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IN VITRO PHARMACOLOGICAL STUDY UPON THE EFFECTIVENESS OF AVAILABLE ANTIVENOM AGAINST *HEMISCORPIUS LEPTURUS* VENOM

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Abstract

The available Razi Institute antivenom is still, empirically, used by intramuscular (IM) or intravenous (IV) administration for the treatment of human scorpion envenoming by 6 medically dangerous scorpion species including *Hemiscorpius lepturus* (*H.lepturus*). The aim of this study was to assess the neutralizing ability of the antivenom upon the hemolysis effects of the venom from *H. lepturus* under *in vitro* conditions in rat. *In vitro* studies were including evaluation of hemolysis potency of the venom on rat washed RBC, assessment of antihemolytic potency of antivenom on rat washed RBC suspension and time profile of antihemolytic effectiveness of antivenom on treated rat washed RBC with venom, respectively. The *in vitro* results showed that the antivenom (at 10µl/ml of rat washed RBC suspension) reduced the concentration-dependent hemolysis of 1% rat washed RBC produced by venom from 71 to 43 % after 24 hours of incubation. Time profile of *in vitro* antihemolytic effectiveness of 10 µl available antivenom against hemolysis produced by 10µg of venom was dependent on the duration of incubation. In conclusion the findings of this study on animal model demonstrate that *in vitro* results were not correlated well with clinical findings in human.

Keywords:
Hemiscorpius lepturus, Scorpion, Venom, Hemolysis, Antivenom.

Introduction

Scorpions are present throughout the world (1, 2). Some known species are endogenous in different parts of the world: *Androctonus* species in North Africa (3), *Leirus quinquestriatus* and *Andrecotonus crassicauda* in Saudi Arabia (4), *Odonthubuthus doriae* in Iran (5) and *Mesobuthus tamulus* in India (6). In Khuzestan province, southwest of Iran, there are several endogenous species of

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scorpions, the best known are *Mesobuthus eupeus*, *Androctonus crassicauda* and *Hemiscorpius lepturus* (7).

H.lepturus is a public health problem in Khuzestan province of Iran, because of hemoglobinuria, hemolysis and dermonecrosis upon envenomation. After a *H. lepturus* sting, various degrees of local toxicity are observed in the majority of the cases which include macular erythema, purpuric changes, bulla, necrosis, and ulcers (8). The neurological effect of *H. lepturus* venom is similar to that of scorpions of the *Buthidae* family. However, they differ in RBCs lysis, which was highly significant when induced by *H. lepturus* venom (9). Different investigations showed comprehensible hemotoxicity including hemoglobinuria, microscopic hematuria, proteinuria or microalbuminuria and/or their break-down on renal function. An *H. lepturus* sting precedes acute renal failure at a frequency great enough to suggest a causal relationship between the sting and renal failure in humans (10, 11, 12). Hemolysis due to envenomation by this scorpion is so common that when a patient is suspected of been stung by a scorpion, the first clinical test is to check the presence of hemoglobin in the patient's urine. Severe hemolysis and secondary renal failure due to a scorpion sting was observed in Khuzestan province, southwest of Iran—a complication that, to our knowledge, has not been well recognized by other scorpion stings (10).

This scorpion is the most venomous of all types of Iranian scorpions and contributes to 95% of scorpion-associated mortalities (12). In Iran, serotherapy by Razi institute multivalent antivenom against 6 common Iranian scorpions (13) is one of the major therapeutic measures used in the treatment of scorpion envenoming for the last 30 years (14). Antivenom was capable to reverse hematological effects caused by Indian red scorpion, *Mesobuthus tamulus* (15). Despite large annual envenomation cases in this area and clinical and experimental results, there is yet no consensus among the clinicians upon the effectiveness of this treatment modality following envenomation arising from this dangerous species. Furthermore, no previous toxico-pharmacological study has specifically focused on studying the effectiveness of antivenom in neutralizing the toxic manifestations of the venom under *in vitro* conditions. The aim of our study was therefore to assess the effectiveness of antivenom therapy in preventing the toxic effects of this venom under *in vitro* conditions in rat.

Materials and methods

Animals

Male adult Wister rats of average weight of 200-300 g were purchased from the animal house of Jundishapur University of Medical Sciences, Ahvaz, Iran. All animals were housed in PVC cages with free access to tap water and hard food pellets. The animals were kept at 23 ± 2 °C and maintained at 12 hourly light/dark cycle, starting at 7 am–7 pm.

H. lepturus venom

Lyophilized *H. lepturus* venom was purchased from Razi institute of Iran (Hessarak, Karaj). Briefly, 50 *H. lepturus* scorpions were trapped from Khuzestan province in Iran and their raw venoms were collected in Razi institute by applying electrical shock (with 128 Hz of frequency and 15V of voltage and were collected into a 1.5 ml sterile Eppendorf tube) on their telsons. The collected venom was milked, lyophilized and stored at -20° C before using. For experiment, an aliquot of the lyophilized venom was reconstituted by the addition of distilled water or PBS solution. Protein content of the venom and multivalent antivenom was determined by Coomassie blue dye-binding method with bovine serum albumin as the standard (16). Briefly,

Protein content was determined by reading absorbance at 280nm.

Iranian polyvalent antivenom preparation
The multivalent scorpion antivenom (5ml ampoules, stored at 2-8 °C) is a pepsin-digested; refined and concentrated preparation obtained from equine hyper immune serum and, according to the manufactures pamphlet, it has a neutralizing potency against the venoms of the 6 endemic Iranian scorpions (*A. crassicauda, Buthotus saulcyi, Buthotus schach, Odontobothus doriae, Mesobuthus eupeus and H. lepturus) (*13*).*

Preparation of rat washed red blood cells (RBC)
Freshly collected blood samples from retinal vasculature of healthy rats, after anesthesia with diethyl ether, were mixed with anticoagulant Alsever's solution (pH 7.4), and centrifuged at 1500 x g for 5 minutes at 4°C. The supernatants were removed by gentle aspiration and the above process repeated twice. One ml of the washed erythrocytes was finally re-suspended in 100 ml of PBS (phosphate buffer saline solution) to make 1% RBC suspension (17). This water-based salt solution was used for maintaining a constant osmolarity and pH. This solution is containing sodium chloride, sodium phosphate, and in some formulations potassium chloride and potassium phosphate.

In vitro evaluation of haemolysis potency of the venom on rat washed RBC
In order to determine the optimum concentration of the venom that produces submaximal hemolysis of 1% rat washed RBC, the following experiment was performed: Increasing concentrations of *H. lepturus* of the lyophilized venom (1, 10 and 50 µg/ml of 1% washed RBCs were in Eppendorf tubes and after 3, 6, 18 and 24 hours of incubation at 37° C, the samples were centrifuged at 1500 x g for 5 minutes at 4° C using Eppendorf centrifuge (Eppendorf centrifuge model 5410, Germany). One percent Triton X-100 in PBS and PBS buffer, with equal incubation periods and volume of washed RBC suspension, were used as positive and negative controls, respectively. Absorbance of the supernatant was measured at 414 nm using spectrophotometric microplate reader (Biotek Instruments) to measure the extent of red blood cell lysis (18). Percentage lysis was calculated as:

$$(Abs_{sample} - Abs_{PBS}) / (Abs_{Triton} - Abs_{PBS}) \times 100$$

where Abs_{sample}, Abs_{PBS} and Abs_{Triton} refer to absorbance in the presence of venom, PBS buffer, and positive control in presence of 1 % Triton X-100 respectively. The means and standard deviations for all groups were determined from triplicate samples.

Antihemolytic effectiveness of antivenom on hemolysis induced by H. lepturus on rat washed RBC
The aims of this series of experiments were to evaluate the antihemolytic effectiveness and to find the minimum effective dose of Razi institute multivalent antivenom that can reduce significantly the hemolysis induced by 10 µg of *H lepturus* venom on 1% rat washed RBC suspension. For this purpose varying concentrations of Razi institute multivalent antivenom (1, 10 and 100 µl) were added separately, in triplicates, to 1 ml of previously prepared 1% rat RBC suspension treated with *H. lepturus* venom and incubated for 24 hours at 37°C. One % Triton-X 100 and PBS solution were used as positive and negative controls respectively.

Time profile of antihemolytic effectiveness of antivenom on treated rat washed RBC with H. lepturus venom

In order to evaluate the time profile of antihemolytic effects of the selected minimum effective concentration of the antivenom on venom-treated RBC suspension the following experiment was carried out: The allocated concentration of Razi institute multivalent antivenom (10 µl) was added to 1 ml of previously prepared 1% rat RBC suspension exposed to 10 µg of *H. lepturus* venom within 24 hours incubation at 37ºC. At different time intervals (3, 6, 18 and 24 hours) the extent of hemolysis was assessed by ELISA, by measuring of the absorbance (in triplicate) at 414 nm and the percentage of hemolysis was calculated and compared. One percent Triton-X 100 and PBS solution were used as positive and negative controls, respectively.

Statistical analysis

The results are expressed as mean ± standard deviation; paired student t-test and analysis of variance followed by Tukey test were used to determine the significant differences between means. $P<0.05$ was considered to be statistically significant. All data are given as mean ± SD (n=4).

Results

In vitro hemolytic activity of H. lepturus venom

The results of this study revealed that the venom produced concentration-dependent hemolytic activities on 1% rat washed RBC suspension. As depicted in Table 1, *H. lepturus* venom at 1, 10 and 50 µg produced 32.1, 71.6 and 77.6 % hemolysis of 1% rat RBCs after incubation for 24 hours at 37°C, respectively. However, no statistically significant differences in hemolytic effects were found between the 10 and 50 µg of venom (71.6% versus 77.6%) by t-test after 24 hours.

In vitro antihemolytic effectiveness of multivalent antivenom on hemolysis induced by the venom

As illustrated in Fig. 1, different concentrations of antivenom had significant effects on prevention of hemolysis. Addition of 10µl of antivenom reduced hemolysis from 71% in the venom treated blood to 43% after 24 hours. There was no significant differences in prevention of hemolysis between 10 µl versus 100 µl of Razi institute antivenom (43% against 38.54%) by t-test ($P>0.05$), therefore the 10 µg concentration was selected for next series of *in vitro* experiments.

Table 1. Percentage of hemolysis of 1 ml of 1% washed rat RBC in relation to duration of incubation after exposure to different concentrations (1, 10 and 50µg) of *H. lepturus* venom. PBS buffer solution and 1% triton X were used as negative and positive controls, respectively.

	Duration of incubation (h)				
Treatment	0	3	6	18	24
PBS buffer	0	3.5	4.2	8.7	10.4
1% Triton X	0	100	100	100	100
1(µl/ml)	0	9.2[a]	20.3[b]	27.5[c]	32.1[c]
10 (µl/ml)	0	14.1[b]	25.7[c]	53.2[c]	71.6[c]
50 (µl/ml)	0	23.4[c]	30.3[c]	60.2[c]	77.6[c]

[a]$P<0.05$, [b]$P<0.01$ and [c]$P<0.001$ between venom-treated and negative control respectively.
The results are mean and standard deviations of triplicates samples for each concentration and exposure time (n=4).

Time profile of in vitro antihemolytic effectiveness of 10 µl *multivalent antivenom against hemolysis produced by* 10 µg of *H. lepturus*

Incubation of 10 µl of antivenom with 1 % rat washed RBCs produced duration of incubation-dependent anti-hemolytic activity, causing 38.4 % reduction after 24 h following simultaneous incubation with 10 µg of the venom. After 3, 6 and 18 h of incubation hemolysis levels, relative to venom alone, were reduced by 23.4 ($P<0.01$), 35 ($P<0.001$)and 41 % ($P<0.001$), respectively (Fig. 2).

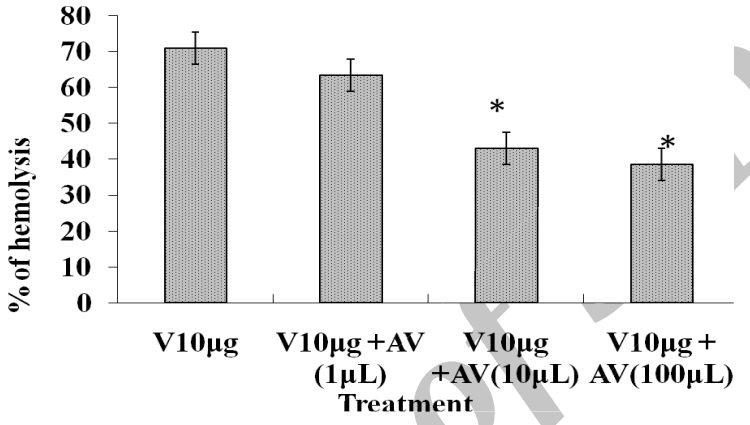

Fig. 1: *In vitro* anti-hemolytic effectiveness of different concentrations (1, 10 and 100 µl) of Razi institute multivalent antivenom on *H. lepturus* venom-treated 1% rat RBC suspension after 24 hours incubation at 37°C. Results are representative of three different experiments carried out and presented as mean ± S.D. (n=4)**.** *P<0.05 between venom and venom + antivenom. V: venom; AV: antivenom.

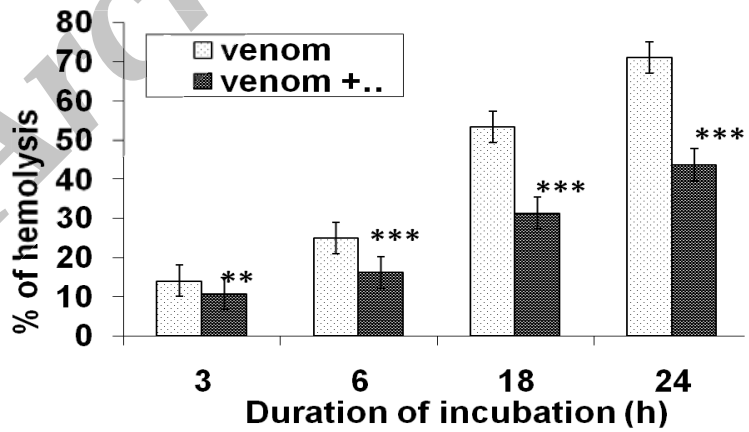

Fig. 2**:** *In vitro* anti-hemolytic effectiveness of 10 µl Razi institute antivenom on hemolysis induced by 10µg *H. lepturus* venom on 1 % washed rat RBC following incubation for different periods (3, 6, 18 and 24 h) at 37°C %. All data are given as mean ± SD (n=4). **P<0.01 and ***P<0.001 between venom and venom-antivenom exposed groups.

Discussion

Envenomation by *H. lepturus*, the most dangerous scorpion of Iran (19) in Khuzestan province is of great concern especially during warm months (9). This study addresses the neutralizing ability of available polyspecific antivenom manufactured and used for the antihemolytic effectiveness of hemolysis induced on rat washed RBC by venom of the most dangerous scorpion of Iran, *H. lepturus* (19). In the present study, 10µg *H. lepturus* venom and 10-100µl antivenom were used, respectively. The dose of venom was adjusted, as the result of a scorpion sting, the same amount is injected. Also 10-100µl of antivenom administration was adjusted to rats, since in the current Iranian protocol, 2 vials of 5 ml antivenom are recommended for an adult person (almost with 70kg weight). Intramuscular administration route was commonly used in health centers of Iran. Moreover, concerning the used dose for both venom and antivenom, the design of study was based on clinical practice and not on the basis of dose-response experiments. So the results may provide conclusive evidence, from clinical point of view.

The protein content determination of venom and antivenom aimed at comparing protein contents. Scorpion venom contains short neurotoxin polypeptides consisting of simple, low-molecular-weight proteins with immunogenic property (2). Thus, identification of immunogenic proteins may allow more clearly defined the efficient antivenom. Protein determination showed that the antivenom contains high antibody content. This amount easily provides effective immunological protection against the *H. lepturus* venom. The details were previously described by Seyedian et al. (20).

There are some species differences in response to envenomation under *in vitro* conditions such as hemolysis. Dog erythrocytes are the most susceptible than other animals to jelly-fish venom then other animals(17). *In vitro* hemolysis induction by *H. lepturus* venom is greater in cow (18) than the other animals (horse, goat and chicken). Our results showed that hemolysis induced by *H. lepturus* venom solution was less potent than that reported for the venom from *Loxosceles adelaida* (21). These variations may reflect differences in the phospholipid composition of the erythrocyte plasma membrane of these species (22, 23), as well as the accessibility of the cellular membrane targets such as negative phospholipids to PLA_2 action (24). Other explanations of this phenomenon may include differences in binding and insertion of toxin molecules into plasma membrane followed by oligomerization to form transmembrane pores, and resulting in colloid osmotic lysis (25). The other possible mechanism that mediates RBC destruction is complement-mediated hemolysis or those activated by sphingomyelinase II (24, 25). The exact way by which *H. lepturus* induces hemolysis of RBC is not yet clearly defined and this deserves further pharmacodynamic and biochemical investigations.

The results of the present study demonstrated that Razi institute multivalent antivenom had *in vitro* antihemolytic efficacy. However, there were no similar correlated results under *in vivo* conditions in human following its intramuscular administration. Hemolysis did not change considerably twenty four hours after 10 and 100µl of antivenom adjacent at the same time of envenomation. This finding suggests that maximum protective effects are obtainable at 10 µl of antivenom.

Hematuria is the first clinical sign of envenomation in patients by this scorpion. Therefore, it was logical that be considered as an end point for assessing the efficacy of the antivenom under *in vitro and vivo* condition. In our study, we

used urinary dipstick method for assessing extent of hemoglobinuria following envenomation in rat and found to be simple, rapid and produced repeatable results, suggesting that this method is a reliable one.

Conclusions

We found that the venom produced hemolysis in a dose-related manner in *in vitro* examinations. Furthermore, considering the ineffectiveness of the antivenom following human administration (*in vivo* conditions), these results suggest that the antivenom is not potent enough in controlling all the manifestations of the envenomation and more potent and purified antivenom preparations need to be manufactured. Therefore, Further *in vivo* pharmacokinetic, clinical studies, antigen-antibody interaction, enzymatic analysis and investigation of the effectiveness of the antivenom following its administration by IM and/or IV routes need to be undertaken in order to fully assess the efficacy of this antivenom and make appropriate recommendations when treating envenomed patients.

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