

## Determination of *in vivo* toxicity and *in vitro* cytotoxicity of venom from the Cypriot blunt-nosed viper *Macrovipera lebetina lebetina* and antivenom production

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**Abstract:** The venomous Levantine viper, *Macrovipera lebetina lebetina* is endemic to Cyprus. The objective of this study was to investigate *in vitro* cytotoxicity, *in vivo* lethality, and antivenom production followed by a re-immunization schedule in mice against *Macrovipera lebetina lebetina* venom. The LD<sub>50</sub> value was estimated as 7.58mg/kg within 24 hours by different venom doses administered intraperitoneally in mice. Freund's complete and incomplete adjuvants were used for first and second immunization of mice in antivenom production. A cell-based assay was performed to determine the effects of *Macrovipera lebetina lebetina* venom and antivenom neutralizing potency on L929 cell viability. The snake venom toxicity and cytotoxicity were examined and comparison of results showed good correlation, the LD<sub>50</sub> value was ten-fold higher than the IC<sub>50</sub> value. The IC<sub>50</sub> value was  $0.62 \pm 0.18 \mu\text{g/mL}$  after 48 hours treatment while the calculated value was  $1.62 \pm 0.25 \mu\text{g/mL}$  for the culture media totally refreshed after two hours treatment with venom. The *in vitro* efficacy of antivenom against *Macrovipera lebetina lebetina* venom was found to be low. This is the first report that describes the *in vivo* and *in vitro* toxic effects of *Macrovipera lebetina lebetina* venom and antivenom production against this species.

**Key words:** *Macrovipera lebetina lebetina*, LD<sub>50</sub>, lethality, snake venom, antivenom.

## INTRODUCTION

Snake envenomation is a major public health issue that provokes thousands of deaths throughout the globe. There are nearly 3,000 different species of snakes found in the world of which approximately 300 are venomous (1-3). The Levantine viper, *Macrovipera lebetina*, is a venomous viper species found, in Cyprus, the Middle East, northern Africa, Cyclades, Turkey and central Asia (4-8). The subspecies *lebetina* seems to be endemic to Cyprus. It is the only snake in Cyprus which can be dangerous to humans (9). As reported by Cesaretli and Ozkan (10), the National Poison Information Center recorded 550 snakebite cases between 1995 and

2004, most of them associated with members of the Viperidae family in Turkey.

Snake antivenom, a type serum, is currently the only effective product for treating the consequences of snakebites, a serious public health problem in many tropical and subtropical countries (3, 11-14). Currently, commercial antivenom is available to neutralize the effects of snake envenomation (3, 15). It can be classified as species specific (monovalent) or effective against several species (polyvalent). Monovalent antivenom is ideal, but cost, lack of availability, and difficulty in accurately identifying offending species makes its use less common (16, 17).

A number of *in vitro* approaches have employed cell-based assays to determine the

effects of test compounds on cell viability. Despite the compositional complexities of snake venoms, an assay of this type could be an interesting alternative in toxicity assessment (18, 19). From an ethical perspective paying special attention to increasing public concern for animal welfare, the investigators of the present study have considered and searched for alternative non animal-based toxicity assays (20).

In this work, *Macrovipera lebetina lebetina* (Linnaeus) venom collected in Cyprus was used to produce antivenom and perform acute toxicity studies in mice. As an alternative *in vitro* method, the neutralizing potency of antivenom and venom-induced cytotoxicity were investigated using cultured mammalian cells.

## MATERIALS AND METHODS

### Venom and Experimental Animals

The experimental protocol was approved by Ege University Local Ethics Committee for Animal Experimentation (process number 2005/35). All tests were performed with pooled venom from *Macrovipera lebetina lebetina*, a venomous snake found in coastal areas of Cyprus. The sexually mature *Macrovipera lebetina lebetina* (Figure 1) specimens used in this study were collected from the Dikmen-Kyrenia region of Cyprus (by B. Gocmen). The specimens were taken alive to the Reptile Biology and Ecology Research Laboratory (Zoology Section, Department of Biology, Ege University) and kept in the terrarium; venom was extracted without applying any pressure to their



**Figure 1.** The Cypriot blunt-nosed viper, *Macrovipera lebetina lebetina*.

venom glands, as described by Tare *et al.* (21). As the venom extracts contained some dead cells, pooled venom was diluted in physiological saline, centrifuged for five minutes at 600 g, and stored at  $-20^{\circ}\text{C}$ .

Male Swiss albino mice (7 to 8 weeks old) weighing 28 to 32 g were purchased from Ege University Experimental Animal Research Center. Mice were maintained in groups of five under standard temperature conditions ( $22 \pm 1^{\circ}\text{C}$ ) with a regular 12-hour light/12-hour dark cycle and were allowed free access to standard laboratory food and water.

### Protein Content Determination

Protein content was assayed in triplicate for each diluted venom sample in saline, using the Bradford method (22) at 595 nm with bovine serum albumin as the standard (Molecular Devices, USA).

### Determination of LD<sub>50</sub>

Swiss albino mice weighing 28 to 32 g were used ( $n = 10$  for each group). The venom dose required to kill 50% of animals within 24 hour (LD<sub>50</sub>) was determined by Probit test using intraperitoneal (IP) administration of physiological saline (control group) and different venom doses.

### Immunogen Preparation

Venom at the above determined LD<sub>50</sub> venom concentration was further diluted five times in physiological saline. Immunogens were prepared by passing diluted venom preparation through a membrane filter (0.22  $\mu\text{m}$  pore size). Filtrate was then homogenized with complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) at a 1:1 (v/v) ratio. All operations were carried out under sterile conditions.

### Immunization

Male Swiss albino (7 to 8 week old) mice were immunized by subcutaneous (SC) injection with CFA (Sigma, USA) venom emulsion and boosted on day 7 with IFA (Sigma, USA) venom emulsion using a similar route. Mice were additionally IP boosted with the same diluted venom without adjuvant on days 14, 21, and 29. Homologous antibody responses were tested on day 31 by ELISA. Finally sera were recovered following terminal heart puncture on day 32.

## Enzyme-Linked Immunosorbent Assay (ELISA)

Indirect ELISA was used to evaluate humoral immune response of hyperimmunized Swiss albino mice. Briefly, 96-well polystyrene plates (Nunc, Denmark) were coated with venom (0.1 µg per well diluted in 100 µL 0.05 M carbonate/bicarbonate buffer, pH 9.6). After overnight incubation at 37°C, plates were washed three times. Mice sera for testing (100 µL/well) were added in appropriate dilutions (1:100, 1:500, and 1:1000) to venom coated wells, and the plates incubated at 37°C for one hour. After washing, 100 µL/well of secondary anti-mouse IgG peroxidase (Sigma, USA) conjugate was added and incubated for one hour at 37°C. The enzyme reaction was developed with the addition of H<sub>2</sub>O<sub>2</sub> (Merck, Germany) and O-phenylenediamine (Sigma, USA) and stopped after 30 minutes of incubation by the addition of 4 M H<sub>2</sub>SO<sub>4</sub> (Riedel-de Haen, Germany) per well. Optical absorbance was measured at 492 nm. Results are expressed as optical density (OD) (mean ± SEM). Background control values were subtracted from the absorbance readings.

## Cell Culture and Maintenance

Mouse fibroblastic (L929) cell-lines were purchased from HUKUK (Animal Cell Culture Collections) of the Foot-and-Mouth Disease Institute, Ministry of Agriculture & Rural Affairs, Ankara, Turkey. The cell lines were maintained in RPMI-1640 medium (Gibco, UK) supplemented with 4% heat-inactivated fetal bovine serum, 1% L-glutamine (Biochrome, Germany), and 1% gentamycine (Biochrome, Germany) in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. The cells were subcultured twice a week.

## In Vitro Cytotoxicity Assay

Determination of *in vitro* venom cytotoxicity was based on a procedure used for general screening of cytotoxic agents. Based on metabolic cell viability, this was performed using a modified MTT [3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide] assay which effects the mitochondrial reductase activity of viable cells (23). After treatment with venom, the survival of viable cells in monolayer culture was determined. Cell line L929 was cultivated for 24 hours in 96-well microplates with 8 x 10<sup>4</sup> cells/mL initial concentration. Cultured cells were then treated with different amounts of the

venom and incubated for 48 hours at 37°C. The same experiment was performed after two hours of incubation with different concentrations of venom and then the culture medium was refreshed and incubated for 48 hours. Growth inhibition was compared with untreated controls to find the venom concentration which inhibited growth by 50% (IC<sub>50</sub>).

The assay is based on the cleavage of MTT, a yellow tetrazolium salt, which forms water-insoluble dark blue formazan crystals. This cleavage only takes place in living cells by the mitochondrial enzyme succinate-dehydrogenase. The water-insoluble dark blue formazan crystals are solubilized with dimethyl sulfoxide. Optical density of the dissolved material is measured at 570 nm (reference filter, 690 nm) with a UV visible spectrophotometer (Molecular Devices, UK).

## Determination of IC<sub>50</sub>

Cytotoxicity was expressed as mean percentage increase relative to unexposed control ± SD. Control values were set at 0% cytotoxicity. Cytotoxicity data (where appropriate) were fitted to a sigmoidal curve and a four parameter logistic model was used to calculate IC<sub>50</sub>, which is the concentration of nanomaterial causing 50% inhibition compared to untreated controls. Mean IC<sub>50</sub> is the concentration of agent which reduces cell growth by 50% under the experimental conditions and is the average of at least three independent reproducible statistically significant measurements. The IC<sub>50</sub> values were reported at ± 95% confidence intervals (± 95% CI). This analysis was performed using GraphPad Prism (San Diego, USA).

## In Vitro Determination of Antivenom Efficacy on L929 Cells

Antivenom efficacy was assessed by mixing 1, 3, and 5 IC<sub>50</sub> of native venom diluted in physiological saline with the same amount of antivenom. The procedure was similar to that described above for IC<sub>50</sub> determination. The venom-antivenom mixture was incubated for 30 minutes at 37°C before treating cells. Cultured L929 cells (as above) were treated with the venom-antivenom mixture and incubated for 48 hours. Following incubation, the survival of viable cells was determined by MTT and growth inhibition was compared with untreated controls. Results are expressed as percentage of survival.



## Morphological Studies

Morphological studies of the cells were performed with an inverted microscope (Olympus, Japan) comparing them with controls 48 hours after treatment with venom-antivenom mixture.

## Data Analysis

Values were presented as means  $\pm$  standard error of the mean (SEM). LD<sub>50</sub> was determined using a Probit test, while curve fits and IC<sub>50</sub> calculation were performed with GraphPad Prism (San Diego, USA). Statistical differences between treatments and controls were tested by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. A value of  $p < 0.05$  was considered statistically significant.

## RESULTS

Venom protein content was determined so that venom doses could be adjusted for all tests. The diluted (1:1000) raw venom protein concentration was 2.09 mg/mL. To calculate venom LD<sub>50</sub> value in order to ascertain the immunization dose for Swiss albino mice, single doses (1, 5, 10, and 20 mg/kg) of venom were administered to mice. Table 1 shows the lethality of *Macrovipera lebetina lebetina* venom IP injected in Swiss mice; 24 hour LD<sub>50</sub> was estimated as 7.58 mg/kg.

Approximately one fifth the estimated 24 hour LD<sub>50</sub> (1.5 mg/kg) was used to immunize mice (100  $\mu$ L/mouse). Only one mouse died when immunized, as a first challenge against the venom. There were no more deaths in the study group. Following first immunization, diluted

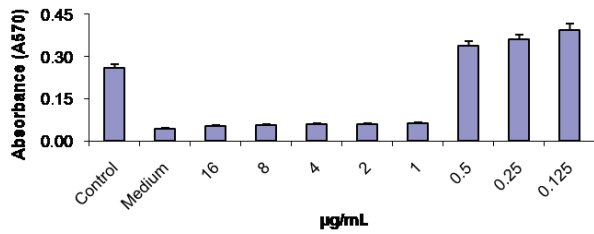
(1:20) sera antibody level was found to be  $0.448 \pm 0.046 - 0.719 \pm 0.051$  OD on day 7 by ELISA. After a second administration and three further injections, antigen mediated ELISA results exhibited immunoreactive properties with the venom, as IgG response (1:100 and 1:500 diluted sera) varied between  $2.336 \pm 0.042$  and  $2.535 \pm 0.031$  on day 32. The immunized Swiss albino mice serum was found to contain high levels of antivenom antibody, which could be useful in the production of antibodies as antivenom.

The cytotoxic effect and IC<sub>50</sub> value of venom on L929 cells were investigated by using different venom concentrations. MTT assay results showed that venom inhibits cell proliferation in a dose-dependent manner (Figure 2). After 48 hour of treatment, IC<sub>50</sub> was  $0.62 \pm 0.18$   $\mu$ g/mL; after two hours of treatment with venom and culture media totally refreshed, IC<sub>50</sub> was  $1.62 \pm 0.25$   $\mu$ g/mL (Figure 3). After 48 hours post-treatment of the venom-antivenom mixture, L929 cell viability was  $65 \pm 1.1\%$ . There were no significant effects on cell viability differences between 1, 3, and 5 IC<sub>50</sub> of native venom-antivenom treated cells ( $p > 0.05$ ) apart from cell morphological changes. There were also morphological changes in L929 cells which were growing logarithmically throughout the treatment with venom and venom-antivenom (Figures 4 and 5). After 48h post-treatment with venom-antivenom, an increased number of rounded cells and growth inhibition were seen in comparison to untreated control cells.

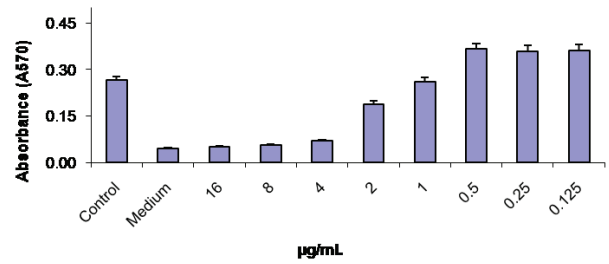
Following incubation of the L929 cell line with venom, various morphological abnormalities were observed. During the preliminary study, treatment with the highest venom dose (4.2  $\mu$ g/

**Table 1.** Lethality of *Macrovipera lebetina lebetina* venom injected intraperitoneally into Swiss albino mice and estimated value of LD<sub>50</sub>

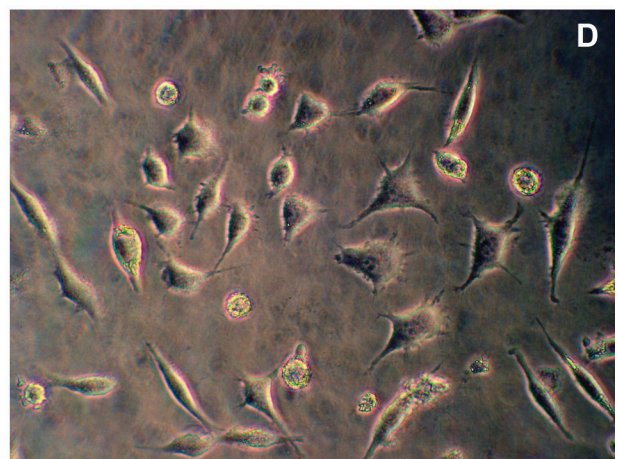
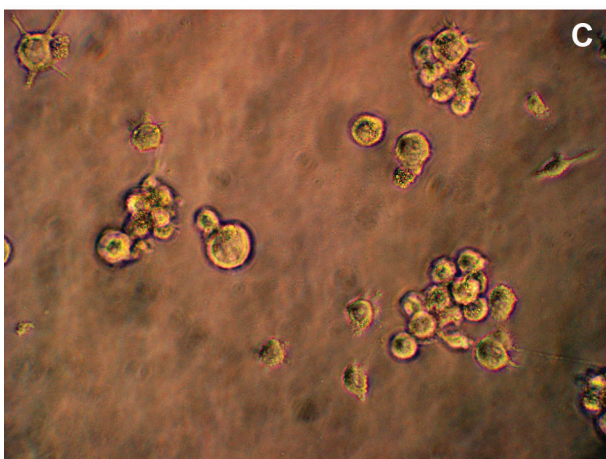
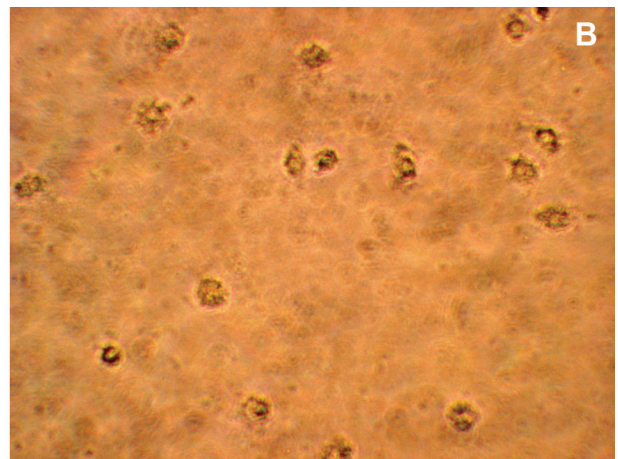
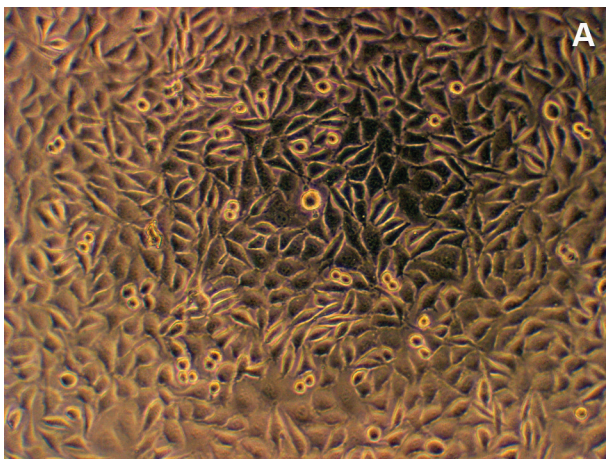
Concentration (mg/kg)	Number of mice	24 hours	
		Dead	Live
Control	10	0	10
1	10	0	10
5	10	2	8
10	10	8	2
20	10	10	0
Estimated LD <sub>50</sub> (mg/kg)		7.58	



**Figure 2.** Cytotoxic effect of *Macrovipera lebetina lebetina* crude venom on L929 cells after 48-hour exposure to different venom concentrations. Cell viability was determined by MTT assay, control was exposed to vehicle only which was taken as 100% viability. Data are expressed as mean  $\pm$  SD.

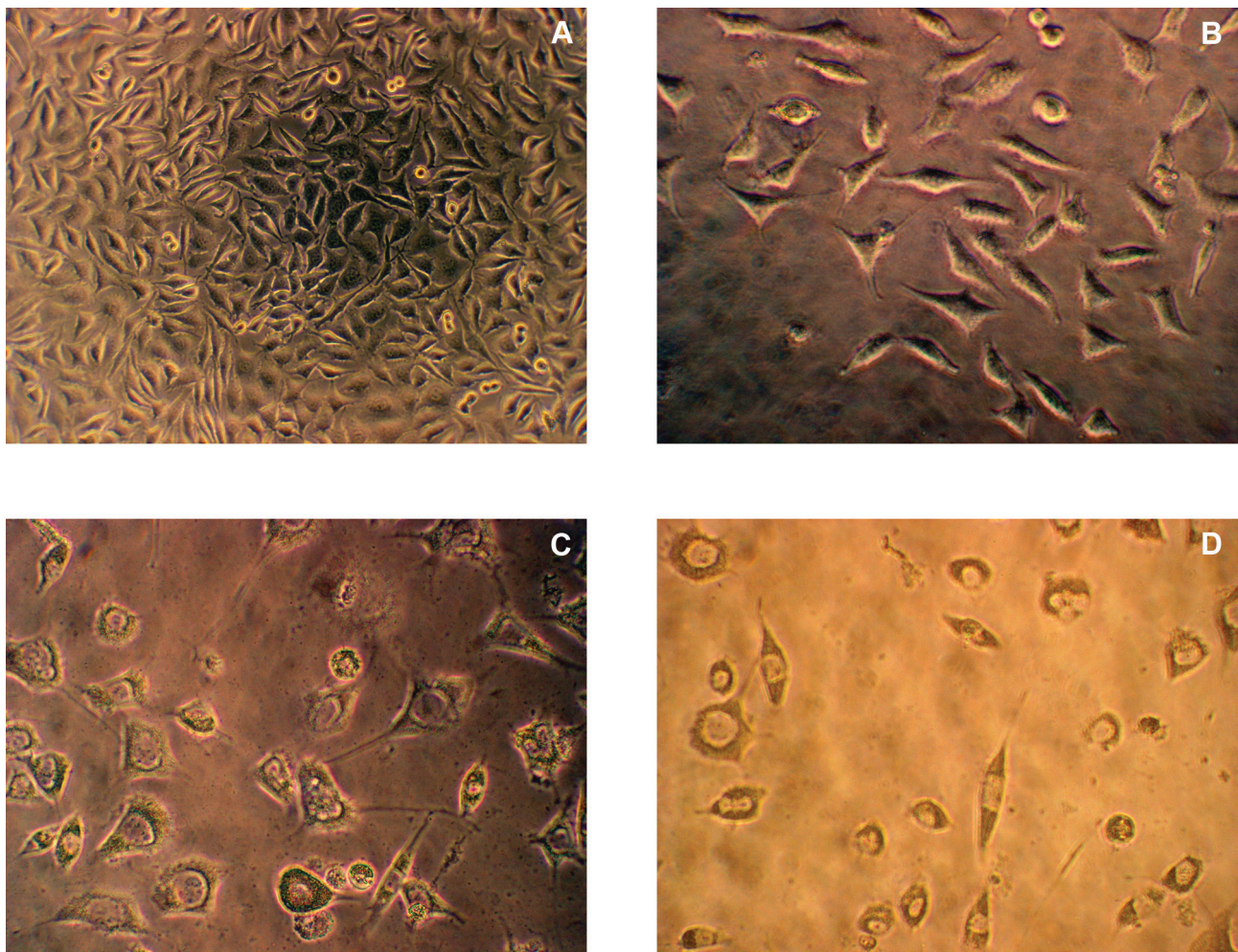


**Figure 3.** Cytotoxic effect of *Macrovipera lebetina lebetina* crude venom on L929 cells after two-hour incubation with different concentrations of venom and medium was refreshed and incubation continued for another 48 hours. Cell viability was determined by MTT assay, control was exposed to vehicle only which was taken as 100% viability. Data are expressed as mean  $\pm$  SD.



**Figure 4.** Morphological changes for L929 viewed by inverted microscope after venom treatment. (A) Untreated cells, (B) cells treated with 4.2 µg/mL, (C) cells treated with 1.1 µg/mL, and (D) cells treated with 0.13 µg/mL (magnification: 20x).





**Figure 5.** Morphological changes for L929 viewed by inverted microscope after venom-antivenom treatment. (A) Untreated cells, (B) cells treated cells with 1  $IC_{50}$  of native venom-antivenom mixture (C) cells treated with 3  $IC_{50}$  of native venom-antivenom mixture, and (D) cells treated with 5  $IC_{50}$  of native venom-antivenom mixture (magnification: 20x).

mL) for two hours killed all the cells whereas treatment at the mildest dose (0.132  $\mu\text{g/mL}$ ) resulted in several cells losing their characteristic appearance and an increased number of rounded cells.

## DISCUSSION

Snake antivenom immunoglobulins are the only specific treatment against envenomation from snakebites. Antivenoms can prevent or reverse most effects of snakebites, and play crucial roles in minimizing mortality and morbidity as toxicity widely differs among species. These preparations are included in the World Health Organization (WHO) List of Essential Medicines and should be a part of any primary health care package where snakebites occur. Currently, there

is an urgent need to ensure the availability of safe, effective and affordable antivenoms, particularly for developing countries, and to improve regulatory control over the manufacture, import, and sale of antivenoms (24).

Currently, snakebites, particularly involving *Macrovipera lebetina lebetina*, comprise an important medical emergency in Cyprus and other areas (9, 25-27). Viper snake venom is highly toxic to humans and vipers are considered one of the most dangerous snakes in the world (28-32). Production of polyvalent antivenom against the toxicity of *Macrovipera lebetina lebetina* should be useful in saving the lives of victims envenomed by these vipers. A cell based assay comparing *in vitro* cytotoxicity and *in vivo* toxicity would also be useful for further research into viperid venoms. The present study

investigated the toxicity, cytotoxicity, antivenom production and *in vitro* neutralizing ability of *Macrovipera lebetina lebetina* snake venom.

Whole snake venom was IP injected into mice in order to establish the LD<sub>50</sub> as previously described (33). Viper venoms are usually toxic to the hematopoietic system. They may act either as an anticoagulant or procoagulant enzyme (28). Hyperimmunization with crude venoms can cause serious side effects that can lead to death (34). The calculated 24-hour LD<sub>50</sub> value allowed us to choose suitable quantities of venom to successfully immunize animals. Production of the specific serum against *Macrovipera lebetina lebetina* venom was done by hyperimmunization using one fifth the LD<sub>50</sub> dose of venom.

One of the mice died a few seconds after the first immunizing inoculation, falling on its back with shock-like symptoms. During the remainder of the experiment, no other study animal died. Subsequent to initial injection, a few mice experienced short periods of lightheadedness or dizziness. However, no local reactions were observed until the end of experiment. The use of CFA and IFA adjuvants respectively in first and second immunization doses generated higher antibody levels with signs of sterile abscess and granuloma formation in a few mice (33, 35). Immunization by IP route was carried out once a week for five weeks and antibody levels began to rise at the end of first week.

The increase in antibody level was achieved by boosting treatment to maintain a high level of specific antibody against the venom antigen. ELISA has been used for detecting venom antigens from different species of venomous animals (11, 36-38). Polyclonal antibodies in antivenoms have been tested to determine specific antivenom antibodies in mice using ELISA. High levels of antivenom IgG obtained with sera from immunized mice have shown good correlation between results by ELISA. These results have shown that high protein content venom can also be used as an immunization agent to get high quality specific antibody in antivenom production.

The pharmacological study of snake venoms and toxins often involves the use of animals or animal tissues (39). Currently, researchers suggest that cell-based assay to examine venom cytotoxicity is an alternative to animal testing (18, 19, 40-42). Our *in vitro* results show that crude venom from *Macrovipera lebetina lebetina*

is highly cytotoxic for cultured fibroblasts causing decreased viability, the disappearance of normal morphological characteristics, rounding up, detachment and death at the highest concentration. Venom treated cells showed an increase in the density of cellular contents with significant obvious deterioration and deformation in a dose-dependent manner.

The relationship between *in vitro* and *in vivo* results correlated very well. Our interpretation of the results suggests that the LD<sub>50</sub> value is approximately ten times greater than the IC<sub>50</sub> value for crude venom from *Macrovipera lebetina lebetina*. Our results agree with those from other authors (40-42). Another important point is that the venom enhances the cytotoxic response of L929 cells when administered for longer periods (48 hours) with an IC<sub>50</sub> value of  $0.62 \pm 0.18 \mu\text{g}/\text{mL}$ . Consequently, cytotoxicity was estimated as  $1.62 \pm 0.25 \mu\text{g}/\text{mL}$  for short term venom exposure (two hours) and total refreshing of culture medium followed by 48-hour incubation.

Since snake venoms are complex mixtures of peptides, proteins, and enzymes, several different types of antibodies are needed to neutralize these toxic substances (29, 34, 43, 44). We also investigated the ability of the antivenom to neutralize the *in vitro* cytotoxicity caused by the venom. We found that the antivenom was unable to fully prevent the venom-induced effects on cell viability when added after preincubation with venom. And, to eliminate the possibility of cytotoxic activity mediated by serum complement, cells were grown and experimental protocols performed in medium containing 4% heat-inactivated (56°C for 0.5 hour) serum. Cell viability was similar for 1, 3, and 5 LD<sub>50</sub> venom-antivenom treated cells. The only differences were in cell morphology. Due to the limited quantity of *Macrovipera lebetina lebetina* venom available, no other venom-antivenom examinations were performed in this study. Other than that, similar cell-based assay results were found by Kalam *et al.* (41) for *Naja* spp. Venom-antivenom neutralizing ability. On the other hand, according to our results and previous studies, the cell-based assay can be useful for examining cytotoxicity levels and the ability of antivenom to neutralize snake venom (18, 19, 41). However, *in vitro* tests are not currently good enough to replace all animal tests as the *in vitro* methodology needs further optimization.



## CONCLUSION

In conclusion, this study indicated that *Macrovipera lebetina lebetina* antivenom production was achieved in a mouse model. Good *in vitro-in vivo* correlation was also obtained between cytotoxicity and venom toxicity data. The *in vitro* neutralization capacity of the serum was not very effective. Meanwhile, results indicated that cell death caused by *Macrovipera lebetina lebetina* venom should be studied on the induction of apoptosis and necrosis. Despite this, further research is necessary for the effective treatment with polyvalent (*Macrovipera lebetina lebetina*) antivenom. Also, to establish a cell-based assay for investigating snake venom, the method needs to be developed and optimized.

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

## ETHICS COMMITTEE APPROVAL

This study was approved by the Ege University Local Ethics Committee for Animal Experimentation (process number 2005/35).

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