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MOLECULAR EVENTS ASSOCIATED WITH *VIPERA LATIFI* VENOM EFFECT ON CONDITION OF HUMAN RED BLOOD CELLS

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Viper venom as a hemolytic biochemical "cocktail" of toxins, primarily cause to the systemic alteration of blood cells. In the sixties and seventies, human erythrocytes were extensively studied, but the mechanical and chemical stresses commonly exerted on red blood cells continue to attract interest of scientists for the study of membrane structure and function. Here we monitor the effect of *Vipera latifi* venom on human erythrocytes ghost membranes using phase contrast microscopy and changes in ATPase activity under snake venom influence *in vitro*. The ion pumps (Na^+, K^+)-ATPase and (Ca^{2+}, Mg^{2+})-ATPase plays a pivotal role in the active transport of certain cations and maintenance of intracellular electrolyte homeostasis. We also describe the action of *Vipera latifi* venom on the free-radical processes in the membrane of erythrocyte ghosts and changes of activity of superoxide dismutase in course of envenomation.

Keywords: *Vipera latifi*, (Na^+, K^+)-ATPase, (Ca^{2+}, Mg^{2+})-ATPases, erythrocyte ghosts, lipid peroxidation, superoxide dismutase.

Introduction. 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. There are 83 snake species in Iran and about 25 of them are venomous [1, 2]. Within 2002–2011 there were about 60 thousands snake bites in Iran that caused 67 mortalities [3]. *Vipera latifi* (also denominated *Montivipera latifi* by some authors) belongs to sister clades of the Middle East complex within the monophyletic Eurasian *Vipera* genus [4]. These snakes are important source of snake bite in Iran each year, leading to severe local effects such as tissue blistering, hemorrhage and necrosis, as well as coagulopathy, especially clotting disorders and hypofibrinogenemia [3, 5]. Venom yield per snake is around 6 ± 2 *mg* and LD50 values for mouse are 3.2–7.9 μg/mouse [1, 5]. Although the venoms of *Vipera latifi* (VL) haven't been yet thoroughly characterized at proteomic level [6], their toxinological profile is quite well studied [3, 5], but the information about correlation with the clinical picture of envenoming still lacks.

Studies on cytotoxic action of viper venoms on human erythrocytes have been carried out since the 1960s [7]. Recent advances in fluorescent and super

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resolution microscopy cause the new wave of re-investigations of cell-invasive mechanisms of snake venoms [8]. After extrusion of nuclei and degradation of endoplasmic reticulum, reticulocytes emerge in the circulation, where they rapidly develop into mature red blood cells (RBC) 8 μm biconcave disks and with 120 days life span [9]. The flexibility of the membrane along with the ability of RBCs to maintain their morphology without a nucleus makes them an excellent model to study membrane mechanics.

Biological production of oxygen free radicals has been known for a long time, but effect of oxidative stress on signal transduction, condition of membranes and cellular functioning have been studied widely only during the last decade. Different investigations have established that oxygen free radicals can cause the phosphorylation and activation of various signaling proteins [10]. In addition, when free radicals react with the polyunsaturated fatty acids in lipids, chain reactions generate radicals in profusion, which leads to alteration of membrane permeability, modification of lipid-protein interactions, and the formation of bioactive degradation products [11].

An extensive defense system against reactive oxygen species (ROS) has been evolved at the cellular level and antioxidants are the first line of defense against ROS. The enzymes, such as superoxide dismutase, catalase and glutathione peroxidases, complete the reduction of ROS to water [12]. In course of the normal functioning of cell both these components are in the dynamic equilibrium, but disturbance of the balance results in pathological changes within the membrane.

In current study we hypothesized that ROS could play an important role in the course of snake venom intoxication. Hence, 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 VL venom. Changes both in lipid composition and protein condition modulate membrane transport processes. RBC from patients with various types of pathology show significant membrane abnormalities [13], and it has been suggested that disorder of some important cell pumps play a role in producing and further spreading of these abnormalities. Sodium pump (Na^+, K^+)-ATPase plays an essential role in cellular function and is altered in a variety of pathological conditions [14]. Theoretically the increasing of intracellular Na^+ would lead to decrease the inward Na^+ gradient, which may drive Ca^{2+} efflux through Ca^{2+}–Na^+ countertransport. The human RBC contains an outwardly directed Ca^{2+} pump, which activity is coupled to phosphorylation and hydrolysis of a 150 *kDa* membrane-bound, Ca^{2+}-activated, Mg^{2+}-dependent ATPase [15]. In the present study erythrocyte ghost (EG) membranes (Na^+, K^+)-ATPase and Ca^{2+}-activated Mg^{2+}-dependent ATPase activities have been analyzed in course of envenomation by the VL venom.

Materials and Methods.

Venom and Chemicals. Iranian VL venom was purchased from “Latoxan” (France); 8-anilino-1-naphthalene sulfonic acid (ANS), oubain and adrenaline were purchased from “Sigma”. Packed red blood cells (PRBC) were obtained from Haemathology Center after Prof. R. Yeolyan (MoH, RA).

Erythrocyte Ghosts. Erythrocyte membranes were obtained by the method of Dodge, Mitchell & Hanahan [16]. Protein was measured by the method of Lowry using bovine serum albumin as a standard. To obtain EG, 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 EG were pelleted by centrifugation at 37000 *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.

(Na^+, K^+)- and (Ca^{2+}, Mg^+)-ATPase Assay in RBC. The activities of the ion motive ATPases: (Na^+, K^+)-ATPase, (Ca^{2+}, Mg^{2+})-ATPase, determined by the method of Hesketh et all [17]. The reaction mixtures for (Na^+, K^+)-ATPase assay contained, *mM/L*: $MgCl_2$ – 2.0; NaCl – 35.0; KCl – 17.5 and Tris-HCl – 10.0; pH 7.4, the ghost suspension in the presence or absence of ouabain (2 *mM*) [15] 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 (Ca^{2+}, Mg^{2+})-ATPase assay contained, *mM/L*: $CaCl_2$ – 17.5; $MgCl_2$ – 2; Tris-HCl – 10.0; pH 7.4 and the ghost suspension. Values were expressed as the difference between ATPase activity in presence of Mg^{2+}, Ca^{2+} and in presence of Mg^{2+} only. Reaction was initiated by the addition of ATP (7 *mM*) and mixture was incubated for 1 *h* at 37°*C*. Reaction was stopped by the addition of 10% (wt/vol.) ice cold trichloroacetic acid (final conc.). The mixture centrifuged at 4000 *g* 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. The activity of each enzyme was expressed as $\mu mol_{P_i}/\mu g_{protein}/min$.

Chemiluminescence Analysis and Lipid Peroxidation. ROS' levels were measured by chemiluminescence (ChL) analysing system 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 malonic dialdehyde (MDA). MDA level of tissues was determined by spectrophotometric measurement [18], 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 three-methin complex dye. MDA concentration was measured at 532 *nm*, using the B01-CT-8 spectrophotometer ("E-ChromTech", Taiwan).

Superoxide Dismutase Activity. Determination of superoxide dismutase (SOD) activity was done using method of the adrenaline autoxidation reaction in pH 10.2 [19]. 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 (adrenokhrom concentration) was preformed after the addition of adrenalin. SOD activity was determined from ratio of the autoxidation rates at the presence and absence of SOD.

Statistical Analysis. For quantitative analysis results are reported as means ±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. Light microscopy of human erythrocytes revealed a normal ~7.5 *μm* biconcave disk structure. The venom addition produced marked changes in size and shape of RBC (Fig. 1), these changes included a formation of characteristic rounded spiky protrusions (echinocyte) followed by increasing of RBC size and final lysis (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 (data not shown). There was a significant decrease of the ghost diameter after envenoming, 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. The first rapid changes of size usually occurred in 1 *min*, and diameter decreased almost on 50%.

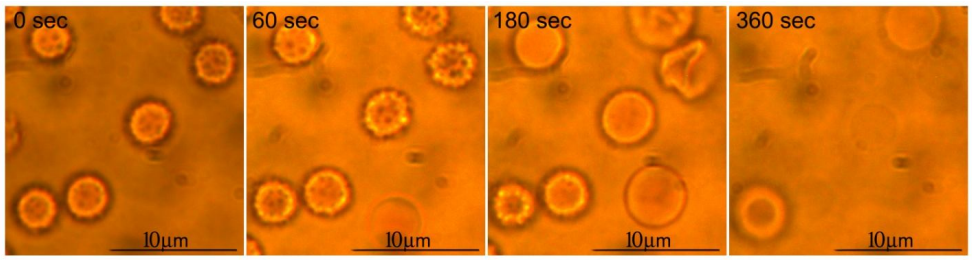

Fig. 1. *Vipera latifi* venom-dependent changes of the intact erythrocytes size and shape is shown. Dried lyophilized toxin of VL was dissolved in Tris-HCl buffer (pH 7.4) with a final concentration of $3 \cdot 10^{-5}$ *M* and 1.1 *μL* of this solution were added to the fluorescent microscope sample.

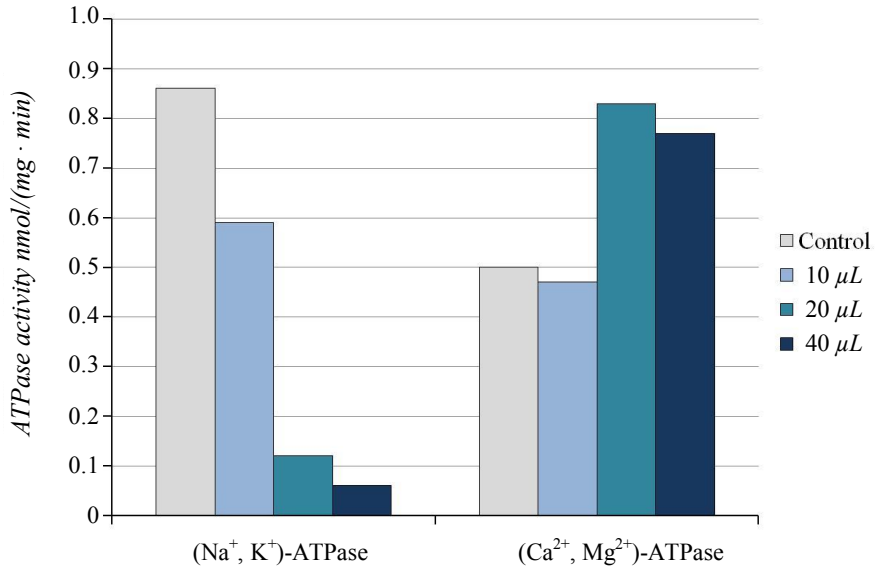

Fig. 2. (Na^+, K^+)- and (Ca^{2+}, Mg^{2+})-ATPase activity in *Vipera latifi* venom-treated erythrocyte ghosts. Enzymatic activity was assayed for three concentrations of snake venom and expressed as μmol_{P_i} released/μg_{protein}/*min*. The columns are the mean ± S.D. (n=6). $p < 0.05$ vs. the control group (ANOVA followed by the Bonferroni test).

The other series of experiments was done with a determination of RBC membrane's main ion transporters activities in course of treatment with VL venom. The *in vitro* treatment of erythrocyte ghosts with VL venom significally inhibit the (Na^+, K^+)-ATPase activity (Fig. 2), which was accompanied by a significant decrease in RBC (Ca^{2+},Mg^{2+})-ATPase activity 10 *min* after addition of the low concentration of venom, compared to control EG (Fig. 2). The treatment with gradually increasing concentrations of venom revealed the dose dependent manner of (Na^+, K^+)-ATPase 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 (Ca^{2+}, Mg^{2+})-ATPase, on the contrary, increases very dramatically in 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.

Moderate decreases in the mean of spontaneous and luminol-induced ChL counts were observed in the course of VL venom *in vitro* processing (Fig. 3), but the difference in luminol-enhanced rapid peak intensity was much more statistically significant in comparison with that of spontaneous one (Fig. 3, right upper corner).

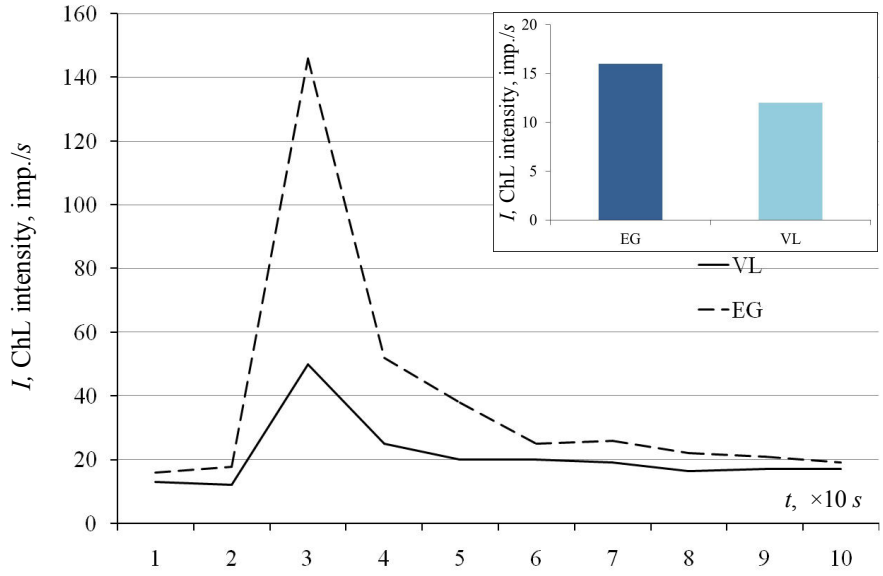

Fig. 3. Changes in the luminal-enhanced and spontaneous ChL levels of EG in the course of *Vipera latifi* venom *in vitro* processing. In above corner spontaneous ChL data are given.

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

This fact is also confirmed by the increasing of SOD activity accompanying these changes in lipid peroxidation processes (Figs. 4, 5). Hence, on the one hand, the suppression of lipid free radical oxidation is taking place in the membranes of erythrocite 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 RBCs,

which are most studied in this sense, the sublethal dose of *Macrovipera lebetina obtusa* (MLO) venom has a radioprotective effect [20].

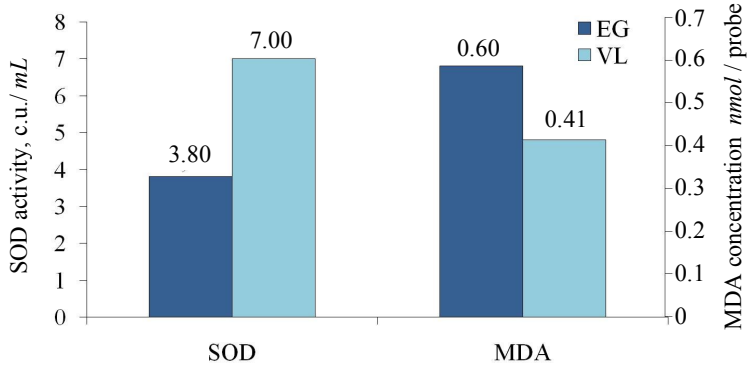

Fig. 4. Changes in the concentration of malonic dialdehide and the superoxid dismutase activity of erythrocyte ghosts in the course of *Vipera latifi* venom *in vivo* processing (first bars are the control, second bars are assays after venom processing).

Erythrocite ghosts were incubated with each assay at 37^oC for a period of 10 *min*. MDA concentration was measured at 532 *nm* after reaction with thio-barbituric acid (TBA) at 100^oC. The adrenochrome concentration was measured at 480 *nm* in assay containing 1*mL* 0.15 *M* Na_2CO_3 buffer with $3\cdot10^{-4}$ *M* EDTA (pH 10.2), 0.5 *mL* suspension of erythrocyte ghosts, 0.7 *mL* 0.005 *M* $KHPO_4$ buffer with $1\cdot10^{-5}$ *M* EDTA (pH 7.8) and 0.4 *mL* $2.25\cdot10^{-3}$ *M* of aqueous solution of adrenaline (pH 2.5).

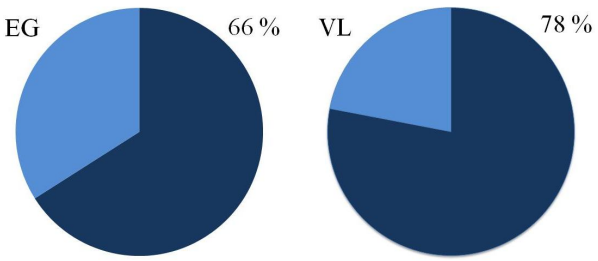

Fig. 5. Changes of the percentage of adrenalin autooxidation by superoxid dismutase in erythrocyte ghosts after viper's venom *in vivo* processing (light pies are the control, dark pies are assays after venom processing).

Discussion. *Vipera 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 these snake venom either by itself or in comparison with other viper venoms of the region become more and more important. Our previous research was devoted to the molecular events associated with *Macrovipera lebetina obtusa* and *Montivipera raddei* venom intoxication and condition of biomembranes [20, 21]. These two species of snakes together with *Vipera 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.

Present study confirmed the direct damage of RBC membranes by VL venom components, and the fast transformation of erythrocytes in course of envenomation.

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 [22]. 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.

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. Moreover, our data for higher concentration of venom let us suggest, that venom components mainly blocking the work of Na^+, K^+ pomp. Together with the damage of lipid packaging these changes cause the drastic increasing of Ca^{2+} cations in the erythrocytes and leading to the actvation of (Ca^{2+}, Mg^{2+})-ATPase work. The present observations on the Ca^{2+} pump ATPase activity in RBC of subjects with intoxication may be taken to suggest that the activity of this membrane-associated pump is not altered irreversible. The Ca^{2+} pump ATPase is the only mechanism, by which RBC 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 RBC (for example, of hypertensive subjects) may reflect a deficit in the ability of certain cells to maintain optimally low intracellular Ca^{2+}. As a conclusion of present experimental observation, we think that *Vipera latifi* 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 increse 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.

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