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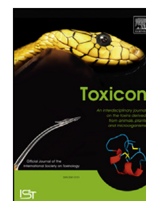

# Camelid antivenom development and potential in vivo neutralization of *Hottentotta saulcyi* scorpion venom

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ABSTRACT

Scorpion envenoming is a serious health problem which can cause a variety of clinical toxic effects. Of the many scorpion species native to Iran, *Hottentotta saulcyi* is important because its venom can produce toxic effects in man. Nowadays, antivenom derived from hyper immune horses is the only effective treatment for sever scorpion stings. Current limitations of immunotherapy urgently require an efficient alternative with high safety, target affinity and more promising venom neutralizing capability. Recently, heavy chain-only antibodies (HC-Abs) found naturally in camelid serum met the above mentioned advantages. In this study, immuno-reactivities of polyclonal antibodies were tested after successful immunization of camel using *H. saulcyi* scorpion crude venom. The lethal potency of scorpion venom in C57BL/6 mice injected intraperitoneally was determined to be 2.7 mg/kg. These results were followed by the efficient neutralization of lethal activity of *H. saulcyi* scorpion venom by injection of antivenom and purified IgG fractions into mice intraperitonelly or intravenously, respectively. HC-Ab camelid antivenom could be considered as a useful serotherapeutics instead of present treatment for scorpion envenomation.

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## 1. Introduction

Scorpion envenoming syndromes are often encountered in numerous tropical and subtropical regions as major medical concerns (Chippaux and Goyffon, 2008). In Iran the largest numbers of scorpion accidents occur mainly in southwestern regions (Pipelzadeh et al., 2007). The toxic effects are of a greater concern in children and the elderly which are either helpless or more prone to adverse effects. Despite many benign scorpion species, a vast number of accidents lead to permanent organ failure or even death (Ismail, 1995; Jalali and Rahim, 2014). The toxicity of envenomation depends on scorpion species, the physiologic reaction of victim and time needed to gain care centers (Saucier, 2004). According to epidemiologic reports, the annual incidence of scorpion accidents in Iran are at least 45,000 cases (Dehghani and Fathi, 2012; Mozaffari et al., 2013) with 19 of these leading to death (Mirshamsi et al., 2011). *Hottentotta saulcyi* or black tailed scorpion is one of the most medically important scorpion species in Iran, Turkey, Syria, and Iraq (Kovařík et al., 2011; Nejati et al., 2014). The venom of this scorpion like those of other *Butdiae* family is composed of heterogeneous mixture of enzymes and considerably toxic polypeptides (Rodriguez de la Vega and Possani, 2005), that are low (4–7 kDa) in molecular masses (Jalali et al., 2005), are associated with neurotoxic effects and have high affinity for voltage gated ion channels (Possani et al., 2000, Possani, 2013). Pharmacokinetic study of scorpion envenoming using radiolabeled toxin has demonstrated high bioavailability (95%) resulting from a nearly complete absorption and distribution of the toxins from the injection site to the peripheral compartments in a short time causing the clinical severity and emergency (Ismail et al., 1994; Krifi et al., 2001). Commercial antivenoms have been used as antidote since 1897 (Chippaux and Goyffon, 1998) and still remain the only life saving option of envenoming induced by scorpion sting. Fabotherapics, $F(ab')_2$-based antivenoms, composed of purified IgG derivatives from hyper-immune horses sera have been used for venom interaction and subsequent elimination of scorpion venom toxic effect (Brown and Landon., 2010; Chippaux, 2010; Latifi and

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Tabatabai, 1979). Several studies indicated that immunotherapy could be medically efficacious in scorpion envenoming when optimized in terms of dose and route of administration (Bon, 2003; Krifi et al., 2001). Successful therapy is aided by speedy administration of antivenom. Recently, camelidae (camels, llamas, alpacas) have been suggested for scorpion antivenom production because like the horse, they produce large volumes of sera and with high neutralizing titer (Cook et al., 2010a). It is more affordable for manufacturers to prepare antivenom from camelid in the tropics where the scorpion envenomation is high. Furthermore, besides heavy and light chain conventional antibodies, *Camelidae* possess “heavy chain only antibodies” (HC-Ab) (Muyldermans, 2001) which are more thermostable (Omidfar et al., 2007), less immunogenic and therefore less likely to induce anaphylactic and serum sickness reactions observed in patients treated with current antivenoms products (Harrison et al., 2006; Herrera et al., 2005). This promising natural antibodies bind with high affinity to their targets of interest particularly those antigens that are unreactive for other immunoglobulin (Muyldermans et al., 2009), appropriate feature to overcome weak immunogenic antigen such as *elapidae* neurotoxin (Pratanaphon et al., 1997). The unique characteristic of variable domain of HC-Abs termed Nanobody (Kolkman and Law, 2010) has speeded up progress in antibody development against various antigens such as toxins (Darvish et al., 2015) enzymes, and even non immunogenic ones (Rahbarizadeh et al., 2011). Recently, several authors which evaluated camel IgG potency for reversal of venom induced toxic effects, suggested that the use of these antibodies may be safer than the conventional treatment (Meddeb-Mouelhia et al., 2003; Cook et al., 2010b). Motivated by these results, we immunized one camel with scorpion crude venom then specific Immunoglobulin (IgG) subclasses including conventional (IgG1) and heavy chain antibodies (IgG2, IgG3) from immune sera were retrieved, purified and characterized. Antigen reactivity of purified polyclonal antibodies was assessed. In consideration of the main concern of antivenom efficacy, in vivo neutralization effectiveness of whole IgG and individual IgG subclasses were evaluated.

## 2. Materials and methods

### 2.1. Venom preparation

The scorpion venom was collected by electrical stimulation of telson. The crude venom was dissolved in sterile double-distilled water and centrifuged at 10,000 × g for 10 min at 4 °C. The precipitate was discarded and the supernatant stored at −20 °C until needed.

### 2.2. HPLC separation of Hottentotta saulcyi venom

500 μg of crude venom was dissolved in 150 μl of solution A (0.0.5% TFA in water) and centrifuged at 10,000 × g for 10 min. The supernatant was then applied to a reverse-phase C18 column (250 × 4.6 mm), and separated on a Knauer HPLC. Proteins were eluted at a flow rate of 1.0 ml/min by a gradient of solution B (0.0.5% TFA in acetonitrile) as follows: 5% B for 5 min, 5%–45% B over 40 min and 45%–60% B over 10 min. The chromatographic run was monitored at 214 and 280 nm, protein fractions were collected, lyophilized, and stored at −20 °C until needed.

### 2.3. Immunization protocol

A male camel (Camelus dromedarius) was used for the antivenom production against scorpion venom. Before the first injection, pre-immune sera were collected and dromedary received subcutaneous doses ranging from 100 μg to 2 mg of scorpion crude venom in physiological saline solution (PSS) at weekly interval. The first injection was performed using equal volume of crude venom and complete Freund's adjuvant and booster injections in incomplete Freund's adjuvant emulsions. After one week of immunization blood sample was taken, serum separated and stored at −20 °C.

### 2.4. IgG subclasses fractionation

IgG subclasses were obtained by differential adsorption on Hitrap-protein A and G (Qiagen) columns as described previously. (Hamers-Casterman et al., 1993). Briefly, 2 ml of immune camel sera were applied onto Protein G column. After washing with 20 mM PBS (pH 7.2), IgG3 and IgG1 successful bound the Hitrap-protein G column, while IgG2 subclass was not adsorbed. The IgG3 was eluted with 0.15 M NaCl, 0.58% acetic acid, pH 3.5. IgG1 fraction was eluted with 0.1 M Glycin-HCL, pH 2.7. The unadsorbed IgG2 fraction was loaded on a protein A column and subsequently eluted by buffer A (pH 4.5). All the purified IgG subclasses were immediately neutralized using 1 M Tris pH 9.0, dialyzed and the absorbance was measured at 280 nm. The purity of IgG fractions was determined on 12% SDS-PAGE under reducing conditions (Sambrook and Russell, 2001). Proteins bands were visualized by staining with Coomassie brilliant blue R-250.

### 2.5. ELISA assessment

After the last boost, purified polyclonal IgG fractions were tested for humoral immune response evaluation by indirect ELISA. Briefly, 96-well Maxisorb plate (Nunc) was coated with crude venom (5 μg per well diluted in 100 μl carbonate buffer pH 9.6). After overnight incubation at 4 °C, residual protein binding sites were blocked with 2% BSA in PBS solution for 1 h at room temperature (RT). After several washing step with PBS-T (PBS 0.1% Tween-20), various amount of IgG fractions were added to duplicated wells and incubated 1 h at RT. To remove non specific antigen–antibody binding, the wells were extensively washed with PBS-T and subsequently bound camel IgGs were detected by rabbit anti-camel antibody (diluted 1:5000, Sigma) and detection of rabbit antiserum was performed with mouse anti-rabbit conjugated to horseradish peroxidase (HRP) (Sigma) diluted 1:3000 in PBS. Finally, enzymatic substrate tetramethylbenzidine (TMB) (sigma) was added. The reaction was stopped after 20 min with 50 μl 2 N sulfuric acid and absorption measured at 450 nm.

### 2.6. In vivo lethality assessment ($LD_{50}$)

The toxic activity of scorpion venom was assessed by preparation of increasing amount of crude venom dissolved in 150 μl PSS and i.p. injection into C57BL/6 mice (18–20 g). Control group received similar volume of PSS without venom. The lethality rate was expressed as median lethal dose ($LD_{50}$) estimated over 24 h after venom injection according to the Probit analysis (Finney, 1971).

### 2.7. In vivo neutralization assays ($ED_{50}$)

The antagonism capacity of polyclonal antibodies were assessed by mixing a constant amount of venom lethal dose (2 $LD_{50}$) and varying amounts of antivenoms or immunoglobulin fractions diluted in PSS, to give a total volume of 150 μl and 200 μl, respectively. The mixtures were pre-incubated at 37 °C for 30 min and injected into groups of four mice. Control groups received 2 $LD_{50}$ of venom pre-incubated with PSS. Antivenom mixtures were injected intraperitoneally whereas IgG fractions were injected into the tail. The neutralizing potency was expressed as median effective dose

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