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ԱԿԱԴ. Լ.Ա. ՕՐԲԵԼՈՒ ԱՆՎԱՆ ՖԻԶԻՈԼՈԳԻԱՅԻ ԻՆՍՏԻՏՈՒՏ

ԱՐԵՍՏԱԿԵՍՅԱՆ ՀՈՎՀԱՆՆԵՍ ՎՐԵԺԻ

MACROVIPERA LEBETINA OBTUSA ՕՁԻ ԹՈՒՅՆԻ ՍՊԵՑԻՖԻԿ ԱԶԴԵՑՈՒԹՅՈՒՆԸ
ՍՐՏԱՄԿԱՆԻ ԿՈՒԼՏԻՎԱՑՎԱԾ ԲՋԻՋՆԵՐԻ ՎՐԱ

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

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NATIONAL ACADEMY OF SCIENCES OF THE REPUBLIC OF ARMENIA
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ARESTAKESYAN HOVHANNES VREZH

SPECIFIC EFFECTS OF MACROVIPERA LEBETINA OBTUSA SNAKE VENOM ON
CULTURED MYOCARDIAL CELLS

SYNOPSIS

of the thesis submitted for
the degree of candidate of biological sciences on the specialty
03.00.09 – Human and Animal Physiology

YEREVAN 2017

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

Գիտական ղեկավար՝

Կ.Գ.Թ., Զ.Ի. Կարաբեկյան

Պաշտոնական ընդդիմախոսներ՝

Կ.Գ.Դ., պրոֆեսոր Զ.Ս. Սարգսյան
Կ.Գ.Թ., դոցենտ Ա.Վ. Ոսկանյան

Առաջատար կազմակերպություն՝

ՀՀ ԳԱԱ Մոլեկուլային կենսաբանության
ինստիտուտ

Ատենախոսության պաշտպանությունը տեղի կունենա 2017թ. հուլիսի 24-ին, ժամը 14⁰⁰-ին, ՀՀ ԳԱԱ Լ.Ա. Օրբելու անվ. Ֆիզիոլոգիայի Ինստիտուտում գործող ՀՀ ԲՈՀ-ի Ֆիզիոլոգիայի 023 մասնագիտական խորհրդի նիստում (0028, Երևան, Օրբելի եղբ. փող. 22):

Ատենախոսությանը կարելի է ծանոթանալ ՀՀ ԳԱԱ Լ.Ա. Օրբելու անվան Ֆիզիոլոգիայի
ինստիտուտի գրադարանում և www.physiol.sci.am կայքում:

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023 մասնագիտական խորհրդի գիտական
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Ն.Է. Թադևոսյան

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

Scientific supervisor:

PhD, Z.I. Karabekian

Official opponents:

Dr. of Biological Sciences,

Professor J.S. Sargsyan

PhD, Associate Professor A.V. Voskanyan

Leading organization:

Institute of Molecular Biology NAS RA

Thesis defense will take place on 24th of July, 2017, at 14⁰⁰, at the meeting of the 023 Physiology
specialized council of RA SCC at the Orbeli Institute of Physiology NAS RA (22 Orbeli Bros. Str.,
0028, Yerevan, RA)

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

The synopsis was submitted on 23th of June, 2017.

Scientific secretary of the 023 specialized council,

PhD



N.E. Tadevosyan

GENERAL DESCRIPTION OF THE STUDY

Relevance of the study. Studies of *Vipera* genus snakes' venom demonstrated a significant difference in the mode of action of their diverse components. Such divergence depends on the prey and the ultimate goal of specific envenomation. The initial analyses of venoms discovered identical families of enzymes and polypeptides among various types of snakes and common principals of their activity. However, a deeper analysis revealed the strict specificity of venom components unique to a given species and even sub-species of snakes. The habitat of a prey and the type of animal used as food greatly influences the composition of the venom. The specific composition of venom ensures certain kind of damages for quick death of prey (Daltry JC et al., 1996). On the whole body level, the prey is being immobilized and disoriented, develops pain syndrome and hypotonic collapse. Destruction of multiple organs and organ systems occurs on the level of functional systems. On a molecular level, venom components act with high specificity on mechanisms of cellular activity of a prey. It is important to mention that the main action of venoms is not only in a direct interaction of venom component with its target but also in an induction of hyperactivity and the generalized reaction of the whole organism to the venom. As opposed to plant poisons, which mainly block a target function in a body of a prey, injected animal venom often recruits prey's defense mechanisms to fight against itself (Wu and Huang, 2003). Many venom components act as bi- or multi-functional agents and depending on the effector site can exhibit enzymatic activity and/or serve as a ligand for different receptors and signaling systems (Lee and Albuquerque, 1979).

The focus of our study is the venom of *Macrovipera lebetina obtusa* (*MLO*) living in Armenia and its effect on the cells of myocardium. This species contains several sub-species, which are characterized by a very specific combination of components (Trummal Ketal., 2005; Kallech-Ziri Olfa et al., 2005; Sanz L. et al., 2008; LimamI. et al., 2010). Along with common enzymes characteristic of *Vipera* species, *MLO* venom contains unique components specific for "obtusa" sub-species, such as disintegrin obtustatin (Sanz L. et al., 2008). This and some other components, at large, define the uniqueness of this venom. *MLO* venom contents metalloproteinases, phospholipase A2, serine proteases, L-alfa amino acid oxidase, few kinds of disintegrins and some other active agents (Sanz L. et al., 2008). The cluster of *MLO* venom components, such as metalloproteinases, obtustatin, C-type lectins and few others are known as cell adhesion inhibition molecules, which are breaking up integrins and cadherins or bind to them (Gutiérrez and Rucavado, 2000; Sanz et al., 2008; Zychar et al., 2010). Therefore, the adhesion affecting properties of *MLO* venom and its action on cell binding in tissue culture was of an interest.

The overarching objective of this study was to investigate the anti-adhesive actions of fermentative systems and active polypeptides of *MLO* snake venom. Specifically, we aim to examine the effect of active venom's molecules on the connection of cell with the extracellular matrix and intercellular connections. Additionally, we aim to examine the survivability of cardiomyocytes (NRCM) and other non-direct targets of the *MLO* venom, such as cardiac fibroblasts (CF) and epithelial HeLa cells. The necessity of such studies is substantiated by the basic mechanisms of the *MLO* snake venom's action. Among well-characterized effects of this venom is it's hemolytic activity on erythrocytes; moreover effect on vascular system, results in damage of histohematic barriers and leakage of plasma proteins into surrounding tissues; and finally *MLO* venom affects hemostasis by aggregating platelets and activating different clotting proteins etc. Among the non-canonical effects of *MLO* snake venom the following processes need to be included – the destruction of cellular membranes, induction of pathologic inflammation, apoptosis, necrosis, ischemia of different organs and organ systems, as well as pre-digestion of prey's tissues.

The understanding of venom's specialized and general action mechanisms on the prey's organism is necessary for it's potential use as therapeutic or diagnostic agent. More importantly, it is necessary to understand the molecular responses of a prey, on order to synthesize easily obtainable and highly specified medications. Such studies usually transferred from *in vivo* investigations on the whole organism level to *in vitro* or even *in silico* studies. Special role is given to genetically

modified animals (knockout mice, chimerical mice that express human MHC molecules, animals with recombinant fluorescent target proteins, etc.) and to cultures of both primary and immortalized cells. This allows us to overcome the long process of “tuning” of the effective dose for prospective medication and allows us to identify the major side effects prior to animal testing.

The specific objectives of this project were:

1. Detecting the general effect of *MLO* snake venom on the morphology of cultured NRCMs, CFs and model epithelial cells.
2. Identifying the morphological and physiological effects of non-lethal doses of *MLO* venom on investigated cell types.
3. Determining the effect of *MLO* venom exposure time on the adhesion properties of tested cells.
4. Correlating the dose and time dependent effects to detachment of *MLO*-treated cells with their subsequent viability.
5. Investigating the role of the PLA2 component of the *MLO* venom on the attachment and viability of tested cells.
6. Investigating the role of *MLO* venom metalloproteinases on the attachment and viability of tested cells.

Scientific novelty. In this study, we demonstrated that crude venom from *MLO* is affecting adhesion properties of NRCM, CF, and HeLa cells. The LD₅₀ of *MLO* venom in mice is 18.4 ug per 1 mouse. To date, there is no information about the concentration of the venom that is reaching the heart after the bite. We estimate it to be in 5-10ug/ml range based on an average adult mouse weight (20-25 grams) and its volume of blood (being about 6-8% of total body weight or 1.5-2ml of blood). These estimates suggest that concentrations of the venom used in this study can be in fact encountered in vivo. Therefore, in this study we systematically investigated the effects of low concentrations of *MLO* venom on aforementioned myocardial cells and epithelial cells.

The results of our studies demonstrated that the *MLO* venom has a significant detaching effect on all tested cell types. Doses up to 1ug/ml are not cytotoxic, which means that the cells are remaining viable upon detachment from the culturing substrate. These cells are capable of reattaching and functioning when venom is removed. Hence, during the course of our investigations we identified a dose of *MLO* venom that affects adhesion properties of aforementioned cells, but does not cause death. This model provides a unique opportunity to study molecular interactions of lasting cellular culture and work out conditions for testing components of *MLO* venom for drug development. These effects are dose and time dependent for all cells.

In an attempt to understand the mechanisms of such activity, we used inhibitors specific for individual components of *MLO* venom. Among those were EDTA-Na₂ chelating agent, which effectively neutralized metalloproteinases of *MLO* venom. The adhesion of tested cells was slightly improved when EDTA-Na₂ inhibited venom was applied to cardiomyocytes, cardiac fibroblasts and HeLa cells. Similar results were obtained with bromophenacyl bromide (BPB)-inhibited venom, where PLA2 enzyme activity was completely blocked. These results indicate that the aggressive detaching activity of *MLO* venom is not delivered by an individual component of the venom, but rather is a combinatorial effect of several active ingredients.

Scientific and practical significance of the work. The results of our studies set off a series of questions that we are planning to address in future. First of all, we are planning to investigate a novel phenomenon – initiation of tachycardia in cardiomyocytes under exposure to non-lethal doses of *MLO* venom. It is known that C type natriuretic protein (CNP) is a part of *MLO* whole venom. CNP promotes natriuresis and diuresis resulting in loss of sodium and water thereby lowering blood volume and blood pressure. These properties of CNP make it a prime suspect in altering the physiology of cardiomyocytes, but direct studies are yet to be done.

Severe detachment was detected as a consequence of *MLO* venom on all three tested cell types. Similar effects were detected on other cells, such as skeletal muscle cells, endothelial cells, when other types of snake venoms were used (Borkow et al., 1995). We discovered that detachment from the substrate occurs prior to cell-to-cell detachments. We are intending to unravel the mechanisms

of this phenomenon. Attachment of cells to culture substrates, which simulates extracellular matrix, is mostly provided by integrins. *MLO* venom contains a series of disintegrins in their multiple forms, the most important being obtustatin. Interaction of each tested cell type with culture substrate and with each other will be the focus of future experiments. On the other hand, the network of synchronously beating cardiomyocytes is created by desmosomes and tight junctions, which have complex composition. Understanding of delayed action of *MLO* venom on these connections will be another focus of future studies.

Unexpectedly, we discovered that very low concentrations of *MLO* venom induce increase in survivability of cardiomyocytes, cardiac fibroblasts and HeLa cells as detected by MTT assay. This is important to decipher for at least two reasons. First, we plan to investigate whether or not there is a real increase in number of cells under these conditions. It is possible that at low levels of stress the proliferative mechanisms are stimulated. If that is the case, this will provide a unique opportunity to stimulate growth of otherwise proliferatively-limited cardiomyocytes for tissue engineering purposes. Second, considering the nature of MTT assay, which measures mitochondrial activity, it is possible that non-lethal doses of *MLO* venom simply accelerate metabolism. We are planning to investigate both of these possibilities.

In addition, we are planning to definitively demonstrate the mechanism of action of individual venom components on detachment, by blocking distinct components of *MLO* venom or their combinations. Our initial experiments indicated the involvement of both PLA2 and metalloproteinases in these processes. However, we detected only slight alterations in venom action when the venom was inhibited by particularized inhibitors. In addition, there are other enzymatic components present in *MLO* venom such as serine proteases. These enzymes are not directly involved in adhesion mechanisms, however they might play a facilitating/combinatorial role in the detected phenomenon and thus will be included in our future studies. Systematic application of all possible inhibitors and testing the effects of their combination will shed more light on *MLO* venom action mechanisms.

Lastly, more cell types will be incorporated in our studies including endothelial cells (HUVEC), as they are exposed to venom before other cells buried deeper in the tissues, liver cells and others in order to evaluate the commonality of *MLO* venom effects.

As another attempt to understand underlying mechanisms of adhesion of NRCM to the substrate, we tested different coatings for attachment of studied cells to well surface. Among those coatings were gelatin, laminin, and fibronectin. Gelatin is a water-soluble natural polymer, which is derived from collagen (Collagen Type I). The key adhesion components of gelatin are α_1 , α_3 , α_5 , and β_1 integrin subunits (Akiyama et al., 1981). Laminin is an important and major component of basal lamina, which has a direct effect on cell differentiation, migration, and adhesion (Aumailley, 2013). The main adhesion molecules of laminin are α_1 , α_3 , α_5 , α_7 , and β_1 (Aumailley, 2013). Fibronectin has numerous important functions, such as cell adhesion, growth, differentiation, and migration (Akiyama et al., 1981). It is also secreted by a variety of cells, in particular, primary fibroblasts and contains the binding part for α_3 , α_5 , α_v , and β_1 integrin subunits (Akiyama et al., 1981). However, despite the differences of coatings, in these experiments, no visual differences were detected in effects of *MLO* crude venom on adhesive properties of aforementioned cells. This indicates that the mechanism of investigated venom action is more general, superseding specific differences in integrins composition of ECM proteins we have tested.

Approbation of the work: The main results of this dissertation have been discussed with experts in the field and presented at seminars and international scientific conferences.

Publications. On the topic of the thesis 3 scientific articles are published.

The structure of the thesis. The thesis consists of an introduction, four chapters, conclusions, future directions and a list of references numbering 200 titles. The thesis is presented in 115 pages, includes 40 figures and 2 tables.

LITERATURE REVIEW

The literature review includes: myocardium, morphological and physiological characteristics of myocardium, cell adhesion, molecular mechanisms of adhesion in myocardium and epithelial cells, snake venom, models used in this study, and venom-cardiomyocyte studies.

MATERIALS AND METHODS

Cells

Primary rat neonatal cardiomyocytes were obtained according to the previously published protocol with modifications. Briefly, beating hearts were excised from 1- and 2-day old Sprague-Dawley rats, rinsed in a cold, calcium- and magnesium-free, Hank's Buffered Salt Solution (CMF-HBSS), and then minced into $\sim 1\text{mm}^3$ pieces. Tissue pieces were incubated overnight at 4°C in fresh CMF-HBSS containing 0.1 mg/ml trypsin. The next day, heart tissue was washed with fresh CMF-HBSS and treated with 0.5ml normal rat serum (NRS). The tissue was then collected in Leibovitz's medium containing 1500U ($\sim 4\text{mg/ml}$) collagenase II and shaken for 30 min at 37°C . The cells were then gently triturated, passed through a cell strainer to remove any undigested pieces, and centrifuged for 5 min at 17.5G . The pellet was resuspended in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% NRS and pre-plated in the 100mm tissue culture dish for an hour to minimize the presence of fibroblasts, which attach more rapidly than myocytes. Unattached cells were then collected, counted and plated in a fibronectin, laminin, or gelatin-coated multiwell cell culture plates at a density of 10^5 cells/cm^2 followed by incubation at 37°C in a 5% humidified incubator for at least 24 hours without being disturbed. Myocytes were then kept under standard culture conditions in DMEM, supplemented with 5% NRS, 10 U/ml penicillin and 1ug/ml streptomycin. Media was changed every other day. Around day three after cell plating, cardiomyocytes form an interconnected confluent network that exhibited rhythmic spontaneous contractions.

Primary rat neonatal cardiac fibroblasts were obtained in parallel with the NRCM isolation. Specifically, the population of cells that rapidly attached during the pre-plating stage of NRCM isolation was cultured for about 7 days . During that time, cells were re-seeded $2\text{-}3$ times to remove accompanying NRCM and, hence, enrich the population of cardiac fibroblasts.

Epithelial cells: HeLa cells were maintained according to the suggested protocol (DMEM supplemented with 10% Human Serum, $1\times$ Pen/Strep) and seeded in a tissue culture treated multiwell plates at a maximum density of $2\times 10^5\text{ cells/cm}^2$.

Venom collection and desiccation. Venom was milked manually as followed. The membrane (polyethylene film, PEF) was stretched over a glass Petri dish. The snake was held behind its head, and the firmness of the grip brings its fangs to the fore. The snake was stimulated to bite through the thin PEF membrane covering the collecting vial, and pressure was applied to the venom glands. The venom was collected in the vial. Membrane was removed and then vial was placed in desiccators for drying. Silica gel was used as desiccant. Venom was kept at a low level of humidity and low temperature. Fresh venom solutions, prepared daily, were used in experiments.

Venom stock solution preparation and further dilutions. The crude venom of *MLO* was collected from local *Macrovipera lebetina obtusa* snakes and stored in the form of lyophilized powder. Powdered *MLO* venom was reconstituted with sterile double distilled water to achieve a stock solution of 1mg/ml and was filter sterilized. This *MLO* stock solution was diluted with complete growth media right before experiments to obtain 0.1ug/ml ; 1ug/ml ; 10ug/ml ; 20ug/ml ; 50ug/ml ; 100ug/ml working concentrations.

Preparation of thermally treated venom. To generate solution of thermally denatured *MLO* venom, 1mg of dry venom was diluted in 1ml pre-warmed sterile water. The 1mg/ml solution was vigorously mixed (Vortex) until complete dissolving. Then the tube with stock solution was placed into boiling water and kept there for 30 min . Thermally treated *MLO* snake venom stock was diluted with complete growth media right before experiments to obtain 0.1ug/ml ; 1ug/ml ; 10ug/ml ; 20ug/ml ; 50ug/ml ; 100ug/ml working solutions.

Preparation of PLA2 inhibited venom. Bromophenacyl bromide (BPB) was used to inhibit the PLA2 of whole *MLO* venom: BPB neutralized venom was prepared as follows. BPB stock solution of 1 mg/ml was prepared prior by diluting it in 1 ml of sterile distilled water and was stored at 4°C. On the day of experiments 20 units of 1mg/ml solution of venom was mixed with one unit of 1mg/ml BPB solution and incubated at 37°C, for 2 hours with periodic mixing, to allow time for BPB to interact with PLA2 and inhibit this enzyme of *MLO* venom. The resulting *MLO*+BPB mixture was diluted with complete growth media immediately before experiments to obtain 0.1ug/ml; 1ug/ml; 10ug/ml; 20ug/ml; 50ug/ml; 100ug/ml final concentrations. The described ratio of venom to BPB was established by estimating the enzymatic activity of PLA2 in whole *MLO* venom (based on molecular weight of the venom and the venoms PLA2 fraction).

Preparation of metalloproteinases inhibited venom. Ethylenediaminetetraacetic acid disodium (EDTA- Na_2) salt was used to inhibit metalloproteinases of whole *MLO* venom. The mixture was prepared as follows: 1mg of dry EDTA- Na_2 was added to 1mg of lyophilized venom, and diluted in one milliliter of pre-warmed sterile distilled water. This stock solution was thoroughly mixed until complete dissolving of both reagents and incubated in the 37°C water bath for 2 hours to allow inhibition to take place. *MLO*+EDTA- Na_2 stock was diluted with complete growth media immediately before experiments to obtain 0.1ug/ml; 1ug/ml; 10ug/ml; 20ug/ml; 50ug/ml; 100ug/ml working solutions.

Venom enzymatic activity testing

Verification of venom caseinolytic activity in vitro. We used our simple and effective test for caseinolytic activity of the venom in assay of milk curdling in vitro. *MLO* venom was added to fresh milk at the ratio of 1:5000. At this ratio fresh venom leads to milk curdling when incubated for 30 minutes at 38°C (in 100% of cases). The control test tubes were filled with pure whole milk, without preservatives. Monitoring of pure milk under the same conditions serves as an internal control and eliminates the errors associated with its spontaneous curdling. Each condition was tested in triplicates.

Verification of venom phospholipase activity in vitro. Venom phospholipase activity was determined by simplified method of chicken yolk coagulation in vitro. Fresh chicken egg yolk was mixed with phosphate buffer (pH 7.4) at the ratio of 1:1 (Yolk Buffer Solution, YBS). Snake venom was added at to YBS at the ratio of 1:5000. Mixture was incubated in at 38°C for 30 min. Next, the tubes with YBS/venom mixture were placed in boiling water for 15 minutes. Control mixture (without the venom) curdles up and changes the color to a lighter shade of yellow. However boiling of YBS/venom mixture does not lead to coagulation and the yolk retains its bright yellow color, which indicated the presence of active phospholipase in the venom. Each condition was tested in triplicates.

Morphological characterization of NRCM, CF, and HeLa cells. In order to determine the qualitative effects of *MLO* crude venom on the morphology of the cultured cells, we used phase-contrast microscopy to monitor cell attachment. All tested cells were seeded at least 24h prior the experiments to obtain 90% confluent monolayers. Considering different proliferative properties of tested cells, NRCM were seeded at 2.5×10^5 cells/cm² and CFs were cultured at 1×10^4 cells/cm², HeLa cells were plated at 3.2×10^4 cells/cm² considering growth properties of each cell type. All experiments were conducted with monolayers reaching ~90% of confluency by the day of treatment. The morphological changes were assessed at 1 and 24 hours after addition of *MLO* venom. All other parameters were kept identical.

Physiological characterization of NRCM

Physiological characterization of contracting monolayers of NRCM consists of two main steps:

1. Fluo-4 AM Loading

Ca^{2+} release in the contracting monolayer of cardiomyocytes was imaged using fluorescence laser scanning confocal microscopy. Unless otherwise specified, loading of NRCM was performed as follows: complete media was removed, monolayers were washed with PBS, and then cultures were loaded with Fluo-4 AM (10μg/ml; Molecular Probes) in either Tyrode or phenol red-free DMEM in order to avoid color associated interference of fluorescent signals. The cultures were

incubated at 37°C for 45 min. The excess of Fluo-4 was removed by washing the monolayers with PBS, after which cells were placed in phenol red free media and analyzed immediately under a confocal microscope. Changes in contractile activity of the cells were recorded as Ca^{2+} transients traces.

2. Recording and analysis of Ca^{2+} Transients/Sparks in NRCM

The changes in contractile activity of NRCMs we examined as changes in intracellular calcium levels using laser scanning confocal microscopy (Leica DMi8 Inverted TCS SPE Laser scanning confocal). As fluctuations in the intensity of calcium indicator Fluo-4. Unless otherwise specified, loading of NRCM was performed as follows: the complete media was removed and the cultures were loaded with Fluo-4 AM (10 $\mu\text{g}/\text{ml}$; Invitrogen) in Tyrode at room temperature for 20-40 minute. Afterwards, cells were placed in fresh Tyrode solution and imaged immediately under a confocal microscope. The following parameters were extracted from calcium transient signals: spontaneous beating rate, amplitude (F1/F0) and peak. The beginning of the upstroke was defined by the initial deflection from baseline.

Investigation of Ca^{2+} fluorescence was done using confocal imaging with a TCS SPE laser scanning system (Leica Microsystems, Germany). Images were taken with a $\times 63$ oil immersion objective (numerical aperture (NA) = 1.4) (Fig.7. to be added). Fluo-4 was excited by 488 nm line of an Argon laser and emission signals over 505 nm were collected. The fluorescent signals represent the relative level of intracellular $[\text{Ca}^{2+}]_i$ and fluorescence intensity indicates the amount of released $[\text{Ca}^{2+}]_i$. We utilized a line scan mode for recording Ca^{2+} transients as sparks in fluorescence. The confocal pinhole was set to render spatial resolutions of 0.4 μm in the horizontal plane and 0.9 μm in the axial direction. Ideally, the detector gain is set at around 700 (no digital gain). Line-scan images were acquired at a sampling rate of 1.54 or 1.92 ms per line, along with the longitudinal axis of the cell. Each line comprises 512 pixels spaced at 0.14 μm intervals. After a sequential scanning, a two-dimensional (2D) image of 512 \times 1000 lines or 512 \times 2000 lines were generated and stored for later analysis. It is not recommended to scan a cell in the same line region for a prolonged time because exposure to laser causes photobleaching of fluorescent dye.

MTT viability assay. A standard methyl-thiazolyl-tetrazolium (MTT) colorimetric assay (Cat. #L11939) was used to assess cell metabolic activity. Briefly, cells were seeded in multiwell microplates at 2×10^5 cells/ cm^2 initial concentration for NRCMs and 1×10^4 cells/ cm^2 for CFs. When the cultured cells form a confluent monolayer they were treated with different venom concentrations and incubated for 24 h. Thereafter, the cultured cells were treated with MTT reagent and incubated for 3 hours until purple precipitate became visible. After incubation the culture medium was removed, and MTT solvent (0.4 μM HCl, 10% TritonX100 in isopropanol) was added to the wells, to dissolve formazan crystals. The plate was shaken for 20 min at room temperature to ensure complete dissolving of formazan crystals. The optical density was measured at 570nm and referenced at 620 nm wavelength using HiPo MPP-96 Microplate Photometer (Biosan). Recorded data were quantified using provided Quant Assay software.

Image analyses. Phase contrast images were collected using Zeiss Telaval 31 inverted microscope. The fluorescent images (static and timeline) were collected Leica DMi8 TCS SPE laser scanning confocal. The changes in recorded contractile activity of the cells were measured as Ca^{2+} transients and analyzed using ImageJ program.

Statistics. MTT assays for NRCM, CF, and HeLa cells viability and attachment experiments included three independent experiments with all conditions run in triplicates. To measure the degree of cells' detachment, at least five different view fields were analyzed. Areas covered with cells were quantitated and related to the areas from which cells detached upon exposure to intact *MLO* snake venom, or thermally-inhibited venom, or PLA2 inhibited venom (BPB-treated), or metalloproteinases inhibited venom (EDTA- Na_2 -treated) at all indicated concentrations and time points. For quantitative analysis of calcium transients, each *MLO* concentration was applied to three different coverslips and Fluo-4 recordings were collected from four to five different fields. All values are expressed as mean \pm SE, with $p < 0.05$ considered statistically significant (*), $p < 0.01$ considered statistically very significant (**), and $p < 0.001$ considered statistically the most

significant (***). Mean values are expressed as a percentage of vehicle control. Statistical analyses were performed using Student's t-test (MS Excel). Representative traces and images are shown.

RESULTS

Correlation of MLO doses with morphological/structural changes on tested cells with viability

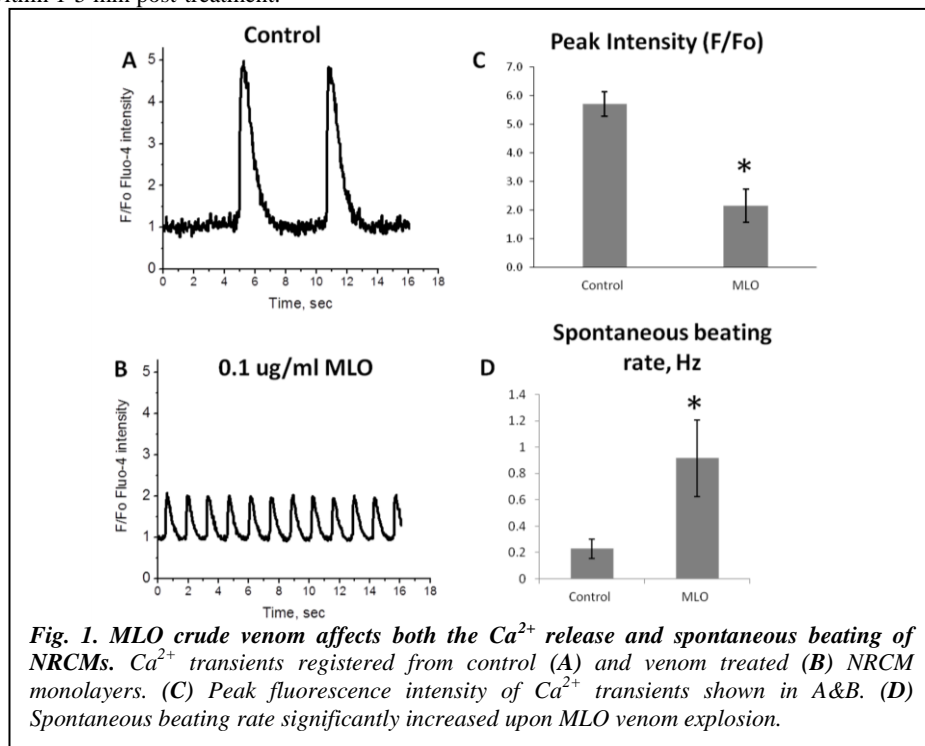
It is known that *MLO* snake venom affects different organs and organ systems. To better understand its effects on cardiovascular system, we decided to assess its effect on cultured cardiomyocytes and other major cells of cardiac muscle. The initial experiments were designed to evaluate the effects of whole *MLO* venom on NRCM, CFs and HeLa cells.

MLO venom effect on NRCM

The *MLO* venom was added to the cultured NRCM at the following final concentrations of 0.1 μ g/ml; 1 μ g/ml; 10 μ g/ml; 20 μ g/ml; 50 μ g/ml; 100 μ g/ml to discover possible reactions such as detachment/reattachment, changes in contraction frequency, action potential amplitude, etc.

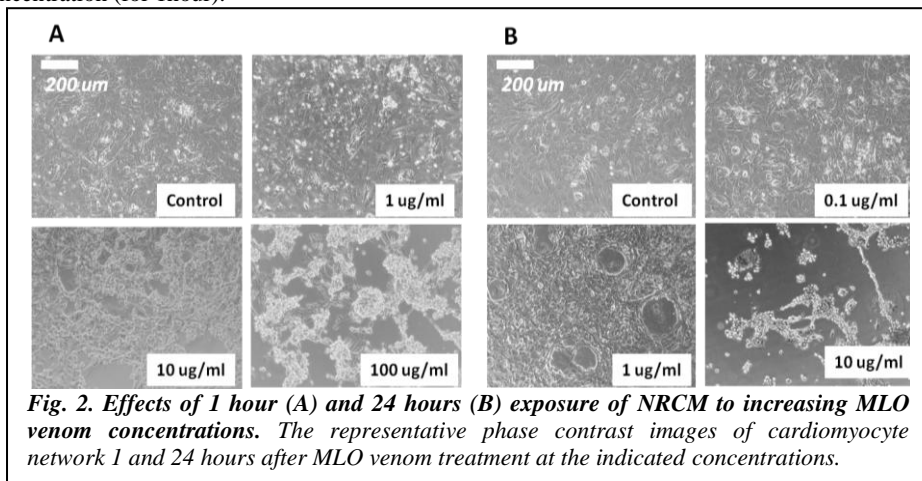
Figure 1A illustrate action potentials registered from un-manipulated control NRCM monolayer, whereas Figure 1B shows similar measurements taken from monolayer of cells treated with the lowest 0.1 μ g/ml concentration. A detailed kinetic analysis of calcium transients revealed associated changes in amplitude and duration of calcium transients. Interestingly, even the lowest dose of venom caused a dramatic change in NRCM contraction amplitude (Fig.1B), decrease in Ca²⁺ release (Fig.1C) and significant increase in NRCM spontaneous beating rate and (Fig.1D). As expected, increased beating frequency led to shorter durations and significantly decreased amplitude (F/F₀) of calcium transients due to the action of bradykinin potentiating/C-type natriuretic (BPP/C-NP) peptide in *MLO* venom.

Cell attachment was minimally affected at both, 1 and 24h of treatment with 0.1 μ g/ml *MLO*. (Fig.2). The rate of NRCM beating also increased upon adding the high dose of 100 μ g/ml *MLO*; however, the rapid contraction was accompanied by detachment from the surface of the culture plate within 1-5 min post-treatment.



Initially, NRCM detached from culture plates as contracting clumps and later (in about 30 min) cells dissociated from each other. After 1-hour, the detached clumps and single-beating cells stop contracting and the monolayer largely disengaged.

At the lower “clinically relevant” concentration of *MLO* (from 0.1ug/ml to 10ug/ml) the NRCM monolayers remain attached which allowed investigation of *MLO* effect at the later time points. Therefore, monolayers were assessed 1 and 24 hours post venom exposure (Fig.2A and 2B respectively). Attachment properties of NRCM were affected by *MLO* venom in a dose-dependent manner. In addition time exposure also has an effect on NRCMs attachment to the substrate and to cell-to-cell contact. Longer exposures times result in higher detachment even at the same *MLO* venom concentrations (Fig.2A vs Fig.2B). In order to quantitatively evaluate the detaching effects of *MLO* venom on NRCMs, areas covered with cells were compared with those w/o cells after exposure to *MLO* venom. As demonstrated in Figure 3A the attachment of NRCM was affected by *MLO* crude venom starting at 1ug/ml concentration and reaching 100% detachment at 100ug/ml concentration (for 1hour).



In order to differentiate between cytotoxic effect of *MLO* venom (causing cell death) and its effect on attachment properties of NRCMs, the viability of described cultures was assessed by MTT viability assay. To proper assess cells' survivability, exclude all non-relevant effects of cell manipulations and set baseline observable facts, we conducted a series of experiments where all three cell types were exposed to thermally-inhibited *MLO* venom at different concentrations. Boiling *MLO* venom for 30 min ensures denaturing of all venom enzymes, but does not rid of other non-proteinaceous elements. Therefore we assessed both the viability and the attachment of cells treated with fully denatured-thermolysed *MLO* venom at all experimental concentrations. Monolayers of NRCMs treated with thermo-destroyed *MLO* venom for 1 and 24 hours post exposure, remain attached and otherwise unaltered (Fig.3A and 3B). The same cultures were assessed by MTT viability assay. The results demonstrated that all attached cells were still live and non-affected by other elements of thermally inhibited venom, which could influence the level of cells survivability. The viability of the NRCMs remained similar after 1 hour (Fig.3A) and 24 hours of treatment (Fig.3B). Similar results were obtained with CFs and HeLa cells (data not shown). Importantly these phenomena correlated with 100% attachment of all cells treated with thermally-inhibited *MLO* venom (Fig.3A and 3B).

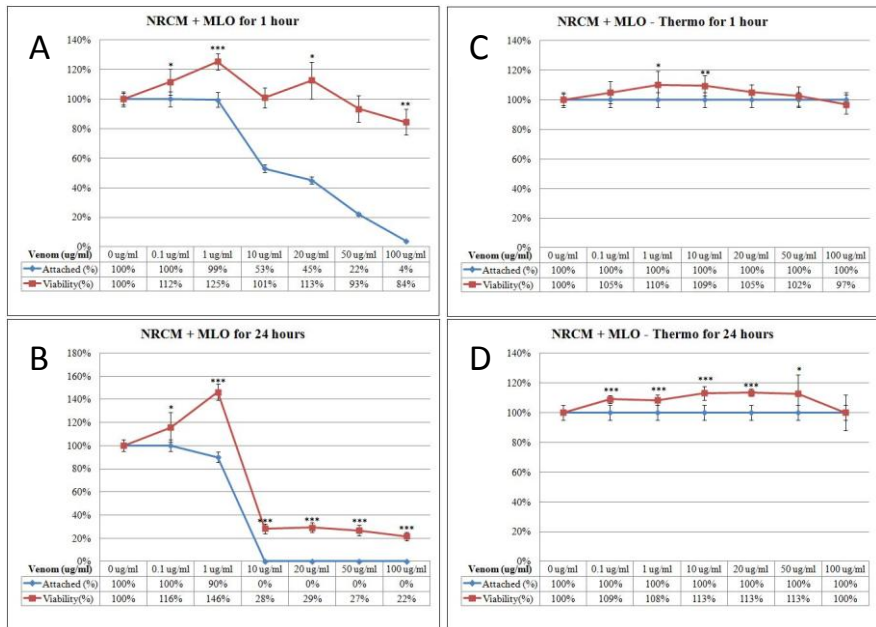


Fig. 3. Attachment and Viability of NRCM is affected by MLO in a dose- and time-dependent manner. Attachment (blue lines) and Viability (red lines) of NRCMs treated with indicated concentrations of either thermally inhibited MLO venom (A&B) or whole venom (C&D). Effect of MLO whole venom on NRCMs attachment and viability were assessed after 1 hour (C) and 24 hours (D) of treatment. For quantification of attachment experiments, the ratio of covered areas to uncovered areas of NRCMs cultured for 1 hour at 0.1ug/ml; 1ug/ml; 10ug/ml; 20ug/ml; 50ug/ml and 100ug/ml concentrations.

Next we tested the effects of native (un-manipulated) *MLO* venom on viability of NRCMs, CFs and HeLa cells. NRCMs treated with venom for one hour remain alive, regardless of applied *MLO* concentrations (Fig.3C). Importantly, survivability of these cells did not correlate with their attachment. More specifically, NRCMs treated with 10ug/ml of *MLO* venom for 1 hour, remained 100% live, but demonstrated 40-50% detachment (Fig.3C). Interestingly when identically treated parallel cultures were kept for additional 23 hours (total of 24 hours of incubation), cell viability was severely lessened (Fig.3D). Specifically, at 24 hours of incubation, all cells treated with 10ug/ml and higher, lost up to 70-80% of their viability. This phenomenon was correlated with 100% detachment of NRCMs treated with high doses of *MLO* venom (Fig.3D).

MLO venom effect on CF

Cardiac fibroblasts are essential part of myocardium. Therefore, the next set of experiments was conducted to evaluate the effects of *MLO* venom on this type of cells. Toward that goal, cell culture media was removed from cultured CFs and the diluted *MLO* venom was added at the final concentrations of 0.1ug/ml; 1ug/ml; 10ug/ml; 20ug/ml; 50ug/ml; 100ug/ml in triplicates. Cells were incubated for 1 hour. The data demonstrated that CFs treated with lowest dose (0.1ug/ml) for 1 and 24 hours, show no visible effects of detachment whereas parallel cultures treated with 100ug/ml were detached even after 1min post-treatment (Fig.4) and more so after 24h incubation.

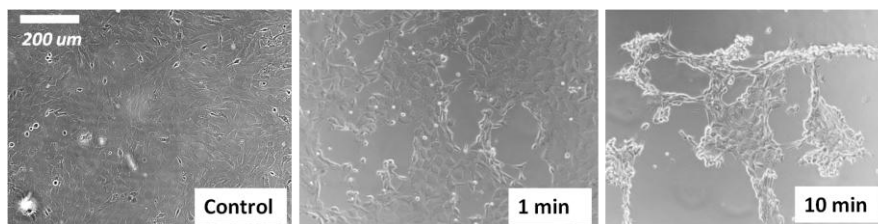


Fig. 4. High concentration of MLO venom is detrimental for CFs attachment to a substrate. CFs (Control) were exposed 100ug/ml of MLO for 1min and 10min.

In order to quantitatively evaluate the detaching effects of indicated concentrations of *MLO* crude venom on CFs, areas covered with cells were compared with the areas without cells after exposure to *MLO* venom for 1 and 24hours (Fig.5A and 5B respectively). The attachment of CF was affected by *MLO* crude venom starting at 1ug/ml concentration and reaching 100% detachment at 100ug/ml concentration. Quantitated data of the attachment properties show that CFs were affected both in dose and in time dependent manner.

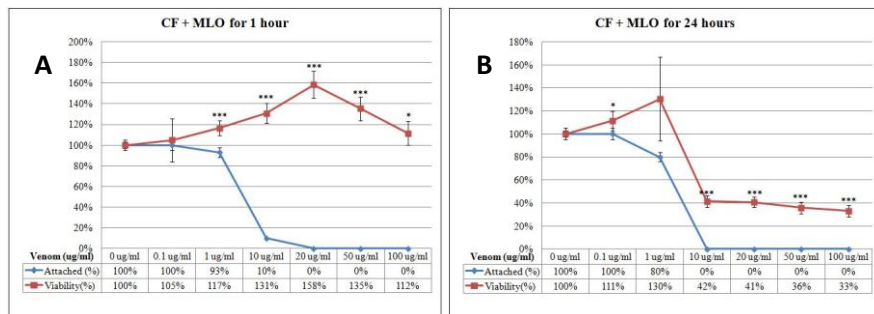


Fig. 5. Attachment and Viability of CFs is affected by MLO in a dose- and time-dependent manner. Attachment (blue lines) and Viability (red lines) of CFs treated with indicated concentrations of whole venom were assessed after 1hour (A) and 24 hours (B) of treatment. For quantification of attachment experiments, the ratio of covered areas to uncovered areas of CFs.

In order to differentiate between cytotoxic effect of *MLO* venom (causing cell death) and its effect on attachment properties of CFs, the viability of described cultures was assessed by MTT viability assay. When viability of treated cells was tested after one hour exposure to the venom, most cells remain alive, regardless of applied *MLO* concentrations. Similarly to NRCMs, survivability of CFs did not correlate with their attachment. More specifically, CFs treated with 10ug/ml of *MLO* venom for 1 hour, remained 100% live (with slight growth up to 130%), but demonstrated 40-50% detachment (Fig.5A). Interestingly when identically treated parallel cultures were incubated for 24 hours, cell viability was dramatically decreased (Fig.5B). More specifically, the first significant difference was observed starting from 10ug/ml and higher concentration, the cells lost up to 60-70% of their viability. This phenomenon was correlated with 100% detachment of CFs treated with high doses of *MLO* venom.

MLO venom effect on HeLa cells

Throughout the study we used HeLa cells as model of epithelial cells. Since myocardium is heavily vascularized and endothelial cells are the first type of cells within cardiac muscle which are exposed to the venom, it was important to identify the effect of *MLO* venom on these type of cells.

For investigation of the effect of *MLO* venom on HeLa cells, culture media was removed and the diluted *MLO* venom was added to the cultured HeLa cells at the final concentrations of 0.1ug/ml; 1ug/ml; 10ug/ml; 20ug/ml; 50ug/ml; 100ug/ml in triplicates. Cells were incubated for 1 hour. The data indicate that HeLa cells treated with lowest dose (0.1ug/ml) for 1 hour, show no visible effects of detachment whereas parallel cultures treated with 10ug/ml and higher concentration reaching 100% detachment after 1 hour of post treatment (Fig.6). In order to quantitatively evaluate the detaching effects of indicated concentrations of *MLO* crude venom on HeLa's, areas covered with cells were compared with the areas w/o cells after exposure to *MLO* venom for 1 and 24hours. Quantitated data of the attachment properties show that HeLa cells were affected both in dose and in time dependent manner.

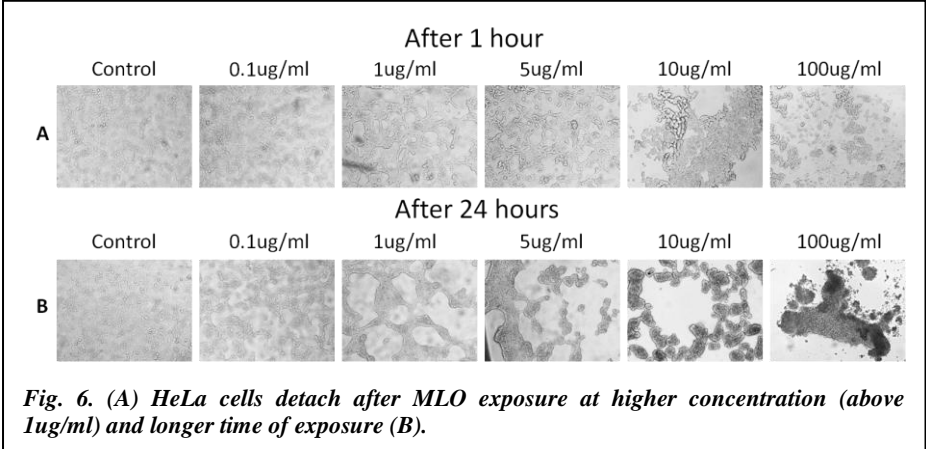


Fig. 6. (A) HeLa cells detach after MLO exposure at higher concentration (above 1ug/ml) and longer time of exposure (B).

As before, to distinguish between cytotoxic effect of *MLO* venom (causing cell death) and its effect on attachment properties of HeLa cells, the viability of described cultures was assessed by

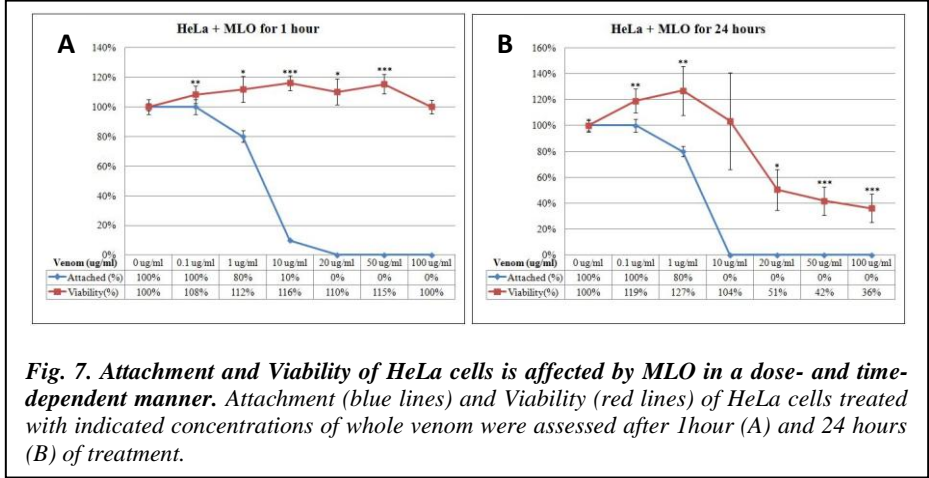


Fig. 7. Attachment and Viability of HeLa cells is affected by MLO in a dose- and time-dependent manner. Attachment (blue lines) and Viability (red lines) of HeLa cells treated with indicated concentrations of whole venom were assessed after 1hour (A) and 24 hours (B) of treatment.

MTT viability assay. When viability of treated cells was tested after one hour exposure to the venom, most cells remain alive, under all applied *MLO* venom concentrations. Importantly, survivability of these cells did not correlate with their attachment (Fig.7A). More specifically, HeLa cells treated with 10ug/ml of *MLO* venom for 1 hour, remained 100% live, but demonstrated 90% detachment. Interestingly when identically treated parallel cultures were kept for total of 24 hours of incubation, cell viability was severely lessened (Fig.7B). Specifically, all cells treated with

20ug/ml and higher, lost up to 50-60% of their viability. This phenomenon was correlated with 100% detachment of HeLa cells treated with high doses of *MLO* venom.

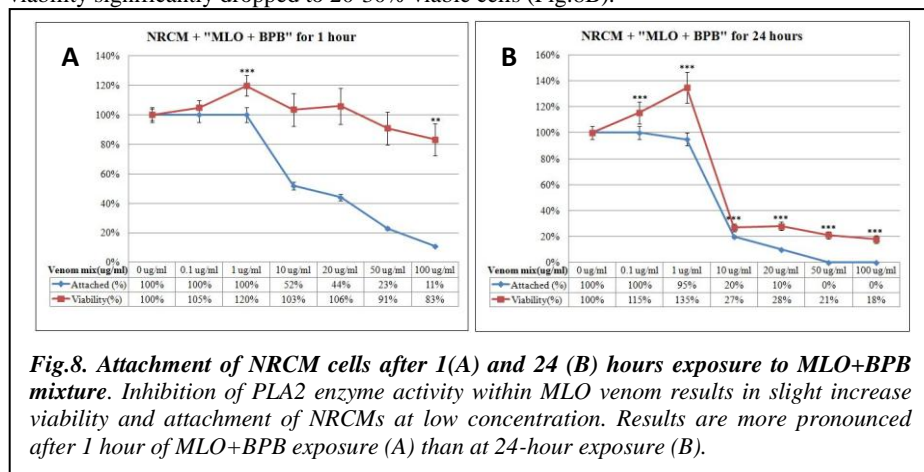
Unraveling the mechanisms of MLO detaching effects

In order to understand the mechanism of detachment and how it affects the viability of the cells we have try to inhibit individual enzymatic components of the *MLO* venom. For this reason we use 2 main inhibitors: one of them BPB was used to inhibit the PLA2 of *MLO* snake venom, the second inhibitor which used in this study was EDTA-Na₂, which inhibits the Metalloproteinases of the *MLO* crude venom.

Effect of MLO+BPB mixture on NRCM

PLA2 activity was inhibited with the use of bromophenacyl bromide (BPB) as described in Materials and Methods section. NRCM treated with MLO+BPB mixture for one hour demonstrated 40-50% detachment under exposure to 10ug/ml the mix (Fig.8A). This trend continued and reached 80-90% detachment rate at 100ug/ml concentration of MLO+BPB mix, while the cell viability remained at 100% (Fig.8A). After incubation of parallel cultures for total of 24 hour, attachment of NRCMs remained unaffected up to the 1ug/ml venom concentration (Fig.8B). This result proves that at least a part of the *MLO* venom activity was inhibited by BPB and attachment of NRCMs was improved compared with the effect of the same concentration of un-inhibited *MLO* (Fig.8B compared with Fig.3B).

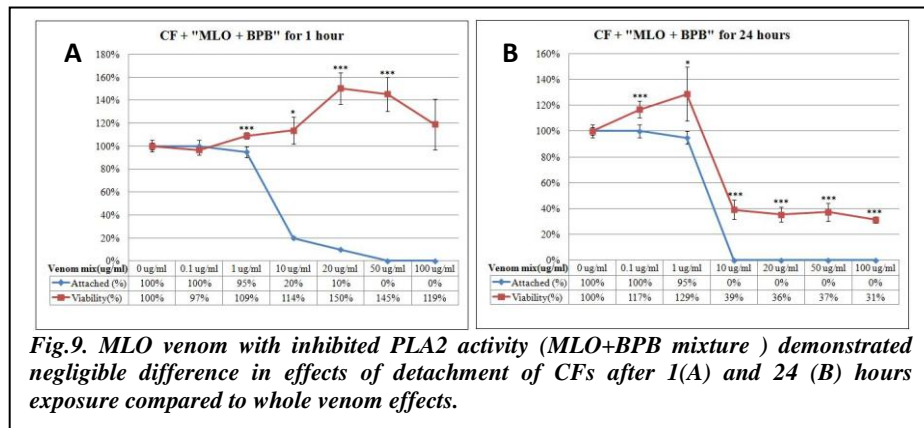
Furthermore viability of the same culture treated with MLO+BPB for 24 hours was also slightly improved for lower venom concentrations, but stating from 10ug/ml MLO+BPB mixture the viability significantly dropped to 20-30% viable cells (Fig.8B).



Effect of MLO+BPB mixture on CF

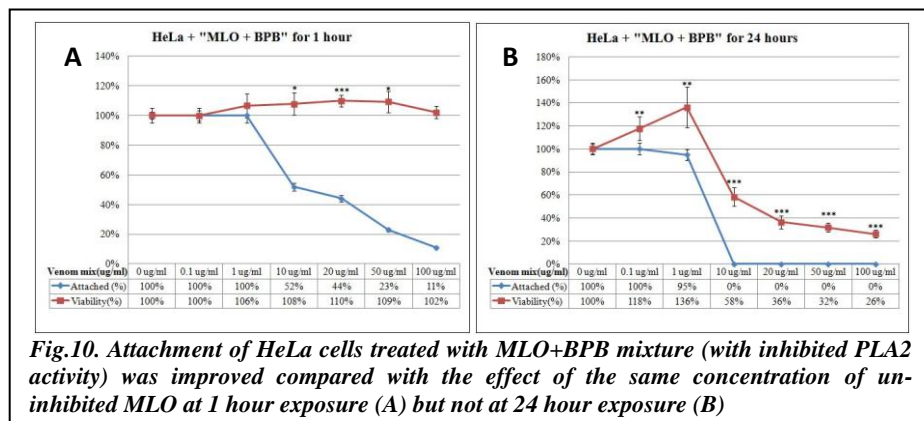
Similar experiments were performed on CFs to assess the BPB inhibitory effects on the action of PLA2 enzyme of *MLO* venom. No changes in attachment were detected at 1 and 24 hours of when cells were exposed to up to 1 ug/ml MLO+BPB mixture. Once CFs were treated 1ug/ml concentration with MLO+BPB for 1 (Fig.9A) or 24 hours (Fig.9B), the detachment was determined to be 5% which is similar to the effect of un-inhibited venom (Fig 5 A&B). However, comparing these results with the parallel effects of un-inhibited *MLO* venom on CFs proves that detachment level of CFs was moderated by the presence of BPB as follows (Fig.9 compared with Fig.5). Starting from a concentration of 10ug/ml of MLO+BPB mixture detachment rate reached 80-100% both after 1 and 24 hours of exposure (Fig.9A and 9B respectively). Remarkably, the cell viability rate remained 100% for the 1 hour treatment (Fig.9A); therefore no correlation exists between detachment and cell viability. After incubation of parallel cultures for total of 24 hours cell viability

dropped by 60-70% (Fig.9B). Taken together this indicates that attachment of CF's is governed by molecules, which are not significantly affected by PLA2 enzyme, whereas survivability of the same cells are.



Effect of MLO+BPB mixture on HeLa

HeLa cells treated with MLO+BPB mixture for one hour demonstrated 5% detachment under exposure to 1ug/ml of the mix for 1 (Fig.10A) and 24 hours (Fig.10B). When put in comparison with un-inhibited *MLO* venom, the degree of detachment of HeLa cells was altered by the presence of BPB. This result confirms that at least a part of the *MLO* venom activity was inhibited by BPB and attachment of HeLa cells was improved compared with the effect of the same concentration of un-inhibited *MLO* (Fig.10 compared with Fig.7). Starting from 10ug/ml MLO+BPB mixture



detachment rate of 1 hour treated cells reached 50-100%, while the cell viability remained 100% (Fig.10A).

Furthermore viability of the same culture treated with MLO+BPB for 24 hours was also slightly improved for lower venom concentrations, but starting from 10ug/ml MLO+BPB mixture the viability significantly dropped to 30-60% viable cells (Fig.10B).

Ethylenediaminetetraacetic acid disodium (EDTA- Na_2) is known chelating agent. As such it acts as effective inhibitor for all metalloproteinases. Therefore to inhibit the metalloproteinases of the *MLO* venom we prepared MLO+EDTA- Na_2 mixture, incubated it for 2hours to allow for the

chelating reaction to take place and test the effects of this mixture on attachment properties and viability of NRCM, CFs and HeLa cells.

Effect of MLO+EDTA-Na₂ mixture on NRCM

Metalloproteinases activity was inhibited with the use of Ethylenediaminetetraacetic acid disodium (EDTA-Na₂) as described in Materials and Methods section. NRCM treated with MLO+EDTA-Na₂ mixture for one hour demonstrated 30-40% detachment under exposure to 10ug/ml the mix.

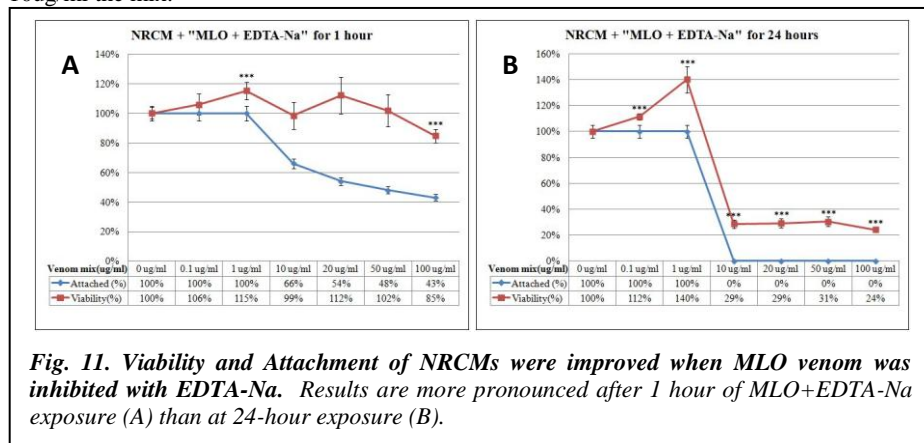


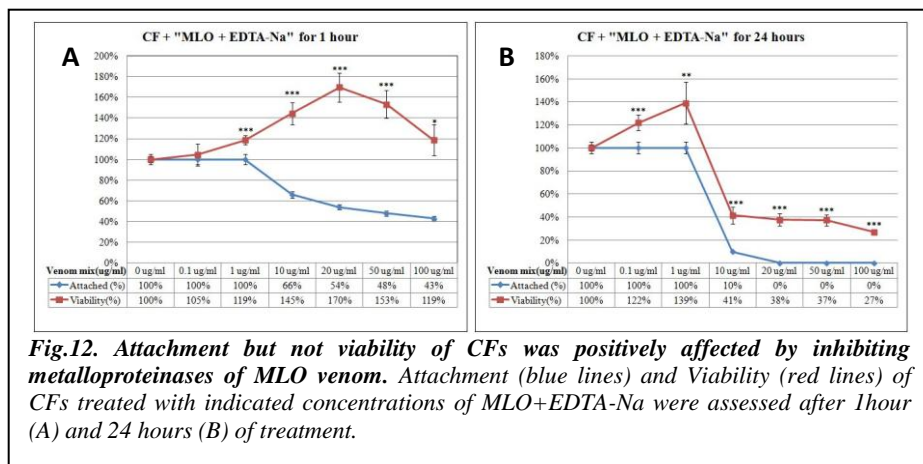
Fig. 11. Viability and Attachment of NRCMs were improved when MLO venom was inhibited with EDTA-Na. Results are more pronounced after 1 hour of MLO+EDTA-Na exposure (A) than at 24-hour exposure (B).

This trend continued and reached 50-60% detachment rate at 100ug/ml concentration of MLO+EDTA-Na₂ mix (Fig.11A). This result proves that at least a part of the *MLO* venom metalloproteinases activity was inhibited by EDTA-Na₂ and attachment of NRCMs was improved compared with the effect of the same concentration of un-inhibited *MLO* (Fig.11A compared with Fig.3A). In the meantime cell viability remained at 100% (Fig.11A). After incubation of parallel cultures for total of 24 hour, attachment of NRCMs remained unaffected up to the 1ug/ml venom concentration (Fig.11B). This result proves that attachment of NRCMs was slightly improved compared with the effect of the same concentration of un-inhibited *MLO* (Fig.11B compared with Fig.3B).

Furthermore viability of the same culture treated with MLO+EDTA-Na₂ for 24 hours was also slightly improved for lower venom concentrations, but starting from 10ug/ml MLO+BPB mixture the viability significantly dropped to 20-30% viable cells (Fig.11B).

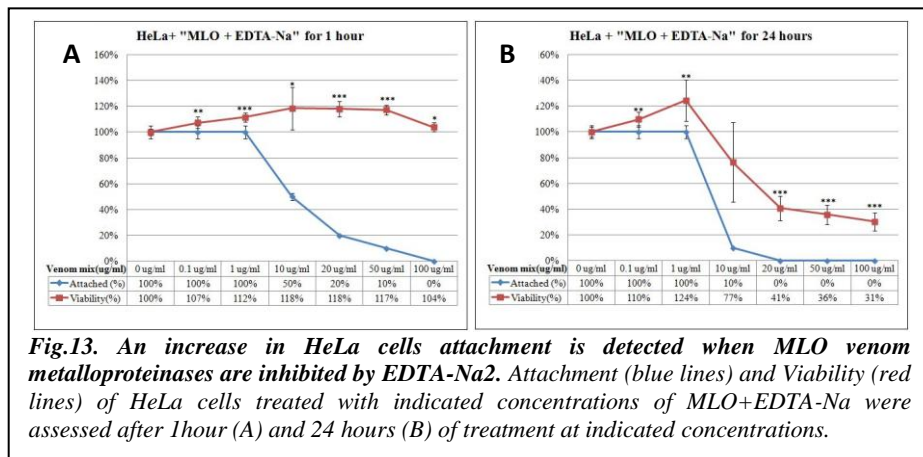
Effect of MLO+EDTA-Na₂ mixture on CF

CFs treated with MLO+EDTA-Na₂ mixture demonstrated no changes in viability properties at 1 and 24 hours across tested concentrations of MLO + EDTA mixture (Fig.12A&B and Fig 5). However, the situation is significantly different when we assessed the attachment of the same cells. The attachment of CFs was significantly increased compared with the attachment of CFs treated with identical concentration of un-inhibited *MLO* (Fig.12A compared with Fig.5A). Specifically, the cell viability rate remained 100% for 1 and 24 hours of treatment for the indicated concentrations (Fig.12A and 12B); therefore there is a direct correlation between inhibition of metalloproteinases activity of *MLO* venom and cell attachment. Remarkably, CFs treated with MLO+EDTA-Na₂ mixture for one hour demonstrated 50% detachment under exposure to 10ug/ml the mix (Fig.12A). This trend continued and reached 100% detachment rate at 100ug/ml concentration of MLO+EDTA-Na₂ mix both after 1 and 24 hours of exposure. Remarkably, the cell viability rate remained 100% for all the concentrations of 1 hour treatment (Fig.12A). Starting from 10ug/ml concentration of MLO+EDTA-Na₂ mixture the viability rate of 24hour treated culture dropped by 60-70% (Fig.12B).



Effect of MLO+EDTA-Na₂ mixture on HeLa

Parallel experiments were performed to assess the role of metalloproteinases enzyme of *MLO* venom by inhibiting them with EDTA-Na₂. Interestingly when dealing with HeLa cells, no changes in viability were detected at 1 and 24 hours across all tested concentrations of MLO+EDTA mixture (Fig.13A&B vs Fig.7). The cell viability rate remained 100% for 1 and 24 hours of treatment for the indicated concentrations. Similarly to CFs, when metalloproteinases activity was inhibited by EDTA-Na₂ and attachment of HeLa cells was significantly increased compared with the effect of



the same concentration of un-inhibited *MLO* (Fig.13A compared with Fig.7A). Therefore, we hypothesize that metalloproteinases directly affect adhesion of HeLa cells to the substrate and to each other. HeLa cells, treated with MLO+EDTA-Na₂ mixture for one hour, demonstrated 50% detachment under exposure to 10ug/ml the mix (Fig.13A). This trend continued and reached 100% detachment rate at 100ug/ml concentration of MLO+EDTA-Na₂ mix both after 1 and 24 hours of exposure. Importantly, the cell HeLa cells remained 100% viable for all the concentrations of 1 hour treatment (Fig.13A). Starting from 20ug/ml concentration of MLO+EDTA-Na₂ mixture, the viability rate of 24-hour treatment culture dropped by 30-50% (Fig.13B).

All in all our data proves the direct effects of *MLO* venom to adherent properties of myocardial cells and HeLa cells. It influences both the adhesion to the extracellular matrix and, differently, the

adhesion of cells to each other. In our working model, closely resembling the estimated concentrations of the venom in the bloodstream of a prey, we found that 1ug/ml concentrations demonstrates the most obvious changes in the tested characteristics of studies cells, namely attachment and viability. This concentration was also found to be the one that does not cause mortality of the cells, but rather simulates a lower level of venom, that is associates with morbidity of a prey and thus is more important to be studied. Table 2 summarizes the results of exposure of all tested cells to whole *MLO* and its inhibited forms for one and 24-hour periods obtained under 1ug/ml venom concentrations to illustrate the most significant results of our studies.

		Exposure time		Treatment type at 1 ug/ml MLO			
				MLO-Thermo	MLO	MLO+EDTA-Na ₂	MLO + BPB
Cell type	NRCM	1 hour	Attachment	100%	99%	100%	100%
			Viability	110%	125%	115%	120%
		24 hours	Attachment	100%	90%	100%	95%
			Viability	108%	146%	140%	135%
	CF	1 hour	Attachment	100%	93%	100%	95%
			Viability	103%	117%	119%	109%
		24 hours	Attachment	100%	80%	100%	95%
			Viability	110%	130%	139%	129%
	HeLa	1 hour	Attachment	100%	80%	100%	100%
			Viability	114%	112%	112%	106%
		24 hours	Attachment	100%	80%	100%	95%
			Viability	116%	127%	124%	136%

Table 1. Summary of attachment and viability data for all types of cells treated with 1ug/ml of each treatment.

CONCLUSIONS

Macrovipera lebetina obtusa is one of the most poisonous snakes in Armenia, which bites causes unavoidable morbidity and sometimes mortality of a prey. Cardiovascular system is one of the neglected targets of this venom. In our studies we develop a model for investigating *MLO* venom effects on cultured myocardial and endothelial cells. In this study we used neonatal rat cardiomyocyte preparation method to address the toxicity of this venom considering the organization of cardiac muscle. Based on the results of presented investigation we made the following conclusions:

- 1) *MLO* venom has a direct affect on cultured myocardial cells such as cardiomyocytes and cardiac fibroblast and epithelial cells, by severely altering their attachment properties.
- 2) *MLO* venom induced detachment is dose and time dependent in all testes cell types.
- 3) The tolerable dose of *MLO* exposure is optimal dose for investigating adhesive properties of myocardial and epithelial cells. This was estimated to be in 0.1 and 1 ug/ml range.
- 4) Cardiomyocytes exposed to tolerable doses of the *MLO* venom demonstrates dramatic increase in contraction frequency, due to C-type natriuretic peptide, which in turn results in significant decrease of contraction amplitude.
- 5) Identified detachment is characterized by specific order of events: initially cells detach from culturing substrate and later cells detach from each other.
- 6) Chelated *MLO* venom causes less detachment in all tested cells types, indicating the involvement of venom metalloproteinases.
- 7) BPB inhibited *MLO* venom is also less detaching for cells, indicating the involvement of PLA2 enzyme of the venom in this process.

The list of published papers on the topic of the thesis

1. **H.Arestakesyan**, Z.Karabekian, N. Ayvazyan. Effect of crude *Macrovipera lebetina obtusa* venom on adhesion properties of cardiomyocytes, cardiac fibroblasts, and epithelial cells. Biological Journal of Armenia, Proceedings of the International Young Scientists Conference “New Trends in Life Sciences”, Dedicated to the 50th Anniversary of the Institute of Molecular Biology NAS RA, September 26-28, 2016, Volume 68, Special Issue, p. 18-22.
2. **H.V.Arestakesyan**. Physiological characterization of neonatal rat cardiomyocytes using laser scanning confocal microscopy. Issues in Theoretical and Clinical Medicine, Journal of Scientific and Practical Medicine, 2017, Volume 20, № 3 (113), p. 49-53.
3. **H.V.Arestakesyan**, Z.I.Karabekian. Adhesion properties of cardiomyocytes and cardiac fibroblasts are affected by *Macrovipera lebetina obtusa* crude venom. Issues in Theoretical and Clinical Medicine, Journal of Scientific and Practical Medicine, 2017, Volume 20, № 3 (113), p. 53-58.

Արեստակետյան Հովհաննես Վրեժի

MACROVIPERA LEBETINA OBTUSA ՕձԻ ԹՈՒՅՆԻ ՍՊԵՑԻՖԻԿ ԱԶԴԵՑՈՒԹՅՈՒՆԸ ՍՐՏԱՄԿԱՆԻ ԿՈՒՆՏԻՎԱՑՎԱԾ ԲՋԻՋՆԵՐԻ ՎՐԱ

ԱՄՓՈՓԱԳԻՐ

Բանալի բառեր. *Macrovipera lebetina obtusa*, Օձի թույն, կարդիոմիոցիդ, սրտային ֆիբրոլիաս, *in vitro* կուկուրա, ադիեզիա, փոքսիկություն

Macrovipera ցեղի օձերի թույնի ուսումնասիրությունները ցույց են տվել, որ թույնի տարբեր բաղադրիչների ազդեցությունների մեխանիզմների մեջ զգալի տարբերություններ կան: Դրանք կախված են զոհի տեսակից և սպեցիֆիկ թունավորման թիրախից: Նախնական հետազոտությունները ցույց են տվել, որ տարբեր տեսակի օձերի թույնում կան ֆերմենտների և պոլիպեպտիդների նույնանման ընտանիքներ: Սակայն ավելի խորն ուսումնասիրությունները ցույց տվեցին թույների բաղադրիչների խիստ որոշակի առանձնահատկություններ, որոնք եզակի են այս կամ այն տեսակի և նույնիսկ ենթատեսակի օձերի համար: Թույնի յուրահատուկ բաղադրությունը առաջացնում է որոշակի վնասվածքներ, որոնք էլ հանգեցնում են զոհի հնարավորինս արագ մահվան (Daltry JC et al., 1996): Չոհն անշարժանում և ապակողմնորոշվում է, ապա զարգանում է ցավային համախտանիշ և հիպոտոնիկ կոլապս, խանգարվում են տարբեր օրգանների և օրգան համակարգերի ֆունկցիաները: Մոլեկուլային մակարդակով թույնի բաղադրիչները բարձր ընտրողականություն են դրսևորում:

Ի տարբերություն բուսական թույների, որոնք զոհի մոտ հիմնականում արգելակում են որևէ թիրախային գործառույթ, կենդանական թույները հաճախ «հարկադրում» են զոհի պաշտպանական մեխանիզմներին պայքարել իր իսկ դեմ (Wu and Huang, 2003): Թույնի շատ բաղադրիչներ հանդես են գալիս որպես երկ- կամ բազմաֆունկցիոնալ նյութեր, և կախված ազդեցության տեղամասից՝ կարող են ցուցաբերել ֆերմենտային ակտիվություն և/կամ ծառայել որպես լիգանդ՝ տարբեր ընկալիչների և ազդանշանային համակարգերի համար:

Macrovipera lebetina obtusa Օձի թույնի ոչ բացահայտ թիրախը սրտանոթային համակարգն է: Թույնի ազդեցությունն ուսումնասիրելու համար մեր հետազոտություններում մշակել ենք կուկտիվացված սրտամկանային և էպիթելային (HeLa) բջիջների մոդելներ: Տոքսիկությունն ուսումնասիրելու համար, հաշվի առնելով սրտամկանի բջիջների կազմակերպվածությունը, հետազոտություններում կիրառել ենք նորածին առնետների սրտերից բջիջների անջատման և կուկտիվացման մեթոդը:

M. lebetina տեսակին բնորոշ ընդհանուր ֆերմենտների հետ մեկտեղ՝ *MLO* թույնը պարունակում է նաև յուրահատուկ բաղադրիչներ (օրինակ՝ օբտուսատոին դիզինտեգրինը), որոնք հատուկ են *obtusa* ենթատեսակին: Այս և որոշ այլ բաղադրիչները միասին որոշում են այս օձի թույնի յուրահատկությունը: Թույնը պարունակում է մետաղալարոտեազներ (*U*Պ), ֆոսֆոլիպազա *A2* (*PLA2*), սերինային պրոտեազներ, *L*-ալֆա ամինաթթվային օքսիդազ, մի քանի տեսակի դիզինտեգրիններ և այլ ակտիվ ազենտներ (Sanz L. et al., 2008): *MLO* թույնի մի շարք բաղադրիչներ (*U*Պ, օբտուսատոինը, *C*-տիպի լեկտինները և այլն), հայտնի են որպես հակաադիեզիվ մոլեկուլներ, որոնք խաթարում են ինտեգրիններն ու կադիերինները կամ կապվում են

դրանք: Մենք ուսումնասիրել ենք ակտիվ թույնի մոլեկուլների ազդեցությունը միջբջջային և բջիջ-արտաբջջային մատրիքս կապերի վրա (հակաադիեզիվ ազդեցություն), ինչպես նաև կարդիոմիոցիտների (ԿՄ), սրտային ֆիբրոբլաստների (ՍՖ) և HeLa բջիջների կենսունակությունը: Թույնի մահացու չափաբաժնի 50%-ը 1 մկան համար 18.4մկգ է: Առ այսօր հայտնի չէ, թե խայթոցից հետո թույնի ինչ չափաբաժին է հասնում սիրտ: Ըստ մեր հաշվարկների՝ այն կազմում է 5-10մկգ/մլ: Հաշվարկը հիմնված է հասուն մկների միջին քաշի (20-25գ) և արյան ծավալի (մարմնի զանգվածի 6-8% կամ 1,5-2մլ) վրա: Այս հաշվարկներից հետևում է, որ աշխատանքում օգտագործված կոնցենտրացիաները, փաստացիորեն, կարող են հանդիպել *in vivo*: Այսպիսով՝ մեր հետազոտություններում մենք համակարգված կերպով ուսումնասիրել ենք MLO թույնի ցածր կոնցենտրացիաների ազդեցությունները նշված բջիջների վրա:

Հետազոտությունների արդյունքները ցույց տվեցին, որ MLO թույնն ունի զգալի հակաադիեզիվ հատկություն: 1մկգ/մլ-ից ցածր չափաբաժինները ցիտոտոքսիկ չեն. բջիջները, չնայած որ պոկվում են սուբստրատից, այնուամենայնիվ, մնում են կենսունակ: Թույնը հեռացնելու դեպքում դրանք ունակ են կրկին ամրանալ սուբստրատին և վերականգնել ֆունկցիաները: Մեր կողմից հայտնաբերվեցին MLO թույնի այն չափաբաժինները, որոնք ազդում են նշված բջիջների ադիեզիվ հատկությունների վրա՝ միևնույն ժամանակ չհանգեցնելով բջիջների մահվան: Օգտագործված մոդելը եզակի հնարավորություն է տալիս ուսումնասիրելու մոլեկուլային փոխազդեցությունները բջջային երկարաժամկետ կուլտուրայում: Այն նաև ստեղծում է հիմքեր MLO թույնի բաղադրիչների ուսումնասիրման համար, ինչը կարող է օգտագործվել դեղամիջոցների արտադրության մեջ:

MLO թույնի ազդեցության մեխանիզմները պարզելու համար մենք օգտագործել ենք թույնի առանձին բաղադրիչներին սպեցիֆիկ ինհիբիտորներ: Դրանցից EDTA-Na₂-ն (խելատացնող նյութ) արդյունավետորեն չեզոքացնում է թույնի ՄՊ: EDTA-Na₂-ով ինհիբիցիայի ենթարկելիս թույնի հակաադիեզիվ հատկությունները փոքր-ինչ նվազել են: Նմանատիպ արդյունքներ են ստացվել նաև բրոմֆենացիլ բրոմիդով (BPB) ինհիբիցիայի ենթարկված թույնի կիրառման դեպքում: BPB-ով լրիվ արգելակվում է թույնի PLA2 ֆերմենտի ակտիվությունը: Ստացված արդյունքները ցույց են տալիս, որ թույնի հակաադիեզիվ ազդեցությունը պայմանավորված է ոչ թե թույնի որևէ առանձին բաղադրիչով, այլ մի քանի ակտիվ բաղադրիչների համատեղ ազդեցությամբ:

СПЕЦИФИЧЕСКОЕ ДЕЙСТВИЕ ЯДА ЗМЕИ *MACROVIPERA LEBETINA OBTUSA* НА КУЛЬТИВИРУЕМЫЕ КЛЕТКИ МИОКАРДА

РЕЗЮМЕ

Ключевые слова: *Macrovipera lebetina obtusa*, змеиный яд, кардиомиоцит, сердечный фибробласт, культура *in vitro*, адгезия, токсичность

Исследования яда рода *Macrovipera* показали значительную разницу в способе действия их различных компонентов. Такая дивергенция зависит от типа добычи и конечной цели специфической интоксикации. Первоначальный анализ ядов обнаружил идентичные семейства ферментов и полипептидов среди различных видов змей и общих принципов их деятельности. Однако более глубокий анализ выявил строгую специфичность компонентов яда, уникальных для данного вида и даже подвида змей. Местообитание жертвы и тип животного, используемого в качестве пищи, сильно влияют на состав яда. Конкретный состав яда обеспечивает определенный вид повреждений для быстрой гибели жертвы (Daltry JC et al., 1996). На организменном уровне жертва обездвиживается и дезориентируется, развивается болевой синдром и гипотонический коллапс. Разрушение нескольких органов и систем органов происходит на уровне функциональных систем. На молекулярном уровне, компоненты яда действуют с высокой специфичностью на механизмы клеточной активности добычи. Важно отметить, что основное действие ядов заключается не только в прямом взаимодействии компонента яда с его мишенью, но и в индукции гиперактивности и обобщенной реакции всего организма на яд.

В отличие от растительных ядов, которые главным образом блокируют целевую функцию в организме жертвы, инъецируемый животный яд часто рекрутирует защитные механизмы жертвы для борьбы с самим собой (Wu and Huang, 2003). Многие компоненты яда действуют как би- так и многофункциональные агенты и в зависимости от эффекторного участка могут проявлять на ферментативную активность и/или служить в качестве лиганда для различных рецепторов и систем сигнализации.

Macrovipera lebetina obtusa - одна из самых ядовитых змей в Армении, укус которой вызывает неизбежную болезнь, а иногда и смерть добычи. Сердечно-сосудистая система является одной из целей действия этого яда. В наших исследованиях мы разрабатываем модель для исследования эффектов *MLO*-яда на культивируемые клетки миокарда и клетки HeLa. В данном исследовании мы использовали метод подготовки кардиомиоцитов новорожденных крыс для изучения токсичности этого яда с учетом организации сердечной мышцы.

Наряду с общими ферментами, характерными для видов *Macrovipera lebetina*, яд *MLO* содержит уникальные компоненты, специфичные для подтипов «*obtusa*», такие как дезинтегрин *obustatin*. Яд *MLO* содержит металлопротеиназы, фосфолипазу A2, сериновые протеазы, L-альфа-оксидазы аминокислот, несколько видов дизинтегринов и некоторые другие активные агенты (Sanz L. et al., 2008). Кластер компонентов *MLO*-яда, таких как металлопротеиназы, окустатин, лектины C-типа и некоторые другие, известны как молекулы ингибирования клеточной адгезии, которые разрушают интегрины и кадгеринины или связывают их. Поэтому адгезия, влияющая на свойства яда *MLO* и его действие на связывание клеток в тканевой культуре, была в центре нашего внимания. В частности, мы исследовали влияние активных молекул ядов на соединение клетки с внеклеточными матрицами и внутриклеточными соединениями, а также выживаемость кардиомиоцитов (NRCM) и других клеток - не прямых мишеней *MLO*, таких как сердечные фибробласты (CF)

и модельные эпителиальные клетки, такие как клетки HeLa. В этом исследовании мы продемонстрировали, что сырой яд *MLO* влияет на адгезионные свойства клеток NRCM, CF и HeLa. LD₅₀ яда *MLO* у мышей составляет 18,4 мкг на мышь. На сегодняшний день нет информации о концентрации яда, который достигает сердца после укуса змеи. Мы оценили его в диапазоне 5-10 мкг / мл, исходя из средней массы взрослой мыши (20-25 г) и объема ее крови (около 6-8% от общей массы тела или 1,5-2 мл крови). Эти оценки позволяют предполагать, что концентрации яда, используемые в этом исследовании, могут быть близки к концентрациям встречающимся *in vivo*. Таким образом, в этом исследовании мы систематически исследовали влияние низких концентраций *MLO* яда на вышеупомянутые миокардиальные клетки и клетки HeLa.

Результаты наших исследований показали, что яд *MLO* оказывает значительное отслаивающее действие на все типы тестируемых клеток. Дозы до 1 мкг / мл не являются цитотоксичными, а это означает, что клетки остаются жизнеспособными, хотя они отделены от культурального субстрата. Эти клетки способны к повторному соединению к субстрату и функционированию при удалении яда. Следовательно, в ходе наших исследований мы определили дозу яда *MLO*, которая влияет на адгезионные свойства вышеупомянутых клеток, но не вызывает их гибель. Эта модель предоставляет уникальную возможность изучить молекулярные взаимодействия долгосрочной клеточной культуры и разработать условия для тестирования компонентов яда *MLO* для разработки лекарственных средств. Описанные эффекты зависят от дозы и времени для всех клеток.

Для того чтоб понять механизмы такой активности мы использовали ингибиторы, специфичные для отдельных компонентов яда *MLO*. Среди них был хелатирующий агент EDTA-Na₂, который эффективно нейтрализует металлопротеиназы яда *MLO*. Адгезия тестируемых клеток была слегка улучшена при применении ингибированного EDTA-Na₂ яда к кардиомиоцитам, сердечным фибробластам и клеткам HeLa. Аналогичные результаты были получены с бромфенацилбромидом (BPB) -ингибированным ядом, где активность фермента PLA2 полностью блокировалась. Эти результаты показывают, что ярко выраженная отделяющая активность яда *MLO* не определяется отдельным компонентом яда, а представляет собой комбинаторный эффект нескольких активных ингредиентов.

