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ԱԿԱԴ. Լ.Ա. ՕՐԲԵԼՈՒ ԱՆՎԱՆ ՖԻԶԻՈԼՈԳԻԱՅԻ ԻՆՍՏԻՏՈՒՏ

ՄԱՐԻԱՄ ՄՈՀԱՄԱԴՎԱՐԶԻ

MONTIVIPERA LATIFI ՕՁԻ ԹՈՒՅՆՈՎ ՊԱՅՄԱՆԱՎՈՐՎԱԾ ԷՐԻԹՐՈՑԻՏԱՐ
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ԽԱՆԳԱՐՈՒՄՆԵՐԸ

Գ.00.09-Մարդու և կենդանիների ֆիզիոլոգիա մասնագիտությամբ
կենսաբանական գիտությունների թեկնածուի
գիտական աստիճանի հայցման ատենախոսության

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NATIONAL ACADEMY OF SCIENCES OF RA
L.A. ORBELI INSTITUTE OF PHYSIOLOGY

MARYAM MOHAMADVARI

MORPHOLOGICAL AND FUNCTIONAL ALTERATION OF ERYTHROCYTE
GHOSTS AND GIANT UNILAMELLAR VESICLES CAUSED BY *MONTIVIPERA LATIFI*
VENOM

S Y N O P S I S

of dissertation for conferring of scientific degree of
Candidate of Biological Sciences
in the specialty of 03.00.09 – Human and Animal Physiology

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Ատենախոսության թեման հաստատվել է ՀՀ ԳԱԱ Լ.Ա. Օրբելու անվ. Ֆիզիոլոգիայի ինստիտուտի գիտական խորհրդում:


Գիտական ղեկավար՝	կ.գ.դ., Ն.Մ.Այվազյան
Պաշտոնական ընդդիմախոսներ՝	կ.գ.դ., պրոֆեսոր Վ.Հ. Սարգսյան
	կ.գ.թ. Կ.Վ.Սիմոնյան

Առաջատար կազմակերպություն՝ ՀՀ ԳԱԱ Հ.Բունիայանի անվան բիոքիմիայի ինստիտուտ

Ատենախոսության պաշտպանությունը տեղի կունենա 2017թ. դեկտեմբերի 26-ին, ժամը 13⁰⁰-ին, ՀՀ ԳԱԱ Լ.Ա. Օրբելու անվ. Ֆիզիոլոգիայի ինստիտուտում գործող ՀՀ ԲՈՀ-ի Ֆիզիոլոգիայի 023 մասնագիտական խորհրդի նիստում (0028, Երևան, Օրբելի եղբ. փող. 22):

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Ատենախոսության սեղմագիրն առաքվել է 2017թ. նոյեմբերի 25-ին:

023 մասնագիտական խորհրդի գիտական
քարտուղար, կենս. գիտ. թեկնածու  Ն.Է. Թադևոսյան

The theme of the dissertation has been approved at the scientific council of the Orbeli Institute of Physiology, NAS RA.

Scientific supervisor:	Dr. of Biological Sciences, N.M. Ayvazyan
Official opponents:	Dr. of Biological Sciences, Professor V.H. Sargsyan PhD, K.V. Simonyan

Leading organization: H. Buniatian Institute of Biochemistry NAS RA

The defense of the dissertation will be held on December 26th, 2017, at 13:00, at the session of 023 Physiology Specialized Council of RA SCC at the Orbeli Institute of Physiology (22 Orbeli Bros. Str., 0028, Yerevan, RA).

The thesis will be available at the library of the Orbeli Institute of Physiology NAS RA, as well as at the www.physiol.sci.am webpage.

The synopsis has been sent on November 25th, 2017.

Scientific Secretary of the 023
Specialized Council,
PhD

 N.E. Tadevosyan

INTRODUCTION

Topic significance. Envenomation by snakes was likely a significant daily concern for our primordial ancestors, and it remains a substantial health issue in many parts of the world; worldwide, it is estimated that nearly 3 million envenomations and 125,000 deaths occur annually [Mackessy S.P., 2010; Fox J. & Gutierrez J.M., 2017]. Morbidity and loss of functions, particularly following viper bites, add to this annual toll, making snakebite an important (though often overlooked) source of human suffering. But venomous animals are much more than just a source of danger to humans. Venomous reptiles have likely existed for well over 120 million years, and in that time, myriad toxins have evolved that allow them to incapacitate, paralyze, kill, and digest their prey with a high degree of efficiency [Kini M., 2010]. The biological potency and specificity of some of these venom toxins is truly astounding, and therein lies much of the attraction for scientists.

Reptile venoms and toxins have a potential for tremendous contribution to treatment of human diseases, and some of this potential has been realized in the production of drugs based on or modeled from venom toxins [Harvey L., 1998, 2016; Huang T.F., 1998]. It is therefore not surprising that reptile venoms contain toxins that can be directed against human cancers, hemostatic disorders, and even diabetes [Steeg P.S., 2010; Kakanj M., et al., 2015]. Further, because many toxins interact with receptors/ligands with a high degree of specificity, they are also an excellent source of novel drug leads and design [Ayvazyan N., et al., 2008, 2012; Ghazaryan N. et al., 2015].

Research goals and tasks. The aim of the present study is to obtain detailed information about the morphological and functional alteration of erythrocyte ghosts and giant unilamellar vesicles caused by *Montivipera latifi* venom in norm and pathology.

Our objective was to determine changes in optical spectroscopic and electrical properties accompanying venom-membrane complexation and study how changes both in lipid composition and protein condition modulates membrane transport processes. The analysis of the free-radical processes, as well as analysis of the activity of antioxidant enzymatic system in the interaction with venom could give new information about mechanisms of venom spreading and action in organism. Also we propose to assess the alteration of the normal work of main ion transporters of RBC membrane in course of treatment with ML venom. Changes both in lipid composition and protein condition modulate membrane transport processes.

Constituted tasks of the research were to:

1. the analysis of the free-radical processes, as well as analysis of the activity of antioxidant enzymatic system in the interaction with venom in normal tissue and S-180 sarcoma tissue.
2. to assess the alteration of the normal work of main ion transporters of RBC (namely, Na⁺,K⁺-ATPase and Ca²⁺-activated Mg²⁺-dependent ATPase) membrane in course of treatment with *Montivipera latifi* venom.
3. to study morphological changes of giant unilamellar vesicles (GUVs) and erythrocyte ghosts after modification with *Montivipera latifi* venom components.
4. to study the influence of *Montivipera latifi* venom on the proliferation of the S-180 sarcoma *in vivo* and *in vitro* using the S-180 sarcoma bearing mouse model DNA-retardation assay

Scientific novelty and applied value of the study. We have chosen two types of membrane models (GUVs and erythrocyte ghosts, EG) and fluorescent probes which being sensitive criteria of the physiological condition of lipids and lipid-contained structures make it possible to visualize the morphological changes of membranes condition in a real-time mode in course of membrane-venom interaction.

In the present study we have shown that *ML* venom has a significant influence on the morphology of artificial lipid membranes and changes the plastic properties of the membrane. Addition of *Montivipera latifi* snake venom apparently witnesses the inhibitory effect of venom on the level of ROS production in red blood cells. The inhibition of (Na^+ , K^+)- and (Ca^{2+} , Mg^{2+})-ATPase activities in venom treated EG conclusively shows that viper venom has a profound restructuring effect on the lateral organization of lipids in model and complex biological membranes. The obtained results are also new in terms of the study of the anticancer activity of the viper venom *in vivo*, since the results of the DNA retardation experiments demonstrated that *ML* venom were able to bind to the genomic DNA of S-180 sarcoma cells and block the electrophoretic mobility of genomic DNA, and furthermore some gene expression. This activity was confirmed by data of chemiluminescence (ChL) intensity, which is suppressed in tumor tissue, our study of both malonicdialdehyde (MDA) and superoxidedismutase (SOD) levels, which are increased in the sarcoma cells, while treated groups demonstrate data, more close to the normal conditions of oxidative processes and antioxidant system.

Main points to present at defense.

1. *Montivipera latifi* venom influences changing of the morphology of the supramolecular structures of giant unilamellar vesicles and erythrocyte ghosts.
2. *in vivo* treatment by *ML* venom results in the S-180 sarcoma growth inhibition.
3. The interaction of the components of *ML* venom with membranes of EG cause the morphological changes of the both GUVs and EG, and the alteration of the normal work of main ion transporters of RBC of the formers.

Work approbation. The main results of the dissertation were discussed at seminars and at international scientific conferences.

Publications. According to experimental data observed in dissertation 4 works, including 3 papers in peer-reviewed journals were published.

Volume and structure of dissertation. The dissertation contains the following chapters: introduction, literature review (Chapter 1), experimental part (Chapter 2), results and discussion (Chapter 3), concluding remarks, conclusions and cited literature (total 180 papers and books). The document consists of 103 pages, 4 tables and 25 figures.

MATERIALS AND METHODS

Reagents and venom Iranian ML venom was purchased from “Latoxan” (France); 8-anilino-1-naphthalene sulfonic acid (ANS), ouabain and adrenaline were purchased from “Sigma”.

Packed red blood cells (RBC) were obtained from Haemathology Center after Prof. R. Yeolyan (MoH, RA).

Artificial membrane models

Phospholipid processing The phospholipid fraction was separated from the bovine brain according to the Muller method (Mueller et al., 1962).

Giant unilamellar vesicles GUVs were prepared by the electroformation method, developed by Angelova et al. (1992). GUVs were formed in a temperature controlled chamber that allows a working temperature range from 20 °C to 50 °C. GUVs were prepared using the following steps: ~2 µl of the phospholipid stock solution was spread on each of the two sample chamber platinum wires. The chamber was then dried for ~1 h to remove any remaining trace of organic solvent (nonane).

The chamber and the buffer (Tris-HCl 0.5 mM, pH 7.4) were separately equilibrated to temperatures above the lipid mixture phase transition(s) (~10 °C over the corresponding transition temperature, which is close to 30⁰C) and then 2 mL of buffer was added to cover the wires. Immediately after buffer addition, the platinum wires were connected to a function generator and a low-frequency AC (alternating current) field (sinusoidal wave function with a frequency of 10 Hz and amplitude of 2 V) was applied for 90 min. The mean diameter of these GUVs should be ~30 µm, as previously reported (Sanchez et al., 2002; Ayvazyan & Ghazaryan, 2012).

Erythrocyte ghosts. Erythrocyte membranes were obtained by the method of Dodge, Mitchell & Hanahan (1956). Protein was measured by the method of Lowry as described (Lowry et al., 1951) using bovine serum albumin as a standard. To obtain erythrocyte ghosts, after the last wash the RBC pellet was mixed with nine volumes of ice-cold lysis buffer (5 mM sodium phosphate) and stirred for 15 min at 0°C. Subsequently, the unsealed erythrocyte ghosts were pelleted by centrifugation at 37 000 × g for 10 min at 0°C. After the centrifugation the ghosts were washed with ice-cold lysis buffer until residual hemoglobin was not visible. The RBC ghosts were suspended in about 0.5 volume of PBS and were kept frozen at -30°C until use.

Fluorescence labeling and microscopy The membrane fluorescence probe ANS (8-anilino-1-naphthalene sulfonic acid) was used for visualization of GUVs and erythrocyte ghosts. 1 µM ANS added to the sample chamber after the vesicle formation and then incubated for 5 min at 25 °C (Verstraeten et al., 2005). Images were collected on a fluorescence microscope FM320-5M (AmScope, USA). The excitation and emission wavelengths for ANS were 360 nm and 490 nm, respectively (Ayvazyan & Ghazaryan; 2012). Phase-contrast imaging was done on the Nikon microscope with 9MP digital camera (AmScope, USA).

Na⁺/K⁺ and Ca²⁺/Mg²⁺ - ATPase assay in RBC The activities of the ion motive ATPases, Na-K-ATPase, Ca²⁺, Mg²⁺-ATPase, determined by the method of Hesketh et al., (Hesketh, J. E., et al., 1978). The reaction mixtures for Na⁺, K⁺-ATPase assay contained 2 mM

MgCl₂, 150 mM NaCl, 17.5 mM KCl, and 10.0 mM Tris-HCl, pH 7.4, the ghost suspension, in the presence or absence of ouabain (2 mM) (Kassák P, et al., 2006) and three concentrations of snake venom (pharmacologically relevant to low, sublethal and lethal concentrations according to the LD₅₀ for mouse). Na⁺,K⁺-ATPase activity was calculated as the difference between the presence or absence of ouabain-sensitive Na⁺,K⁺-ATPase activity. The reaction mixtures for Ca²⁺, Mg²⁺ ATPase assay contained 300 μM CaCl₂, 2 mM MgCl₂, 10.0 mM Tris-HCl, pH 7.4 and the ghost suspension. The values were expressed as the difference between ATPase activity in presence of Mg²⁺, Ca²⁺ and in presence of Mg²⁺ only. The reaction was initiated by the addition of ATP (7mM) and mixture was incubated for 1h at 37°C. The reaction was stopped by the addition of 10% (wt/vol) ice cold trichloroacetic acid (final conc.). The mixture centrifuged at 4000g for 5 min after standing 20 min at 4°C. The supernatant used for the estimation of inorganic phosphate liberated according to standard method of Fiske and Subbarow (Fiske CH, and Subbarow Y. 1925). The activity of each enzyme was expressed as nmol Pi/mg protein/min.

S-180 sarcoma mouse model and administration The S-180 sarcoma cells were transplanted subcutaneously into the right axilla of each mouse. When the tumor grew to 100-300 mm³ we randomly divided mice into 3 groups: norm, model (tumor control), and S-180 sarcoma group treated with ML venom. Each group contained 5 mice. The mice were administered as follows: model group, PBS; one ML venom treated group, each animal received small doses of ML (10 μg/mouse) and control. All solutions dissolved in PBS and administered daily by intra-tumor injection (50 μl) for 5 days. Twenty-four hours after the last drug administration, all animals were weighed and sacrificed by cervical dislocation. All procedures were done according to our institution's animal care rules and the IACUC's ethical guidelines for Decapitation of Unanesthetized Mice and Rats (<http://www.utsouthwestern.edu/utsw/cda/dept238828/files/469088.html>). The LD₅₀ of ML venom for mouse is: LD₅₀ [μg] – 5.7 ±0.7 (Latifi M., 2009).

Antitumor activity assay in vivo The antitumor activity was expressed as inhibitory rate (%) and was calculated as [(A-B)/A]*100%, where A and B was the average tumor weight of the model and that of treated group, respectively.

DNA retardation assay A DNA retardation assay was employed to evaluate the DNA-binding ability of venom and was performed as previously reported (Wang et al., 2012). The genomic DNA was extracted from S-180 sarcoma cells by using a genomic DNA extraction kit (precellys tissue DNA kit, PeQlab, Germany). The DNA concentration was measured by using a spectrophotometric method (Eppendorf BioPhotometer plus, Germany) and then equal amounts of genomic DNA were mixed with different concentration of *Montivipera latifi* venom at a 1:1 (vol:vol) ratio for 30 min. Next, 1% agarose gel electrophoresis (PerfectBlue™ Horizontal Mini Gel System, PeQlab, Germany) was used to detect the migration of DNA bands under a UV illuminator (E-BOX VX2-VILBER LOURMAT, PeQlab, Germany). DNA levels were quantified by density analysis with the use of Image J software, and DNA-binding rate (%) was calculated as [1-(A/B)]*100%, where A is the average density of the electrophoretic band and B is the total of the genomic DNA band (Wang et al., 2012).

Chemiluminescence Analysis and Lipid Peroxidation Non-purebred white rats were decapitated. Then the sarcoma was removed and homogenized for 5 min by the homogenizator of Potter-Elvehjem in Tris –HCl buffer (pH 7.4) with a final concentration of 20 mg/ml.

Reactive oxygen species (ROS) levels were measured by a ChL analysing system: intensities of tissue homogenates were measured on a quantometric device equipped with a photomultiplier, which is an ultra fast single photon counter with spectral sensitivity range of 380-630 nm. All the experiments were performed by Junior LB 9509 portable tube luminometer (BERTHOLD Technologies, Germany).

Lipid peroxides are unstable and decomposed to a complex series of compounds. The most abundant compound is malonicdialdehyde (MDA). The MDA level of tissues was determined by spectrophotometric measurement (Stalnaja and Garishvili, 1985), using the TBA-test, based on the reaction of a chromogenic reagent, thio-barbituric acid (TBA) with MDA at 100°C and two molecules of MDA reacting with one molecule of TBA to yield a stable threemethin complex dye. MDA concentration was measured at 532 nm with the CT-2600 spectrophotometer (CT-ChromTech, Taiwan).

Superoxide Dismutase Activity Determination of superoxide dismutase (SOD) activity was done using method of the adrenaline autoxidation reaction in pH 10.2 (Zaqaryan A. Ye., 2003). The method is based on the inhibition of adrenochrome formation in epinephrine autoxidation in aqueous alkaline solution (pH>8.5) to yield a chromophore with a maximum absorbance at 480 nm, using the B01-CT-8 spectrophotometer. Kinetic measurement of the 480 nm absorbance change (adrenochrom concentration) was preformed after the addition of adrenalin. The SOD activity was determined from ratio of the autoxidation rates at the presence and absence of SOD.

Statistical Analysis For quantitative analysis of chemiluminescence intensity a Student's test was used to compare differences at each time point, considering $P < 0.05$ as significant. All data were presented as mean \pm SEM (n=number of experiments). For quantitative analysis of ATPase activity values, results are reported as means \pm SEM. The significance of differences between the means was assessed by ANOVA followed by Bonferroni's test when various experimental groups were compared with the control group. A value of $P < 0.05$ indicated significance.

RESULTS AND DISCUSSION

Morphological alterations of ghost membranes

Light microscopy of human erythrocytes revealed a normal $\sim 7.5 \mu\text{m}$ biconcave disk structure. The venom addition produced marked changes in size and shape of RBC (Fig 1), these changes included a formation of characteristical rounded spiky protrusions (echinocyte) followed by increasing of RBC size and final lyses (if the concentration of venom is pharmacologically relevant to the sub-lethal and lethal doses). The erythrocyte ghosts are spherical and have a bigger size compare to the normal RBC (Fig. 2A). There was a significant decrease of the ghost

diameter after envenoming (Fig. 2B), but interestingly this decrease was reversible and the secondary increasing of erythrocyte ghosts' size was observed at all times, when the processing with venom was done with a lower concentration of venom (Fig 2C). The second addition of the same amount of venom led to the shrinking of the erythrocyte ghosts again, but this time the effect is irreversible (Fig. 2G-I). The first rapid changes of size usually occurred in 1 minute, and diameter decreased almost on 50%. The one interesting observation was that erythrocyte ghosts with abnormal primary small size could be shrinking until the complete lyses even after the first addition of the low concentration of venom (Fig. 2A-C, right).

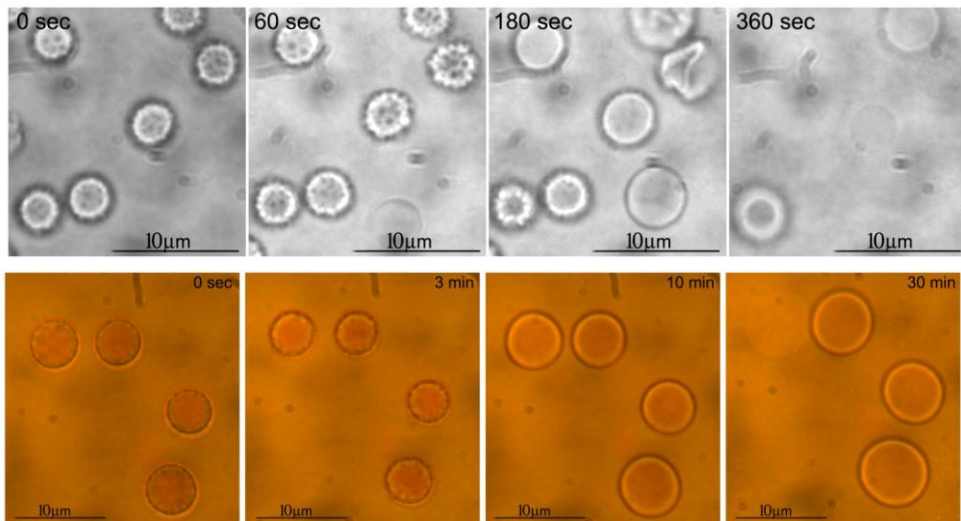


Fig. 1. *Montivipera latifi* venom-dependent changes of the intact erythrocytes size and shape is shown. Dried lyophilized toxin of ML was dissolved in Tris -HCl buffer (pH 7.4) with a final concentration of $3 \cdot 10^{-5}$ M, and $1.1 \mu\text{l}$ of this solution were added to the fluorescent microscope sample.

The solubilization process of erythrocyte ghosts by the ML venom was observed also with phase contrast and fluorescence microscopy. A solution of $1.1 \mu\text{l}$ of venom was injected with a micropipette placed close to the selected ghosts. After contact with the venom solution, all erythrocyte ghosts observed were partially damaged, as detected by a clear decrease in vesicle size. Fig 3 shows one such example observed under phase contrast microscopy. After only 10 s of venom injection, part of the ghosts is detached and then solubilized in a few seconds (Fig. 3B-C). In this picture it is clear that erythrocyte membrane has lost the initial membrane lipid/protein ratio and sugar asymmetry responsible for the high optical contrast seen in the first snapshot. The last snapshot (40 s) shows the treated condition of the RBCs, which are clearly smaller than the original vesicles and shows a re-thickening of the membrane (**Fig. 3D**).

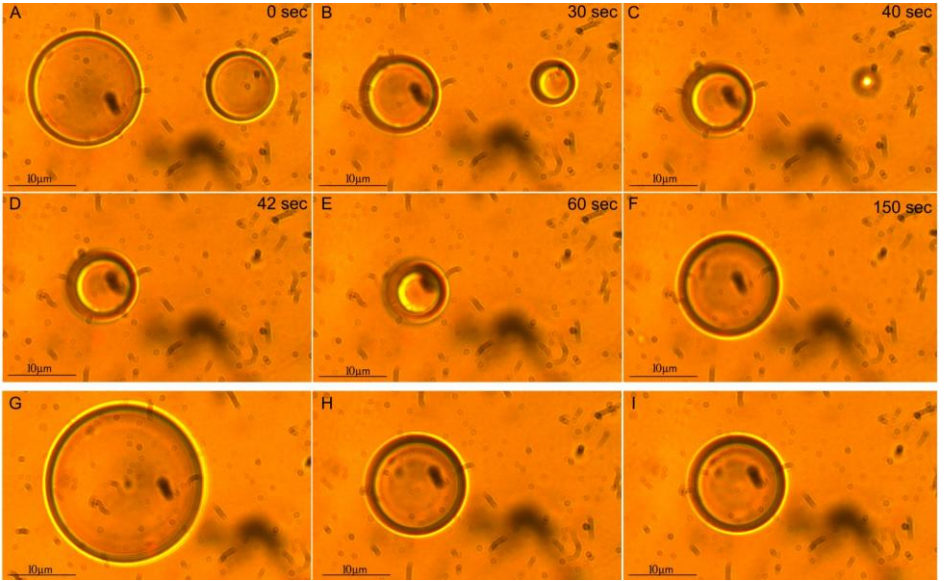


Fig. 2. *Montivipera latifi* venom-dependent changes of the erythrocyte ghosts size is shown.

Erythrocyte ghosts were prepared as described in Section 2.4. Dried lyophilized toxin of ML was dissolved in Tris -HCl buffer (pH 7.4) with a final concentration of 3×10^{-5} M, and **1.1µl** of this solution were added to the microscope sample (A-F), and once again after three minutes (G-I).

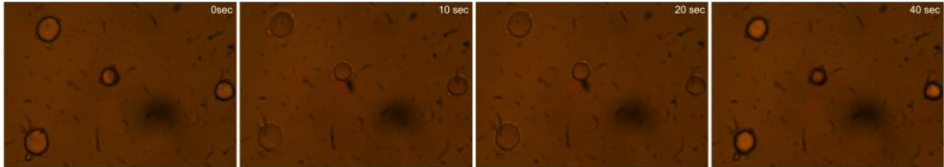


Fig.3. The phase-contrast image of erythrocyte ghosts, in the course of ML low concentration venom-treatment.

Erythrocyte ghosts were prepared as described in Section 2.4. Dried lyophilized toxin of ML was dissolved in Tris -HCl buffer (pH 7.4) with a final concentration of 3×10^{-5} M, and **1.1µl** of this solution were added to the microscope sample.

For fluorescent microscopy the membrane fluorescence probe ANS (8-anilino-1-naphthalene sulfonic acid) was used to visualize erythrocyte ghosts. ANS integrates into the membrane and emits with sufficient intensity to follow the changes produced on the RBCs after the addition of the venom. One selected representative sequence is shown in Fig. 4: erythrocyte ghost show a homogeneous distribution of the fluorescent probe used, ANS, attesting that the

lipid content of the erythrocyte membrane did not exhibit detectable phase separation (see snapshots at 0 and 60s in **Fig. 4**). The main effect caused by ML venom was the dramatically decrease of erythrocyte size without any detectable changes of shape and fluorescence intensity.

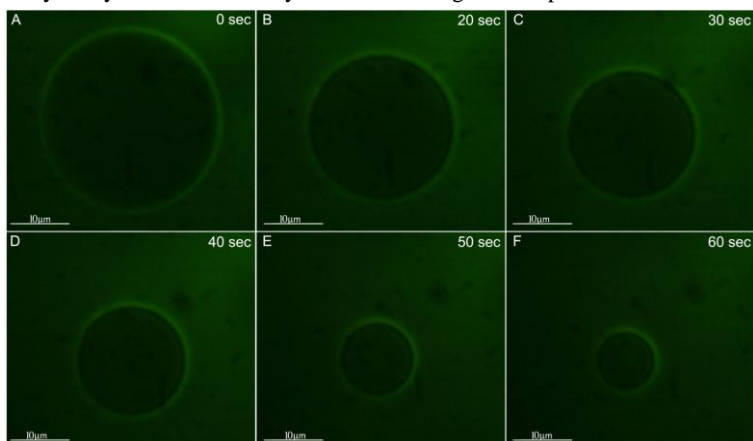


Fig.4. Time-dependent decreasing of the ANS-containing erythrocyte ghosts, in the course of ML venom processing (60 sec).

ANS labeling was done as described in Section 2.5. Dried lyophilized toxin of ML was dissolved in Tris -HCl buffer (pH 7.4) with a final concentration of 3×10^{-5} M, and **1.1µl** of this solution were added to the microscope sample.

Visualization of GUV morphological changes by fluorescence measurements

The phospholipid extract were used to form GUVs, which retained the complexity in lipid composition of the original cells adequately mimicking the erythrocyte ghost's size and curvature. On the other hand, we have a quite simple and commonly used biomimetic system free of its protein components. GUVs also were visualized with the membrane fluorophore ANS during experiments on ML venom hydrolysis. Fig. 5 shows the drastic decreasing of liposomes in course of modification with a lethal concentration of venom at 21 °C in the liquid phase.

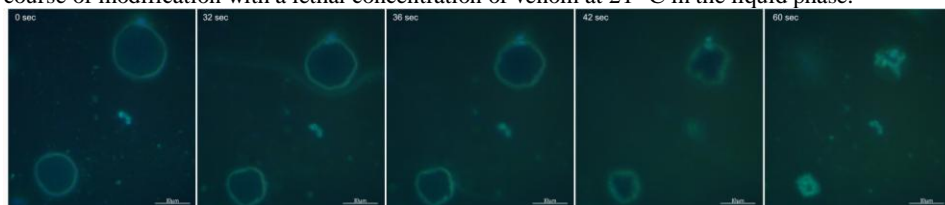


Fig. 5. Rapid changes of the fluorescent intensity of the ANS-containing GUVs in the course of ML venom processing (60sec).

ANS labeling was done as described in Section 2.5. GUVs were prepared according to the electroformation method in a temperature-controlled chamber containing Tris -HCl buffer (pH 7.4). Dried lyophilized toxin of ML was dissolved in the same buffer (Tris -HCl, pH 7.4) with a final concentration of 3×10^{-5} M, and **2.2µl** of this solution was added to the fluorescent microscope sample.

The insertion of venom leads to the very fast shrinking and corrugation of GUVs membrane, after which the vesicle shrinks until nothing (last snapshot). But intriguingly the lower concentrations of venom (sub-lethal) didn't cause any changes at all.

Na⁺/K⁺ and Ca²⁺/Mg²⁺ - ATPase activity in venom-treated erythrocyte ghosts

The other series of experiments was done with a determination of RBC membrane's main ion transporters activities in course of treatment with *Montivipera latifi* venom. The in vitro treatment of erythrocyte ghosts with ML venom significantly inhibit the Na⁺/K⁺ - ATPase activity (Fig. 6), which were accompanied by a significant increase in RBC Ca²⁺/Mg²⁺ - ATPase activity 10 min after addition of the low concentration of venom, compared to control erythrocyte ghosts (Fig. 6). The treatment with gradually increasing concentrations of venom revealed the dose dependent manner of Na⁺/K⁺-ATPases activity inhibition for so called sub-lethal and lethal concentrations (pharmacologically relevant to the half of LD50 dose and LD50 dose, respectively). For these concentration of venom the activity of Ca²⁺/Mg²⁺-ATPase, on the contrary, increasing very dramatically compare with the control data. For higher concentration it was impossible to obtain a countable data because of the fatal damage of membrane lead to the complete lysis of erythrocyte ghosts.

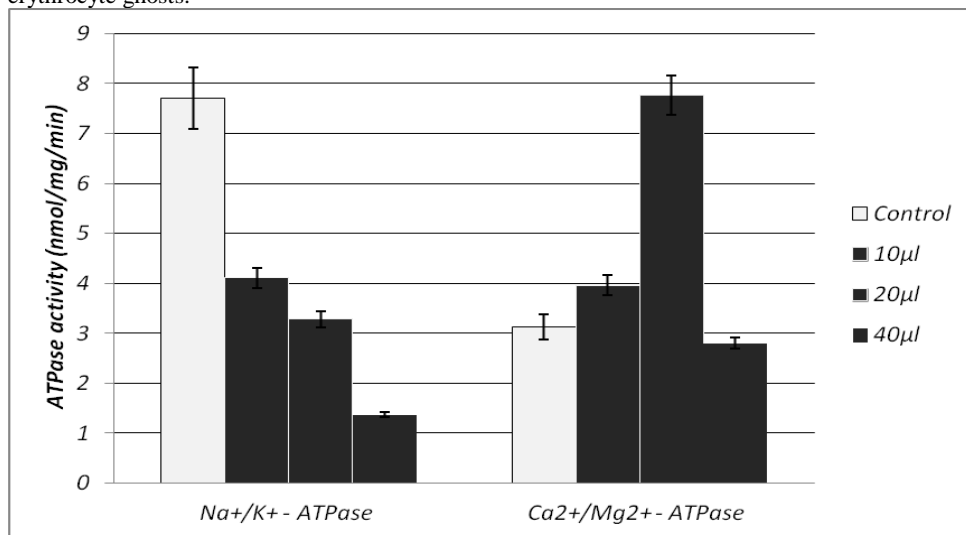


Fig.6. Na⁺/K⁺ and Ca²⁺/Mg²⁺ - ATPase activity in *Montivipera latifi* venom-treated erythrocyte ghosts.

Enzymatic activity was assayed as described in Section 2.6 for three concentrations of snake venom and expressed nmol Pi released/mg protein/min. The columns are the mean±S.D. (n=6). P<0.05 vs. the control group (ANOVA followed by the Bonferroni test).

Free radical processes in RBC under the influence of *Montivipera latifi* venom

Moderate decreases in the mean of spontaneous and luminol-induced ChL counts were observed in the course of ML venom *in vitro* processing (Fig. 7), but the difference in luminol-enhanced rapid peak intensity was much more statistically significant in comparison with that of spontaneous one (Fig. 7, right upper corner).

This fact is also confirmed by the increasing of SOD activity accompanying these changes in lipid peroxidation processes (Fig. 8). Hence, on the one hand, the suppression of lipid free radical oxidation is taking place in the membranes of erythrocyte ghosts, on the other hand, under venom action considerable activation of SOD activation occurs and, thus, intensification of antioxidative processes. According to literature data, the clinical pictures of viper's venom influence are essentially different for different tissues, but it is known that in mammals RBC, which are most studied in this sense, the sublethal dose of *Macrovipera lebetina obtusa* venom has a radioprotective effect (Ayvazyan N. et al., 2012).

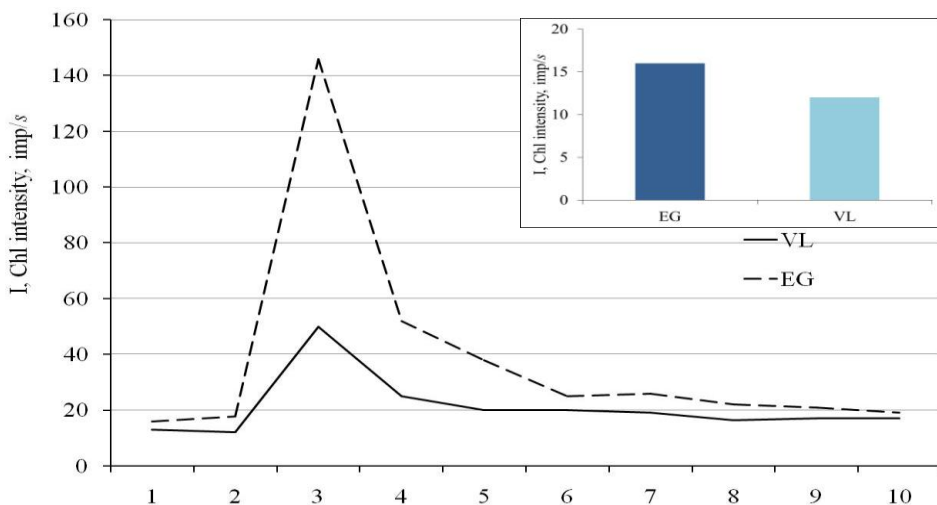


Fig. 7. Changes in the luminal-enhanced and spontaneous ChL levels of EG in the course of *Montivipera latifi* venom *in vitro* processing. In above corner spontaneous ChL data are given.

Dried lyophilized toxin of ML was dissolved in Tris-HCl buffer (pH 7.4) with a final concentration of $3 \cdot 10^{-5} M$, and 0.2mL of this solution were incubated with each assay at $37^{\circ}C$ for a period of 10 min.

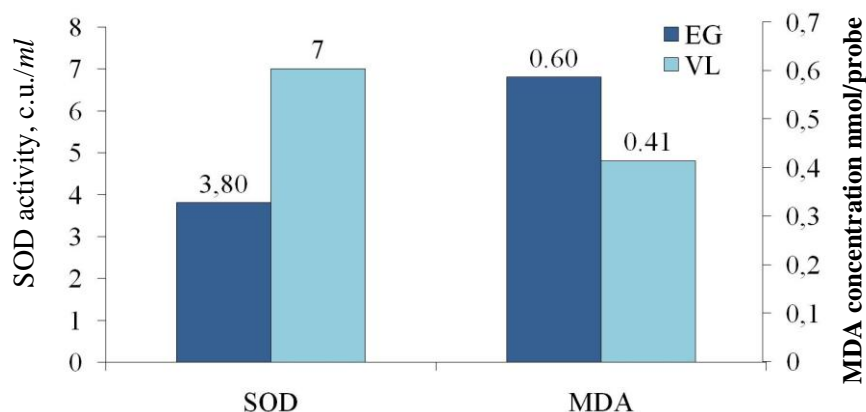


Fig. 8. Changes in the concentration of malonicdialdehyde and the superoxidedismutase activity of erythrocyte ghosts in the course of *Montivipera latifi* venom *in vivo* processing (first bars are the control, second bars are assays after venom (VL) processing).

Macrovipera latifi venom inhibited the growth of S-180 sarcoma in mice

As shown in **Fig. 9** ML venom influence on S-180 sarcoma was noticeable as compared with control group. The antitumor activity index shows the suppression of tumor growth with the inhibitory rate of approximately 25%, while the significant weight loss in the S-180 sarcoma-group mice was not observed indicating that there was no notable side effect on body weight during treatment (**Table 1**).

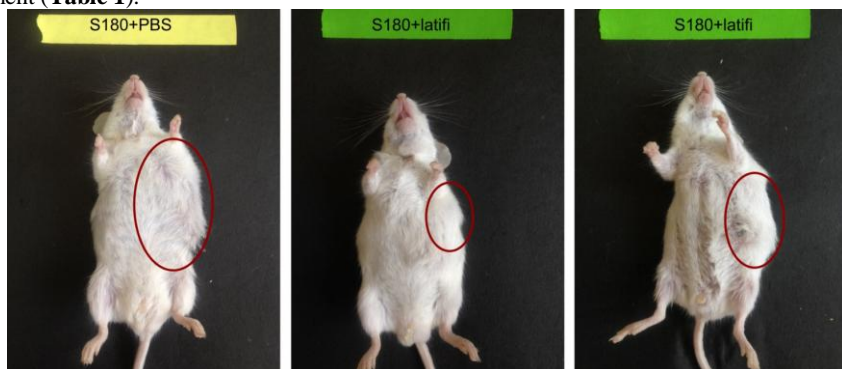


Fig. 9. Effective suppression of sarcoma growth in S-180 bearing mice was induced by ML venom. Each group contained 5 mice. The mice were administered as follows: modelgroup, PBS; venom treated group, each animal received small doses of ML (10 µg/mouse). All solutions were dissolved in PBS and were administered daily by intra-tumor injection (50 µl) for 5 days. Twenty-four hours after the last drug administration, all animals were weighed and sacrificed by cervical dislocation. Tumor growth was significantly suppressed in mice treated with ML venom compared to the control group. The red circle indicates the location and size of the S-180 sarcoma.

Table 1. Body weight changes of S-180 sarcoma bearing mice with and without ML treatment. The total body weights of the S-180 treated mice were similar to those of the model group, during treatment.

Sample	Day	1	2	3	4	5
Norm		17.86	18.36	19.38	19	19.6
Model Group (S-180+PBS)		23.84	24.13	24.27	25.4	26.6
Treated Group (S-180+ML)		24.18	23	23.12	25	23.74

Sarcomas are defined as a type of cancerous connective tissue tumors (Dean and Whitwell, 2009). As results of different oxidative loads vary in tissues, the total antioxidant capacity varies. Individual tissues acquire their own antioxidant composition based on the oxidizing courses that it most likely would endeavor (Muller et al., 1996). Tumors arising in bone and soft tissue share common characteristic features (to not only common mesenchymal origin but also the anatomical surrounding). Soft tissue sarcomas are extra-compartmental or found in an anatomical region that is not blocked off by anatomical barriers. The inhibitory effect of one of the *Macrovipera lebetina obtusa* venom components obtustatin is already shown on the melanoma (Brown et al., 2008) and the same Crocker sarcoma (Ghazaryan N. et al., 2015). However, we've been interested to try some other viper venom in this regard.

S-180 sarcoma DNA **S-180 DNA with ML venom (mg/ml)**
1 **0.5** **0.25** **0.125**

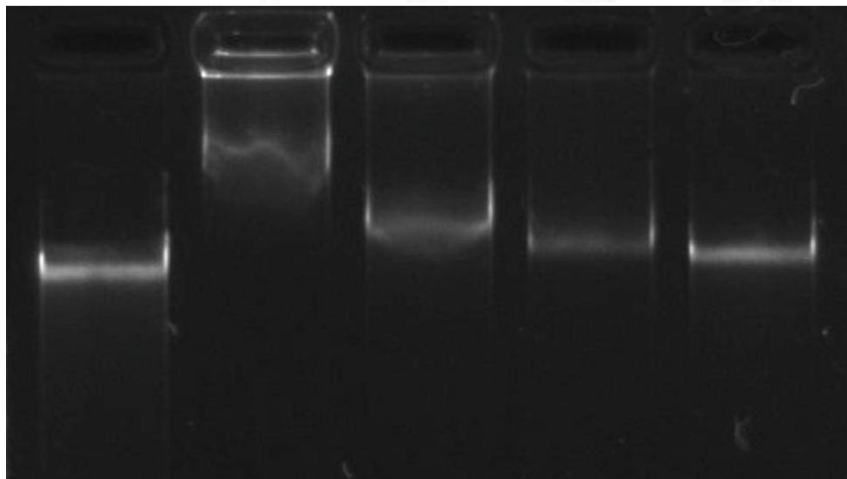


Fig.10. Retarded electrophoretic migration of ML venom treated genomic DNA from S-180 sarcoma cells. Photograph of gel retardation assay of genomic DNA from S-180 sarcoma cells treated with ML venom. Genomic DNA was extracted from S-180 sarcoma cells treated with ML venom (1, 0.5, 0.25 and 0.125 mg/ml) for 30 min and electrophoretically analyzed for 1h. These results demonstrated that ML venom were able to bind genomic DNA in S-180 sarcoma cells.

Montivipera latifi venom binding to genomic DNA of the S-180 sarcoma cells

Electrophoretic gel mobility shift assays were performed to characterize the binding of ML to whole genomic DNA in more detail (Fig. 10), because they provide information on the electrophoretic stability, mobility and fluorescence intensities of the venom components and DNA complexes at once. A reduction of the electrophoretic mobility of DNA was observed upon an increase of the concentration of venom pre-stained with DNA samples. Of note, at low concentrations the bands were sharp, but started to become diffuse and less intense at dose above 0.5 mg/ml, which may indicate partially denatured or degraded DNA, eventually by ML venom components. The sample in gel with highest concentration of venom is not a band at all but rather a smear. While bands with intensities depending on the protein concentration can be attributed to DNA and protein-DNA complexes, with 'DNA' referring to double-stranded DNA, bands with smearing intensities represent a single-stranded DNA. This can be attributed to the formation of partially single stranded DNA during gel electrophoresis, so we can say that *ML* venom might exert its inhibitory effect on S-180 sarcoma cells by binding genomic DNA and blocking gene expression, and even damage its packaging.

Results of the Reactive Oxygen Species production and Superoxide dismutase activity

The importance of lipid peroxidation processes in different cancers is already known: the imbalance between the pro-oxidants and antioxidants in favor of the former gives rise to oxidative stress that has been proven to lead to carcinogenesis (Battisti et al., 2008; Chang et al., 2008; Wauquier et al., 2009). While moderate decreases in the mean of spontaneous chemiluminescence counts were observed in the course of S-180 growth, the treatment by ML venom *in vivo* lead to the particular increase of the ROS production in organism almost till the normal level (**Fig. 11**). The lipid peroxidation, which is the particular case of the whole oxidative processes in tissues, in contrary, increases even more under venom components influence (**Fig. 12**). This reaction could be quite expectable, taking into consideration the high percentage of phospholipase A2 in venom of vipers.

This fact is also confirmed by the increasing of SOD activity accompanying the similar changes in ROS production processes (**Fig.12**). Hence, on the one hand the intensification of lipid free radical oxidation is taking place in the membranes of tissue cells, on the other hand under venom action considerable suppression of SOD occurs and thus inhibition of antioxidative processes. Previously we've shown a radioprotective effect of the sublethal doses of two other species of *Viperidae* snakes venom in the mammal's red blood cells, but according to literature data, the clinical pictures of viper's venom influence are essentially different for different tissues and for blood (Ayvazyan N.M. et al., 2012).

Free radicals attack lipids mainly polyunsaturated fatty acids, giving rise to lipid peroxides that play an essential role in cell division regulation (Battisti et al., 2008). As a result MDA formed from lipid peroxidation functions as a tumor promoter and co-carcinogenetic agent and has the ability to hinder the role of antioxidant enzymes. The direct correlation between lipid peroxidation and cell proliferation with increased lipid damage in highly proliferated cells has been noted (Niedernhofer et al., 2003). Although ChL intensity is suppressed in tumor tissue, in our study both MDA and SOD levels are increased in the sarcoma cells, while treated groups demonstrate data, more close to the normal conditions of oxidative processes and antioxidant

system which is in an agreement with data concerning the oxidative stress of patients suffering from bone and soft tissue sarcomas (Nathan et al., 2011).

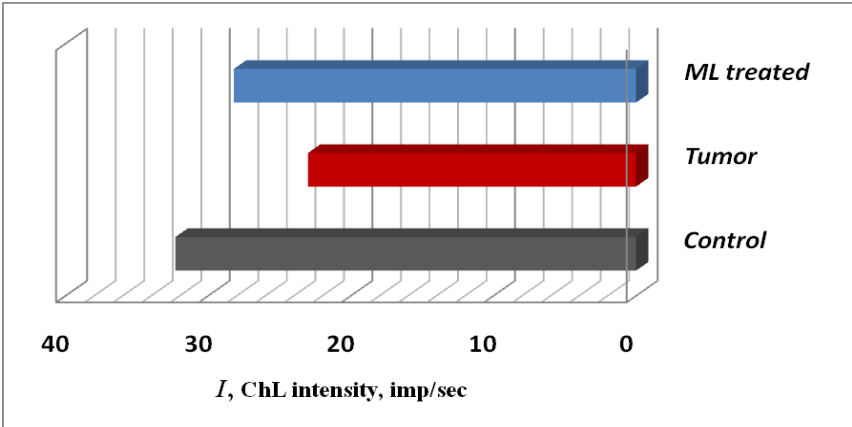


Fig. 11. Changes in the spontaneous chemiluminescence levels of S-180 sarcoma tissue in the course of *Montivipera latifi* venom in vivo processing.

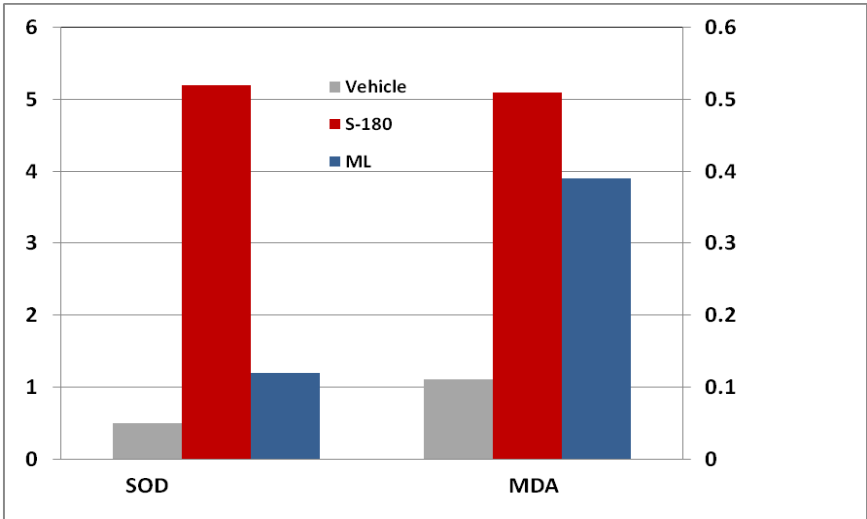


Fig.12. Changes in the concentration of malonicdialdehyde and the superoxide dismutase activity of S-180 sarcoma tissue in the course of *Montivipera latifi* venom in vivo processing (first bars –control, second bars – tumor assays, third bars –tumor assays after venom processing).

Conclusions and perspectives

The snakebites with viper venom produced extensive systemic alterations, including tissue blistering, edema, hemorrhage and necrosis, as well as hypotension shock and coagulopathy, especially clotting disorders and hypofibrinogenemia (Kakanj et al., 2015; Sanz et al., 2008; Ayvazyan and Ghazaryan, 2012). The damage concern primarily blood cells and the resistance of erythrocytes to the envenomation in this respect became quite crucial issue.

Montivipera latifi is a very endemic species in Iran and any investigations of its toxinology and envenomation effects are very scarce, but the distribution of these snakes in the country is quite wide, so the studying of this snake venom either by itself or in comparison with other viper venoms of the region becomes more and more important. The previous research of our lab was devoted to the molecular events associated with *Macrovipera lebetina obtusa* and *Montivipera raddei* snakes' venom intoxication and condition of biomembranes (Ayvazyan and Ghazaryan, 2012; Ayvazyan et al., 2012). These two species of snakes together with *Montivipera latifi* are the most pharmacologically important *Viperidae* snakes of Iran and detailed analysis of their membranotropic properties quite vital both for antivenom production in the country and opening of new perspectives in drug design and development.

The present study confirmed the direct damage of red blood cell membranes by ML venom components, and the fast transformation of erythrocytes in course of envenomation. It also showed that morphological changes of red blood cells caused by venom injection could be reversible depend on the venom concentration in media. The study underlines that drastic changes during venom treatment occur both in the lipid composition of membranes and the main protein systems within the membranes, like ATPase complexes. These functional alterations could be also caused by the changes in lipid bilayer packaging in the RBCs membrane, its plastic properties, fluidity and microviscosity. The inhibition of Na^+/K^+ and $\text{Ca}^{2+}/\text{Mg}^{2+}$ - ATPase activities in venom-treated erythrocyte ghosts conclusively shows that viper venom has a profound restructuring effect on the lateral organization of lipids in model and complex biological membranes. Moreover, our data for higher concentration of venom let us suggest, that venom components mainly blocking the work of Na^+/K^+ pump. Together with the damage of lipid packaging these changes cause the drastic increasing of Ca^{2+} cations in the erythrocytes and leading to the activation of $\text{Ca}^{2+}/\text{Mg}^{2+}$ - ATPase work. The present observations on the Ca^{2+} pump ATPase activity in RBCs of subjects with intoxication may be taken to suggest that the activity of this membrane-associated pump is not altered irreversibly. The Ca^{2+} pump ATPase is the only mechanism by which RBCs maintain low intracellular Ca^{2+} concentration and an important means by which other cells accomplish this. Maintenance of low intracellular Ca^{2+} concentration is critical for normal cell function and viability. It is known, that even 20% decrease in activity of the Ca^{2+} pump ATPase in the RBCs (for example, of hypertensive subjects) may reflect a deficit in the ability of certain cells to maintain optimally low intracellular Ca^{2+} (Farber, 1981). Current observation of the enhanced activity of $\text{Ca}^{2+}/\text{Mg}^{2+}$ - ATPase could be an attempt to indirectly counteract the Ca^{2+} overload, i.e. Ca^{2+} extrusion through the $\text{Na}^+/\text{Ca}^{2+}$ exchanger operating in forward mode. Based on these considerations, we suggest that the enhanced activity of Ca^{2+} pump ATPase seen after treatment with ML venom may be a protective mechanism aimed at preserving erythrocyte ghosts cytoskeletal function during membrane damage. This conclusion agrees with our previous observation, that erythrocyte membrane damage could be reversible for low concentrations of venom, but irreversible for the increased dose of toxins. As a conclusion of present experimental observation, we think that ML venom components damaging the lipid bilayer of erythrocyte membrane, which cause the multiple

defects in the bilayer packaging and, hence the outwardly directed efflux of K^+ ions from erythrocyte and increase inward of Ca^{2+} ions gradient. It seems to us logical to suggest that Ca^{2+}/Mg^{2+} -ATPase is not significantly damaged by the venom components, but because of this dramatic changes of cation exchange through RBC membrane, the activity of Ca^{2+} pump ATPase used to increase almost twice compare to the normal conditions. Clearly, as well as its numerous benefits, there are limitations to studying mechanisms of action of molecules on erythrocyte ghosts and GUVs, as there are with any model systems. But such a complex experiments of erythrocyte ghosts in comparison with lipid vesicles as a negative control without protein components, seems to us very perspective for future investigations.

Free radicals are, in general, highly reactive and extremely short-lived, that is why they are elusive and hard to be detected. In order to confirm their production, we often have to search for end products or by products of radical, induced reactions, examining the reaction “path” of the radicals. The ChL analysis currently is the only direct way to detect and measure free radicals in the real time mode, so, the complex approach is the best way for studying reactions that produce free radicals and cause oxidative damage.

Our results show the correlation between the data of the TBA-test and ChL analysis, which are significantly, witness the inhibitory effect of venom on the level of ROS production in red blood cells. Very likely, as we believe, marked influence is due to the recently found disintegrins: a group of cysteine-rich peptides occurring in *Crotalidae* and *Viperidae* snake venoms (Calvete JJ et al., 2009, 2016). As it is known, the cysteine-containing substrates are strong antioxidants. The purified components demonstrate more toxic effect than the content of whole venom and have zinc-chelated sequences. The results obtained here allow us to suppose that in this 10 min treatment with snake venom the toxicant exercises its harmful effect also on the protein components of the cellular membrane, because during exposure time the activity of SOD enzyme exhibits some fast changes in its specific activity to the dramatic increase almost twice compare to the normal condition.

Our previous results have shown the changes of red blood cells and GUV membranes condition and properties under *in vitro* and *in vivo* influence of the *Macrovipera lebetina obtusa* venom (Ghulikyan L. et al., 2016). The addition of *ML* venom to mixed-lipid giant unilamellar vesicles from brain lipids of rats and erythrocyte ghosts shows noticeable changes, both distortions in the vesicle membrane and shrinking of the vesicle size. These effects are likely to disturb the local packing structure of the lipids and fluidity of the bilayer (Ayvazyan and Ghazaryan, 2012; Ghazaryan et al., 2013, Ghazaryan et al., 2015). We’ve also monitored the effect of *Macrovipera lebetina obtusa* and *Montivipera raddei* venom on ATPase activity in human erythrocytes ghost membranes *in vitro* (Kirakosyan G. et al., 2016). So here, we decided to study whether crude *ML* venom also shows the antitumor influence on the sarcoma model as it was reported earlier by N. Ghazaryan et al. (2015b) for *Macrovipera lebetina obtusa*.

Sarcomas are defined as a type of cancerous connective tissue tumors (Dean and Whitwell, 2009). As results of different oxidative loads vary in tissues, the total antioxidant capacity varies. Individual tissues acquire their own antioxidant composition based on the oxidizing courses that it most likely would endeavor (Muller et al., 1996). Tumors arising in bone and soft tissue share common characteristic features (to not only common mesenchymal origin but also the anatomical surrounding). The inhibitory effect of one of the *Macrovipera lebetina obtusa* venom components obtustatin is already shown on the melanoma (Brown et al., 2008) and the same Crocker sarcoma. However, we’ve been interested to try some other viper venom in this regard.

As clear from our results, *ML* venom also able to suppress the S-180 sarcoma growth with 25% at the doses of 10 µg/mouse, which is less than the similar data for *Macrovipera lebetina*

obtusa and obtustatin, but still quite essential. This activity was confirmed by the results of the DNA retardation experiment demonstrated that *ML* venom were able to bind to the genomic DNA of S-180 sarcoma cells and block the electrophoretic mobility of genomic DNA, and furthermore some gene expression. The results of our DNA retardation experiment demonstrated that *ML* venom components could bind to genomic DNA from the S-180 sarcoma cells and suppress its electrophoretic mobility in a dose-dependent manner. These data indicated that higher doses of venom lead to genomic DNA double strand reaming and further degradation.

Free radicals attack lipids mainly polyunsaturated fatty acids, giving rise to lipid peroxides that play an essential role in cell division regulation (Battisti et al., 2008). As a result MDA formed from lipid peroxidation functions as a tumor promoter and co-carcinogenetic agent and has the ability to hinder the role of antioxidant enzymes. The direct correlation between lipid peroxidation and cell proliferation with increased lipid damage in highly proliferated cells has been noted (Niedernhofer et al., 2003). Although ChL intensity is suppressed in tumor tissue, in our study both MDA and SOD levels are increased in the sarcoma cells, while treated groups demonstrate data, more close to the normal conditions of oxidative processes and antioxidant system which is in an agreement with data concerning the oxidative stress of patients suffering from bone and soft tissue sarcomas (Nathan et al., 2011).

Snake bites are an endemic public health problem in Iran, both in rural and urban area. Iranian herpetofauna is quite rich by venomous snakes, including the representatives of the *Elapidae*, *Crotalidae* and *Viperidae* families. Hence, the possibility to isolate active, single components of this venom as a prototype of the specific inhibitor for cancer cells seems very perspective, but, on the other hand, there are very interesting new results witnesses that synergic action of venom components and their effect in concert within the crude venom content could be more potent. In this current stage of our experiments the results suggested that *ML* venom might be a potential candidate for the treatment of sarcoma, and might prevent tumor growth *in vivo* with low toxicity, which possibly could be concerned with the work of some disintegrins in venom composition.

CONCLUSIONS

The following conclusions were made based on experimentally obtained data:

1. It has been shown *in vivo*, that treatment by *ML* venom results in the S-180 sarcoma growth 25% reduction.
2. It has been shown *in vitro* by EMSA that *ML* venom components could bind to genomic DNA from the S-180 sarcoma cells and suppress its electrophoretic mobility in a dose-dependent manner.
3. It has been shown with the help of fluorescence probes (ANS and pyrene) that *Montivipera latifii* venom causes the damage of the membrane of erythrocyte ghosts with following lysis and induces dose-dependent shrinking of GUVs or leakage of liposomes without changing the shape of GUV.

4. It has been discovered that the *Montivipera latifi* venom causes the alteration of the erythrocyte ghosts' membrane Na^+/K^+ -ATPase system work, but not of the Ca^{2+} pump ATPase.
5. It has been shown that although ROS generation intensity is suppressed in tumor tissue, in our study both MDA and SOD levels are increased in the sarcoma cells, while treated groups demonstrate data, more close to the normal conditions of oxidative processes and antioxidant system.

LIST OF PUBLICATIONS AS A PART OF DISSERTATION TOPIC

1. L. Ghulikyan, M. Mohamadvarzi, G. Ghukasyan, A. Kishmiryan, N. Zaqaryan, G. Kirakosyan & N. Ayvazyan, Molecular events associated with *Vipera latifi* venom effect on condition of human red blood cells. **PROCEEDINGS OF THE YEREVAN STATE UNIVERSITY**, 2016, № 2, p. 43–50.
2. G. Kirakosyan, M. Mohamadvarzi, L. Ghulikyan, N. Zaqaryan, A. Kishmiryan, & N. Ayvazyan, Morphological and functional alteration of erythrocyte ghosts and giant unilamellar vesicles caused by *Vipera latifi* venom. **Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology**, 2016, V. 190, pp 48–53 (DOI: 10.1016/j.cbpc.2016.08.006).
3. N. Ayvazyan, M. Mohamadvarzi, G. Kirakosyan, L. Ghulikyan, N. Zaqaryan, Morphological and functional alteration of human erythrocytes caused by some iranian vipers' venom: novel glance at the old problem. **Acta Physiologica**, June 2016, 217: 42.
4. M. Mohamadvarzi, Recognition of *Vipera latifi* venom components by the S-180 sarcoma DNA and its anti-tumor activity. **Journal of Scientific and Practical Medicine**, 2017, Volume 21, N 4, pp. 31-36.

ՄԱՐԻԱՄ ՄՈՀԱՄԱԴՎԱՐՁԻ

MONTIVIPERA LATIFI ՕՁԻ ԹՈՒՅՆՈՎ ՊԱՅՄԱՆԱՎՈՐՎԱԾ ԷՐԻԹՐՈՑԻՏԱՐ ՍՏՎԵՐՆԵՐԻ և ՀՍԿԱ ՄԻԱԼԱՄԵԼ ՎԵՋԻԿՈՒԼՆԵՐԻ ՄՈՐՖՈՖՈՒՆԿՑԻՈՆԱԼ ԽԱՆԳԱՐՈՒՄՆԵՐԸ

Ամփոփագիր

Հանգուցային բառեր՝ *Montivipera latifi*, թաղանթային մոդելներ, էրիտրոցիտար ստվերներ, Կրոկերի սառկոմա, ազատ ռադիկալային գործընթացներ:

Իժերի թույնն՝ որպես տոքսինների հեմոլիտիկ կենսաքիմիական “կոկտեյլ”, հիմնականում առաջացնում է արյան բջիջների համակարգային փոփոխություններ և վնասվածքներ: Վերջին տարիների հետազոտություններն ապացուցում են, որ օձի թույները հանդես են գալիս ոչ միայն որպես կարևոր գործիքներ ուսումնասիրելու համար տարբեր ֆիզիոլոգիական գործընթացների մոլեկուլային հիմքը, այլև՝ որպես թերապևտիկ գործոնների բնական նախատիպերի յուրօրինակ պահոց են բավականին լայն սպեկտրով հիվանդությունների բուժման համար: Իժերի թույնի օնկոստատիկ ազդեցությունը բացատրվում է անգիոգենեզն արգելակելու ունակությամբ: Մեր հետազոտությունների նպատակն է եղել բացահայտել մկների մոտ *Montivipera latifi* օձի թույնի արգելակիչ ազդեցությունը S-180 սարկոմայի աճի վրա *in vitro* և *in vivo* պայմաններում: Կիրառվել են ուռուցքի աճի ինտենսիվության որոշման ինչպես դասական մեթոդներ, այնպես էլ՝ մոլեկուլա- կենսաբանական և կենսաքիմիական այլ մոտեցումներ (քիմյումինեսցենտային վերլուծություն, հյուսվածքների լիպիդների պերօքսիդային օքսիդացման և հակառադիկալային պաշտպանության համակարգի ֆերմենտների ակտիվության համեմատական ուսումնասիրություն, հելում ԴՆԹ-ի շարժունակության դանդաղեցման վերլուծություն (EMSA)): Արդյունքները թույլ են տալիս խոսել այն մասին, որ *Montivipera latifi* օձի թույնի 10 մկգ/մուկ չափաբաժնով ամենօրյա ներարկումը հստակորեն ճնշում է ուռուցքի աճը 25 % -ով: Թույնով մշակելուց հետո հելում ԴՆԹ-ի շարժունակության արագության փոփոխությունը (EMSA մեթոդ) վկայում է կայուն ԴՆԹ-սպիտակուց կոմպլեքսների առաջացման մասին: Վերջինս կարող է համապատասխան գեների էքսպրեսիայի ճնշման պատճառ լինել: Փորձարարական խմբերում լիպիդների պերօքսիդային օքսիդացման մակարդակը և օքսիդացման գործընթացների ազատ ռադիկալային ֆոնը բարձրացել է՝ մոտենալով նորմային: Այդ իսկ պատճառով, օրգանիզմում սուպերօքսիդի համուտագի բարձր ակտիվությունը ևս նորմալավորվել է:

МАРИАМ МОХАМАДВАРЗИ

МОРФОФУНКЦИОНАЛЬНЫЕ НАРУШЕНИЯ МЕБРАН ГИГАНТСКИХ
УНИЛАММЕЛЯРНЫХ ВЕЗИКУЛ И ЭРИТРОЦИТАРНЫХ ТЕНЕЙ ПРИ ВОЗДЕЙСТВИИ
НЕ НИХ ЯДОМ *MONTIVIPERA LATIFI*

РЕЗЮМЕ

Ключевые слова: *Montivipera latifi*, мембранные модели, эритроцитарные тени, саркома Крокера, свободнорадикальные процессы.

Яд гадюковых, являясь таким биохимическим «коктейлем» токсинов, в первую очередь вызывает обширные повреждения клеток крови. Исследования последних лет доказали, что токсины змеиных ядов проявили себя не только в качестве важных инструментов для изучения молекулярных основ различных физиологических процессов, но также как уникальную природную кладовую прототипов терапевтических агентов для лечения самого широкого спектра патологий. Онкостатический эффект ядов гадюковых объясняют способностью ингибировать ангиогенез. Наши исследования ставили своей целью выявить ингибирующее влияние яда *Montivipera latifi* на рост саркомы S-180 у мышей *in vitro* и *in vivo*. Применялись как классические методы определения интенсивности роста опухоли, так и методы молекулярно-биологических и биохимических подходов (хемилюминесцентный анализ, сравнительное исследование перекисного окисления липидов тканей и активности ферментов системы антирадикальной защиты, анализ на замедление подвижности ДНК в геле, EMSA). Результаты позволяют говорить о стойком подавлении роста опухоли на 25% цельным ядом *Montivipera latifi* при ежедневных инъекциях в дозах 10 мкг/мышь. Изменение скорости миграции ДНК в геле после обработки ядом (метод EMSA) свидетельствовал об образовании стабильных ДНК-белковых комплексов, что может быть причиной подавления экспрессии соответствующих генов. Перекисное окисление липидов и свободнорадикальный фон окислительных процессов в экспериментальных группах повышался, приближаясь к норме, в связи с чем повышенная активность супероксиддисмутазы в организме также нормализовалась.