THE NEW ARMENIAN MEDICAL JOURNAL
Vol.5 (2011), No 3, p.4-10

FREE RADICAL OXIDATION AND CONDITION OF MEMBRANES FROM BRAIN LIPIDS OF VERTEBRATES IN THE COURSE OF MACROVIPERA LEBETINA OBTUSA VENOM INTERACTION

Ayvazyan N.M.[1], Zaqarian A.E.[2], Ghazaryan N.A.[1]

[1] L.A. Orbeli Institute of Physiology, National Academy of Sciences of Armenia, Yerevan, Armenia
[2] Department of Biophysics, Yerevan State University, Yerevan, Armenia

Abstract

The present study was undertaken to elucidate the antioxidant effect of *Macrovipera lebetina obtusa* venom on brain lipids of different vertebrates, as well as to reveal how the plastic properties of model membranes from native lipids of vertebrates change in the course of venom processing. The monitoring of free-radical activity of nervous tissues of vertebrates by chemiluminescence analysis, by comparison with thio-barbituric acid (TBA) test has shown that venom interacts with membranes of nervous cells and leads to a tendency to decrease oxidation level of their lipid components depending on the degree of polyunsaturation. It was also demonstrated that electroporation and electropermeabilization of bilayer lipid membranes from native lipids of vertebrate's brain's increased more than twice in media of 0.1 M potassium chloride. Intriguingly, zinc ions added to the experimental media lead to a decrease of the toxic injuries, in contrast with other bivalent ions (Ca^{2+}, Mg^{2+}).

Keywords: *chemiluminescence, ,lipid bilayer, electroporation, vertebrates, venom.*

INTRODUCTION

Snake venoms have gained considerable attention in recent years as a treatment used in pharmacology and for their application in disorders of nervous system function and blood diseases [*Orlov B., Valceva I., 1977; Harvey A., 1997; Khalaji N. et al., 2010*]. There is still a considerable need for basic descriptive work on venoms and toxins, as the venoms of many species are wholly unknown, and many high-throughput techniques are not yet sufficient at detecting subtle aspects of structure-function differences in many molecules that share a common structural fold but have very different pharmacologies. During the last quarter of a century, the study of snake venom biochemistry has evolved very quickly, but in the scientific literature the main emphasis has been on the specificity of venom action on the circulatory system of an organism with corresponding clinical manifestations [*Orlov B., Gelashvili D., 1985; Mackessy S., 2010*]. As far as the detailed molecular and biochemical changes are concerned, the pathological mechanisms of the harmful interactions of zootoxins with systems of cell activity in different organs and the whole organism remain problematic. Theories about the synergist influence of different venom components are formulated as a number of observations [*Newcomb R. et al. 1998; Peter M. et al., 1998*]. The fact that has received some attention is the difference between the toxic activity of purified venom components and that of the same components in the whole venom [*Kardong K., 1996; Bdolah A. et al., 1997*]. In the venom of *Macrovipera lebetina obtuse (MLO)* a specific toxin was not identified, but only a collection of enzymes, which are widely used in a number of pharmacological treatments (namely: Lebetox, Reptinaz, Vipratox, Viprosal, etc.). Many recent investigations had shown the influence of factors from venom on cell membranes of mammals. Most of the studies concern the function of phospholipase A_2 (PLA_2) in the venom of *MLO* and its action on lipids of cell membranes [*Ohkura N. et al., 1997; Sagane K. et al., 1998*]. It is known that purified PLA_2 has only 1% of the toxic activity in venom. The most important role in the intoxication process is ascribed to low molecular weight metal proteases (such as obtustatin, lebein desintegrins), but the scientific literature on this issue is contradictory [*Masuda Sh. et al., 1998;*

Address for Correspondence:
L.A. Orbeli Institute of Physiology, NAS (Armenia)
22 Orbeli Street, 0028, Yerevan, Armenia
Tel.: (+374 94) 105425
E-mail: ghazaryan-n@rambler.ru

Sagane K. et al, 1998]. The opinions about the problem of *MLO* intoxication might show that we know exactly neither the underlying mechanism nor the respective determining factors [*Siigur E. et al., 1996*].

The present study was in part prompted by an interest in the changes of the lipid condition, which take place in native membranes under the influence of *MLO* venom. Free radical oxidation as a sensitive criterion of the physiological condition of lipids and lipid-containing structures became a highly motivated choice for us [*Ayvazian N. et al., 2003*]. Study of the above-mentioned processes, on the one hand, and modeling plastic properties of lipid bilayers in the interaction with venom, on the other hand, could provide important information.

MATERIAL AND METHODS

Tissue Processing: Four species of vertebrates: crucian carp (*Carassius carassius*), marsh frog (*Rana ridibunda*), caucasian agama (*Stellio caucasicus*), and non-purebred white rats were decapitated. Then the brains were homogenized for 5 *min* by homogenizator (Potter-Elvehejm) in Tris -HCl buffer (pH 7.4) with a final concentration of 20 *mg/mL*. Dried lyophilized toxin of *MLO* was dissolved in the same buffered saline with a concentration of $3 \cdot 10^{-5}$ 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*.

Phospholipid Processing: Lipids fractions were isolated from the brains of above species of vertebrates by the Kates method [*Kates M., 1972*]. Then a vacuum pump was used to remove the chloroform-methanol mixture. For the chemiluminescence (ChL)-analysis it was incubated with venom solution and held at a constant temperature of 37°C for 10 min. Then lipid sediments were dissolved in nonane (3% solution).*Chemiluminescence Analysis*: Reactive oxigen species (ROS) levels were measured by a ChL analyzing system: intensities of tissue homogenates and lipid solutions were measured on a quantometric device equipped with FEU-140 (Russia) photomultiplier with the spectral sensitivity range of 300-800 *nm.* The system contains a photon detector, ChL counter, water circulator and 32 *bit* IBM personal system. A cooler circulator is connected to the FEU-140 photon detector to keep the temperature at 50°C. This ChL analyzisng system is extremely sensitive, capable of detecting as little as 10-15 *W* of radiant energy. ChL intensity was measured in an absolutely dark chamber in *impulse/sec* mode [*Ayvazian N. et al., 2002*]. The graphical and statistical analysis of data was done by LabView program (National Instruments, USA) [*Zaqarian A. et al., 2006].*

Lipid Peroxidation: **L**ipid peroxides are unstable and decomposed to a complex series of compounds. The most abundant compound is malone dialdehyde (MDA). The MDA level of tissues was determined by spectrophotometric measurement **[***Stalnaja I., Garishvili T., 1985***]** using the thio-barbituric acid (TBA)-test based on the reaction of a chromogenic reagent, TBA, with MDA at 100°C: two molecules of MDA react with one molecule of TBA to yield a stable threemethin complex dye. MDA concentration was measured at 532 nm using the SF-46 spectrophotometer (Russia).

Bilayer Membranes; The lipid bilayer membranes (BLM) were formed from the lipid fractions of rat and bovine brains on a teflon aperture by means of the Muller method **[***Mueller P. et al., 1962***].** The electrical parameters of lipid bilayers were measured on an electrometric device equipped with a Keithley-301 (USA) differential feedback amplifier operating on a fixed voltage mode. The potential of membrane rupture recorded in the experiments with shielded camera was taken as the threshold value of the voltage applied; 0.1 M KCl served as ionic media.

Statistical Analysis: **F**or quantitative analysis of chemiluminescence intensity and electrical parameters of BLMs, 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 ±SEM (n=number of experiments).

RESULTS

At analysis of MDA accumulation in brain tissues of the above-mentioned vertebrates in norm and after processing by snake venom, we observed the divergence of its amount in brain of poikilothermal and homoiothermal vertebrates (Figure 1). On the one hand, the suppression of lipid free radical oxidation takes place in the last case, while on the other hand the considerable activation of MDA formation is detected in nervous tissues of fishes, frogs and lizards under venom action; and thus intensification of peroxidative processes occur.

According to literature data, the clinical pictures of viper's venom influence are essentially different for warm-blooded and cold-blooded organisms. Mo-

rover, it is known that in mammals, which are most studied in this sense, the sublethal dose of *MLO* venom has a radioprotective effect [*Suzuke K., et al., 1997*].

Earlier we showed how the processes of free radical oxidation in brain of vertebrates changed in the course of phylogenetic development [*Zaqarian A. et al., 2000*; *Ayvazian N. et al., 2002*]. Then we investigated the spontaneous ChL of brain tissue homogenates of four species of vertebrates before and after the processing with snake venom (Figure 2). In this experiment the antioxidant effect of venom was observed for all vertebrates, except *Carrassius carrassius*. On the contrary, the ChL response of the latter increases in the course of intoxication.

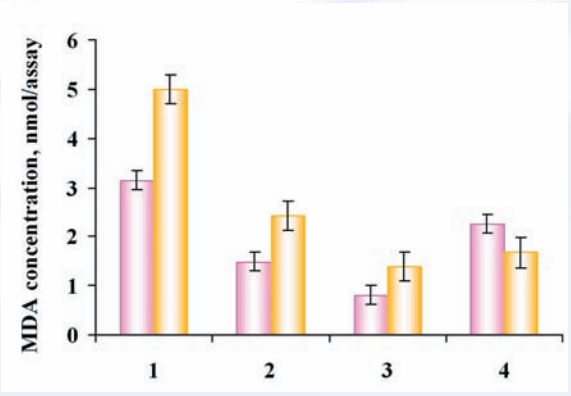

Figure 1. *Changes of lipid peroxidation level in brain lipids of vertebrates under Macrovipera lebetina obtusa venom influence.*

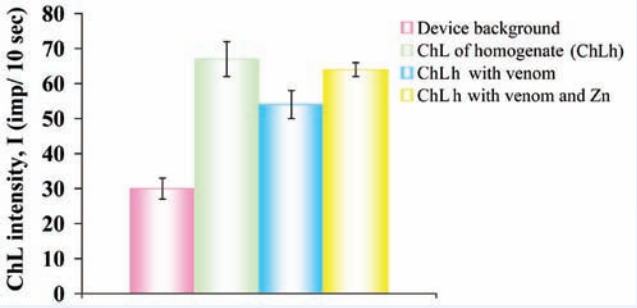

Figure 2. *The effect of Macrovipera lebetina obtusa venom and zinc ions on spontaneous chemiluminescence of mammalian brain.*

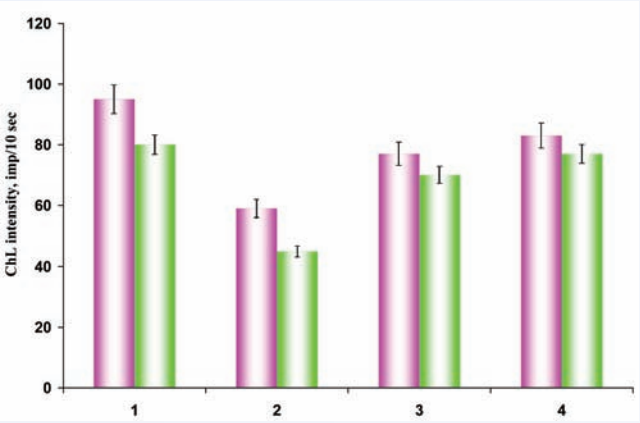

Figure 3. *Changes of spontaneous ChL-intensity level in brain lipids of vertebrates under Macrovipera lebetina obtusa venom influence.*

Interestingly; the antioxidant effect disappeared if Zn ions were added (Table 1), and the influence of zootoxin on ChL of purified lipid fractions demonstrated the antioxidant effect for all studied classes of animals (Figure 3). At the same time the own ChL of the solvent (nonane) only slightly exceed the background of quantometric device. So, it could not exert much influence on the course of ChL analysis.

Thus, the intent of lipid interaction with ChL-decreasing components, as we suggest, directly depends on the degree of unsaturation of lipids. For example, fish brain lipids are rich in polyunsaturated fatty acids, unlike amphibian and reptile lipids. The last two have a low index of unsaturation in combination with a very high level of cholesterol, which is known as a so-named "structure" antioxidant [*Avrova N., 1999; Zabelenskii S., et al., 2000*].

In the next series of experiments we studied the action of the MLO venom on the electrical parameters of bilayer membranes, which can represent adequate models for native cell membranes [*Zaqarian A.,et al., 2005*].

Table 2 presents electrical parameters of artificial bilayers in a 0.1 *M* solution of potassium chloride. Addition of viper venom decreased the electrical resistance of BLM more than twice (the acting portion was 0.2 *mL* of venom added to both sides of the membrane). It is of interest that in this case introducing $ZnCl_2$ in potassium media leads to restoration of the primary value of BLM's electrical resistance. In the electrical field the stability of BLM from mammalian brain lipids decreases to an extent before processing.

DISCUSSION

Macrovipera represents a genus of terrestrial and oviparous venomous vipers that inhabit the semideserts and steppes of North Africa, the Near and Middle East, and the Milos Archipelago in the Aegean Sea. Four species are currently recognized, *M. deserti*, *M. lebetina*, *M. mauritanica*, and *M. schweizeri* [*McDiarmid R. et al., 1999*]. *Macrovipera lebetina obtusa* (West-Asian blunt-nosed viper, Levant viper,

Table 1.

The intensity of spontaneous chemiluminescence of tissue homogenates and brain lipids of some vertebrates in norm and after venom processing

Species	n	ChL (*imp*/10 *sec*)			
		homogenate	G*	lipids	L*
Carassius carassius	10	124 ± 15.7	175 ± 21.7	95 ± 4.18	80 ± 2.35
Rana ridibunda	10	67 ± 4.47	56 ± 1.24	59 ± 6.86	45 ± 2.13
Stellio caucasicus	7	55 ± 3.75	49 ± 2.26	77 ± 2.78	70 ± 1.88
Rattus rattus	10	58 ± 4.18	43 ± 2.78	83 ± 2	77 ± 3.15

Notes: $P<0.05$ by Student's *t*-test relative to the corresponding control;
G* - brain homogenate processed by toxin;
L* - brain lipids processed by toxin.

Mountain adder) is a large (reaching about 2 *m* in length) venomous viper subspecies found in dry, rocky, mountainous areas between 1000 and 2500 *m* elevation between central Turkey and northern Pakistan (Kashmir). It is now likely that certain subspecies of *M. lebetina* (*M. l. cernovi*, *M. l. lebetina, Macrovipera lebetina obtusa, M. l. transmediterranea*, *M. l. turanica*) will be elevated to valid species status [*Mallow D. et al., 2003*]. Earlier it has been shown that venom composition keeps information on the evolutionary history of congeneric taxa and hence venomics may aid in taxonomy [*Calvete J. et al., 2007*] and estimate the similarity between the venom proteins of *M. l. obtusa* and *M. l. transmediterranea* [*Sanz L. et al., 2008*].

In accordance with clinical manifestations, the picture of *Macrovipera lebetina obtusa* (MLO) toxin action on tissue membranes is characterized by the development of local edema, which gradually increases with the venom spreading in blood. Until now this effect was attributed to the content of venom of PLA_2, the enzyme catalyzing hydrolysis of fatty acids from the sn-2 position of membrane phospholipids [*Tatulian S., 2001; Kudo L. et al., 2002*].

Table 2.

Electrical properties of bilayer lipid membranes (BLM_s) in media of 0.1 *m kcl* and $ZnCl_2$ under influence of the *Macrovipera lebetina obtusa* venom.

Media (0.1 M)	R_m (*Ohm*)			Breaking potential
	BLM	+ venom	$+Zn^{2+}$	
KCl	$5x\ 10^{11}$	$1.3x\ 10^{11}$	$5x\ 10^{11}$	340
$ZnCl_2$	$5x\ 10^{11}$	$2.5x\ 10^{10}$	-	390

Notes: Each group contained 20 BLMs from four brains. $P>0.01$ by Student's *t*-test relative to the corresponding control.

Our results show some differences between the data of the TBA-test and ChL-analysis is limited, as we suppose, by methodical specification of TBA-test, which depends 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, influences the level of ChL intensity. Thus, as we believe, the specificity of action of the MLO toxin component [*Bazaa A. et al., 2005*], which is responsible for the radioprotection effect, can be determined by the level of monounsaturated fatty acids. Therefore, it mostly affects rat brain lipids consisting of monounsaturated and saturated fatty acids (mainly neuron and stearic fatty acids). Very likely; marked influence is due to the recently found disintegrins: a group of cysteine-rich peptides occurring in *Crotalidae* and *Viperidae* snake venoms [*Gould R. et al., 1990; Barbouche R. et al., 1996; Huang T., 1998; McLane M. et al., 1998; Calvete J. et al., 2003*]. As it is known, the cysteine-containing substrates are strong antioxidants. The purified components demonstrate more toxic effect than the content of a whole venom and have zinc-chelated sequences [*Eble J.A., et al., 2003; Moreno-Murciano M. et al., 2003*]. 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 [*Marcinkiewicz C. et al., 2003*]. Its role in the picture of intoxication is not clear. We think, probably, the observed effect of Zn ions on nervous tissue could account for the speed of intoxication (so-named

"spreading-effect"), which is very characteristic of vipers venom.

We have demonstrated the utility of biophysical methods in assessing the changes of lipid properties under the influence of the venom of MLO.

Summing up the above mentioned, we conclude that MLO viper's venom, being very complicated in its chemical composition of biologically active substances, interacts with membranes of nervous cells and leads to a tendency to decrease in the oxidation level of its lipid components depending on the degree of polyunsaturation. This lower level of free radical oxidation processes has to be paralleled with the changes in activities of members of antioxidant defense system of an organism (such as superoxide dismutase, catalase and glutathione peroxidases) [*Marcinkiewicz C. et al., 2003*]. Therefore, in further studies, it would be necessary to check the consequences of intoxication on the activities of these enzymes.

As soon as the venom interaction with lipids induce the proper changes of bilayer interface, the orientation of lipids in flat BLMs is not sufficient to allow an insertion of proteins into the membrane [*Sanchez S.A. et al., 2002*]. However, our data on electroporation and electropermeabilization of BLMs in the course of intoxication indicate that the influence of venom content could change the plastic properties of bilayer, and concomitantly, its microviscosity and fluidity. At the next stage of our research we shall try to combine the giant unilammeliar vesicles formation technique (the curvature and size of which are more relevant for biological membranes) and fluorescence microscopy for deciphering of these questions.

Acknowledgements: *This work was particularly supported by Grant # 1362-NS from the ANSEF Office.*

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