Original Article

Determination of the Median Lethal Dose and Electrophoretic Pattern of *Hottentotta saulcyi* (Scorpiones, Buthidae) Scorpion Venom

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Abstract

Background: In this study, we investigated the lethal potency, electrophoretic protein pattern and in vivo effects of *Hottentotta saulcyi* scorpion venom in mice.

Methods: Scorpions were collected at night, by using a UV lamp from Mardin Province, Turkey. Venom was obtained from mature *H. saulcyi* scorpions by electrical stimulation of the telson. The lethality of the venom was determined by i.v. injections using Swiss mice. In vivo effects of the venom were assessed by using the intraperitoneal route (ip) injections into mice (20±1g) and monitored for 24 h. The protein profiles of the scorpion venom were analyzed by NuPAGE® Novex® 4–12 % gradient Bis-Tris gel followed by Coomassie blue staining.

Results: The lethal assay of the venom was 0.73 mg/kg in mice. We determined the electrophoretic protein pattern of this scorpion venom to be 4, 6, 9, 31, 35, 40, 46 and 69 kDa by SDS-PAGE. Analysis of electrophoresis indicated that *H. saulcyi* scorpion intoxicated mice exhibited autonomic nervous system symptoms (tachypnea, restlessness, hyperexcitability, convulsions, salivation, lacrimation, weakness).

Conclusions: *Hottentotta saulcyi* scorpion venom includes short-chain neurotoxins and long-chain neurotoxins according to the electrophoretic protein patterns. The stings of *H. saulcyi* scorpion must be considered of risk for humans in the southeastern region, Turkey.

Keywords: Scorpion, *Hottentotta saulcyi*, Venom, In vivo Effects

Introduction

In the world, approximately 2091 species and 197 genera of scorpions are described (Rein 2013), currently distributed in 15–19 families (Soleglad and Fet 2003, Prendini and Wheeler 2005). Scorpion envenomation is a major public health problem in developing countries especially in tropical and subtropical regions (Özkan et al. 2011).

Cases of scorpion envenomation into humans are common in Turkey due to its geographical location, climate and socioeconomic structure (Özkan et al. 2004, Özkan and Kat 2005). Therefore, scorpion envenomation is common in several regions of the country, especially in Southeastern Anatolia (Adıguzel et al. 2007, Al et al. 2009, Boşnak et al. 2009, Özkan et al. 2008, 2011). In the southern region of Turkey, scorpion stings and envenomation cases are a major public health problem, especially in Southeastern Anatolia (Altınkaynak et al. 2002, Adıguzel et al. 2007, Boşnak et al. 2009). Children are at greater risk of developing severe cardiac, respiratory and neurological complications (Altınkaynak et al. 2002, Boşnak et al. 2009).

So far, the most medically important and dangerous scorpion species responsible for envenomings described in Turkey are: *Androctonus crassicauda*, *Leiurus quinquestriatus* (now *Leiurus abdullahbayrami*), *Mesobuthus*

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gibbosus and *Mesobuthus eupeus*, all of which belong to the Buthidae (Uçar et al. 2005, Boşnak et al. 2009, Adıguzel 2010, Özkan et al. 2011). *Hottentotta* is one of the most widely distributed genera of the family Buthidae, with species present throughout Africa, the Middle East and Asia (Kovařík 2007, Yağmur et al. 2008). The minor numbers of *H. saulcyi* scorpion sting cases were reported in Iran (Dehghani and Fathi 2012) while the scorpion sting was not reported in Turkey.

In Turkey, *H. saulcyi* was first recorded in Mardin Province (Crucitti, Vignoli 2002) and later observed in Batman, Şırnak, and Hakkâri provinces, by Yağmur et al. (2008). Adıguzel (2010) reported that there is no data related to venom toxicity and in vivo effects of *H. saulcyi* scorpion species in Turkey.

In the present work, we studied the toxicity of *H. saulcyi* from Mardin Province of Turkey. The lethality and effects of the venom were assayed in mice and the protein profiles (molecular weight) of the venom determined.

Materials and Methods

Scorpions and venom

This study was approved by the Ethic Committee of Refik Saydam Public Health Agency, Ankara, Turkey under process number 33/13.11.2009.

Scorpions were collected at night, by using a UV lamp from Eskikale Village, in Mardin Province of the Southeastern region in 05.09.2009, Turkey (Fig. 1). Avoiding scorpion cannibalism, captive scorpions were housed in individual plastic boxes at the Department of Entomology, Faculty of Veterinary Medicine, Ankara University, Turkey. The scorpions were fed with crickets or cockroaches and received water daily.

Venom was obtained from mature *H. saulcyi* scorpions from Mardin Province by electrical stimulation of the telson (Özkan and Filazi 2004). Obtained venom from scorpions was collected into an Eppendorf tube. The venom was dissolved with sterile double-distilled water and centrifuged at 15,000 rpm for 15 min at 4 ºC (Özkan and Filazi 2004). The precipitate was discarded and the supernatant was stored at -20 ºC until use. Protein concentrations were determined using a BCA kit (Pierce, USA) with BSA as the standard.

Experimental Animals

Swiss mice of both sexes (20±2g) were employed to determine the median lethal dose (LD_{50}) by intravenous (iv) Route of administration. They were bred in the animal facility of the Refik Saydam Public Health Agency (RSPHA). The animals were housed under controlled temperature (20±2 °C), with a 12:12 light/dark schedule and were fed commercial rodent pellets and water *ad libitum* throughout the experiment.

Determination of the median lethal dose

The lethality of the venom was determined by iv injections as described by Behrens and Karber (1935) sing Swiss mice. Five mice per each dose group were injected iv with increasing volume doses of venom (11.22, 13.02, 14.80, 16.63, and 18.40 µg/ 19g mouse), diluted in 0.2 ml physiological saline solution (PSS: 0.85% NaCl). An equivalent volume of PSS was injected into five mice as negative control group. The mice were observed for 24 h after venom injection. Deaths occurring during the first 24 h after injection were recorded in order to determine the median lethal dose. The lethality was expressed as the median lethal dose (LD_{50}). Experimental protocols for animal experiments were approved by the ethical committee of the RSPHA.

Evolution of the experimental envenomation

The experimental envenomation was asses-

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sed by using the intraperitoneal route (ip) injections into mice (20±1g). One group of mice (n: 6) was injected i.p. with 3 LD_{50} doses of *H. saulcyi* venom (2.2 mg/kg), diluted in 0.5 ml PSS. The symptoms were monitored for 24 h after venom injection in order to assess the results of experimental envenomation.

Gel electrophoresis of the venom

Polyacrylamide gel electrophoresis of venom sample was carried out following the Laemmli (1950) method. For separation of proteins, a sample was run on NuPAGE® Novex® 4–12 % gradient Bis-Tris gel (Invitrogen Corparation, USA) in MES SDS Running Buffer (Invitrogen: 50 mM MES, 50 mM Tris-HCl, 1 % SDS, 1.025Mm EDTA) using Xcell SureLock Mini Cell (Invitrogen) following standard manufacturer protocol. SeeBlue® Plus2 Pre-Stained Standard (Invitrogen, LC5925) was run in parallel in order to calculate the molecular weights of proteins. Detection of proteins was carried out initially by Coomassie blue staining. The gel was then scanned and molecular weights of the proteins were calculated with Molecular Imaging Software (Kodak MI).

Results

Scorpions and venom

All scorpions were observed as being very aggressive in the course of all keeping milking time. A colorless watery secretion was obtained during capturing and was followed by more viscous milky droplets or ejaculate (mucous accompanied with the venom) during stimulation. After centrifugation of whole venom, the supernatant was of a more viscous form. The protein content of the venom sample was 2.3 mg/ml.

Lethal potency of the scorpion venom

The median lethal dose for *H. saulcyi* scorpion venom was determined in mice. The LD_{50} of the scorpion venom was found to be 0.73 mg/kg by iv injection route.

Assessment of the experimental envenomation after venom injection

When intoxicated ip with 2.1 mg/kg (3 x LD_{50} iv doses) of venom from *H. saulcyi*, the mice showed the following signs of intoxication: Immediately following venom injection, mice showed intense and long-term squeaking (indicating pain), jumping and later restlessness, aggressive behavior, fight, tachypnea, deep dyspnea, weakness, convulsions, paralysis and coma resulting in death. But none of the animals exhibited any hypersalivation or lacrymation symptoms after venom injection by ip route. However, these signs were observed in mice after iv venom injection.

Determination of protein profiles

The protein profiles of *H. saulcyi* scorpion venom were analyzed by NuPAGE® 4–12 % gradient Bis-Tris gel followed by Coomassie blue staining. Proteins of the venom (30 µg) were determined to be between 3 and 188 kDa on electrophoresis on gradient gel as shown in (Fig. 2). Eight different protein bands with molecular masses of 4, 6, 9, 31, 35, 40, 46 and 69 kDa were detected in the venom sample. The proteins in venom secretion were more strongly determined to be 4, 6, 9, 46 kDa than other bands after staining by Coomassie Blue.

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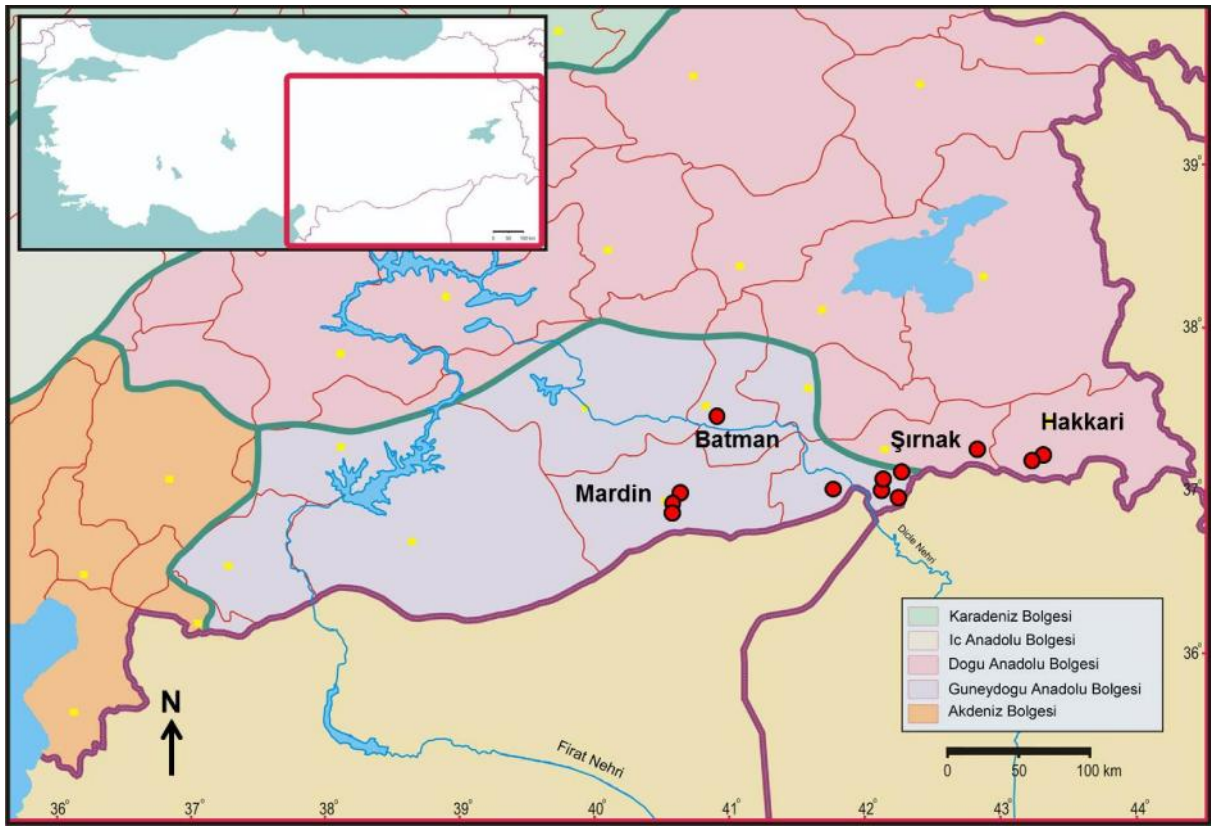

Fig. 1. Distribution (red circles) of *Hottentotta saulcyi* in Southeastern Anatolia region (Mardin, Batman, Şırnak, Hakkâri provinces), Turkey. The scorpions were captured in Mardin

Fig. 2. The proteins of venom (I) of *Hottentotta saulcyi* (A) from Mardin Province were separated by using 4–12 % gradient gel electrophoresis (B). Molecular weight (II): 188 kDa Myosin, 98 kDa Phosphorylase, 62 kDa BSA, 49 kDa Glutamic Dehydrogenase, 38 kDa Alcohol Dehydrogenase, 28 kDa Carbonic Anhydrase, 17 kDa Myoglobin Red, 14 kDa Lysozyme, 6 kDa Aprotinin, 3kDa Insulin, B Chain (SeeBlue® Plus2 Pre-Stain)

Discussion

Scorpions do not harbour any agent capable of causing disease. However, scorpions can cause serious health problems by stinging humans, most of the time, they use their venom to protect themselves (Adıguzel 2010).

Scorpion sting is a life-threatening emergency, especially in children and older individuals who suffer from respiratory and/or cardiovascular diseases. The clinical manifestations of scorpion envenomation are predominantly sympathetically and parasympathetically mediated, depending on the scorpion (Ismail 1995, Boşnak et al. 2009). On the other hand, scorpion species responsible for the stings often are unclear, and this is due to the lack of knowledge of health professionals regarding the scorpion species and the fact that the scorpion is neither seen

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nor identified in most scorpion sting cases (Latifi et al. 1979, Shahbazzadeh et al. 2009). The types of scorpions are categorized as yellow or black scorpion by patients in the southeastern part of Turkey. Therefore in vivo studies are required to be performed to determine health effects and toxicity of scorpion species' venom. Up to now, no data has been found about toxicity and the effects of *H. saulcyi* scorpion venom from Turkey. In our current study, we have determined the lethal potency and health effects in mice and the protein profiles of *H. saulcyi* scorpion venom. To our knowledge, this is the first work on protein profiles and the toxicity of the venom of *H. saulcyi* in Turkey.

Androctonus crassicauda, Mesobuthus phillipsii, H. saulcyi, Compsobuthus matthiesseni of the Buthidae family, *Scorpio maurus* of Scorpionidae and *Calchas birulai* of the Iuridae family have been recorded in Mardin Fauna up to now (Crucitti, Vignoli 2002, Yağmur et al. 2008a, 2008b, Kovařík et al. 2011). *Hottentotta saulcyi* is distributed in Mardin, Batman, Şırnak, and Hakkâri Provinces of the southeastern part of Turkey (Yağmur et al. 2008c) (Fig. 1) and neighbouring countries, Syria, Iran and Iraq (Kovařík et al. 2007).

In southern Anatolia region of Turkey, as in numerous tropical countries, envenomation by scorpion stings is a major public health problem since most of medically important species in scorpion fauna of Turkey are found in this region. In Khuzestan Province of Iran, the prevalence of *H. saulcyi* sting is 3.35 % (Dehghani et al. 2009, Dehghani and Fathi 2012) while no epidemiologic data have been found on *H. saulcyi* scorpion sting in Turkey (Adıguzel 2010). On the other hand, the largest numbers of scorpion sting cases were reported in the Southeastern Anatolia region (30.4%) of Turkey. Mardin Province (18%) has one of the highest incidences of scorpion stings in the Southeastern Anatolia region (Özkan et al. 2008). In Batman, Siirt, Mardin, Şırnak and Hakkâri provinces of the Southeastern Anatolia region of Turkey, epidemiological and clinical studies reported that local and systemic symptoms were seen after scorpion sting (Soker and Haspolat 2000, Boşnak et al. 2009). On the other hand, Al et al. (2009) stated that 120 patients who were older than 16 years old by scorpion stung did not exhibit cardiac dysfunction, myocardial damage and deaths secondary to major systemics envenoming.

During the course of the experimental envenomation, the effects of the venom in mice, its toxicity showed characteristics similar to those described for other medically important scorpion species (*A. crassicauda, M. gibbosus, M. eupeus*, and *L. abdullahbayrami*) in Turkey. In our study, similar autonomic nervous system symptoms (sympathetic signs [tachypnea, restlessness, hyperexcitability and convulsions] and parasympathetic signs [salivation, lacrimation, and weakness]) were observed in envenomed mice. Therefore, *H. saulcyi* could be considered as a potential scorpion responsible for severe envenomation and probably death in humans.

Scorpion venom contains simple proteins with short neurotoxin and low-molecular-weight polypeptides. Additionally, scorpion venom also contains serotonin, which is thought to contribute to the pain associated with scorpion envenomation (Adam and Weiss 1958). Medically important scorpion stings are almost universally characterised by intense local pain. Systemic effects occur in a smaller proportion of scorpion sting depending on various factors (Luca and Meier 1995). In the current work, interestingly, we observed intense and long-term squeaking in envenomed mice according to our experience. To our knowledge, no data have been found on envenomation by scorpions in human. However, squeaking in mice indicated pain, therefore the venom of *H. saulcyi* can be considered to be rich in terms of serotonin.

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The geograpical origin of scorpions have an important role with regard to toxicity of venom. Latifi and Tabatabai (1979) determinated that LD_{50} of *Buthotus saulcyi* (=*H. saulcyi*) scorpion venom from Iran was 0.95 mg/kg while Hassan (1984) stated this to be 1.01 mg/kg in the same country by iv injection route. In our investigation, the median lethal dose of *H. saulcyi* scorpion venoms was found to be 0.73 mg/kg by iv injection route.

Scorpion venoms can be classified into two groups according to their molecular sizes, and long-chain and short-chain neurotoxins. Among these more-studied groups are the short-chain neurotoxins that present 3,000 to 4,400 Da and act on potassium or chloride channels. Long-chain neurotoxins that have 6,500 to 8,500 Da, act mostly on sodium channels (Possani et al. 1999, Possani et al. 2000, Rodriguez de la Vega and Possani 2004, 2005). In our study, we have exhibited the electrophoretic protein pattern of *H. saulcyi* venom to be between 4 and 69 kDa by gradient gel. Four protein bands with molecular masses of 4, 6, 9 and 46 kDa are more strongly detected than other protein bands in the venom sample. Analysis of electrophoresis indicated that *H. saulcyi* scorpion venom possesses both short-chain neurotoxins and long-chain neurotoxins according to the electrophoretic protein patterns.

Conclusion

Hottentotta saulcyi scorpion venom has neurotoxin proteins and lethal potency in mice. Therefore, the stings of *H. saulcyi* scorpion must be considered of risk for humans in the southeastern region, Turkey. Scorpion envenomation in the Southeastern Anatolia region is an increasing public health problem. Even if the scorpion is not captured, each victim should be asked about the sting history. All patients and especially pediatric patients should also be admitted to the hospital.

In further studies, the identification of the scorpion may be considered as a useful clinical and epidemiological tool in determining the incidence and risk of scorpion envenomations. Therefore, the effects of this species in victims should be described and strengthened with the epidemiological and clinical studies in these regions. In addition, the monovalent *A. crassicauda* antivenom has commonly been used in Turkey up to now. Therefore, neutralization experiments should be carried out to test the usefulness of monovalent antivenom on the venom of *H. saulcyi* scorpion.

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