Cytotoxic Activities of the Crude Venoms of *Macrovipera lebetina lebetina* from Cyprus and *M. l. obtusa* from Turkey (Serpentes: Viperidae) on Human Umbilical Vein Endothelial Cells

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Abstract: In this study, we used human umbilical vein endothelial cells (HUVEC) as an in vitro model to compare the cytotoxic activities of the venoms of two *Macrovipera lebetina* subspecies, *M. l. obtusa* from southern Anatolia and *M. l. lebetina* from northern Cyprus. Well-established 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was preferred to assess the cytotoxicity. Our results showed that venom reduced cell viability both in a time and dose-dependent manner. The cytotoxic effect of *M. l. lebetina* venom on HUVEC is reported for the first time in the present study.

Keywords: hemorraghe, HUVEC, levantine viper, toxinology.

Kıbrıs’tan *Macrovipera lebetina lebetina* ve Türkiye’den *M. l. obtusa* (Serpentes: Viperidae) Ham Zehirlerinin İnsan Kordon Veni Endotel Hücreleri Üzerindeki Sitotoksik Etkileri

Öz: Bu çalışmada, Kuzeý Kıbrıs’tan *Macrovipera lebetina lebetina* ve güneydoğu Anadolu’dan *M. l. obtusa* örneklerine sahip iki *M. lebetina* alt türüne zehirlerinin (venomlarının) sitotoksik aktivitelerini karşılaştırmak için in vitro bir model olarak insan kordon veni endotel hücreleri (HUVEC) kullanıdık. Sitotoksik etkisinin değerlendirilmesinde iyi bilinen bir yöntem olan 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromür (MTT) testi tercih edilmiştir. Çalışmanın sonuçları her iki zehrin de hücre canlanması zamanına ve doza bağlı olarak azalttığı görülmüştür. *M. l. lebetina* zehrinin HUVEC üzerindeki sitotoksik etkisi ilk kez bu çalışmada rapor edilmiştir.

Anahtar kelimeler: hemoraji, HUVEC, koca engerek, toksinoloji.

1. Introduction

Snake venom is a molecular cocktail stored in venom glands and consists mainly of proteins and peptides that are secreted from the specialized cells of the venom gland. Enzymes such as serine proteinase, metalloproteinase, phospholipase A2 (PLA2), L-aminoo acid oxidase (LAO), hyaluronidase, acetycholinesterase, nucleotidase and other proteins/peptides such as disintegrin, C-type lectin (CLP), neurotoxins, cysteine-rich secretory protein (CRISP), bradykinin potentiating peptide (BPP), vascular endothelial growth factor (VEGF) and nerve growth factor (NGF) are prominent molecules found in snake venoms. Snake venoms have various biological activities including cytotoxic/anticancer, antimicrobial, anticoagulant, procoagulant, antiplatelet activities and neurotoxic effects (Chipaux, 2006; Mackessy, 2010; İçi & Demiralp, 2012).

*Macrovipera lebetina* (Linnaeus, 1758), blunt-nosed viper is the largest venomous viper species in Turkey and Cyprus. Its distribution ranges from northern Africa to Pakistan and from the Gulf of Oman to the Caspian Sea and Dagestan (Russia) with different subspecies. According to the latest accepted systematics, *M. l. obtusa* (Dwiguwski, 1832) subspecies has a distribution in Anatolia whereas nominate taxon, *M. l. lebetina* (Linnaeus, 1758) occurs in Cyprus (Mallow, Ludwig, & Nilson, 2003). This species is distributed in southern, southeastern, eastern, and northeastern Anatolia in Turkey, (Mallow et al., 2003; Budak & Göçmen, 2008; Sarıkaya, Yildiz, & Sezen, 2017).

Venomous snakebite is a neglected but important public health problem (Williams et al., 2010). Viperid venoms have proteins that interfere with the coagulation cascade and generally cause tissue damage resulting in hemorrhage (Chipaux, 2006). *M. lebetina* is one of the medicinally important vipers (Stümpel & Joger, 2009) in Anatolia and its venom affects the human hemostatic system, causes bleeding, edema, and necrosis (Göçmen, Arkan, Özbek, Mermer, & Çiçek, 2006). Understanding the pathology of venomous snakebites will help develop more effective treatments. Vessel endothelial cells are one of the main targets of snake venoms (Baldino, Janora, Yamanouye, Zorn, & Moura-da-Silva, 2010). In this study, we aimed to assess the cytotoxic activity of the crude venom of *M. l. lebetina* from Cyprus against human umbilical vein endothelial cells (HUVEC) as an in vitro model in comparison to *M. l. obtusa* venom from southeast Turkey.

2. Materials and Methods

2.1. Snake Venoms

Crude venom was extracted by letting vipers bite paraffin-covered laboratory beaker from two adult *M. l. obtusa*...
(both male from Şanlıurfa and Diyarbakır provinces, southeastern Turkey) and two adult M. l. lebetina (one male, one female) from Turkish Republic of Northern Cyprus. Vipers were collected during the field trips between 2004 and 2009 and fed in terrariums. M. l. obtusa individuals were collected in April and May whereas M. l. lebetina individuals were collected in April and July. After extraction, venom samples were centrifuged at 2,000 × g for 10 min at 4°C, supernatants were immediately frozen and lyophilized using a benchtop freeze-dryer (Millrock Technology).

2.2. Determination of the protein concentration

Protein concentrations of the reconstituted venom samples (3.8 mg/ml for each subspecies) were determined by the Bradford’s Coomassie blue-based method using 96-well microtiter plate. Bovine serum albumin (BSA) was used as calibration standard and all standards and samples were measured at 595 nm wavelength using a multi-plate reader spectrophotometer (SpectraMax, Molecular Devices). All the samples and standards were measured in triplicate and mean values were used.

![Figure 1. The cytotoxic effect of M. l. lebetina and M. l. obtusa crude venoms on HUVEC at different times.](image)

2.3. Cell Culture Conditions

Human umbilical vein endothelial cells (HUVEC) (provided by Dr. Erkan YILMAZ, Ankara University Biotechnology Institute) were grown in M-199 medium (Lonza) supplemented with 10% fetal calf serum (HyClone), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma) and 2 mM L-glutamine at 37°C in a humidified incubator with 5% CO₂. After initial culturing, 2 × 10⁴ cells were seeded in 96-well cell culture plates in 100 µl of growth medium and incubated for 24 h to adhere. Lyophilized venom samples were reconstituted in deionized water and diluted using medium. 100 µl of venom samples were added to wells at a final concentration between 3-24 µg crude venom/ml and incubated for 3, 16 and 24 h. Only deionized water was added to negative control wells.

2.4. Cytotoxicity Assay

Assessment of the cytotoxicity was done using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, which measures mitochondrial reductase activity (Mosmann, 1983). For this purpose, MTT dye (Sigma) was reconstituted at 5 mg/ml concentration and added to wells. The plates were incubated in the cell culture incubator for 3 h and 1 N HCl-20% isopropanol solution was added to dissolve formazan crystals. Measurement was done using a spectrophotometer at 570 nm wavelength. Six replicate wells were used for negative control and each venom concentrations and mean values were used for calculations. The viability percentages of the cells were calculated according to the following formula:

\[
\text{% Viable cells} = \left(\frac{\text{The absorbance of the treated cells} - \text{the absorbance of the blank}}{\text{The absorbance of the control} - \text{the absorbance of the blank}}\right) \times 100
\]

Inhibition of the growth by 50% (IC₅₀) was calculated using % viability values by plotting the data as sigmoidal curve and using a four parameter logistic model. The viability of negative control wells were set to 100%.

3. Results

Protein concentrations of stock venom solutions were found similar, which were 2.5 µg/µl for M. l. lebetina and 2.4 µg/µl for M. l. obtusa. Dose and time-dependent cytotoxic activity was observed for both venom samples with similar reduction rates. The highest venom dose (24 µg/ml) of M. l. lebetina and M. l. obtusa was reduced the cell viability by 49.0% and 45.7%, respectively after 3 h
treatment (Fig. 1). The results of 16 and 24 h treatments were similar but the % inhibition values were much higher than 3 h treatment. The highest venom dose (24 µg/ml) of M. l. lebetina and M. l. obtusa was reduced the cell viability by 65.0% and 68.3%, respectively after 16 h; whereas this value is 72.7% for both venoms after 24 h treatment (Fig. 1). The IC50 values for 24 h were calculated as 7.32 and 6.28 µg/ml for M. l. obtusa and M. l. lebetina venoms, respectively (Table 1). The IC50 value of M. l. lebetina venom at 24 h was slightly decreased compared to 16 h, while the value of M. l. obtusa venom did not change significantly between 16 and 24 h.

Table 1. Calculated IC50 values of M. l. lebetina and M. l. obtusa crude venoms for their cytotoxic activities on HUVEC.

| Taxa         | 16 hours | 24 hours |
|--------------|----------|----------|
| M. l. obtusa | 7.30     | 7.32     |
| M. l. lebetina| 7.19     | 6.28     |

4. Discussion

Snake venoms contain various proteins interacting with vessel endothelium and as; a result of this interaction, persistent bleeding can be seen after a viper bite (Baldo et al. 2010). Edema, hemorrhage, and necrosis are the typical symptoms of a venomous bite of M. lebetina in humans (Göçmen et al., 2006). M. lebetina venom causes intracellular hemorrhage, mononuclear cell infiltration, and cellular degeneration in liver, kidney, and heart tissues of mice (Yücel, Ağan, & Hayretdağ, 2019). Histological investigations on mouse capillary blood vessels after injection of Bothrops asper venom also showed that Viperid venoms cause endothelial cell degeneration leading gaps in capillaries and hemorrhage (Moreira, Gutiérrez, Borkow, & Ovadia, 1992). The results of the present study showed that the venoms of two subspecies of M. lebetina have similar time and dose-dependent cytotoxic effect against HUVEC. The aforementioned activity of M. l. lebetina (subspecies of M. lebetina occurring in Cyprus) was reported for the first time with this study. Islands are isolated ecosystems and geographical variation can lead significant difference in the activity and toxicity of the venoms (Glenn, Straight, Wolfe, & Hardy, 1983). Therefore, it is important to compare the activities of venoms from different geographical origins. Although some vipersid venoms do not possess strong cytotoxic activity against endothelial cells (Borkow, Lomonte, Gutiérrez, & Ovadia, 1994), our results showed that M. l. lebetina and M. l. obtusa venom could damage vessel endothelium in bite cases, corroborating previous studies (Borkow et al., 1994; Kakanj, Ghazi-Khansari, Mirakabadi, Daraei, & Vatanpour, 2015).

Macrovipera lebetina venom contains many bioactive proteins and peptides that interfere with blood coagulation cascade and cause tissue damage. Main protein/peptide families identified in the venoms of different subspecies of M. lebetina are as follows: metalloproteinase, serine proteinase, PLAS, LAAO, hyaluronidase, nucleotidase, disintegrin, CLP, CRISP, VEGF, NGF and BPP. Most abundant proteins in M. lebetina venom are serine and metalloproteinases and PLAS (Sanz, Ayvazyan, & Calvete, 2008; İlçi & Demiralp, 2012; Siigur, Aasplöiu, & Siigur, 2019). The observed cytotoxicity in the present study is a result of the combined activities of these proteins.

In addition to their role on the pathology of a venomous snakebite, bioactive proteins of M. lebetina venom interfere with various molecular pathways. Their specific activities make these proteins interesting tools which may have therapeutic and diagnostic potential. Metalloproteinases purified from M. lebetina venom have fibrinogenolytic and factor X activating properties (Siigur et al., 2019). Additionally, a heterodimeric metalloproteinase purified from the venom of M. lebetina is known to induce apoptosis in HUVEC (Trummal et al., 2005). This protein inhibits the endothelial cell adhesion to extracellular matrix proteins such as fibrinogen, fibronectin, vitronectin, and collagen I and IV. Snake venom metalloproteinases also cause local tissue damage and hemorrhage (Gutiérrez & Rucavado, 2000). Serine proteinases purified from the venom of M. lebetina show fibrinogenolytic, factor V activating and bradykinin-releasing activities. PLASs in snake venoms can possess neurotoxic, myotoxic, hemotoxic, anticoagulant, antiplatelet, and antibacterial activities. Disintegrins are another important protein family which is found especially in Viperid venoms. They are antagonists of various integrin receptors. Both monomeric and dimeric disintegrins have been purified and identified in M. lebetina venom (Siigur et al., 2019). Due to their antiplatelet and anticancer activities, disintegrins found in snake venoms have therapeutic value (Calderon et al., 2014). Additionally, metalloproteinases, LAAOs, PLASs and CLPs in snake venoms show anti-cancer activities by interacting with various pathways (e.g. apoptosis induction) (Calderon et al., 2014).

Venom proteins of M. l. lebetina and M. l. obtusa were compared previously by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and significant differences were found. Protein spots of M. l. obtusa venom were identified by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry and the most prominent differences were seen in PLAS region (İlgı & Demiralp, 2012). However, we did not observe remarkable difference between two subspecies regarding the cytotoxic activities on HUVEC cells. The main protein family responsible for the hemorrhage, endothelium, and local tissue damage in viper venoms is metalloproteinases (Gutiérrez & Rucavado, 2000; Baldo et al. 2010; Panfoli, Calzia, Ravera, & Morelli, 2010). Therefore, further studies should be performed especially focusing on the proteinases of M. l. lebetina and M. l. obtusa venoms.

Crude venom of M. l. obtusa showed dose-dependent cytotoxicity against some cancer cell lines, kidney epithelial cells from African green monkey (Vero), and human embryonic kidney 293 cells (HEK-293) in previous studies (Samel, Trummal, Siigur, & Siigur, 2012; Ozen, İlçi, Yağcı, Göçmen, & Nalbantsoy, 2015; Jahromi, Mirakabadi, & Kamalzadeh, 2016; Süzergöz et al. 2016; Oghalaie, Kazemi, Yalçin, Goçmen, & Nalbantsoy, 2015). Therefore, further studies should be performed especially focusing on the proteinases of M. l. lebetina and M. l. obtusa venoms.
lower IC50 values (6.28 and 7.32 μg/ml) in the present study. Such intra-specific variation in snake venom composition and activity can be seen depending on the geographical origin of the samples (Glen et al., 1983; Alape et al. 2008). Our study showed that endothelial cell disruption can contribute to the pathology of venomous bites caused by both Anatolian and Cypriot M. lebetina.

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References

Alape, J.M., Ghazi Jahromi, H.E., Mirakabi, S., Gutiérrez, J.M., & Rucavado, A. (2000). Snake venom metalloproteinases: Tissue distribution and in situ hydrolysis. PLoS Neglected Tropical Diseases, 4(e6), e727. https://doi.org/10.1371/journal.pntd.0000277

Borkow, G., Lomonte, B., Gutiérrez, J.M., & Ovadia, M. (1994). Effect of various Viperidae and Crotalidae Snake Venoms on Endothelial Cells In Vitro. Toxicol, 32, 12, 1689-1695. https://doi.org/10.1016/0300-9783(94)90370-1

Budak, A., & Göçmen, B. (2008). Herpetoljoji. İzmir, Türkiye, Ege Üniversitesi Yayını, Feni Fakültesi Yayıncısı No: 194, 226 pp.

Calderon, L.A., Sobrino, J.C., Zaque, K.D., & de Moura, A.A., Grabner, A.N., Mazzi, M.V., Marcussi, S., ... Soares, A.M. (2014). Antitumoral activity of snake venom proteins: New trends in cancer therapy. BioMed Research International, 2014, 201639, 1-19. http://dx.doi.org/10.1155/2014/201639

Chippaux J-P. (2006). Snake Venoms and Envenomations. Florida, USA, Krieger Publishing Company, 287 pp.

Glenn, J.L., Straitj, R.C., Wolfe, M.C., & Hardy, D.L. (1983). Geographical variation in Crotalus scutulatus scutulatus (Mojave rattlesnake) venom properties. Toxinicon, 21(1), 119-130. https://doi.org/10.1016/0041-0143(83)90055-7

Göçmen, B., Arıkan, H., & Yildiz, M.Z. (2012). Determination of in vivo toxicity and in vitro cytotoxicity of venom from the Cypriot Blunt-nosed viper Macrovipera lebetina lebetina and antivenom production. The Journal of Venomous Animals and Toxins including Tropical Diseases, 18(2), 208-216. http://dx.doi.org/10.1016/j.jvt.2013.02.001

Göçmen, B., Arıkan, H., Özbel, Y., Mermer, A., & Çiçek, K. (2006). Clinical, physiological and serological Observations of a Human Following a Vipera lebetina obtusa (Ophidia: Viperidae) crude venom. Frontiers in Life Science, 8(4), 363-370. https://doi.org/10.1080/21553769.2015.1053862

Panfili, I., Calzia, D., Raveza, S., & Morelli, A. (2010). Inhibition of Hemorrhagic Snake Venom Components: Old and New Approaches. Toxins, 2, 417-427. https://doi.org/10.3390/toxins20400417

Samel, M., Trummal, K., Siigur, E., & Siigur, J. (2012). Effect of HUVEC apoptosis inducing proteinase from Vipera lebetina venom (VLAIP) on viability of cancer cells and on platelet aggregation. Toxicon, 60, 648-655. https://doi.org/10.1016/j.toxicon.2012.03.033

Sanz, L., Ayvazyan, N., & Calvete, J.J. (2008). Snake venoms of the Armenian mountain vipers Macrovipera lebetina lebetina and Vipera radaei. Journal of Proteomics, 71, 198-209. https://doi.org/10.1016/j.jprot.2008.05.003

Söndergar, B., Yildiz, M.Z., & Sezen, G. (2017). The herpetofauna of Adana province (Turkey). Commagene Journal of Biology, 1(1), 1-11. https://doi.org/10.31954/commagene.391784

Siigur, J., Aaspöll, A., & Siigur E. (2019). Biochemistry and pharmacology of proteins and peptides purified from the venoms of the snakes Macrovipera lebetina subspecies. Toxicon, 158, 16-32. https://doi.org/10.1016/j.toxicon.2018.11.294

Stumpel, N., & Joger, U. (2009). Recent advances in phylogenoy and taxonomy of near and Middle Eastern Vipers—an update. ZooKeys, 39, 179-191. https://doi.org/10.3897/zookeys.31.261

Süzeroglu, F., Içgi, N., Cavus, C., Yildiz, M.Z., Coskun, M.B., & Göçmen, B. (2016). In vitro cytotoxic and proapoptotic activities of Anatolian Macrovipera lebetina obtusa (Terciopelo). Experimental and Molecular Medicine, 48, 1-11. https://doi.org/10.1016/s0140-6736(16)30733-5

Williams, D., Gütscher, M.J., Harrison, R., Warrell, D.A., White, J., Winkel, K.D., & Gopalakrishnakone, P. (2010). The Global Snakebite Initiative: an antidote for snake bite. Lancet, 375, 89-91. https://doi.org/10.1016/s0140-6736(09)61534-4