five fractions of *G. blomhoffii* venom, with the most active fraction **4**. Antiaggregant activity was demonstrated by thirteen fractions of *G. halys*, with the most active fractions **16** and **17**, and eleven fractions of *G. blomhoffii*, with the most active fractions **9**, **10**, and **11**.

Thus, the effect of whole venom from both copperhead species on platelet hemostasis is the result of the opposing effects of many protein components

Table 1. Effect of whole venom and fractions of *G. halys* and *G. blomhoffii* on ADP-induced human platelet aggregation (n = 7)

ADP-platelet aggregation		
Substance	*G. halys* venom and fractions	*G. blomhoffii* venom and fractions
Parameter	Amplitude, %	
Control	80.7±1.45	68.5±1.39
Whole venom	52.7±2.14•	39,7±1.56•
Fractions		
1	100.4±1.12*	67.3±1.19
2	101.6±0.98*	50.1±0.77•
3	92.8±1.54*	63.8±1.43•
4	16.9±0.77•	clot
5	72.6±2.11•	84.2±1.15*
6	clot	94.4±2.01*
7	clost	81.3±2.13*
8	40.3±1.02•	33.9±1.59•
9	clot	>100
10	100.5±2.23*	>100
11	88.1±0.98*	>100
12	80.5±1.32	65.5±1.28
13	33.5±1.52•	64.9±1.87
14	83.0±2.41	80.0±2.15*
15	92.6±0.87*	67.6±1.89
16	>100	***75.3±1.76****
17	>100	75.8±1.22*
18	56.9±0.43•	70,9±1,33
19	48.3±1.54•	97.8±1.76*
20	82.5±1.98	71.4±1.55
21	99.8±0.87*	76.7±0.99*
22	***94.5±0.94****	52.5±1.88•
23	107.6±1.23*	70.1±1.51
24	73.9±2.19•	—
25	99.3±0.89*	—
26	90.0±0.73*	—

Results for fractions containing $sPLA_2$ are shown in bold italics; * – differences are statistically significantly higher as compared to the control ($p < 0.05$); • – differences are statistically significantly lower as compared to the control ($p < 0.05$).

of the venoms. $sPLA_2$-containing *G. halys* fraction **22** and *G. blomhoffii* fraction **16** demonstrated a moderate antiaggregant effect.

Plasma hemostasis. The results of the plasma hemostasis study of *G. halys* and *G. blomhoffii* venom fractions in the APTT test are presented in Table 2.

Table 2 shows that whole *G. halys* and *G. blomhoffii* venoms exhibited an anticoagulant effect, which was statistically higher compared with control ($p < 0.05$) and was manifested by prolonging the clot formation time in the APTT test. The majority (19 of 26) of *G. halys* venom fractions, as well as whole venom, exhibited a pronounced anticoagulant effect to varying degrees. Fraction **18** components inhibited plasma hemostasis via the intrinsic pathway most strongly. The procoagulant effect demonstrated only four fractions of the *G. halys* venom, with the maximum (irreversible) effect observed in fractions **16** and **17**. The $sPLA_2$-containing fraction **22** of the *G. halys* venom also exhibited a weak procoagulant effect (Fig. 1).

Since most viper $sPLA_2$ are characterized by inhibition of blood coagulation via the intrinsic mechanism [2, 9], it can be assumed that the opposite effect of $sPLA_2$ from *G. halys* venom is associated with the presence of some procoagulant, possibly a serine protease [10–12]. This assumption is supported by Zhong et al. [13]. They demonstrated the anticoagulant effect of recombinant, highly purified $sPLA_2$ from the venom of this copperhead snake, associated with its catalytic activity.

Both whole venom and most fractions (11 of 23) of *G. blomhoffii* exhibited anticoagulant activity, with the maximum effect observed in fractions **8** and **12**. Only four fractions (**1**, **9**, **10**, and **11**) demonstrated the procoagulant, irreversible effect. The fraction containing $sPLA_2$ (fraction **16**) exhibited anticoagulant activity. The increase in coagulation time in the presence of this fraction was approximately 1.5-fold.

The anticoagulant activity observed in some snake venoms $sPLA_2$ is due to inhibition of the prothrombinase complex as a result of $sPLA_2$ binding to factor Xa, a component of this complex [14]. Among 12 viperid venoms (*Viperidae*) studies in [15], the catalytically active isoform of alkaline $sPLA_2$ from the venom of the rattlesnake *Crotalus durissus terrificus* possessed the most potent anticoagulant activity. Var inhibited the anticoagulant activity of the components of the studied snake venoms [16], confirming the contribution of phospholipase activity to the anticoagulant effect. Therefore, it seemed reasonable to evaluate the effect of Var on the $sPLA_2$-mediated anticoagulant effect of whole *G. halys* and *G. blomhoffii* venoms in the APTT and PT tests.

Table 2. Effect of whole venom and fractions of *G. halys* and *G. blomhoffii* on plasma hemostasis in the APTT test (n = 7)

APTT, s		
Substance	*G. halys* venom and fractions	*G. blomhoffii* venom and fractions
Control	38.4±0.84	37.5±0.99
Whole venom	47.5±0.51*	46.6±0.27*
Fractions		
1	41.6±0.49*	27.3±1.22•
2	43.0±1.12*	40.5±1.34
3	40.3±0.89	41.1±1.77
4	49.4±0.97*	40.4±0.86
5	45.3±1.03*	41.5±1.29
6	51.0±0.63*	73.5±1.68*
7	41.7±1.02*	127.5±2.32*
8	40.8± 1.11	>200
9	45.3±0.91*	clot
10	44.5±0.88*	clot
11	45.6±2.01*	clot
12	110.3±2.45*	>200
13	76.6±1.43*	74.4±1.59*
14	44.4±0.99*	45.5±0.87*
15	41.7±0.78*	49.1±1.76*
16	clot	***57.2±1.89****
17	clot	48.4±1.33*
18	>400	52.8±1.81*
19	64.6±1.14*	48.6±1.55*
20	43.9±0.98*	36.9±0.74
21	71.1±1.01*	39.8±1.46
22	***19.9±1.21•***	40.0±1.33
23	31.6±0.87•	38.8±2.08
24	71.7±0.98*	—
25	46.3±1.54*	—
26	40.9±0.76	—

Results for fractions containing $sPLA_2$ are shown in bold italics; * – differences are statistically significantly higher as compared to the control ($p < 0.05$); • – differences are statistically significantly lower as compared to the control ($p < 0.05$).

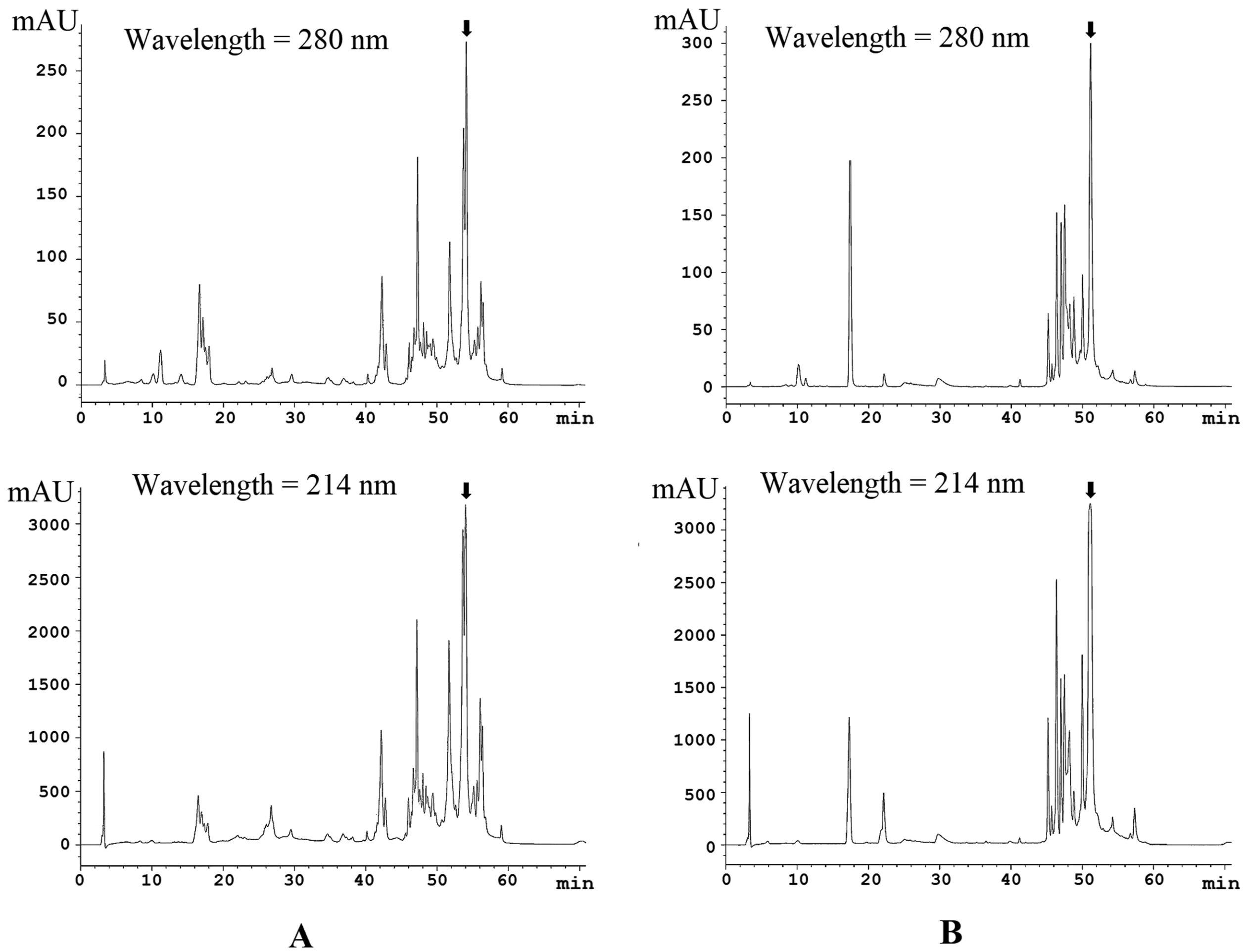

Figure 1. Reversed-phase chromatography of *G. halys* (**A**) and *G. blomhoffii* (**B**) venom with absorption detection at 214 nm (bottom row) and 280 nm (top row). The target fraction, corresponding to $sPLA_2$, is marked with a black arrow.

Table 3 presents data on the effect of whole *G. halys* and *G. blomhoffii* venoms on plasma hemostasis parameters in the absence and in the presence of the $sPLA_2$ inhibitor Var.

As can be seen from Table 3, Var in combination with the venom of both *G. halys* and *G. blomhoffii* enhanced the inhibition of the intrinsic pathway of human blood coagulation, prolonging the clot formation time in the APTT test ($p < 0.05$). The paradoxical effect of more potent increase in the anticoagulant activity of the venoms in the presence of $sPLA_2$ inhibitor Var, which was more pronounced in *G. blomhoffii* compared to *G. halys* ($p < 0.05$), could be attributed to enhanced interaction of $sPLA_2$ with factor Xa of the hemocoagulation cascade after complex formation of between the enzyme and its inhibitor. This is supported by the literature data on the possibility of manifestation of biological effects of inactive $sPLA_2$ from snake venom due to protein-protein interactions [17]. In the PT test, Var in combination with the venom of either *G. halys* or *G. blomhoffii* had no effect on the extrinsic pathway of human blood coagulation.

Table 3. Effect of whole venoms of *G. halys* and *G. blomhoffii* on plasma hemostasis parameters in APTT and PT tests without the addition of the $sPLA_2$ inhibitor Var and in its presence

APTT, s		
Substances	*G. halys*	*G. blomhoffii*
Whole venom	47.9±1.99	47.6±1.46
Var	46.8±1.54	47.0±1.03
Whole venom +Var	54.3±2.24*	69.7±1.83*^
PT, s		
Whole venom	14.5±1.28	13.9±1.55
Var	13.7±2.10	13.1±0.89
Whole venom +Var	12.6±1.12	13.4±1.11

* – Differences are statistically significant compared to whole venom, $p < 0.05$; ^ – differences are statistically significant between samples with whole venom and Var, $p < 0.05$.

CONCLUSIONS

1. A study of the effects of *G. halys* and *G. blomhoffii* venoms and their protein fractions on ADP-induced human platelet aggregation revealed a proaggregatory effect of whole venoms and an antiaggregatory effect of $sPLA_2$-containing fractions.

2. Whole *G. halys* and *G. blomhoffii* venoms inhibited the intrinsic pathway of human blood coagulation in the APTT assay. The paradoxical effect of even higher increase in the anticoagulant activity of *G. halys* and *G. blomhoffii* venoms by the $sPLA_2$ inhibitor Varespladib may be due to increased interaction of sPLA2 with factor Xa of the hemocoagulation cascade after complex formation between the enzyme and its inhibitor.

3. Whole venoms of *G. halys* and *G. blomhoffii*, as well as the $sPLA_2$ inhibitor Varespladib contained in the venom, did not affect the extrinsic pathway of human plasma coagulation in the PT test.

FUNDING

The study was performed without external funding.

COMPLIANCE WITH ETHICAL STANDARDS

The study was approved by the Local Ethics Committee of the Pavlov First Saint Petersburg State Medical University (Protocol no. 307 dated October 20, 2025).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ВЛИЯНИЕ СЕКРЕТОРНЫХ ФОСФОЛИПАЗ A_2 ЯДОВ *GLOYDIUS BLOMHOFFII* И *GLOYDIUS HALYS* НА КОМПОНЕНТЫ ГЕМОСТАЗА *IN VITRO*

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Целью данного исследования было изучение воздействия ядов *Gloydius blomhoffii* и *Gloydius halys* и их фракций на тромбоцитарный и плазменный гемостаз, а также оценка возможности ингибитора секреторной фосфолипазы A_2 (сФЛA_2) Varespladib нейтрализовать коагулопатическую активность, обусловленную сФЛA_2 в составе данных ядов. Установлено, что проагрегантное действие на тромбоцитарный гемостаз цельных ядов *G. halys* и *G. blomhoffii* является результирующей разнонаправленного действия многих белковых компонентов ядов, а содержащие сФЛA_2 фракции оказывали антиагрегантное действие. Цельные яды *G. halys* и *G. blomhoffii* и Varespladib, добавленный к ним, не влияли на внешний путь гемокоагуляции, но тормозили внутренний путь активации свёртывания крови человека. Парадоксальный эффект ещё большего увеличения антикоагулянтной активности ядов *G. halys* и *G. blomhoffii* ингибитором сФЛA_2 Varespladib может быть следствием усиления взаимодействия сФЛA_2 с фактором Xa каскада гемокоагуляции после образования комплекса фермента с его ингибитором.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: фосфолипаза A_2; Varespladib; гемостаз; *Gloydius blomhoffii*; *Gloydius halys*

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