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Dynamic Changes in Lipid Peroxidation and Antioxidant Level in Rat's Tissues with *Macrovipera lebetina obtusa* and *Montivipera raddei* Venom Intoxication

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Abstract

We investigated the balance of free radicals in different tissues (liver, heart, brain and muscle) of rats in course of *in vivo* and *in vitro* processing by *Macrovipera lebetina obtusa* (MLO) and *Montivipera raddei* (MR) snake venoms. Chemiluminescence (ChL) levels were examined in tissue assays after incubation (at 37˚C for a period of 10 min) with venom for *in vitro* experiments and in tissue assays isolated of 10 min after venom injection for *in vivo* experiments. The TBA-test was also performed to confirm the free radical expression. The activities of antioxidant enzymes (such as superoxide dismutase and glutathione peroxidase) in isolated tissues were detected by spectrophotometry. During the *in vitro* processing chemiluminescence levels of tissue homogenates significantly decreased, while in course of *in vivo* intoxication the level of ChL was elevated in brain and liver; lipid peroxidation also increased in brain tissue, but there was no significant balance change in other tissues; the activity of superoxide dismutase mainly correlated with changes of free radical balance during intoxication. On the contrary, the activity of glutathione peroxidase showed the reverse tendencies to change. We suggest that free radicals and their oxidative stresses may play a role in the early stage of intoxication causing the so-named "spreading-effect", which is very characteristic for the venom of vipers.

Keywords

Chemiluminescence, Lipid Peroxidation, Superoxide Dismutase, Glutathione Peroxidase, *Macrovipera lebetina obtusa*, *Montivipera raddei*

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1. Introduction

Venoms produced by snakes of the family *Viperidae* (vipers and pit vipers) contain proteins that interfere with the coagulation cascade, the normal haemostatic system, and tissue repair [1] [2]. Human envenomation is often characterized by clotting disorders, hypofibrinogenemia, and local tissue necrosis. *Viperidae* venoms may contain well over 100 protein components [3] [4]. Most of these proteins can be found in the venom of other snakes. However, only a few protein families, including enzymes (serine proteinases, Zn^{2+}-metalloproteinases, L-amino acid oxidase, group II PLA2) and non-enzymes (disintegrins, C-type lectins, natriuretic peptides, myotoxins, CRISP toxins, nerve and vascular endothelium growth factors, cystatin and Kunitz-type proteinase inhibitors) are unique for *Viperidae* venom with respect to their biological effects. The medicinal value of snake venoms has been known from ancient times [5] [6]. In general, snake venoms are medicinally effective at very low doses and their therapeutic properties are achieved by mechanisms which are different from those of known therapies. With the advent of biotechnology, the efficiency of venom treatments has been substantiated by purifying components of venom and delineating their therapeutic properties. The short RTS-disintegrin obtustatin from *M. l. obtusa* venom is the first discovered $\alpha 1\beta 1$ antagonist [7]. It specifically blocks the interaction of the $\alpha 1\beta 1$ integrin with collagens IV and I *in vitro* with IC50s of 2 nM and 0.5 nM, respectively, and angiogenesis *in vivo*, and thus may represent a lead molecule for the development of novel anti-angiogenic therapeutic strategies to treat aggressive cancers [8]. On the other hand, the potential therapeutic value of *M. raddei* venom for regenerating damaged peripheral nerves has been reported [9]. In addition, studies on the underlying mechanisms of membrane/peptide binding are of considerable theoretical interest in the field of molecular evolution, in the development of new research tools and drugs of potential clinical use, and for antivenom production strategies.

Importance of oxygen for functioning of various types of biological membranes is hard to overrate. Although the biological production of oxygen free radicals has been known for a long time, the effects of oxidative stress on signal transduction and cellular functioning have only been studied widely during the last decade. Various studies have established that oxygen free radicals can cause the phosphorilation and activation of numerous signaling proteins [10] [11]. In addition, when free radicals react with the polyunsaturated fatty acids in lipids, chain reactions generate radicals in profusion, which results in alteration of membrane permeability, modification of lipid-protein interactions, and the formation of bioactive degradation products [12]. Proteins can be modified, resulting in carbonyls' level increase, oxidized methionines, and/or cysteines, protein hidrophobicity etc. These modifications as a result change the enzymes activity.

At the cellular level an extensive defense system against reactive oxygen species (ROS) has been evolved and antioxidants are the first line of defense against ROS. They comprise of enzymes, such as superoxide dismutase, catalase and glutathione peroxidases, which complete the reduction of ROS to water [13]. During the normal functioning of cell both these components are in the dynamic equilibrium, but disturbance of the balance leads to pathological changes in membrane.

We hypothesized that ROS might play an important role in the course of snake venom intoxication and study of the free-radical processes as well as in the activity of antioxidant enzymatic system in the interaction with venom; this could give new information about mechanisms of venom spreading and action in organism.

2. Experimental Procedure

2.1. Animals

Adult outbread male rats (weighing 180 - 220 g) were used in all experiments; the animals were kept in a 12-h light: dark cycle at 22˚C ± 2˚C and fed with laboratory chow and tap water *ad libitum*. Experiments were carried out between 08:00 and 09:00 AM. All procedures were done according to our institution's animal care rules and the IACUC's ethical guidelines for Decapitation of Unanaesthetized Mice and Rats (http://www.utsouthwestern.edu/utsw/cda/dept238828/files/469088.html).

2.2. Tissue Processing

Crude *Macrovipera lebetina obtusa* (*MLO*) and *Montivipera raddei* (*MR*) venoms were collected by milking snakes, vacuum dried at ambient temperature and stored at 4˚C until use. The venoms were tested for its ability to induce supramolecular changes in rats after short-term (10 min) intramuscular injection of the venom (0.35 mg/kg approx. 0.5 LD 50), by chemiluminescent examination of some organs (liver, heart, brain and muscle).

We tried to compare the data of *in vitro* and *in vivo* experiments. After decapitation, tissues were homogenized for 5 min by homogenizator of Potter-Elvehjem in Tris-HCl buffer (pH 7.4) with a final concentration of 20 mg/ml. For *in vitro* experiments dried lyophilized viper venoms were dissolved in the same buffered saline with a concentration of 3×10^{-5} M. For chemiluminescence analysis, both control assay of tissue and assay with added venom (3:0.2 ml) were incubated at 37˚C for a period of 10 min.

2.3. Chemiluminescence Analysis

Reactive oxigen species' (ROS) levels were measured by a ChL analysing system: intensities of tissue homogenates and lipid solutions were measured on a quantometric device, equipped with FEU-140 (Russia) photomultiplier with diapason of spectral sensitivity by 300 - 800 nm. The system contains a photon detector, ChL counter, water circulator and 32 bit IBM personal system. For registration of ChL-data with simultaneous statistic analysis a computational system based on LabView program was elaborated (National Instruments, USA) [14]. A cooler circulator is connected to the FEU-140 photon detector to keep the temperature at 50˚C. This ChL analyzing system is extremely sensitive, capable to detect as little as 10 - 15 W of radiant energy. ChL intensity was measured in an absolutely dark chamber by impulse/sec mode [15]. All experiments were performed also by Junior LB 9509 portable tube luminometer (BERTHOLD Technologies, Germany).

2.4. Lipid Peroxidation

Lipid peroxides are unstable and decomposed to a complex series of compounds. The most abundant compound is MDA. The MDA level of tissues was determined by spectrophotometric measurement [16], 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 SF-46 (Russia) spectrophotometer.

2.5. SOD Activity

Determination of superoxide dismutase (SOD) activity was done using method of the adrenaline autooxidation reaction in pH = 10.2 [17]. 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 SF-46 (Russia) spectrophotometer. The kinetic measurement of the 480 nm absorbance change (adrenokhrom 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.

2.6. Glutathion-Peroxidase Activity

Determination of glutathion-peroxidase (GP) activity was obtained through a technique consisting in the measure of NADPH oxidation by glutathione reductase at 340 nm [18]. The unit of ferment activity is a quantity of enzyme, necessary for oxidation of 1 μmol of reduced glutathione in 1 min. The whole peroxidase activity in the marked tissues was determined as described elsewhere [19] using a technique based on the inhibition of gvayacol products formation at 470 nm.

2.7. Statistical Analysis

For quantitative analysis of chemiluminescence intensity and antioxidant enzymes activities, a Student's test were used to compare differences at each time point, considering $P < 0.05$ as significant. All data were presented as mean ± SEM (n = number of experiments).

3. Results

Moderate decreases in the mean of ChL counts were observed in all tissues in the course of MLO and MR venoms *in vitro* processing (**Figure 1**), but the difference in heart and muscle tissues was not statistically significant in comparison with that of controls.

Results of the free radical scavenging effect of the snake venom extract in the *in vitro* assay, and in the TBA-test assay, showed a significant *in vitro* antioxidant activity (**Table 1**). In both assays the extract expressed

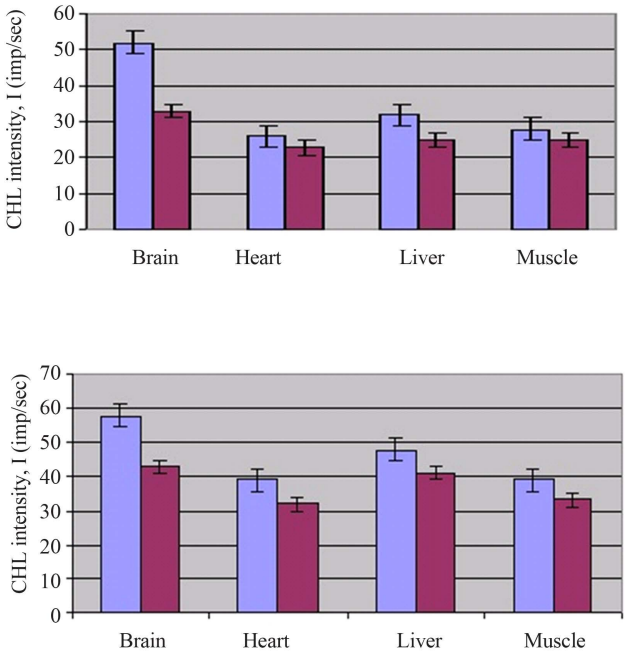

Figure 1. Changes in the spontaneous chemiluminescent levels of tissues in the course of viper's venom *in vitro* processing (above—*Macrovipera lebetina obtuse*; below—*Montivipera raddei*; blue bars—control, purple bars—assays after venom processing). Dried lyophilized toxin of MLO and MR was dissolved in Tris-HCl buffer (pH 7.4) with a final concentration of 3×10^{-5} M, and 0.2 ml of this solution were incubated with each assay at 37˚C for a period of 10 min.

Table 1. Changes in the spontaneous chemiluminescent levels and concentration of malonic dialdehide of tissues in the course of viper's venom *in vitro* processing.

Tissue	ChL intensity (imp/sec)		MDA concentration (nmoles/assay)	
	Control	*+Venom*	*Control*	*+Venom*
Brain	58 ± 4.18	43 ± 2.78	23 ± 0.08	1.67 ± 0.05
Heart	39 ± 3	32 ± 1.12	16 ± 0.03	0.56 ± 0.04
Liver	48 ± 3.91	41 ± 2.55	27 ± 7.06	1.2 ± 0.1
Muscle	39 ± 1.58	33 ± 1.22	15 ± 0.04	0.53 ± 0.04

Dried lyophilized toxin of MLO was dissolved in Tris-HCl buffer (pH 7.4) with a final concentration of 3×10^{-5} M, and 0.2 ml of this solution were incubated with each assay at 37˚C for a period of 10 min. $P < 0.05$.

a high antioxidant activity, but this reaction is not the same in the case of *in vivo* intoxication. The data of ChL-analysis indicate antioxidant effect of zootoxins on heart and muscle tissues after their treating with venoms, but the oxidative processes in brain and liver after intoxication are remarkably increased (**Figure 2**).

When we analyzed the accumulation of MDA in different tissues of the mammals in norm and after processing by snake venom, we observed the divergence of its account in all tissues and liver (**Figure 3**). On the one hand the suppression of lipid free radical oxidation is taking place in the last case, on the other hand in nervous tissues, heart and muscles under venom action considerable activation of MDA formation occurs and thus intensification of peroxidative 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, which are most studied in this sense, the sublethal dose of MLO venom has a radioprotective effect [20].

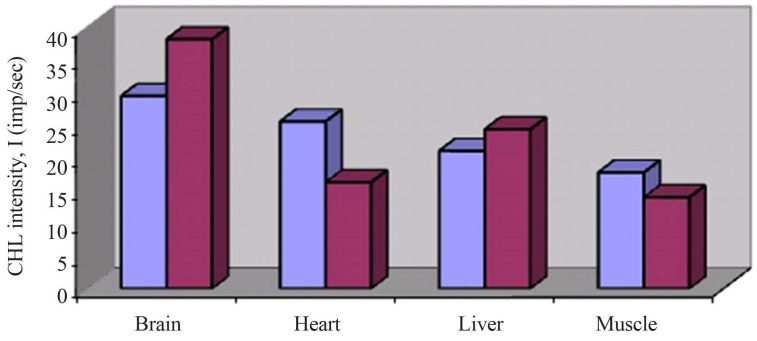

Figure 2. Changes in the spontaneous chemiluminescent levels of tissues in the course of viper's venom *in vivo* processing (blue bars—control, purple bars—assays after venom processing). Tissues of rats were homogenized for 5 min by homogenizator of Potter-Elvehjem in Tris-HCl buffer (pH 7.4) with a final concentration of 20 mg/ml after short-term (10 min) intramuscular injection of the venom (0.35 mg/kg approx. 0.5 LD 50).

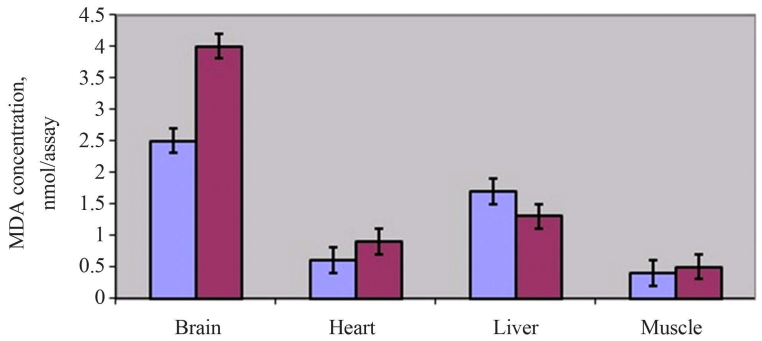

Figure 3. Changes in the concentration of malonic dialdehide of tissues in the course of viper's venom *in vivo* processing (blue bars—control, purple bars—assays after venom processing). Tissues of rats were homogenized for 5 min by homogenizator of Potter-Elvehjem in Tris-HCl buffer (pH 7.4) with a final concentration of 20 mg/ml after short-term (10 min) intramuscular injection of the venom (0.35 mg/kg approx. 0.5 LD 50). MDA concentration was measured at 532 nm after reaction with thio-barbituric acid (TBA) at 100˚C.

This fact is also confirmed by the increasing of SOD activity accompanying these changes in lipid peroxidation processes (**Figure 4**).

Another common and widely accepted method of detecting free radical production in the course of intoxication is by checking for levels of glutathione peroxidase that is intimately involved in some of the body's most important antioxidant defense systems. GP levels have typically been measured in brain, heart, liver and muscle tissues and have shown decrease after venom injection in brain and muscle, with concomitant increases in heart and liver (**Figure 5**). This decrease in GP activity can account for the raised levels of reduced glutathione, as it is the main substrate regulating its activity. Our results demonstrated that in experimental model of *in vivo* intoxication by viper venom oxidative stress is present because of the reduction of antioxidant enzyme activity and increased lipoperoxidation. This data is in good correlation with the levels of whole peroxidase activity in rat's tissues after viper biting (**Figure 6**). Only in the heart tissue the contradictory tendency was observed: whole peroxidase activity is significantly decreased, while the activity of GP shows the weak increasing.

4. Discussion

Free radicals are elusive and hard to be detected, because they are (generally) highly reactive and extremely short-lived. In order to confirm their production or clarify their function, investigators often have to search for end products or by products of radical-induced reactions, examining the reaction "path" of the radicals. The

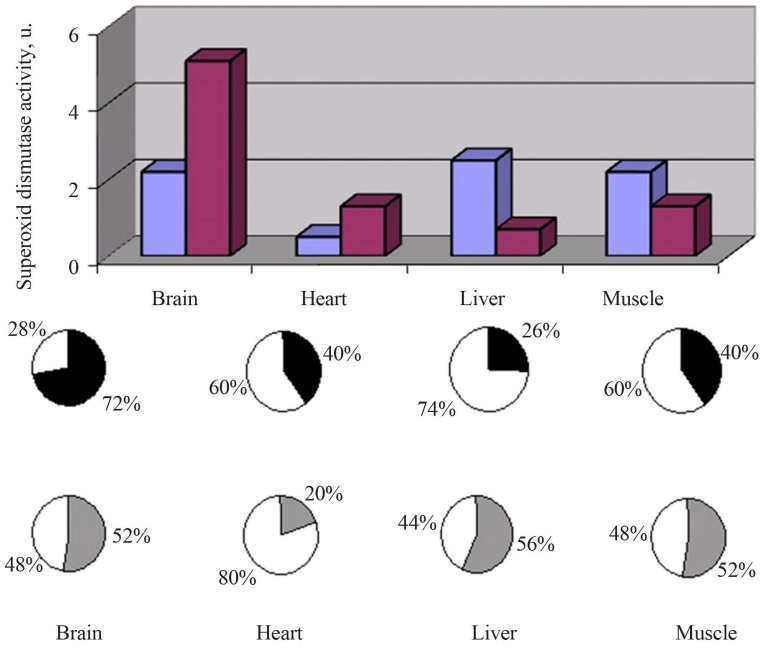

Figure 4. Changes of the superoxid dismutase activity (above) and percentage of adrenalin autooxidation by superoxid dismutase (below) in rat's tissues after viper's venom *in vivo* processing (blue bars—control, purple bars—assays after venom processing). The adrenochrome concentration was measured at 480 nm in assay containing 1 ml 0.15 M Na_2CO_3-buffer with 3×10^{-4} M EDTA (pH 10.2), 0.5 ml supernatant of studied tissue, 0.7 ml 0.005 M $KHPO_4$-buffer with 1×10^{-5} M EDTA (pH 7.8) and 0.4 ml 2.25×10^{-3} M of aqueous solution of adrenaline (pH 2.5).

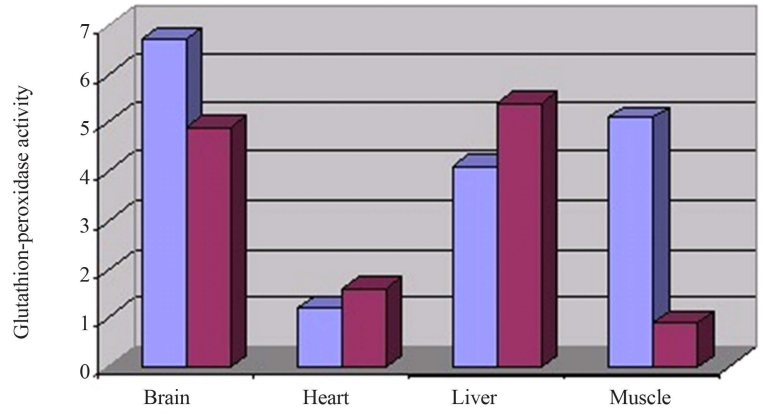

Figure 5. Changes of the glutathion-peroxidase activity in rat's tissues after viper's venom *in vivo* processing (blue bars—control, purple bars—assays after venom processing). The NADPH oxidation by glutathione reductase was measured at 340 nm in assay (2 ml) containing 0.05 M phosphate buffer (pH 7.4), 10^{-3} M EDTA, 0.12×10^{-3} M NADPH, 0.85×10^{-3} M glutathione reductase and homogenate of studied tissue. The unit of ferment activity is a quantity of enzyme, necessary for oxidation of 1 mkmol of reduced glutathion in 1 min.

only direct way to detect and measure free radicals in the real time mode is by chemiluminescence analysis. The complex approach is the best way for the overall systems, conditions, and reactions that produce free radicals and cause oxidative damage to be fully elucidated and substantiated.

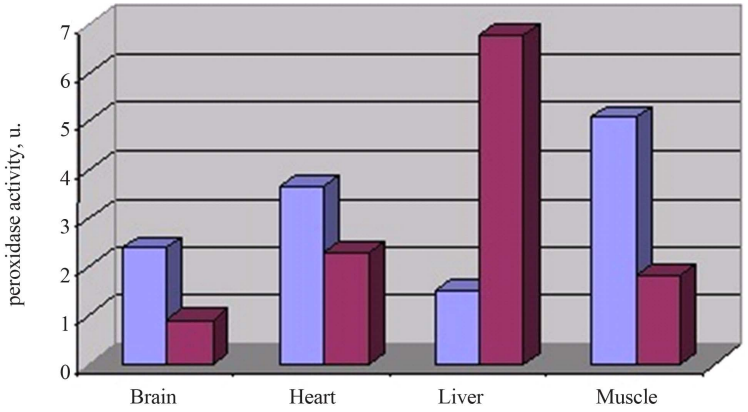

Figure 6. Changes of the whole peroxidase activity in rat's tissues after viper's venom *in vivo* processing (blue bars—control, purple bars—assays after venom processing). The whole peroxidase activity was measured at 470 nm in assay containing 1 ml gvayacol, 1 ml H_2O_2, 1 ml $NaHPO_4$-buffer (pH 7.6) and 0.2 ml of supernatant of studied tissue.

In accordance with clinical manifestations, the picture of MLO toxin action on tissue membranes is characterized by the development of local edema, which gradually increasing as far as the venom spreading in blood. Till now this effect was attributed to the content of venom of PLA_2, the enzyme catalyzing the hydrolysis of fatty acids from the sn-2 position of membrane phospholipids [21] [22].

Our results show some differences between the data of the TBA-test and ChL-analysis, limited, as we suggest, by methodical specification of the TBA-test, which is depending on the participation in the reaction of MDA formation only by di- and polyunsaturated fatty acids, but not monounsaturated ones. Nevertheless, in the course of ChL analysis the product of monounsaturated fatty acids-hydroperoxides—influence the level of ChL intensity. Thus, as we believe, the specificity of action of the MLO toxin component [23], which is responsible for the radioprotection effect, can be determined by the level of monounsaturated fatty acids. That is why this mostly affects rat brain lipids, consisting of monounsaturated and saturated fatty acids (mainly neuron and stearic fatty acids) [24]. Very likely; marked influence is due to the recently found disintegrins: a group of cysteine-rich peptides occurring in Crotalidae and Viperidae snake venoms [25]-[29]. 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 [30]-[32]. One key component of MLO venom microelements is Zn. In the composition of venom its concentration is a magnitude higher than that of bivalent ions [33]. Its role in the picture of intoxication is not clear. We think probably the observed effect of Zn ions on nervous tissue affects by snake venom in the intact organism could account for the speed of intoxication (so named "spreading-effect"), which is very characteristic for the venom of vipers.

The results obtained here allow us to suppose that in this 10-minute treatment with snake venom the toxicant exercises its harmful effect on the cellular membrane, because during exposure time, the activity of SOD and peroxidase antioxidant enzymes exhibits some fast changes in their specific activity, as if there were a "preamble," in which oxygen-derived free radicals, by intoxication with venom, are inside the antioxidant enzymatic systems possessed by different tissues.

5. Conclusion

Our experiments revealed that snake venom intoxication in organism could reduce or enhance ROS generation in different tissues. The tissues might promote their own antioxidant enzyme activities to cope with the increased oxidative stresses and effectively lower the amount of lipid peroxidation; however this tendency is true for SOD, but not for the glutathione-peroxidase. It has been indicated that alteration in the specific activity of antioxidant enzymes in the cell or tissues, in addition to the increase in lipoperoxidation (determined by an increase in MDA) of the fatty acid of cellular membranes, is indicative for oxidative stress, which is mainly characterized by the excessive production of free radicals derived from oxygen or by the decrease in antioxidant mechanisms [10] [12] [13] [15]. Further studies of ROS in the course of viper venom intoxication would be

needed to elucidate the exact mechanism involved in tissues response and, therefore, to provide the possibility of using viper venom's components for disease with ROS elevation. These findings may significantly contribute to a better understanding of the physiopathology in the course of intoxication with snake venom. Our study is also relevant to the therapeutic potential of *Macrovipera lebetina obtusa* and *Montivipera raddei* venoms.

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