

Original Article

Purification of the Immunogenic Fractions and Determination of Toxicity in *Mesobuthus eupeus* (Scorpionida: Buthidae) Venom

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Abstract

Background: Scorpions stings are a health problem in many parts of the world. *Mesobuthus eupeus* (Buthidae) is the most prevalent species in the Middle East and Central Asia. Definition of toxicogenic and immunogenic characteristics of the venom is necessary to produce antidote. In this study, the noted properties of *M. eupeus* venom were evaluated.

Methods: Venom was obtained by milking *M. eupeus* scorpions for lyophilization. Toxicity was determined after injecting the venom to albino mice and calculating LD₅₀. Polyclonal antibodies against *M. eupeus* venom were obtained from immunized rabbits. The CH-Sepharose 4B column was used for isolating the specific antibodies. 10 mg of the affinity-purified antibodies were conjugated with a CH-Sepharose 4B column and *M. eupeus* venom was applied to the column. The bound fragments were eluted using hydrogen chloride (pH: 2.5). Crude venom and affinity-purified fractions of the venom were analyzed by SDS-PAGE technique.

Results: Lethal dose (LD) was 8.75, 11.5 and 4.5 mg/kg for IP, SC and IV respectively. The LD₅₀ of *M. eupeus* venom was 6.95 mg/kg. The crude venom had 12 detectable bands with molecular weights of 140, 70, 50, 33, 30, 27, 22, 18, 14, 10 kDa and two bands less than 5 kDa. The affinity-purified venom presented eight bands. The 27 kDa band was clearly sharper than other bands but 70, 18, 10 and one of the less than 5 kDa bands were not observed.

Conclusions: Contrary to popular belief, which know scorpion venom as non-immunogenic composition, the current study was shown that the most fractions of the *M. eupeus* are immunogenic.

Keywords: *Mesobuthus eupeus*, Scorpion, Venom, Immunogenic, Toxicogenic

Introduction

Scorpions have existed on earth about 400 million years ago (Ozkan et al. 2007). The scorpion stings are a major threat to human and animal health especially in tropical regions (Bawaskar et al. 2012, Warrell 2012). Annual rate of scorpion stings is 1.2 million, and the mortality rate is about 3250 per year. Children are more vulnerable to scorpion envenomation and the highest death rate is observed in this age group (Chippaux and Goyffon 2008).

Scorpions belong to the phylum Arthropoda,

class Arachnida, order Scorpiones. 1500 described species of scorpions are included 70 genera and 6 families. 50 species are dangerous for human (Keskin and Koc 2006) where Buthidae family is the most venomous of them (Shirmardi et al. 2010).

Iranian scorpion (sting agents) species are classified in Buthidae and Scorpionidae families with 16 genera and 25 species (Dehgani et al. 2009). The limited number of dangerous species are found in Iran (Sagheb et al. 2012). *Mesobuthus eupeus* is a species be-

longing to the Buthidae family and commonly known as the lesser asian scorpion or the mottled scorpion. It was found in the Middle East and Central Asia and is responsible for many cases of envenomation in these regions (Karatas 2003, Sadeghian 2003, Dehghani and Khamehchian 2008).

Mesobuthus eupeus is the most common species in Iran. Its venom contains several toxin fractions, which may cause a number of scorpion sting symptoms (Tuuri and Reynolds 2011, Sagheb et al. 2012).

Scorpion venom consists of many biological compounds which affects vertebrate and invertebrate organisms (Upadhyay and Ahmad 2008).

Scorpion venom composes of short-chain peptides with low molecular weight (Adiguzel 2010), which elicit a strong immunogenic reaction in the host (Corzo et al. 2001). As yet, about 400 toxic peptides have been detected in scorpion venoms but it has been estimated that 100,000 distinct peptides exist in scorpion venom (Karatas 2003).

Serotherapy is the only effective treatment against scorpion stings and has been an issue of discussion in the last decade (Boyer et al. 2009, Duarte et al. 2010).

Based on previous reports, approximately 42500 scorpion stings occur in Iran annually (Dehghani and Fathi 2012). In Iran, the scorpion antivenom is made through the process of injecting horses with a mixture of six different scorpion venoms including: *Hemiscorpius lepturus*, *Buthotus saulcyi*, *B. schach*, *Odonobuthus doriae*, *M. eupeus* and *Androctonus crassicauda* (Razi Vaccine and Serum Research Institute, Karaj, Iran).

Many investigations were performed to improve the quality of antidote against scorpion venom. Study of the immunological properties of venom is critical for antivenom development as much as better (Inceoglu et al. 2006). Moreover the detection of antigenic proteins is very important in the field of toxicology and parasitology (Kalapothakisa

et al. 2001). So development of specific antibodies against immunogenic fragments of the venom can effectively improve therapeutic alliance. Gel electrophoresis, electro-focusing or liquid chromatography are used to detect protein patterns of venoms (Escoubas et al. 2002, Pimento et al. 2003).

The current study was conducted to investigate the immunogenic and toxicogenic properties of the *M. eupeus* venom.

Materials and Methods

Venom preparation

Mesobuthus eupeus scorpions were collected with UV light at night from different parts of the Khuzestan Province (31°19–32°73N, 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

The protein content of venoms was determined by the absorbance at 280nm with Bovine Serum Albumin (BSA) as standard.

Toxicity determination

All experiments were performed according to the guidelines of the ethical committee of the Faculty of Veterinary Medicine of Tehran University, Iran (National Ethics Advisory Committee 2006).

For toxicity determination, increasing concentrations of the venom were injected subcutaneously (SC), intraperitoneally (IP) and intravenously (IV) to albino mice. Following treatment with venom solution, animals were monitored for 24 hours, and the number of dead animals was recorded at the end of the

experiment, then, LD was calculated. LD₅₀ was determined using the Spearman-Kaerber method. Briefly, 35 mice were divided into 7 groups of 5 mice each. Appropriate venom concentrations were prepared to cover the full range between zero and 100% of induced animal mortalities. Different doses (175, 160, 145, 130, 119, and 109 µg) of the venom stock solution were prepared and injected intraperitoneally (IP). An equivalent volume of buffer was injected into 5 mice as a negative control group. Deaths were scored up to 24h and LD₅₀ was then calculated.

Production of polyclonal antibody

Outbred New Zealand white male rabbits were acclimatized to room temperature at 18 °C for two weeks former to immunization. Preimmune sera was attained throughout this period. The immunization plan and programmes of immunization were the alike as those detailed previously. In initial immunization, three rabbits were each injected intradermally with 250 µg of venom in 0.5 ml of PBS emulsified with 0.5 ml of complete Freund's adjuvant by a multiple injection method (10 sites/ rabbit) (Inceoglu et al. 2006). These first injections were pursued by three sets of booster injection. Booster injections were made at 2nd, 4th and 6th weeks with 130 µgr 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 Ascoli's test. Finally, after 10 days, the immunization blood was directly collected into sterilized glass tubes without any anti-coagulants and allowed to clot in cold. Serum was pipette out and centrifugated 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 and then subjected to an affinity column conjugated with venom. The column was prepared by conjugating 20mg of venom with 7ml of activated CH-Sepharose 4B. Cyanogen bromide activation was performed by the method of Cuatrecasas (March et al. 1974).

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.

Purification of immunogenic peptides of venom

The fractions, including the exact antibodies were merged, dialyzed against Borate buffer pH 8.4, overnight and used for another affinity column. Ten mg of this affinity purified antibody conjugated with a CH-Sepharose 4B column and 5mg of *M. eupeus* venom were applied to it. The bound proteins were eluted as before.

SDS-PAGE analysis of the venom

The protein profiles of crude venom as well as the affinity fractions (purified venom) were analyzed by SDS-PAGE (Laemmli 1970), the concentration of acrylamide was 15%. Proteins were stained with 1% coomassie blue R 250. Molecular mass standard (Vivantis, product No: PR0602) was run in parallel in order to calculate molecular weights of the proteins. Then, the gels were photographed and molecular weights of the proteins were calculated.

Results

Venom lethal dose (LD) was assessed by either subcutaneous, intraperitoneal or IV injection using 18±2g albino mice. LD was 8.75, 11.5 and 4.5mg/ kg of the body weight of albino mice for IP, SC and IV, respectively.

The median lethal dose (LD₅₀) of *M. eupeus* venom was 6.95mg/ kg with IP injection.

Proteins of the venom were determined to be between 5 and 140 kDa on electrophoresis on 15% polyacrylamide gel. The crude venom had 12 detectable bands with molecular weights of 140, 70, 50, 33, 30, 27, 22, 18,

14, 10 kDa and two bands less than 5 kDa. The affinity-purified venom presented eight bands. The 27 kDa band was clearly sharper than other bands but 70, 18, 10 and one of the less than 5 kDa bands were not observed (Fig. 1).

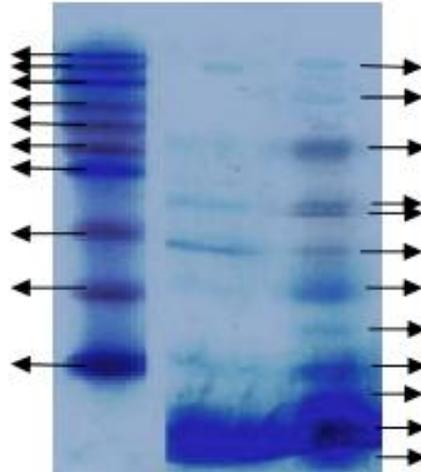


Fig. 1. The SDS-PAGE analysis of *Mesobuthus eupeus* scorpion venom. From right Lane 1: Marker proteins (175, 130, 95, 70, 62, 51, 42, 29, 22 and 14 respectively). Lanes 2 and 3: Electrophoretic pattern of the immunogenic fractions present in the venom (140, 50, 33, 30, 27, 22, 14, 5 kDa) and crude venom (140, 70, 50, 33, 30, 27, 22, 18, 14, 10, 5, 5 kDa) respectively.

Table 1. The variations of protein in *Mesobuthus eupeus* venom

Protein bands (kDa)	140	70	50	33	30	27	22	18	14	10	Fever than 5	Total number of protein bands
A	+	+	+	+	+	+	+	+	+	+	++	12
B	+	-	+	+	+	+	+	-	+	-	+-	8

A) Venom samples B) Immunogenic fractions of venom

Discussion

In the present investigation, we determined the in vivo toxic effects of the venom of *M. eupeus*. The venom of *M. eupeus* appears to be more toxic when injected intravenously. This phenomena could be associated to different toxicokinetics of the three injection methods.

Additionally, we studied the electrophoretic protein pattern of the crude venom, and immunogenic fractions of the venom. The results clearly displayed that most of *M. eupeus* venom fragments were immunogenic. Our results also showed that scorpion toxins were proteins with various molecular

weights, which induce both toxicological and immunological reactions *in vivo*. We also developed an approach toward application and refining of the immunogenic fractions of *M. eupeus* venom.

Previous studies in Iran determined 4.5 mg/kg (Zayerzadeh et al. 2012) and 1.45 mg/kg (Hassan 1984) as the median lethal dose (LD₅₀) of *M. eupeus* venom. Another study was calculated the median lethal dose of *M. eupeus* venom 0.18mg/ kg via intracerebroventricular (ICV) injection (Ozkan and Carhan 2008). In our study, LD₅₀ of the venom was 6.95mg/ kg via IP injection.

Diverse studies reported various numbers of protein bands with different molecular weights for scorpion venoms. Molecular weights of *Iurusdufourieus asiaticus* specie venom were determined 14–205 kDa with individual variations (Turkey) (Keskin and Koc 2006). A similar study on *Tityus pachyurus* specie suggested 14–97 kDa venom proteins using electrophoresis (SDS-PAGE) method (Latin America) (Barona et al. 2004). They developed three anivenom which prominently reacted with low molecular weight fragments. The most of venom proteins molecular weights of *M. eupeus* were 12–112 kDa (Ozkan and Carhan 2008). We determined protein fragments from 5 to 140 kDa. One study showed that the venom of *M. gibbosus* consisted of 19 protein bands with molecular weight from 6.5 to 210 kDa (Ucar and Tas 2003). Protein bands with molecular weight of 28, 30, 33, 68 and 98 kDa were detected in the venom of the captive male *M. gibbosus* from the same biotope during the summer (Turkey, Mugla Province) (Ozkan and Ciftci 2010). The causes of disagreement between studies may be due to the effects of the sex, geography, and hormonal condition of scorpions, which all alter feeding manners and result in venom creation with diverse molecular weights. In the current study, 12 protein bands were detected in *M. eupeus* scorpion venom.

Variations in the biochemical and immunological contents of the various scorpion venoms must be considered to realize clinical signs, produce efficient antivenoms and determine optimal dosage (El-Hafny et al. 2002, Calvete 2010).

Recognition and comparing of the MesoLys-C amino acid sequence of three major species scorpion is used for detecting phylogenetic relationships of various scorpion species. For example, MesoLys-C isolated from *M. eupeus* of Khuzestan exhibited the highest and the lowest sequence similarities with *M. gibbosus* and *M. cyprius*, respectively (Eskandari and Khoonmirzaei 2011).

The ability of heminecrolysin to suppressing the major physiopathological effects of *H. lepturus* envenomation may be due to elicit high titer of specific IgGs (Borchani et al. 2011).

The antigenicity studies of iberiotoxin of Eastern Indian scorpion demonstrated whole protein was not necessary to stimulate the immune system, because a small fragment of the venom protein called the antigenic determinant was adequate for eliciting the immune response (Gomase et al. 2009). A study performed by Garcia et al. (2003) approved this statement.

Gazarian et al. (2005) realized that no immunity was developed against scorpion venom during evolution. Because of no evolutionary relationship between humans immunity and scorpion venom, scorpion venoms can be suitable candidates for immunogenic probes (March et al. 1974). Because of completely distinct phylogenetics properties of two noted entities, any structural changes of scorpion venoms can followed and probably manipulated for inactivation of their antigenic activity (Gazarian et al. 2005).

Contrary to popular belief, which know scorpion venom as non-immunogenic composition, the current study was shown that the most fractions of the *M. eupeus* were immunogenic. Further investigations are necessary to explain more details of these immu-

nogenic fractions and to detecting lesser toxic fragments, which, improves the quality of the antidotes and helps vaccines designing.

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