

RESEARCH ARTICLE

Serum TNF- α levels reflect the clinical severity of envenomation following a *Hemiscorpius lepturus* sting

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ABSTRACT. *Hemiscorpius lepturus* (*H. lepturus*), found in south-western areas of Iran and south of Iraq, is considered to be the most dangerous scorpion in the region, and poses a significant risk to the health of the indigenous population due to the unique, clinical manifestations associated with its sting. In the present study, 36 patients from the Khuzestan province in the southwest of Iran, displaying varying degrees of envenomation following an *H. lepturus* scorpion sting, were admitted to hospital. Serum levels of interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor- α (TNF- α) were measured using double-ligand, enzyme-linked, immunosorbent assay (ELISA) kits, and were compared with 30 healthy controls and ten age-matched patients stung by the *Mesobuthus eupeus* (*M. eupeus*) scorpion, a less dangerous species that produces primarily neuro-toxic manifestations. Blood samples from *M. eupeus* and *H. lepturus* victims were taken on admission, and from *H. lepturus*-stung patients six hours after serotherapy with multivalent anti-venom. When compared to healthy volunteers, with the exception of TNF- α , significantly higher serum cytokine levels were measured in patients following *M. eupeus* envenomation. However, all three groups of *H. lepturus*-stung patients showed significantly, and in a severity-related manner, higher mean values for all the interleukins that were measured, including TNF- α , when compared with *M. eupeus*-stung cases. Six hours after serotherapy, there was a greater reduction in cytokine and TNF- α levels in patients classed as having mild symptoms, in comparison with patients classed as having moderate to severe symptoms. The results of the present study suggest that, unlike *M. eupeus*, the toxic manifestations observed following being stung by *H. lepturus* are associated with increased serum TNF- α levels and correlate positively with the clinical severity of the symptoms. Furthermore, serotherapy is only effective when administered to mild cases of *H. lepturus* scorpion envenomation.

Key words: *Hemiscorpius lepturus*, *Mesobuthus eupeus*, TNF- α , IL-1, IL-6, IL-8

Hemiscorpius lepturus (*H. lepturus*), is found in the south western areas of Iran, and is considered to be the scorpion that is the most dangerous to human health in this part of the world [1]. The signs and symptoms of stings from this species are usually more severe in children [2-4]. The clinical symptoms following *H. lepturus* envenomation are unique. They differ considerably from those caused by stings from other types of scorpion, and result in renal, hepatic, haematological disorders and necrotic skin reactions [5]. These effects are believed to be due to its cytotoxic nature [1-4]. Patients stung by the *H. lepturus* scorpion do not experience pain, but can exhibit signs and symptoms involving the central and autonomic nervous as well as the respiratory and cardiovascular systems, which can lead to death [4]. On the other hand, patients stung by

Mesobuthous eupeus (*M. eupeus*), similar to other species of the Buthidae family, primarily show signs and symptoms of autonomic nervous system over-activity, and experience severe pain at the sting site.

Analysis of scorpion venom from different species has shown that they are composed of a mixture of many toxic proteins and enzymes with diverse and complex pharmacological effects. Patients stung by scorpions such as *Tityus serrulatus* and *Tityus discrepans* who develop systemic inflammatory response syndrome, also show an imbalance in cytokines levels. This is believed to play a major role in the pathogenesis of envenomation [6-8].

The major, pro-inflammatory cytokines linked to severely scorpion-envenomed patients are TNF- α , IL-1, and IL-6 [8]. Furthermore, it has previously been suggested that

there is a close relationship between IL-6 levels and mortality amongst seriously ill patients who exhibit signs of respiratory distress syndrome and multiple organ failure [9-11]. Moreover, the cell- and antibody-mediated immune activity is dependent on IL-8 [12]. Activated macrophages are known to release a broad spectrum of cytokines such as IL-1 and TNF- α [13, 14]. Furthermore, these cytokines induce the production of other pro-inflammatory cytokines such as IL-8 from local fibroblasts and endothelial cells at the sting site [15, 16].

Although previous studies have shown a close, positive correlation between the serum IL-1 and IL-6 levels and the severity of envenomation [17-19], there are no studies that aim to study specifically the changes in cytokine levels in patients envenomed with *H. lepturus*. The aims of the present study were three-fold; firstly, to determine the serum levels of IL-1, IL-6, IL-8 and TNF- α following *H. lepturus* envenomation among patients with different degrees of clinical severity; secondly, to compare the levels of these cytokines with those patient envenomed by *M. eupeus*, and thirdly, to assess the effectiveness of anti-venom therapy in reducing serum cytokine levels of following envenomation.

DONORS AND METHODS

Patients

The present study included 46 (36 *H. lepturus* and 10 *M. eupeus*), age-matched, envenomed patients, who were admitted to the Abozar Jundishapur University hospital in Ahvaz during the summer of 2008. All patients had suffered a scorpion sting; captured scorpions were identified by trained health-care personnel. Control values for the cytokines measured were taken from 30, healthy, age-matched subjects. The *H. lepturus*-envenomed patients were grouped into three classes according to the severity of their symptoms [4], mild (patients with fever and vomiting); moderate, (included patients who, in addition, presented with at least two of following symptoms: hypertension, tachycardia, psychomotor agitation, haematuria, apparent skin manifestation at the sting site), and blood cell disorders (thrombocytopenia, haemoglobinuria); and severe (who, in addition to the signs and symptoms seen in the moderate cases, presented with at least two of the following symptoms): seizure, coma, pulmonary oedema, haematuria, cardiac failure, apparent renal failure such as proteinuria and oliguria, severe skin manifestations, reduction in haemoglobin level below 10 g/dL, and blood cell disorders [4]. This grading of severity is based on previous investigations, and guidelines written by expert physicians in the area. The second group consisted of patients who had been stung by another dangerous scorpion found in Iran, *M. eupeus*, and were matched for age.

All patients, regardless of the type of scorpion involved, were injected IM with two, 5ml, multivalent, anti-venom injections on admission, and were hospitalized under supervision for eight or 48 h following an *M. eupeus* and *H. lepturus* sting, respectively. The polyvalent anti-venom, prepared by the Razi Institute in Karaj, Iran, acts against the six of the most dangerous scorpions in Iran, including *H. lepturus* and *M. eupeus* [20]. This treatment was

administered in accordance with the Iranian treatment protocol for victims stung by the *H. lepturus* scorpion. The protocol of the study was approved by the Ethics Committee of Jundishapur University. All persons participating in this study provided informed, written consent.

Blood collection

Blood samples, from all patients, were taken immediately after admission and 6 h later, and from *H. lepturus*-envenomed patients following two, IM injections of anti-venom. Blood was collected into sterile syringes, transferred to test tubes containing 3.8% sodium citrate and centrifuged at 4,000 g for 10-15min at room temperature. The plasma was stored at -20°C until use.

Cytokine assays

The serum cytokines levels (IL-1, IL-6, IL-8 and TNF- α), of all samples taken from patients and from healthy, non-smoking volunteers, were determined using a double-ligand ELISA kit (Amersham Bioscience, USA), according to the manufacturer's instructions. The samples from scorpion-stung patients were taken at admission and 6 h following administration of anti-venom from *H. lepturus*-stung patients. The concentrations of cytokines were extrapolated from standard curves and expressed as pg of cytokines/mL serum. Patients with a previous history of chronic inflammatory conditions such as arthritis, asthma or cardiac diseases were excluded from this study.

Statistical analysis

Results were expressed as mean \pm SEM. The statistical significance of differences within groups was analyzed using Student's t-test. Variation among groups was analyzed using the Tukey-Kramer rank correlation coefficient. Data were considered significant statistically when $p < 0.05$.

RESULTS

The levels of cytokines found in *M. eupeus*-envenomed patients were significantly increased relative to their corresponding values among healthy volunteers. The highest and lowest significant increases were observed for IL-1 and IL-8, producing respectively a 10-fold ($p < 0.001$) and 84.7% ($p < 0.01$) increase relative to the control healthy group (table 1). While there was a non-significant (16.2%) increase in the levels of TNF- α , the relative changes in the serum cytokine and TNF- α levels in *M. eupeus*-stung patients were significantly smaller in the serum of patients stung by *H. lepturus*. In patients severely envenomed by *H. lepturus*, the maximum increase, relative to *M. eupeus*-stung patients, recorded for TNF- α was 148.5% ($p < 0.001$), and the levels of IL-6 and IL-8 increased by 106 and 105 %, ($p < 0.001$), respectively (table 1). In mildly *H. lepturus*-envenomed patients, the smallest increase was recorded for IL-6 (16.8%). The increases in remaining cytokine and TNF- α levels were similar and ranged from 24 to 30% ($p < 0.01$) (table 1). Overall, there was a close correlation between the severity of envenomation and the levels of TNF- α (table 1).

Administration of anti-venom significantly change serum cytokine levels in *H. lepturus*-envenomed patients. More

Table 1
Comparison of the serum cytokines levels (pg/mL) among victims stung by *M. eupeus* and *H. Lepturus*, and their levels 6 hr after serotherapy among *H. lepturus*-stung patients in comparison with control, healthy, age-matched volunteers.

Cytokine	Healthy control	<i>M. eupeus</i> (% of controls)	<i>H. lepturus</i> on admission (% of <i>M. eupeus</i>)			<i>H. lepturus</i> 6 hr after serotherapy (% of before serotherapy)		
			Mild	Moderate	Severe	Mild	Moderate	Severe
IL-1	2.32 \pm 0.2	26.8 ^{***} \pm 1 (+1056)	34.6 \pm 1.1 (+29)	50.8 \pm 0.9 (+89)	55.3 \pm 1.2 (+106)	30.8 \pm 2.1 (-11)	47.4 \pm 0.8 (-6.7)	51.9 \pm 1.1 (-2.5)
IL-6	14 \pm 0.15	35.9 ^{**} \pm 0.8 (+151)	41.8 ^{***} \pm 0.9(+16.6)	61.7 ^{***} \pm 0.90(+72)	73.9 ^{***} \pm 0.9 (+105)	34.9 ^c \pm 1 (-16.5)	56.4 ^b \pm 1 (-8.6)	70.5 \pm 1.2 (-4.7)
IL-8	14.9 \pm 0.2	27.5 ^{**} \pm 0.9 (+84.7)	35.8 ^{**} \pm 0.7 (+30)	51 ^{***} \pm 0.77 (+86)	64.7 ^{***} \pm 1.2 (+134)	31.2 ^b \pm 0.8 (-13)	46.6 ^b \pm 0.6(-8.5)	54.9 \pm 1.3 (-15)
TNF- α	22.4 \pm 0.24	26 \pm 1 (+16.2)	32.5 [*] \pm 0.7 (+24)	59.9 ^{**} \pm 1.6 (+130)	64.7 ^{***} \pm 1.2(+148.5)	28.4 \pm 0.8(-12.5)	61.4 \pm 1.6 (-2.5)	61.3 \pm 1.1 (-5.2)

* p<0.05, ** p<0.01, *** p<0.001 between control *M. eupeus* and *H. lepturus* stung patients; ^a p<0.05, ^b p<0.01, ^c p<0.001 between before and 6 h after serotherapy

mildly envenomed cases responded with a reduction in IL-1 and IL-6 levels of between 11% and 16% respectively (p<0.001), and, following anti-venom therapy, the levels of IL-8 and TNF- α among mildly *H. lepturus*-envenomed patients were also reduced significantly (p<0.01) (table 1). There was a general trend for cytokine levels to decrease following anti-venom therapy among all of the patients stung by *H. lepturus*, however, the greater the severity of envenomation, the less effective was the anti-venom therapy. The lowest reduction was in IL-1, which showed only a modest, 2.5%, non-significant reduction compared with a 12.5% reduction in mildly envenomed cases (table 1). A summary of the demographic characteristics of both patient groups stung by *H. lepturus* and those stung by *M. eupeus* are presented in table 2, and clearly demonstrate the differences in the signs and symptoms, outcome and clinical management of these patients (table 2). These findings further support the notion that cytokine levels seem to be related to observed toxic manifestations. For ethical and legal reasons, we could not measure the cytokine levels in patients not administered the anti-venom since, regardless of the type of scorpion involved, once the diagnosis of scorpion sting is established, physicians are obliged to administer the anti-venom.

DISCUSSION

A sting from *M. eupeus* produces severe pain, and is usually treated symptomatically, and with serotherapy. The majority of patients are discharged early, with no serious, post-sting complications. However, patients stung by *H. lepturus* do not report pain and usually refer late with haematuria, which may progress to severe renal complications leading to shock and death, especially if the symptoms are severe. In the present study, we measured the serum IL-1, IL-6, IL-8 and TNF- α levels for the first time in patients who had been stung by *H. lepturus*. The levels of these cytokines were compared with those measured for patients stung by *M. eupeus*. Serum samples were taken once, on admission, for *M. eupeus*-stung patients, and twice for patients stung by *H. lepturus*. The first was taken immediately on admission and the second 6 hr after anti-venom therapy with the polyvalent anti-venom available. The serum cytokine levels of both groups were compared with age- and sex-matched, healthy volunteers. The results showed that, with the exception of TNF- α , there was a significant increase in the cytokines among the *M. eupeus* patients. However, the extent of the increase in serum cytokines, including TNF- α , was significantly greater, and was related to the severity of envenomation, among patients stung by *H. lepturus*. Furthermore, anti-venom therapy was partially effective in reducing the cytokine levels only in mild cases of envenomation.

Several previous studies have demonstrated an association between proinflammatory cytokines and the pathological responses resembling systemic inflammatory response syndrome in human scorpion-envenomation [17-19]. However, the types and levels of cytokines varied with the species of scorpion under investigation. In the present study, we observed the same phenomenon, in that all the cytokines studied, namely IL-1, IL-6, IL-8 and TNF- α , were increased. This correlated with the severity

of envenomation by *H. lepturus*, while in patients stung by *M. eupeus*, the extent of this increase was less, and was limited to IL-1, IL-6 and IL-8, with no significant increase in TNF- α . The signs and symptoms of envenomation by *H. lepturus*, even when mild, were more severe than those for other scorpions, especially *M. eupeus*.

Treatment of human envenomation by *H. lepturus* with the available anti-venom has been difficult, unpredictable and always carried a risk of failure (between 1 to 5%). Although we did not measure the venom concentration in our patients, previous clinical observations [4] suggest that the severity of envenomation is related to the age of the victim (more severe in children), time of sting (more severe for stings occurring early evening), site of envenomation (more when on the face or trunk), and the size of the scorpion (more when the scorpion is mature). All of these variables are important factors for determining the serum venom concentration, and hence the development of severe forms of envenomation. Previous studies have linked the severity of envenomation with serum venom concentration [18]. In our opinion, one exacerbating factor for the development of the severe symptoms and hence the higher mortality rate seen with this scorpion, is the late referral for medical care as a consequence of the absence of pain at the sting site [4].

Although administration of anti-venom resulted in varying degrees of reduction in the levels of cytokines studied, the percentage reduction being relatively greater in milder cases, it failed to normalize the levels of these cytokines. These remained similar to or more than those measured for untreated patients stung by *M. eupeus*. Therefore, as far as these cytokines are concerned, the findings suggest that serotherapy for *H. lepturus* with the anti-venom that is currently available is not an effective treatment for controlling the inflammatory responses associated with increases in serum cytokine levels. These findings may explain the underlying reasons for treatment failure, especially among children.

Similar to other studies, these results showed an overall correlation between the cytokine levels and severity of envenomation. However, unlike cases of *M. eupeus*-envenomation, patients stung by *H. lepturus* showed significant increases in TNF- α levels that were related to the severity of the envenomation. This finding has both clinical and immunological implications, since many of the unique clinical features of envenomation [4] might be associated with this potent pro-inflammatory mediator. Previous studies have suggested the serum IL-6 level is an important factor for predicting the outcome of envenomation by *Tityus serrulatus* [18], and has been associated with the severity of envenomation. Although we can not rule out the role of other cytokines, our results suggest that TNF- α is more important for the assessment of envenomation status, and may possibly be used as a tool for predicting the outcome of treatment in patients stung by *H. lepturus*.

Furthermore, the significant, severity-related increase in serum TNF- α levels in patients stung by *H. lepturus* suggests a selective stimulatory action of the venom from this scorpion on the immune system. This observation deserves more detailed studies and may pave the way for a better understanding the mechanism of the unique toxicity associated with this scorpion, which includes dermal necrosis, renal failure and haemolysis, the hallmarks of *H. lepturus* envenomation. TNF- α is a potent proinflammatory mediator with a range of pleiotropic effects including proliferation of neutrophils and fibroblasts, increase in prostaglandin synthesis, up-regulation of adhesion molecules and promotion of cytotoxicity [21-23]. Furthermore, previous clinical [2, 5], and experimental reports [3] have emphasized the cytotoxic nature of the venom of this scorpion, and this effect seems to be associated with TNF- α .

In addition to TNF- α , the serum levels of other cytokines also increased in proportion to the severity of envenomation, which suggests that the clinical manifestations

Table 2

Demographic characteristics of healthy control and scorpion-stung subjects, and the main clinical manifestations and outcome of treatment among patients stung by *M. eupeus* and *Hemiscorpius lepturus* with varying degrees of severity of envenomation.

Group	Gender	Age (yrs)	Main clinical manifestations	Outcome
<i>H. lepturus</i> : mild (n=12)	6 M/ 6F	9-12	Transient haematuria, mild anaemia, mild fever and nausea and vomiting. Limited localised reactions	Mostly discharged within three days; no deaths
<i>H. lepturus</i> : moderate (n=12)	5M/7F	9-11	Haematuria which lasted up to five days; reduction in haemoglobin level up to 10.5 g/100ml and haematocrit value, ranging from 25 to 31%, increase in bilirubin level above 1 mg/100 mL, oedema and greater dermonecrotic reactions	Discharged between seven to 14 days; no deaths
<i>H. lepturus</i> : severe (n=12)	6M/6F	9-11	Dark bloody urine (haemoglobinuria); severe dermonecrosis (one required surgical debridement); severe haemolytic anaemia (two cases required blood transfusion); oliguria and anuria (one case requiring peritoneal dialysis); CNS manifestations (agitation, convulsions and stupor). Cardiac and respiratory dysfunction	Mostly discharged after three weeks. Two patients died as a result of sudden respiratory arrest
<i>M. eupeus</i> stung cases (n=10)	5M/5F	9-12	Moderate to severe pain at the sting site	Treated with local lidocaine injection; discharged within three hours
Healthy control	5M/5F	9-12	—	—

associated with the toxic manifestations are not solely related to this cytokine. The highest percentage increase was observed for serum IL-1 levels. Similarly, other studies have shown significant increases in IL-1, IL-6 and TNF- α following envenomation by *Tityus serrulatus* [6, 8], *L. quinquestriatus* [17], *Androctonus australis hector* [23] and *Centruroides noxius* [12].

The cytokines most frequently reported to mediate the inflammatory process are IL-1b, IL-6 and TNF- α [24] all of which were found to be released in high amounts in our patients. The level of increase in IL-1 in mildly envenomed, *H. lepturus*-stung patients, relative to both healthy and *M. eupeus*-stung subjects was more than 15-fold and 29 % respectively. IL-1 is known to be released by macrophages. Previous studies in which large amounts of IL-1 and TNF- α are released report systemic inflammatory response syndrome (SIRS), septic shock and death [25]. Since the extent of increase in these cytokines has also been reported by other investigators, and the clinical manifestations associated with this scorpion being different, it seems that other mediators, such as kinins [18], might also be involved. This hypothesis needs to be tested in further studies. Taken together, these findings suggest that TNF- α may act independently or in concert with other cytokines, as well as other proinflammatory mediators, to produce the unique toxic manifestations associated with *H. lepturus* envenomation.

Relative to healthy subjects, the lowest increase seen in the other cytokines was found for serum IL-8 levels following *M. eupeus* envenomation (84%). However, like other cytokines in patients stung by *H. lepturus*, the extent of the increase in this cytokine was also associated with the severity of envenomation (more than two- to four-fold in mildly and severely envenomed patients, respectively). IL-8, a member of the chemokine family, is a proinflammatory mediator released by a variety of proinflammatory cells including macrophages, and is responsible for early immune and inflammatory responses resulting in activation of leukocytes and recruitment of a variety of proinflammatory cells such as monocytes, lymphocytes, mast cells and eosinophils [26]. Our previous experimental study [3] showed profound aggregation of various leukocytes, with marked extravascular migration of inflammatory cells at the skin site and in profound structural changes in the nephrons [3]. These observations suggest that IL-8 is possibly implicated in this damaging effect.

What evidence do we have that supports our hypothesis for the involvement of TNF- α in the inflammatory responses seen following a *H. lepturus* sting? A variety of cellular responses have been previously attributed to TNF- α , ranging from inflammation [14], to cellular death [27]. In addition, different clinical and experimental studies suggest a correlation between cardiac failure and TNF- α levels [28-30]. Furthermore, cytotoxicity due to TNF- α has been observed in cultured liver cell lines [31]. Therefore, it seems that the production of TNF- α may be responsible for the inflammation of remote organs, inducing for example, acute lung injury and liver function abnormalities, as reported both in human and animal studies [1, 4]. However the main limitations of this study include the small sample size and short follow-up period for our patients. Therefore, before the clinical relevance of these findings can be appreciated fully, a similar, larger and randomized study needs to be performed.

In conclusion, as with other scorpion stings, the manifestations of envenomation by *H. lepturus*, and, to a lesser extent, those of *M. eupeus*, are mediated by an increase in cytokine levels, with signs and symptoms that resemble systemic inflammatory response syndrome. However, only TNF- α seems to have contributed to the severity of the envenomation. In addition, serotherapy was found not to be effective in reversing the increase in the levels of the cytokines measured, which increased more significantly according to the severity of the envenomation in patients stung by *H. lepturus*. This observation emphasizes the importance of the type and amount of cytokine released, other measures, such as early administration of glucocorticosteroids, and the use of more specific agents such as monoclonal antibodies. Anti-TNF- α therapy may have a potential benefit in attenuating the severity of envenomation associated with this dangerous scorpion.

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