THE DEVELOPMENT AND EVALUATION OF EFFECTIVENESS OF ANIMAL DERIVED MONOVALENT ANTIVENOM AGAINST *MACROVIPERA LEBETINA OBTUSA* (MLO) VENOM

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The article describes the processing and development of animal-derived monovalent antivenom against MLO venom according to several protocols, which is a significant problem for public health and the Ministry of Health of Armenia. The developed product is already showing quite high effectiveness and after several experiments, it will be possible to derive the antivenom satisfying international standards which will be much cheaper and accessible compared with Market rivals.

Snake venom – vipers – antivenom – gyurza – Macrovipera lebetina obtusa

Հոդվածում նկարագրված է մի շարք պրոտոկոլների համադրությամբ MLO օձի թույնի դեմ կենդանական ծագման, մոնովալենտ հակաթույնի մշակումը և ստացումը, ինչը բավականին արդիական խնդիր է ներկայիս ՀՀ առողջապահության համար։ Ստացված արգասիքը ցուցաբերում է բավականին բարձր արդյունավետություն և որոշակի կլինիկական փորձարկումներից հետո հնարավոր կլինի ստանալ միջազգային ստանդարտներին համապատասխանող բուժամիջոց, որն ավելի մատչելի և հասանելի կլինի մրցակից պատրաստուկներից։
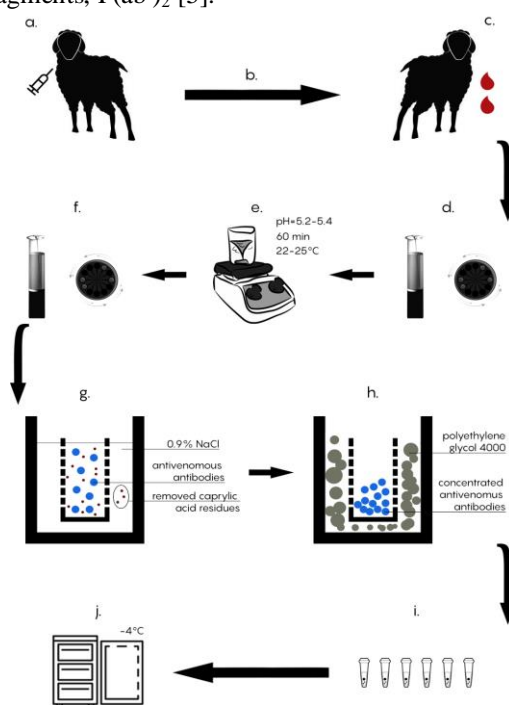
Օձի թույն – իժեր – հակաթույն – գյուրզա – Macrovipera lebetina obtusa

В статье описана разработка и получение моновалентного противоядия животного происхождения против яда змеи MLO с использованием сочетания нескольких протоколов, что является актуальной проблемой для нынешнего здравоохранения РА. Полученный продукт показывает довольно высокую эффективность и уже после определенных клинических испытаний можно будет получить соответствующее международным стандартам лекарственное средство, которое будет более доступным по сравнению с конкурентными препаратами.

Змеиный яд – гадюки – противоядие – гюрза – Macrovipera lebetina obtu

The struggle against snake bites is one of the most important problems imposed on public health [6,11], every year several thousand people die because of snake bites, more than that remain lifelong disabled. World Health Organization classifies snake bites as a neglected tropical disease, that is more prevailing in Asia, Sub-Saharan Africa,

Latin America, and Oceania. The only scientifically proven treatment for snake bites is the injection of specific antitoxins [11]. General classes of snake antivenom production developed around a decade ago with very little innovation, mainly relaled to the introduction of modern technical equipment. Snake antivenoms represent immunoglobulins derived from serum of immunized animals (complete immunoglobulin molecules or Fab fragments, F(ab′)$_2$ [5].

Fig. 1. The stages of specific antivenom development against MLO venom:
a) hyperimmunization; b) 2-week break; c) blood harvesting; d) serum separation;
e) precipitation with caprylic acid; f) supernatant separation; g) supernatant dialysis;
h) dialysate concentration; i) separate into the eppendorf vials; j) storage.

Viperidae snake bites produce notable morbidity and mortality and have a significant impact on health care. These snakes are widespread throughout Eurasia, especially Southeast (the Caucasus including Armenia, Turkey, Iran, etc.).

In Armenia, the majority of snake bites are due to *Macrovipera lebetina obtusa* (MLO), which is a subtype of viper family [2,4,10]. According to the data provided by the Ministry of Health of Armenia (MOHA), during timeframe of January to June of 2019, there were recorded 49 cases of snake bites with one mortal case. Despite this devastating impact on public health, MOHA does not purchase and provide medical institutions in Armenia with antivenoms. For a period of several years, antivenom of quite high quality produced by Institute of Immunology of Zagreb were available in Yerevan [7], unfortunately, the institution stopped production of the antivenom. Currently, antivenom is available only in two medical institutions in Armenia: Muratsan University Hospital and Armenia Medical Center. The available antivenom is imported by a private organization (Armen Farm) from Uzbekistan and according to it's itinerary, it is a polyvalent antivenom against *Vipera lebetina, Echis carinatus* and *Najae oxiana*

snake bites. *Vipera lebetina* snake involves 5 subspecies (*M. l. Cernovi, M. l. lebetina, M. l. obtuse, M. l. transmediterranea, M. l. turanica*) [1], which differs in their venom composition. Taking into account the fact that in Uzbekistan, there is only one subtype of viper (*M. l. turanica*), also frequent dissatisfaction of clinicians with the effect of antitoxin, there is huge concern regarding the quality and effectiveness of currently available antivenom.

Considering these facts, implementation of the production of Armenian antivenom and development of antivenom production guidelines will have a significant impact on public health.

This article represents methodological protocol of antivenom (AV) development against MLO snake, it corresponds to already quite effective protocol of antivenom production against *Vipera ammodytes* [3] and satisfies WHO current instructions of antivenom production (fig.1) [11].

Materials and methods. *The development of animal-derived experimental antivenom against MLO venom (MLOv)*. The animals are chosen according to their availability, the ease and expenses of their care and nutrition and also according to the probability of the antivenom to cause anaphylactic reactions. The animals were medically examined against various infections and diseases. The optimal method of development and production of the animal-derived experimental antivenom implemented on 1.5-2 years-old male sheep (40kg) in accordance with the Council Directive 2010/63/EU of the European Parliament, the Council of 22 September 2010 on the protection of animals used for scientific purposes, and approved by the Committee of Ethics of Yerevan State Medical University (YSMU) (Yerevan, Armenia).

The animal was kept in constant light (07.00-19.00) and temperature (25±2°C) and provided with food and water according to its needs. To minimize stress, the animal was under continuous nursing.

Hyperimmunization

The hyperimmunization of the animal was done with subcutaneous injections of multiple and increasing dosages of venom of *Macrovipera lebetina obtusa* snake. The MLO venom bought from Latoxan (France). Hyperimmunization included two stages. During the first stage, the animal was injected with MLO venom; to decrease the chance of tissue damage with venom and adjuvant, the injections made in two different anatomical regions. The corresponding dosages of venom dissolved into adjuvant, sodium alginate (Medisar LLC). The use of adjuvant increases the antibody formation, due to the fact, that adjuvant makes venom depots and slows venom absorption into the systemic circulation, giving the immune system opportunity to constantly produce antibodies [8].
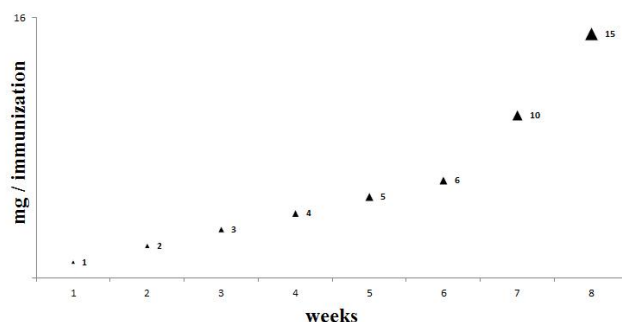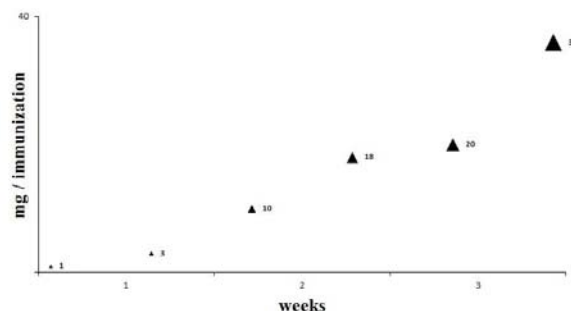

Fig. 2. 1st stage of hyperimmunization of the ovine with MLO venom

The solutions envisaged for hyperimmunization, were repaired immediately before the injection. In the room temperature sodium alginate dissolved into the 0.9% NaCl until reaching the

1.25% (mass/volume) during 15 min. Thereafter MLO venom dissolved into the 1.5 ml of the solution (fig. 2). During the second stage of hyperimmunization, the MLO venom without adjuvant was injected into animal multiple times. The venom dissolved into sodium chloride 0.9% solution and injected according to fig. 3. After the last injection, 2-week break was taken to allow the organism to produce enough quantity of antibodies.

Fig. 3. 2-nd stage of hyperimmunization of the ovine with MLO venom

Thereafter, 1-2 times a week 100-150 ml of blood was collected using aseptic equipment to avoid sample contamination. The collected blood immediately centrifuged for 15 min in 3000 rpm, the blood cells were separated, and the serum kept in the freeze (maximum 2 weeks) until the antibodies separated and purified.

To conserve the adequate titer of antibodies in animal, subsequently, they were immunized according to the second stage of hyperimmunization.

The separation and purification of antibodies using caprylic acid

The serum containing IgG antibodies were subsequently processed by caprylic acid to separate these antibodies. During this stage, every 100 ml serum slowly mixed with 5.27 ml caprylic acid (5 % w/v, analytical grade, ≥99,5 %, Carl Roth) with magnetic stirrer in room temperature (22-25°C). The mixing was continued for one hour and maintaining solution pH in 5.2-5.4 range using 4N NaOH. Subsequently, this solution centrifuged for 15 min in 3000 rpm (BECKMAN, GS-6R centrifuge) and the sediment was separated. To separate this sediment from the remnants of caprilic acid it was dialyzed for 36 hours using 1.35 L 0.9 % NaCl, during this time the NaCl solution changed every 9 hours. Dialysis subsequent yield purified IgG antibodies which were condensed 3 times using polyethylene glycol 4000. The final condensed solution containing IgG antibodies were divided in eppendorf vials and refrigerated (-4°C) until subsequent experiments.

The assessment of effectiveness and cross-reactivity of monovalent antivenom developed against MLO venom

In the scope of evaluating the effectiveness of antivenom, $5LD_{50}$ dosage of MLO venom lethality was neutralized with antivenom [7]. The experiments were undertaken on the white mice (18-20 g) grown in the vivarium of Institute of Physiology after L. Orbeli. Experimental animals were divided into 5 groups of four mice. Mice of control group injected with exclusively venom solution, the rest of groups injected with $5LD_{50}$ dosage of venom pre-incubated with different dilutions of antivenom (Dilution Factor (DF): 8, 4, 2, and without dilution). Each mouse in the control group was injected with $5LD_{50}$ dosage (92 µg/mouse) of venom [7] dissolved in 0.5 ml of 0.9% sodium chloride solution. In case of the rest groups, the same dosage dissolved into 250µl 0.9% sodium chloride solution, thereafter mixed with above-mentioned dilutions of antivenom in 1:1 ratio and incubated for 30 minutes in 37°C. Centrifugation (5 min, 3000 g) and separation of the sediment was undertaken, and each mouse was injected with 0.5 ml of this solution. During a 24-hour period after injections mice were observed to record the lethal cases.

The Spearman–Karber method [12] used to calculate the median effective dose (ED_{50}), which constitute the amount of undiluted antiserum (in mL) efficient for neutralizing the lethality of used venom dose in 50 % of animals.

Using ED_{50} value, Protective efficacy (R) was calculated and represent the amount of LD_{50} possible to be neutralized with 1 ml of undiluted antivenom.

Immunodiffusion Assay

Double diffusion test according to Ouchterlony and Nilsson [9] used to study antigenic relationships between the different antigens. Holes 5 mm in diameter were blown in horizontal gels containing 1.2% agarose in $1\times$ PBS. Protein fractions of several types of viper venom and cobra venom (20 μl) were placed in the peripheral wells and AV in the central well. Diffusion was let to proceed for 24 h at 37 °C. The gel was then cleaned with saline and dried. The precipitin line was visualized with Coomassie Brilliant Blue.

Results and Discussion. As already mentioned, we have evaluated the effectiveness of our developed antivenom to neutralize lethal dosage of MLO venom, the data is shown in fig. 4. The mice in control group were dead immediately after injection of $5LD_{50}$ dosage of venom, whereas in the groups were antivenom was used, the percentage of mortality decreased opposite to the dilution of the antivenom, particularly the antivenom diluted 2 times displayed complete neutralization of venom lethal toxicity. The ED_{50} of this antivenom compiles **0.05257** ml/mouse or **2.6285** ml/kg. This data allowed calculating the antivenom Protective efficacy (R), which equals to **76**.

	Injection volume (ml/mouse)	Antivenom volume (ml/mouse)	Number of mice			Percentage of deaths (%)
			Died	Lived	Total	
MLOv+AV	0.5	0.25	0	4	4	0
+AV (DF=2)	0.5	0.125	0	4	4	0
+AV (DF=4)	0.5	0.625	2	2	4	50
+AV (DF=8)	0.5	0.03125	3	1	4	75
MLOv	0.5	-	4	0	4	100

The ED_{50} was calculated according to Spearman and Karber [13]:

$$ED_{50} = logED_{50} = logX_{100} - \frac{logDF}{n} - (\sum t{-}n/2)$$

$$= -1.2925$$

$$ED_{50} = 10^{-1.27925} = \boxed{\begin{array}{l} 0.05257 \text{ ml/mouse*} \\ 2.6285 \text{ ml/kg*} \end{array}}$$

ED_{50} = the 50% effective dose. $logX_{100}$ = log dose giving 100% survival and having 100% survival for all higher doses. log DF = the log dilution factor (the log dose interval is constant). n = # mice used at each dose level. t = #mice alive at each dose level. Σ = the sum of mice surviving at every dose level.

*The ED_{50} is the effective volume of AV that will protect 50% of the mouse population when injected with $5LD_{50}$s.
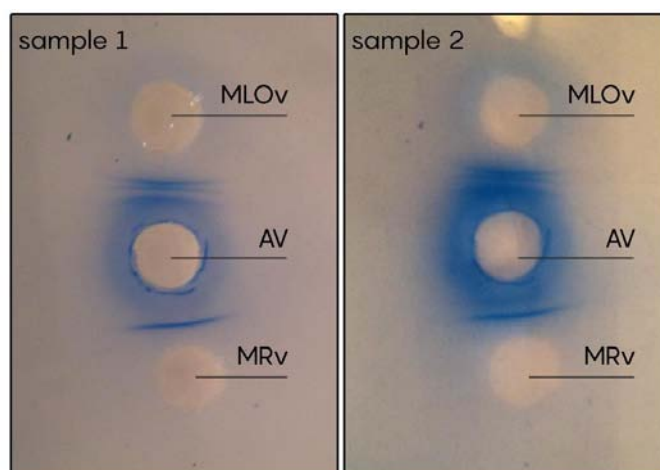
$$R = (T_V{-}1)/ED_{50}$$
$$= (5{-}1)/0.05247$$
$$= \textbf{76}$$

T_V- is the amount of LD_{50} injected one mouse

Fig. 4. Effective dose fifty (ED_{50} = ml/mouse(ml/kg)/5LD50) assay of AV

The results of experiments of AV cross-reactivity are shown in fig. 5. It is evident that when anti-MLO IgG experimented against crude MLO venom, a wide pattern shown in fig. 5 (top well) was obtained pointing coalescence of antigens. Whereas the same AV was tested against the venom of *Montivipera raddei* one precipitin line was formed.

Pic. 5. Agar gel diffusion (AGD) test using autoclaved extraction antigens and different snake venoms. AV: antiserum against MLO, MLOv: *Macrovipera lebetina obtusa* venom, MRv: *Montivipera raddei* venom.

Obtained results indicated that the antivenom against development MLO venom described in this article display quite high effectiveness and target the components of viper venom that complie the toxic potential of venom. Yet these results are still not enough for the final assessment of the effectiveness of the product and some several set of pre-clinical investigations are need to be implemented before the clinical trial, we could insist already that the production of antivenom in Armenia is necessary, especially taking into consideration that it is not only cheaper and accessible, than its' imported vertions, but also have the specificity and quality corresponding to international standards.

Acknowledgments

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