

Experimental Kinetic Analysis of *Mesobuthus Eupeus* Scorpion Venom Absorption by ELISA

ZOHREH HOSSEINI¹, MASOUD GHORBANPOOR², MOHAMMAD KHOSRAVI^{2,*}, MANSOUR MAYAHI³

¹ DVM Student, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran

² Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran

³ Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran

Abstract

Background: A satisfactory therapeutic of envenomation requires accurate evaluation of the kinetic parameters of venoms. In this study, an AC-ELISA was developed for detection of *Mesobuthus eupeus* scorpion venom levels in the sera of experimentally envenomed mice. The aim was to establish a correlation between these levels and the amount of injected venom in different intervals after treatments.

Methods: Eighteen adult male N-mary mice were randomly divided into 6 equal groups; the first five groups received 180, 150, 100, 50 and 25 µg of *M. eupeus* venom per mouse respectively by SC route. The 6th group received PBS and was kept as control. At intervals of 15 and 30 minutes and 1, 2, 4, 6, 24 and 48 hours after treatment, the blood was obtained and its serum was assayed for detection of scorpion venom by an in-house AC-ELISA technique.

Results: One hour after injection of 25µg and 150µg *M. eupeus* venom, the highest serum concentrations of the venom were recorded as 12.6% and 19.2%/mL of the injected venom, respectively. The venom was detectable in serum within 15 minutes until 24 hours after injection.

Conclusion: The maximum time that the venom was detectable in serum is dependent on the level of the injection. In all venom received mice, the highest serum levels of the venom were recorded 1 hour after envenomation. The sensitivity of the designed ELISA was at least 19.5 ng. The indirect AC-ELISA is capable of identifying the circulating venom.

Keywords: ELISA; Kinetics; Mice; Scorpion; Venom

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INTRODUCTION

Scorpion envenomation is a health problem in many regions, especially in tropical and subtropical parts (1). Scorpions include 70 genera and 6 families, of which, 50 species are dangerous for human (2). The family of *Buthidae* contains the most toxic genera such as *Androctonus*, *Mesobuthus*, *Centruroides*, *Leiurus* and *Tityus* (3). *Mesobuthus eupeus*, widely spread species of the *Mesobuthus* genus, is a polymorphic scorpion species commonly known as the lesser Asian scorpion or the mottled scorpion (4).

Due to the complexity of *scorpion venom components*, scorpion sting leads to a range of problems in victims. The clinical symptoms of scorpion envenomation are various according to some conditions such as the scorpion species, the venom composition, the victim's age and physiological response to the venom (5). These various disorders need specific medical treatments in stung persons; but as the scorpion species cannot be identified, the same maintenance treatment is considered. This could endanger the patients'

lives and prolong the therapeutic process or at least raise the costs (6).

The identification of the scorpion species is not always possible and the signs of envenoming are not adequate to implicate the use of specific antivenom therapy in some cases of scorpion envenomation. The lack of a good laboratory method for the detection of venom may contribute to the ineffective treatments (7). The bioassays for detection of venoms by their neurotoxic or hemorrhagic activity are expensive, time consuming and nonspecific (8). In these cases, immunodiagnostic assays, that have noticeable clinical and investigational applications, could be a useful tool. The enzyme-linked immunoassay (ELISA) and radioimmunoassay give sufficient specificity and sensitivity for these targets. ELISA permits the measurement of the venom levels in circulation system and tissues (9). The evaluation of venom concentration in victim's serum and prevalence information can help health care institutions to suitably allocate their antivenoms (10). ELISA is regularly considered as a suitable assay for the venom detection, because of the usual detection limit of 10 to 100 ng, running time of 30 to 45 minutes, cost

*Correspondence to: Mohammad Khosravi; PhD, Assistant Professor, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran.
Tel: +989376211901, Email: m.khosravi@scu.ac.ir
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benefit, being relatively easy to perform, stable reagents and diversity of used samples as wound aspirate, serum and urine (8).

The kinetics data are necessary for the development of new diagnostic assays. The important factors that would be considered are victim's body weight, time of envenomation and concentration of venom (9). A good relationship between serum venom levels and clinical symptoms was established by Krifi et al. (11). Also the kinetics information is essential for a suitable treatment that is related to the promptness of antivenom administration and antivenom dosage (12). The symptomatic treatment of envenomed victims without giving consideration to the kinetics of venom and their variability among envenomed patients can lead to the failure of treatment approach. Overall, a satisfactory therapeutic of envenomation requires accurate evaluation of the kinetic parameters of scorpion venoms. In this study, an ELISA was developed for detection of *Mesobuthus eupeus* scorpion venom levels in the sera of experimentally envenomed mice. The aim of this study was to establish a correlation between these levels and the amount of injected venom in various intervals.

METHODS

Venom preparation

M. eupeus scorpions were collected with UV light at night from different parts of the Khuzestan Province (31°19'–32°73'N, 48°41'–49°4' E, with an area of 63,238 km²) in South West of Iran and were milked by electric stimulation at the end of the tail. The freeze-dried venom was dissolved in distilled water and then dialyzed against distilled water at 4 °C for 48 hours. After dialysis, the venom solution was centrifuged at 1500rpm for 15 minutes, and the supernatant was collected.

Protein assay

All of the protein concentrations were determined by the Bradford assay with Bovine Serum Albumin (BSA) as standard.

Production of polyclonal antibody

Three Breed chickens and three New Zealand white male rabbits were acclimatized to room temperature at 18 °C for two weeks before immunization. Preimmune sera were attained throughout this period. In initial immunization, animals were injected intradermally with 250 µg of venom in 0.5 of PBS emulsified with 0.5 ml of complete Freund's adjuvant by a multiple injection method (10 sites/rabbit) (13). These first injections were pursued by three sets of booster injection. Booster injections were made at 2nd, 4th and 6th weeks with 130 µ gr of immunogen, 0.5ml of PBS and 0.5ml of incomplete Freund's adjuvant at two sites in both thighs intramuscularly. The existence of antibodies in serum was determined through immunodiffusion and Dot-ELISA test. Finally, 10 days after the last immunization, blood was directly collected into sterilized glass tubes without any anti-coagulants and allowed to clot in cold. Serum was pipetted out and centrifuged at 1500 rpm for 10 minutes and then isolated in a sterilized vial and stored at 4 °C for bioassay tests.

Purification of polyclonal antibody against venom

Polyclonal antibody against venom was first purified by ammonium sulfate precipitation (50% saturation for the final

solution) and dialyzed in PBS, then it was subjected to an affinity column conjugated with venom. The column was prepared by conjugating 20 mg of venom with 7ml of activated CH-Sepharose 4B. Cyanogen bromide activation was performed by the method of March et al. (14). Antibody was eluted from the column with 0.1M glycine pH 2.5 and fractions were collected and neutralized immediately by adding an appropriate amount of 1 M tris-pH 9 to each fraction.

SDS-PAGE and DOT-ELISA analysis

Purity of polyclonal antibody was analyzed by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) at the concentration of %11 according to the Laemmli method (15). Proteins were stained with %1 Coomassie blue R-250 and their affinity against venom were confirmed by DOT-ELISA before being used in the next steps.

ELISA optimization

The optimum concentration of ELISA reagents including chicken (trapper: Ab1) and rabbit (detector: Ab2) specific polyclonal antibody, serum dilution, and conjugated anti-rabbit antibody dilution were determined by checkerboard titrations. Polypropylene 96-well microtiter plates were coated with 33 µg/ml of Ab1 (100 µL/well) in carbonate bicarbonate coating buffer pH 9.6 and incubated overnight at 4 °C. All the subsequent washing cycles were performed 3 times with PBST (PBS containing 0.05 tween 20) and one time with PBS containing phosphate buffered saline (PBS) pH 7.2, after each step. Blocking of the wells was achieved by overnight incubation at 4 °C with 200 µl per well of PBS containing 4% powdered skim milk and 0.08% tween 20. Different preparations of *M. eupeus* scorpion's venom (ranging from 12.5 µg/ml to 195 ng/ml, divisor: 2 diluted in normal mice sera) were added to rows (A to G) and normal mice sera were added as blank (100 µl/well) and incubated 45 minutes at room temperature. Following another washing cycle, 100 µl of Ab2 (20 µg/mL) in PBS containing 1% skim milk was added to each well and incubated 45 minutes at room temperature and the ELISA plate was washed again as previously described. Goat anti-rabbit IgG-HRP conjugate (Immuno Chemistry Technologies company, USA, HRP AffiniPure Goat anti-Rabbit IgG Fc, Catalog Number: 6293), freshly diluted 1:20000 and 100 µL was added to the wells. After 45 min incubation at room temperature, the plate was washed again and 75 µL of tetramethylbenzidine substrate solution was added to the wells and incubated for 10 min. The reactions were stopped by addition of 75µl of 0.1M H₂SO₄ and finally, optical density (OD) values were measured at 450 nm with ELISA reader.

Assessment of ELISA

All experiments were performed according to the guidelines of the ethical committee (16). Eighteen N-mary mail adult mice were distributed into 6 equal groups (n=3) of A to F. Those in the control group (A) received 500 µL of ultra-pure water administered via subcutaneous injection into the inguinal region using a disposable 1mL hypodermic syringe. Each of the mice in the experimental groups received by identical route, 500µL of a solution containing 180 µg group B, 150 µg group C, 100 µg group D, 50 µg group E and 25 µg group F, of scorpion venom dissolved in ultra-pure

water. At intervals of 15 and 30 minutes and 1, 2, 4, 6, 24 and 48 hours after treatment, venous blood was sampled and sera (50 µl/well) were blinded assayed for scorpion venom antigenemia using the ELISA technique described above.

Data analysis

In order to quantitate the amount of present venom in serum samples, a titration curve was constructed by plotting the venom concentration against absorbance value for each of scorpion venom. Known amounts of venom spiked with mice serum from normal controls were included with each test to obtain a titration curve. Descriptive and inferential statistical methods were applied to data analysis.

RESULTS

Reagents optimization

The purified specific antivenom antibody of chickens and rabbits were tested by DOT-ELISA (Figure 1 and 2). After three sets of immunization with *M. eupeus* venom, at the same concentration of specific antibody (100 µg/mL), rabbit's antibody (Ab2) has a detectable higher affinity than chicken's (Ab1). The Ab1 and Ab2 were selected as trapper and detector antibody, respectively.

ELISA optimization

After the checkerboard assessment, the flowing concentration of reagents were selected for final ELISA preparation; Ab1: 33 µg/mL, Ab2: 20 µg/mL, conjugated goat anti rabbit IgG: 1/20000 dilution (Figure 3).

Kinetics of venom absorption

The five concentrations of venom (25, 50, 100, 150 and 180 µg) were injected SC to 18±4 gr weight mice. The highest serum concentration of the injected venom ranges from 12.6 (150µg) to 19.2 (25µg) % / mL, one hour after treatments. Firstly, the venom was detected in serum 15 minutes and finally 24 hours after treatments. Also, the

maximum venom levels in serum were detected 1 hour after envenomation (Table 1). The sensitivity of the designed ELISA was at least 19.5 ng.

DISCUSSION

Very small quantities of antigen and antibody could be detected by ELISA. The required reagents are cost benefit and stable and diagnostic testing can be performed at a reasonable time. The venom levels in various tissues or liquid samples could be defined with a good designed ELISA kit for use in the field (17). However, various assays were tested for this purpose; in 1957, the detection of cobra venom in tissues of a bitten individual was performed by precipitin test (18). In the early 1970s, Radioimmunoassay was developed for snake venom detection (19). Firstly, snake venom was detected with ELISA in serum of experimental animals and human victims by Theakston et al. (20). Since then, ELISA became the first choice for this point than any other tests such as immunodiffusion or radioimmunoassay. The current ELISA assay detected at least 19.5 ng of scorpion venom in mice sera. Various detection limits were reported for employment of ELISA in experimental animals or human victims of snake, scorpion, bee or spider envenomation. As the difference between samples, these reports are in wide range from 0.9 ng/ml, 2.5 ng, 3 ng, 3.9 ng and 17.1 ng (11, 21-24). Sikarwar et al. reported a DOT-ELISA that has the positive results from a minimum venom concentration of 1953ng/ml for *Naja naja sumatrana* and *Calloselasma rhodostoma* venom and 977ng/ml for the cobra venom fraction (25). Also the RPA skin test, 15 min after SC injection of 100µg *M. eupeus* venom, showed 84.44% sensitivity and 100% specificity (6). The amount of venom that a sting may inoculate is about 0.1–0.6 mg. The developed ELISA in current study, 15 min after injection of 25µg *M. eupeus* venom, detected envenomed animals. These results suggested the appropriateness of this assay for experimental or field utilization.

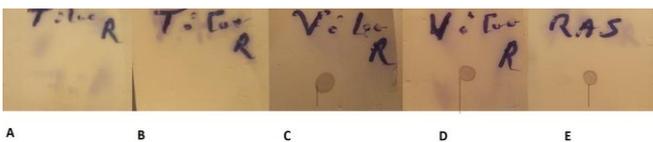


Figure 1. DOT-ELISA for evaluation of the purified rabbit's antibody. The tetanus toxoid were used as negative controls (A and B). Two dilutions of rabbit's specific antibody (C, D) were tested against *M. eupeus* venom. The rabbit's hyperimmune sera were used as positive control (E).

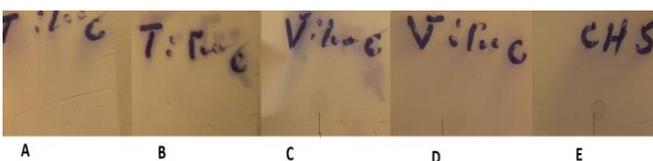


Figure 2. DOT-ELISA for evaluation of the purified chicken's antibody. The tetanus toxoid was used as negative controls (A and B). Two dilutions of chicken's specific antibody (C, D) were tested against *M. eupeus* venom. The chicken's hyperimmune sera were used as positive control (E).

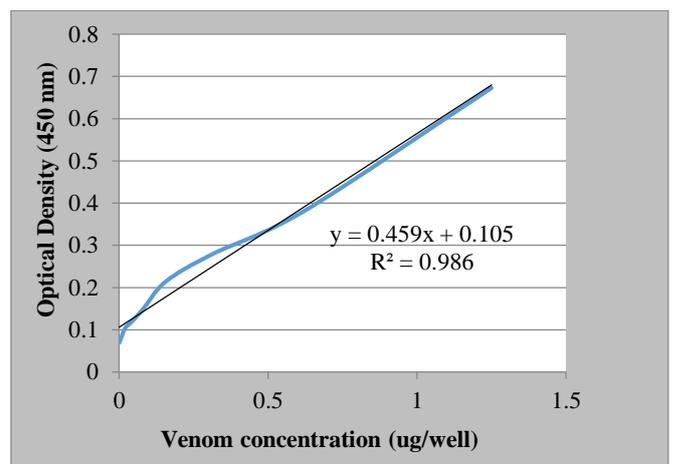


Figure 3. Standard curve of ELISA for detection of *M. eupeus* venom. The *M. eupeus* venom ranging from (1.25µg/ml to 19.5ng/ml) was diluted in normal mice sera. The optical densities were assayed by ELISA reader. The OD results were correlated with the venom concentrations (R²=0.986).

Table 1. The concentration (mean±standard error) of five doses of *M. eupeus* venom in experimental envenomed mice sera samples in different intervals.

180 µg	150 µg	100 µg	50µg	25µg	Time / Dose
0.281 ± 0.027	0.273 ± 0.025	0.395 ± 0.05	0.201 ± 0.048	0.122 ± 0.032	15 min
0.790 ± 0.033	0.551 ± 0.042	0.608 ± 0.068	0.338 ± 0.038	0.127 ± 0.024	30 min
1.355 ± 0.27	0.953 ± 0.06	0.634 ± 0.042	0.375 ± 0.078	0.243 ± 0.012	1 hr.
0.621 ± 0.053	0.134 ± 0.036	0.057 ± 0.03	0.067 ± 0.024	0.031 ± 0.018	2 hrs.
0.205 ± 0.07	0.065 ± 0.013	0.053 ± 0.017	0.022 ± 0.02	0.021 ± 0.01	4 hrs.
0.038 ± 0.01	0.054 ± 0.01	—	—	—	6 hrs.
0.033 ± 0.01	—	—	—	—	24 hrs.
—	—	—	—	—	48 hrs.

The symbol (—) indicates the undetected items.

The various researches reported the different times for venom to reach its peak concentration. In agreements to the current study, Paniagua et al. reported that components of venom reached the bloodstream during the first 60 minutes after SC injection of 5 mg *Coral* snake venom into sheep (26). Santana et al. reported after SC injection of 200 /100 µg *Tityus serrulatus* venom, the maximum plasma concentrations were reached at 60 min (27). However, in disagreements, the maximum *Tityus serrulatus* scorpion venom levels in experimentally envenomed mice occurred at 30 min in serum. Venom decreased rapidly in serum and in all other organs after 2 hours, until 8 hours that the venom was not detectable thereafter (7). Also, the IM injection of 0.5 mg/kg *Naja sumatrana* to rabbits showed that the serum levels of the venom declined rapidly within the first 1 h (28). After SC injection of 1 mg/kg *Crotalus durissus terrificus* venom to dogs, the maximum serum levels of venom were noticed 2 hr after envenomation and the venom concentration was ranged between 5 and 90 ng/ml (29). The use of different animals, venom and injection route in experiments are possible reasons for disagreements with the current study and the others. After SC injection of 10 µg *Crotalus* snake venom, the peak concentration occurred at 15 min, which declined after 30 min. No toxin was detected after 60 min (23). The current research shows that the venom can be detected in serum until 24 hours after envenomation in severe envenomation condition; however, the last time that it could be found out is dose-dependent. In agreements, *Naja naja karachiensis* venom was cleared from circulation system of rabbits after 24 hours (30). Also, Gomez et al. reported that venom amounts of less than 40 ng are detected in the injected sites until 24 hours after envenomation (31).

LIMITATIONS

The main limitation of this study might be physiological differences between mice and different animals or a human, which hesitant physicians to use the results in the treatment of human or animal's envenomation.

CONCLUSION

One hour after subcutaneous treatments, the highest serum concentrations of the venom were recorded. Also, the venom was detectable in serum within 15 minutes until 24 hours after

subcutaneous injection. The sensitivity of the developed ELISA was at least 19.5 ng. The last time that the venom was detectable in serum is dependent on the level of the injected venom. These findings suggest the best time that antivenom should be administrated is the first hour after envenomation.

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Conflict of interest: None to be declared.

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