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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: hemorrhage, 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, Kuzey Kıbrıs'tan *Macrovipera lebetina lebetina* ve güneydoğu Anadolu'dan *M. l. obtusa* olmak üzere iki *M. lebetina* alt türünün zehirlerinin (venomlarının) sitotoksik aktivitelerini karşılaştırmak için in vitro bir model olarak insan kordon veni endotel hücreleri (HUVEC) kullandık. Sitotoksik etkinin değerlendirilmesinde iyi bilinen bir yöntem olan 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromür (MTT) testi tercih edilmiştir. Çalışmamızın sonuçları her iki zehrin de hücre canlılığını zamana ve doza bağlı olarak azalttığını göstermiştir. *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 A_2 (PLA_2), L-amino acid oxidase (LAAO), hyaluronidase, acetylcholinesterase, 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 (Chippaux, 2006; Mackessy, 2010; Igci & 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* (Dwigubski, 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, Yıldız, & 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 (Chippaux, 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, Arıkan, Özbel, 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 (Baldo, 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*

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(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.

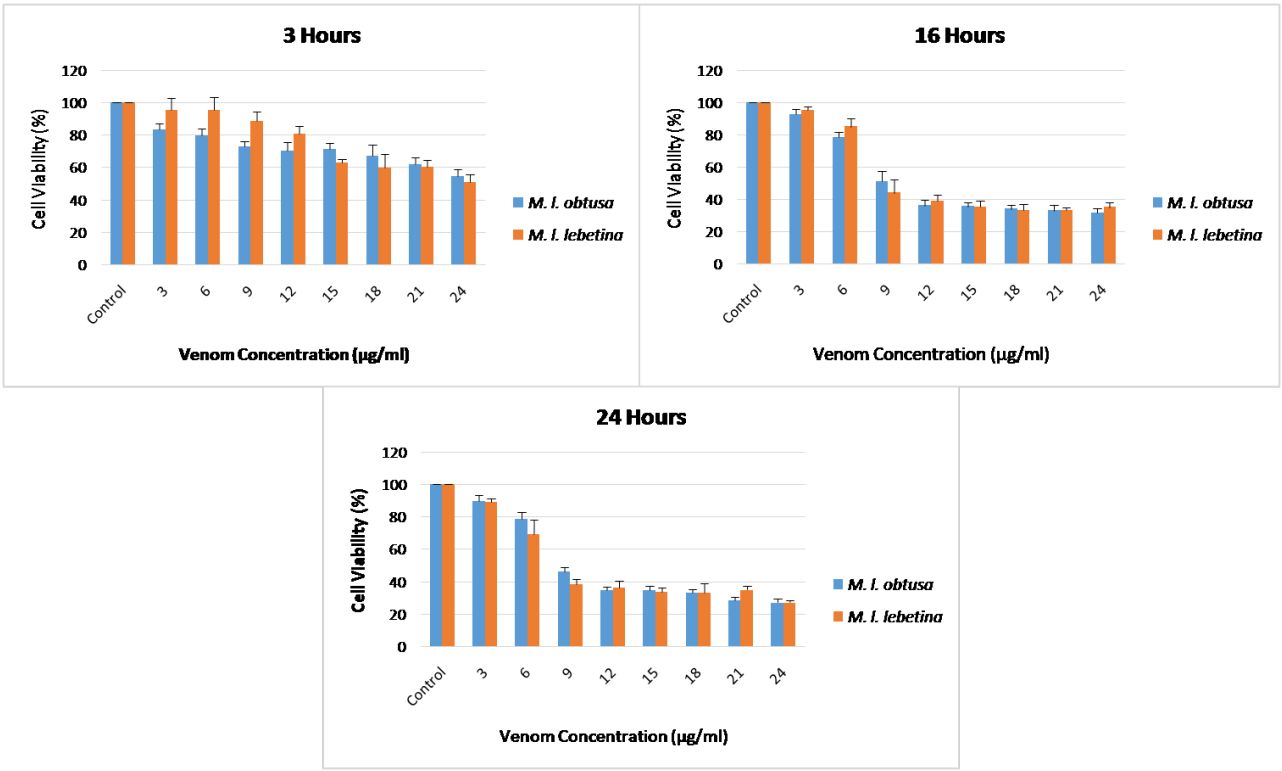

Figure 1. The cytotoxic effect of *M. l. lebetina* and *M. l. obtusa* crude venoms on HUVEC at different times.

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 x 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:

% Viable cells = [(The absorbance of the treated cells) – (the absorbance of the blank)] / [(The absorbance of the control) – (the absorbance of the blank)] × 100

Inhibition of the growth by 50% (IC_{50}) 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 IC_{50} 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 IC_{50} 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 IC_{50} values of *M. l. lebetina* and *M. l. obtusa* crude venoms for their cytotoxic activities on HUVEC.

	Hours	
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 viperid 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, PLA_2, LAAO, hyaluronidase, nucleotidase, disintegrin, CLP, CRISP, VEGF, NGF and BPP. Most abundant proteins in *M. lebetina* venom are serine and metalloproteinases and PLA_2s (Sanz, Ayvazyan, & Calvete, 2008; İğci & Demiralp, 2012; Siigur, Aaspõllu, & 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 fibrin(ogen)olytic 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 fibrin(ogen)olytic, factor V activating and bradykinin-releasing activities. PLA_2s 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, PLA_2s 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 PLA_2 region (İğci & 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 embriyonic kidney 293 cells (HEK-293) in previous studies (Samel, Trummal, Siigur, & Siigur, 2012; Ozen, İğci, Yalçin, Goçmen, & Nalbantsoy, 2015; Jahromi, Mirakabadi, & Kamalzadeh, 2016; Süzergöz et al. 2016; Oghalaie, Kazemi-Lomedasht, Zareinejad, & Shahbazzadeh, 2017). Moreover, dose-dependent cytotoxic activity of *M. l. lebetina* venom against L929 mouse fibroblast cell line was also reported previously (Nalbantsoy et al., 2012). Kakanj et al. (2015) studied the cytotoxic effect of Iranian *M. lebetina* on HUVEC cells and determined the IC_{50} value as 11.77 µg/ml after 24 h incubation using MTT assay. Venoms of Anatolian and Cypriot *M. lebetina* were found to be more potent with

lower IC_{50} 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 (Glenn et al., 1983; Alape-Girón 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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