Short communication

An in vitro comparative study upon the toxic properties of the venoms from Hemiscorpius lepturus, Androctonus crassicauda and Mesobuthus eupeus scorpions

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Abstract

The aim of the present study was to compare the toxic effects of the venoms from Hemiscorpius lepturus (H. lepturus), Androctonus crassicauda (A. crassicauda) and Mesobuthus eupeus (M. eupeus). For this purpose, three in vitro models were employed to compare the toxic effects of various concentrations of the venoms from these three scorpions, namely: hemolytic potential using human RBCs, phospholipase activity using Saubouraud's dextrose agar (SDA) supplemented with 2% egg yolk and lactate dehydrogenase (LDH) enzyme releasing effect using K562 leukemia cell line. In addition, the neutralizing effectiveness of the antivenom against these toxic properties was assessed. The results showed that, unlike the venoms from A. crassicauda and M. eupeus, the venom from H. lepturus produced dose-dependent lysis of human RBCs and showed phospholipase activity. However, all the tested venoms showed variable degrees of LDH releasing properties. The venom from H. lepturus had highest and the venom from M. eupeus had the lowest LDH releasing effect. The antivenom effectively inhibited all the tested toxicities. In conclusion, these results suggest that the venoms from the studied scorpions have variable toxic properties, which may explain the underlying reason for the differences in their clinical manifestations. In addition, the antivenom was effective in neutralizing all the tested toxic effects.

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1. Introduction

In Khuzestan, a south-western province of Iran, there are two families of scorpions belonging to Buthidae and Scorpionidae families. Of the Buthidae family Androctonus crassicauda (A. crassicauda) and Mesobuthus eupeus (M. eupeus) and from Scorpionidae (recently renamed Lithodidae) family Hemiscorpius lepturus (H. lepturus) are toxic to human. The frequency of envenoming by these scorpions stings varies throughout the year. The highest incidence is during late March and extends to late October with a peak in June, coinciding with scorpion reproduction (Pipelzadeh et al., 2007). Scorpionism due to H. lepturus constitutes a medical emergency due to the fact that it is one of the most toxic scorpion species in the world. Although the incidence of scorpionism due to H. lepturus accounts for 10–15% of all annually reported scorpion envenomation in
this province, yet almost 90% of scorpion-associated deaths are attributed to this scorpion (Pipelzadeh et al., 2007). The toxic manifestations due to this scorpion is greatly different from those reported for the other two scorpions, in that while the victims experience no pain at the sting site, therefore refer late for medical care, these patients develop delayed hematuria which may progress into severe renal and cardiovascular failure. While stings due to A. crassicauda and M. eueps produce severe pain and patients refer early for medical care and show no signs of hematuria nor other serious toxic effects.

The majority of envenoming scorpion species belonging to Buthidae family, known as “Old World” scorpions, contain neurotoxins with high affinity to muscle and nerve voltage-gated sodium channels. It is due to this property that, when present at high concentrations in the circulation, produce variable toxic effects on nervous, cardiovascular and respiratory systems. Treatment of envenomation due A. crassicauda and M. eueps is usually symptomatic. In M. eueps stung cases relief of pain, with local administration of lidocaine, is the only clinical management. While in A. crassicauda envenomed cases, which produces a mixed sympathetic and parasympathetic overstimulation, in addition to administration of polyvalent antivenom other supportive measures are usually undertaken. However, up to now there is no generally agreed protocol for treatment following envenomation with H. lepturus scorpion other than administration of polyvalent antivenom at admission and follow up of patients who are normally hospitalized, kept under close observation and treated symptomatically (Radmanesh, 1990). The responses among H. lepturus envenomated patients vary widely and once they progress to severe toxicities not much can be done (Ahmadi-zadeh and Razi Jallali, 2006; Pipelzadeh et al., 2006).

Elucidating the toxic properties of venoms can pave the way to better understanding of the nature of the toxicity and can aid in improvement of their clinical management.

No previous study has been specifically designed to compare and characterize the nature of toxicity of these venoms under similar experimental conditions. The aim of the present study was, therefore, to compare toxic properties these venoms in terms of their hemolytic (using human washed RBCs) (Pipelzadeh et al., 2006) cytotoxic, by assessing the extent of lactate dehydrogenase (LDH) leakage on K562 leukemia cell line (Decker and Lohmann-Matthes, 1988) and phospholipase activity, using Saubouraud’s dextrose agar (SDA) media supplemented with egg yolk (Price et al., 1982). In addition the effectiveness of the available polyvalent antivenom in neutralizing the toxic effects of these venoms was undertaken.

2. Materials and methods

2.1. Preparation of stock solution of the venoms

The venoms of H. lepturus, A. crassicauda and M. eueps were collected following milking by electrical stimulation of the telsons of the captured scorpion, freeze-dried and stored under refrigeration conditions. On the day of the experiments, stock solutions of the venoms were prepared by dissolving the lyophilized venom in normal saline at 1mg/ml concentration.

2.2. Comparison of hemolytic potential of the venoms from H. lepturus, M. eueps and A. crassicauda

Blood samples from three healthy non-smoking volunteers were collected in heparinized test tubes, washed three times with normal saline and subjected to centrifugation at 3000 rpm for 2 min between each washing, from which 2% RBC suspension was prepared.

Ten μl of 10× serially diluted venom solutions of the three lyophilized venoms were added separately to each of the blood samples in Appendorf tubes containing 1 ml of 2% RBC suspension and incubated at 37 °C for 24 h to give final venom concentration of 10, 1 and 0.1 μg/ml. The tubes were centrifuged in Appendorf centrifuge (Hettich Mikro 22R, Zentrifugen, Germany), for 3 min at 3000 rpm and the degree of RBC lysis was assessed by measurement of the optical density of the supernatant at 405 nm wavelength. The results were compared with negative none-treated control and a positive control (100% lysis) in which normal saline was substituted with distilled water. The % of lysis was calculated using the following relationship:

\[
\text{% of RBCs lysis} = 100 - \frac{(\text{OD}_{\text{max}} - \text{OD}_n) - (\text{OD}_{\text{v}} - \text{OD}_n)}{(\text{OD}_{\text{max}} - \text{OD}_n)}\times 100
\]

where ODmax, ODv and ODn are the ELISA optical density readings of positive control (distilled water-treated, 100% lysis), venom exposed and none-treated RBC respectively. The final volumes of the negative and positive controls were adjusted by addition of 10 μl of normal saline.

2.3. Assessment of antivenom efficacy in neutralizing the hemolytic action of the venom from H. lepturus

The effectiveness of the polyvalent antivenom, prepared by Razi Institute of Iran, raised against the venoms from 6 endemic Iranian scorpions (A. crassicauda, B. Saulcyi, B. schach, O. doriae, M. eueps and H. lepturus) (Jalali et al., 2011a,b), in inhibiting lysis of RBCs that was caused by 10 μg H. lepturus was assessed in the presence of increasing concentrations (10, 30, 60 and 100 μg/ml of the venom and the % of lysis was assessed after 24 h of incubation at 37 °C with 2% washed RBCs. The results were compared with venom- and negative non-treated-RBC suspension controls respectively. The final volumes of the negative and positive controls were adjusted by addition of similar volumes of normal saline. The % of inhibition of lysis was calculated using the following relationship:

\[
\text{% of inhibition of lysis} = \frac{(\text{OD}_{\text{vav}} - \text{OD}_n) - (\text{OD}_{\text{v}} - \text{OD}_n)}{(\text{OD}_{\text{v}} - \text{OD}_n)} \times 100
\]

where ODvav, ODv and ODn are the ELISA measured optical densities of venom-antivenom mixture, venom alone and none-treated negative control respectively.
2.4. Comparison of the phospholipase activity of the venoms from H. lepturus, M. euepeus and A. crassicauda and evaluation of effectiveness of the polyvalent antivenom

Phospholipase activity was assessed according to the method suggested by Price et al. which was originally used for detection of phospholipase released by C. albicans. The test media contained 65 g SDA, 58.4 g NaCl and 5.5 g CaCl₂. The medium was dissolved in 980 ml of distilled water and sterilized at 121 °C for 12 min. Egg yolk was centrifuged at 5000 g for 30 min. The supernatant was removed and added to cooled medium (45–50 °C) at concentration of 2%, mixed, dispersed in plates and kept under refrigeration conditions (4 °C) until used.

Various volumes of venom solutions (10, 25 and 50 μl) were inoculated onto the media and incubated at 37 °C. The diameter of the zone formed (reflecting the extent of presence phospholipase activity) was measured after 4 days of incubation.

In order to assess the inhibitory effectiveness of the available polyvalent antivenom on the phospholipase activity of the venoms, 10, 25 and 50 μl of the venom solutions was premixed for 15 min with 50 μl of the polyvalent antivenom and then inoculated in the media and assessed similarly.

2.5. Preparation of cell culture

K562 leukemia cell line (Pasteur Institute, Tehran) were grown in DMEM (Dulbecco’s Modified Eagle’s Medium) in 75 ml cell culture flask, supplemented with 10 % fetal bovine serum, 1% penicillin-streptomycin solution in a humidified atmosphere of 5% CO₂, 95% air at 35 °C. The passage was maintained between 20 and 25. For experimental purposes, cells were transferred in 96-well plates and used at optimal cell count of 5000 cells/50 μl. Cells were allowed to attach for 24 h before exposed to the venom solutions.

2.6. LDH leakage assay for comparison of the cytotoxic potential of the venoms from H. lepturus, M. euepeus and A. crassicauda on K562 leukemia cell culture and assessment of antivenom effectiveness

For cytotoxicity estimation, the procedure for measurement of LDH was followed as directed manufacturer’s instruction (Cytotoxicity Detection Kit plus- cat no. 04 744 926 001, Roche). In brief, triplicates of 25 μl of double-diluted venom solutions (giving a final concentration of 8.8, 4.4 and 2.2 μg/ml) were transferred into wells that contained 50 μl of cell culture (5000/50 μl) plus 75 μl of RPM assay media. The reaction was initiated by addition of 100 μl of freshly prepared reaction mixture and incubated at 37 °C. The reaction was stopped by addition of 100 μl of stopper solution after 4 h of incubation. Control values for background (RPM assay solution media only) and spontaneous (non-treated cell culture in assay media), were subtracted from the readings. While for positive control, the cells were exposed to 5 μl lysis solution and undergone similar treatment (considered as maximum leakage). The % of LDH activities of various venom solutions were measured using ELISA reader at 492 nm (Tecan-Sunrise Touchscreen, Austria) using the following equation:

\[
\% \text{ of LDH activity} = \frac{\text{ODv} - \left[ \text{ODbg} + \text{ODspont} \right]}{\text{ODmax} - \left[ \text{ODbg} + \text{ODspont} \right]} \times 100
\]

where ODv, ODbg, ODspont and ODmax are the measured ELISA readings of venom-treated at different concentrations, background (assay solution), spontaneous release from cell culture in RPM media and maximum readings measured following exposure to lysis solution respectively.

In order to assess the inhibitory effectiveness of the antivenom on LDH activity induced by the venoms, 25 μl of the double diluted venom solutions were premixed with 25 μl of antivenom, replacing part of assay media, for 15 min, and transferred into wells that contained 50 μl of cell culture (5000/50 μl) and 50 μl of RPM assay media. The procedure as above was repeated. The % of inhibition was calculated using the following relationship:

\[
\% \text{ of inhibition of LDH activity} = \frac{\text{ODexp} - \left( \text{ODbg} + \text{ODspont} \right)}{\text{ODv} - \left( \text{ODbg} + \text{ODspont} \right)} \times 100
\]

3. Results

3.1. Hemolytic properties of the venoms from H. lepturus, M. euepeus and A. crassicauda scorpions

After 24 h of incubation at 37 °C with various concentrations of scorpion venoms, only those exposed to H. lepturus venom produced lysis of washed human RBCs. The degree of lysis of RBCs was dose-dependent. The highest % of lysis was observed with 10 μg/ml which produced 80% lysis of RBCs after 24 h of incubation relative to the positive control group exposed to distilled water (Fig. 1).

3.2. Effectiveness of the polyvalent antivenom in neutralizing the hemolytic effect of H. lepturus venom

The antivenom was found to cause a concentration-dependent inhibition of lysis of human RBCs induced by
10 μg/ml of *H. lepturus* scorpion venom. Complete inhibition of lysis was achieved with 100 μl/ml of the antivenom following premixing with the venom for 15 min (Fig. 2).

3.3. Phospholipase activity of the venoms from *H. lepturus*, *M. eupeus* and *A. crassicauda* scorpions and the inhibitory effectiveness of antivenom

Similar to the previous finding, only the venom from *H. lepturus* showed phospholipase activity. This effect was found to be concentration-dependent producing 0.8, 1.5 and 2.5 cm of deposition zone around the inoculation point with 10, 25 and 50 μg of the venom, respectively (Fig. 3).

Premixing of varying volumes of the polyvalent antivenom (10, 25 and 50 μl) with 50 μg of the venom produced volume of antivenom-dependent reduction in phospholipase activity: the zone around the inoculation was 2/C60.15, 1/C60.1 and 0/C60.1 cm with 10, 25 and 50 μl of antivenom respectively. A typical zone of phospholipase activity produced by 50 μg of the venom and its complete inhibition by 50 μl antivenom is shown in Fig. 3.

3.4. LDH releasing properties of the venoms from *H. lepturus*, *M. eupeus* and *A. crassicauda* scorpions and neutralizing effectiveness of the polyvalent antivenom

Unlike the results from the previous two experiments, the venoms from all the three scorpions produced LDH releasing effects on K562 leukemia cells. However, the extent of this activity varied with the type of the tested venom. At equal concentrations, the venom from *H. lepturus* produced significantly higher LDH activity than the other venoms (*P < 0.5*) (Table 1). The venom from *H. lepturus*, at 8.8 μg/ml of cell culture mixture produced 98% LDH releasing activity relative to high control, while the venoms from *A. crassicauda* and *M. eupeus*, at similar concentration, produced 80 and 43% LDH activity respectively (*P < 0.05*). The descending order of LDH activity was found to be in the following order: *H. lepturus* > *A. crassicauda* > *M. eupeus*.

On the other hand, the polyvalent antivenom (25 μl) was most effective in inhibiting *M. eupeus* induced LDH release. The descending order of effectiveness of the antivenom was found to be *M. eupeus* > *A. crassicauda* > *H. lepturus* (Table 1). Complete inhibition of LDH activity was achieved for 2.2 μg/ml of *M. eupeus* venom when premixed with 25 μl antivenom while the same quantity of antivenom reduced *H. lepturus* venom LDH activity by 64% (Table 1).

4. Discussion

The results from this study showed that the RBCs were more sensitive to hemolytic action of *H. lepturus* venom with production of 80% lysis of 2% of RBC and 98% of LDH releasing activity of at 10 and 8.8 μg/ml respectively compared with its phospholipase activity, which occurred at a higher concentration of 50 μg. Although the sensitivity and specificity of the experimental protocols in detecting the toxic effects under investigation were not same, these observations suggest that the venom from *H. lepturus* has concentration-dependent toxic effects on different body tissues. These findings come in agreement with previous in vivo biochemical and organ toxicity studies on venoms from *H. lepturus* and other scorpion species. Among the biochemical changes reported is the increase in serum LDH level following *H. lepturus* (Ahmadizadeh and Razi Jallali, 2006), *L. quinquestriatus* (Meki and El-Deenn, 2002)
species have different toxic properties. Observations suggest that the venoms from various scorpion species, such as Hadrurus concolorus, Pandinus imperator, Palmamneus gravimanus, and Tityus serrulatus, are reported with a concentration-dependent manner over 24 h and 4 days of incubation. However, in support of this observation, the present study has demonstrated that hemolysis of RBCs as a side effect that develops 2 days after venom injection. This syndrome, in addition to increase in serum LDH activity following experimental envenomation by H. lepturus venom and other active constituents may be involved in the dermonecrotic effects reported for other scorpion venoms.

Previous clinical studies showed that the signs and symptoms of hematuria, the most serious and common side effect that develops 2–3 days after H. lepturus envenomation (Radmanesh, 1990). The reason for this is not clear. However, in support of this observation, the present in vitro study has demonstrated that hemolysis of RBCs as well as phospholipase activity progressed gradually in a concentration-dependent manner over 24 h and 4 days of incubation respectively. In addition, a recent study showed a significant increase in serum TNF-α among patients stung with H. lepturus compared with M. eupeus venom (Jalali et al., 2011a,b). These observations suggest that the hemolytic activities of this venom are mediated by both direct and indirect actions that lead to production of a cascade of biochemical and immunological processes. The exact mechanism that leads to hemolysis of RBCs requires more detailed studies.

All the venoms from the three studied scorpions showed different concentration-dependent LDH releasing potencies. This observation is supported by a previous in vivo study in which a significant increase in serum LDH enzyme following experimental envenomation by H. lepturus (Ahmadizadeh and Razi Jallali, 2006). Increase in LDH level is associated with necrosis and thus seems to have an important role in the dermonecrotic effects reported following H. lepturus sting (Radmanesh, 1998). Renal injury and necrosis, a characteristic feature of H. lepturus envenomation with detrimental clinical outcome, can develop following obstruction of renal tubules by products of lysed RBC. Although we can not rule out direct nephrotoxic effects, more recent studies demonstrated that patients stung with this dangerous scorpion show manifestations of hemolytic uremic syndrome (Valavi and Alemzadeh Ansari, 2008). This syndrome, in addition to increase in serum LDH level which is largely derived from ischemic or necrotic tissue cells, (Moake, 2002) is characterized by thrombocytopenia, acute renal failure and presence of fragmented erythrocytes and buff cells on the blood smear. Endothelial cell injury appears to be the primary event in the pathogenesis of this condition and is the main cause of acute renal failure in children (Valavi and Alemzadeh Ansari, 2008).

In this study, we tested the neutralizing effectiveness of the antivenom. The results showed that the antivenom was effective in neutralizing all these toxic effects. However, the neutralizing concentration of the antivenom needed was directly related to the potency of the venoms in producing these toxic effects. The antivenom, on ratio basis, was least effective in neutralizing lytic action of RBCs and most effective against phospholipase activity. While the venom: antivenom ratio needed to neutralize the phospholipase activity was 1:1, 50% of LDH releasing effect was reduced at 1:3, while 50 and 100% inhibition of hemolysis of RBCs was obtained at 1:3 and 1:10 ratios respectively. These findings suggest that the phospholipase activity is partially responsible for the hemolytic activity produced by H. lepturus venom and other active constituents may be involved (Borchani et al., 2011). Furthermore, these observations suggest when treating patients envenomed by H. lepturus, we need to give a dose that is a least 10 fold higher than the amount of the venom in circulation. However, the optimum dose and route of administration of the antivenom need to be assessed further under in vivo conditions.

Despite its broad range of serious toxic manifestations, no pain is sensed following envenomation with H. lepturus scorpion (Radmanesh, 1998). The reason for this discrepancy is not clearly known. However, it is generally accepted that the mechanism of sensation of pain is mediated by release of substances that act directly on nerve endings, or lower the firing threshold for pain sensation, which include potassium, acetylcholine, histamine, serotone, prostaglandins, and bradykinine (Dray, 1995). One or combinations of the following possible reasons may be proposed: Firstly, the sting of H. lepturus scorpion is very small (<1 mm) relative to both M. eupeus and A. crassicauda (>3 mm) in length. It is possible that the injected venom does not penetrate the underlying dermal layers rapidly to activate the pain sensing nerves. Secondly, the venom from H. lepturus, while seemingly lacking alpha toxins commonly present in other scorpion venoms, has strong enzymatic activity, containing phospholipase, as shown by the present findings, as well as gelatinolytic, caseinolytic, hyaluronidase (Seyedian et al., 2010) and,

Table 1

<table>
<thead>
<tr>
<th>Scorpion</th>
<th>% of LDH activity of various concentrations (µg/ml) of the venom in the absence of the antivenom</th>
<th>% of LDH activity of various concentrations of the venom following 15 min premixing with 25 µl of antivenom</th>
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<tr>
<td>H. lepturus</td>
<td>98 ± 2&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt; 49 ± 1&lt;sup&gt;a&lt;/sup&gt; 28 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44 ± 0.9&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt; 19 ± 0.3&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt; 10 ± 0.2&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>A. crassicauda</td>
<td>80 ± 2 43 ± 1 21 ± 0.5</td>
<td>33.7 ± 0.1&lt;sup&gt;b&lt;/sup&gt; 9 ± 0.2&lt;sup&gt;b&lt;/sup&gt; 6 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>M. eupeus</td>
<td>43 ± 1 35 ± 1 12 ± 0.5</td>
<td>12.3 ± 0.4&lt;sup&gt;b&lt;/sup&gt; 6.7 ± 0.1&lt;sup&gt;b&lt;/sup&gt; 0 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup> <sup>b</sup> 0.05 between H. lepturus and both A. crassicauda and M. eupeus.

<sup>b</sup> P < 0.05 LDH activity in the presence and absence of antivenom.
more recently, sphingomyelinase D-like enzyme named heminecrolysin (Borchani et al., 2011). All of these enzymes have the capacity to cause necrosis of pain sensing tissues before they are activated. Thirdly, this venom caused the release of morphine-like substances that cause inhibition of pain sensation. Fourthly, it has a tetrodotoxin-like component that inhibits activation of ion channels that are responsible for the release of pain mediators (Narahashi et al., 1964). Prudently, further studies on the exact mechanism(s) that mediate this effect need to be assessed in separate studies.

What is the relevance of these findings in clinical practice? Although conclusions from the results obtained from these in vitro conditions need to be carefully guarded, it is generally accepted that improvement of therapy against envenomation arising from different venomous animals, depends upon how much we know about the toxic properties of the venom under investigation both experimental and clinical settings. The results of this in vitro toxicological study were found to be positively correlated with the clinical picture of more severe signs and symptoms of envenomation arising from H. lep- turus. Unlike the venoms from M. eupeus and A. crassicauda, the venom from H. lep- turus has hemolytic and phospholipase activity and is significantly more cytotoxic. The results showed the experimental methods selected were useful in terms of their simplicity, rapidity and more importantly, spared the use of animals. The antivenom was found effective in neutralizing all the studied toxic effects and is a suitable agent in treat- ment of envenomation, especially against H. lep- turus scorpion. Therefore, it seems that, under clinical settings, the antivenom is an effective treatment modality, provided the patients refer early for medical care and receive a dose that is at least 10 fold of the venom in circulation.

This in vitro study is merely a small step forwards not only it facilitates better understanding of the nature of toxicity but also aids in finding alternative in vitro methods that may be used in preliminary characterizing the toxicity of venoms and assessment of effectiveness of the antivenom. It also helps to implementation of the principle of reduce, refine and replace. Prudently other studies similar to those carried out on the venom from A. crassicauda (Caliskan et al., 2006) designed for characterization of venom components from this dangerous scorpion need to be carried out and the components that are responsible for these activities need to be identified.

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Conflict of interest statement

All the authors have no conflict of interest to disclose.

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