

Full Article

An in vitro Comparative study upon the Hemolytic, Thrombogenic, Coagulation parameters and Stability properties of the *Hemiscorpius lepturus* Venom

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ABSTRACT

Hemiscorpius lepturus belonging to Hemiscorpiidae family is the most venomous of all types of scorpion existing in south west of Iran causing hemoglobinuria and dermal lesions by envenomation. We compare the hemolytic pattern upon time in different domestic animals upon time according to their different sphingomyelin contents. In addition other in vitro hematologic parameters, platelet lysis, coagulation changes and finally preservative factors (temperature, pH, proteases) are discussed. The hemolytic activity was inhibited significantly by heating at 100 °C for 60 minutes (26%) and reached 38% via incubation with papain (10U/ml) while retained over a pH range of 4-11. Horses and sheep have the lower (61%) and upper (100%) rate of hemolysis. Calcium and magnesium ions could increase rate of hemolysis and EDTA solution had significantly decreasing effect. The venom significantly changed in vitro coagulation factors (PT and APTT) from base line levels and had no effect on platelet lysis. It seems that our venom belongs to metalloproteinases due to potentiation effects of bivalent cations (calcium and magnesium) and ghost cell formation in our study indicating hemoglobin efflux.

Keywords: *Hemiscorpius lepturus*, Stability Hemolysis, Ghost cell, Metalloenzyme

INTRODUCTION

Hemiscorpius lepturus scorpions, locally known as Gaodim (tail of the cow), are widely distributed in southwestern part of Iran especially Khuzestan province (Shahbazzadeh *et al* 2009, Heidarpour *et al*

2012). Envenomation by this scorpion is not painful and clinical manifestations in stung patients include bloody urine due to hemoglobinuria or hematuria, dermonecrotic reactions and in minority of cases acute renal failure leading to death following disseminated intravascular coagulation especially in infants (Radmanesh 1990, Pipelzadeh *et al* 2007, Vazirianzadeh

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et al 2012). The venom contains hemolytic activities and its metalloproteinases, like gelatinase, caseinase and hyaluronidase, facilitate its proteolytic aspects (Seyedian et al 2010). Recent developments, like discovering Hemitoxin, Hemicalcin and Heminecrolysin (Srairi-Abid et al 2008, Shahbazzadeh et al 2007), have led to further studies on the biological properties of this scorpion. The aim of our study is to find red blood cell destructive mechanism(s) of this venom in different species. In addition, this experiment has been done on patients with various hemoglobinopathies present in Iran. These are: G6PD deficiency (9.8 and 15.51%), beta thalassemia patients (0.5% to 0.9% of all deaths of children under 5 in low or middle income countries) and sickle cell disease in south provinces of Iran. Finally evaluation of the thrombogenic and platelet destruction of this venom, as well as its related stability conditions has been studied.

MATERIALS AND METHODS

Hemiscorpius lepturus venom and multivalent antivenin collection and preparation. *Hemiscorpius lepturus* venom (Razi institute, Karaj, Iran) was used. Briefly, the raw venoms from captured scorpions were collected by applying electrical shock on their telsons. The collected venom was pooled, lyophilized and stored at -20 °C before use. An aliquot of the lyophilized venom was reconstituted by addition of Normal Saline or Phosphate Buffer Saline. The multivalent scorpion antivenin (5ml ampoules, stored at 2-8 °C) is a pepsin-digested; refined and concentrated preparation by double saline precipitation obtained from equine hyper immune serum. The protein content of this product was 3.6mg/ml with a neutralizing capacity of 26LD₅₀/ml.

Chemicals and drugs. Trypsin from Bovine pancreas and Papain solution from *Carica* were purchased from Sigma (St. Louis). All chemicals used were of the purest grade available.

Preparation of washed red blood cells. Freshly collected blood samples from non smoker healthy humans and other domestic animals (cats, horses and

sheeps) were mixed with anticoagulant EDTA solution (pH 7.4), and centrifuged at 1,500×g for 5 minutes at 4 °C. The supernatants were removed by gentle aspiration and the above process was repeated twice. One ml of the washed erythrocytes was finally re-suspended in 100 ml of Normal Saline to make 1% RBC suspension (Kang et al 2009).

In vitro evaluation of hemolytic potency of the venom on human and other species (cats, horses and sheeps) washed RBC. *H. lepturus* venom (10 µg/ml) in 1% washed RBCs of different species were prepared in Eppendorf tubes and after time intervals (1, 6, 12, 21 and 24 hours) of incubation at 37° C, the samples were centrifuged at 1,500×g for 5 minutes at 4° C using Eppendorf centrifuge (Hettich EBA 12R, Germany). One percent Triton X-100 in PBS and PBS alone, with equal incubation periods and volume of washed RBC suspension, were used as positive and negative controls respectively. Human erythrocytes that were treated by this venom (10 µg/ml), fixed with 95% methyl alcohol for three minutes staining with Wrights solution and photographed by inverted microscope (Nikon Y-THRL-Japan) at 24 hours post envenomation. Absorbance of the supernatant was measured at 490 nm using ELISA spectrophotometer (Stat fax 2100). Percentage lysis was calculated as: $(\text{Abs sample} - \text{Abs PBS}) / (\text{Abs Triton 1\%} - \text{Abs PBS}) \times 100$ (Ribeiro et al 2007). The mean and standard deviation for all groups were determined from triplicate samples.

Effects of bivalent cations and EDTA on the hemolytic activity of *Hemiscorpius lepturus* venom. The effects of these agents (Calcium and Magnesium ions plus EDTA) were studied by adding Calcium Acetate, MgCl₂. (6H₂O) and EDTA solution in distilled water to *Hemiscorpius lepturus* venom and assessment of hemolytic activity. Calcium and magnesium ions in addition to EDTA solution were tested upon erythrocytes suspensions at a final concentration of 0.1, 0.25, 0.5 and 1mM.

Stability of *H. lepturus* venom upon temperature and acidity changes. *Hemiscorpius lepturus* venom prepared in Normal Saline was incubated at different

conditions of temperature and acidity to evaluate hemolytic changes of this venom on 1% human washed red blood cells after 24 hours incubation at 37°C. Briefly each aliquot of the venom was incubated at the temperatures of 4, 20, 60 and 100 °C for 60 minutes, respectively. The indicated venoms (10µg) were added to 1% red blood cell suspension (1ml) to evaluate retained hemolytic potencies following 24 hours incubation at 37 °C. The pH dependency of the venom was performed by incubation of *H. lepturus* venom at different pH values (1, 4, 7, 9 and 11) on ice for one hour and assessment of its hemolytic activity.

Evaluation of the changes on blood coagulation induced with Hemiscorpius lepturus venom. This assay was performed according to (Sivan *et al* 2007) with some modifications. Fresh human blood was collected in to a test tube containing 3% sodium citrate solution in a proportion of 1:9(v/v). The plasma was separated by centrifugation (2000rpm) for 5 minutes after mixing the sample gently. About 0.1 ml of calcium chloride (1%) was added in to the 0.2 ml of plasma and mixing was done at 37 °C. A solid clot was observed after 3 minutes showed that our sample was suitable for the next examination. The *H. lepturus* venom was prepared in Normal Saline at concentrations of 10 and 100 ×10µg/ml to perform our coagulation studies. The test samples were added to the test tubes containing 0.2 ml of plasma with or without the addition of 0.1 ml of calcium chloride and observed for changes. The experiments were repeated three times. For determination of in vitro coagulation changes induced by *H. lepturus* venom, samples of pooled human plasma from nonsmoking donors were incubated at 37 °C with 10 or 50 µg/ml. Assays of prothrombin time (PT) and activated partial thromboplastin time (APTT) were performed with different concentrations of venom (0, 10 and 50µg/ml). Our experiment was repeated with normal saline with same experimental condition as control.

Effect of Proteases on hemolysis. The effects of proteases (Trypsin and Papain) on hemolysis by *H. lepturus* venom were studied (Feng *et al* 2010). They were incubated for 30 minutes at 37°C with venom at

final concentrations of 5.0, 10.0 and 20.0 units/ml. Treated venoms were assayed upon erythrocytes as mentioned before.

In vitro platelet lysis. Supernatant rich platelet plasma was obtained by centrifugation (1, 200 grounds for 15 min at 4 °C.) from fresh citrated human blood at room temperature in Bushehr Transfusion Center. The effects of *H. lepturus* venom on platelets were evaluated by incubating increasing concentration of venom (2.5, 5 and 10µg) with 100µl of plasma at 37 °C for 5 minutes. Negative and positive controls of cytotoxicity were prepared by incubating platelets with saline and 1% triton X-100 respectively. After centrifugation at 2000g for 10 minutes, lactate Dehydrogenase activity was measured in supernatants according to previous methods (Bergmeyer & Bernt, 1974) with Lisa300 (French) device. All experiments were performed in triplicates.

Statistical analysis. The results are expressed as mean± standard deviations. The significance between different groups was studied by the analysis of variance (ANOVA) and Tuckey test when the values were statistically significant (P<0.05).

RESULTS

Hemolytic effects of *H. lepturus* venom. In vitro hemolytic effects of this venom had a gradual activity which led to total hemolytic occurrence 24 hours following our experiment. According to Figure 1, the

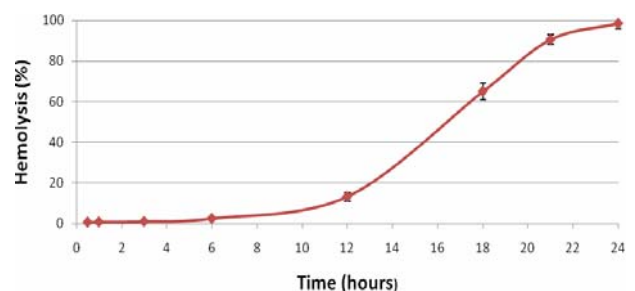


Figure 1. In vitro hemolytic potencies of *Hemiscorpius lepturus* venom (10µg/ml) on 1% human red blood cell suspension upon time at 37 °C. Results are representative of triplicate experiments and presented as Mean ±SD.

peak hemolytic effects of this venom were between 12 and 18 hours. Hemolytic activity upon time in different

species were approximately like human washed red blood cells following treatment with *H. lepturus* venom (10 μ g/ml) (Figure 2). Sheeps and horses washed red blood cells were the most and least susceptible samples among them.

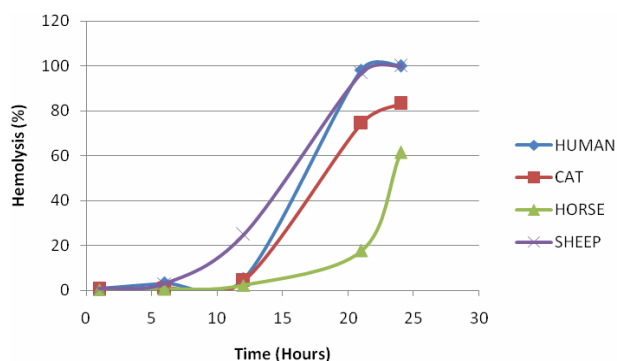


Figure 2. In vitro hemolytic effects of *H. lepturus* venom (10 μ g/ml) on different species one percent red blood cell suspension upon time. The mixture of red blood cell suspension plus *H. lepturus* venom after incubation at 37 °C were centrifuged at 1500 \times g for 5 min at 4 °C upon time (1, 5, 12, 21 and 24 hours) and its absorbance was measured at 490nm using Elisa reader (Stat fax 2100).The results are expressed as mean \pm S.D. (n=3).

Stability of *H. lepturus* venom. Thermal stability investigation of *H. lepturus* venom showed that it could be affected by increasing of environmental temperature (Figure 3a). The hemolytic activity was significantly disappeared by heating at boiling point but not affected by incubation at 4 °C and finally 20 °C. The *H. lepturus* venom was treated at various pH conditions from 1 to 11 for 60 minutes on ice. According to Figure 3b, the hemolytic activity was significantly decreased at pH= 1 but not changed after treatment in other conditions.

Effects of divalent cations and EDTA. The results of hemolytic activity after treatment with divalent cations as Ca²⁺, Mg²⁺ and EDTA solution after 12 hours is shown in figure 4. With the concentration increase of calcium and magnesium ions from 0.1mM to 1 mM, the hemolysis (%) was changed from 88.2 \pm 3.4 to 98.4 \pm 2.3 and 92.1 \pm 3.5 to 98.9 \pm 4.2 respectively, and the treated sample with venom (10 μ g/ml) was 18.4 \pm 2.4 (not shown). The hemolytic activity of *Hemiscorpius lepturus* after incubation with different concentrations of a chelator like EDTA solution (0.1, 0.25, 0.5 and 1 mM) was performed to further confirm the metalloproteinase nature of hemolytic effects. The

hemolytic activity was significantly decreased from treated sample (18.4 \pm 2.4) to 5.2 \pm 0.92.

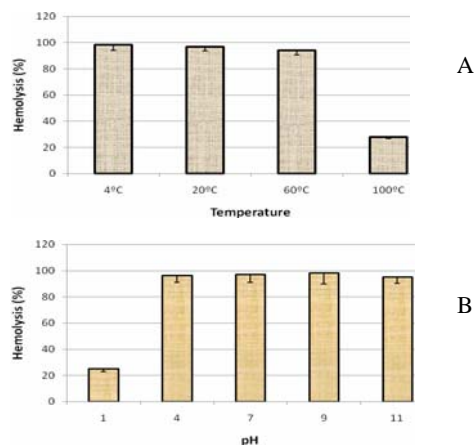


Figure 3. Temperature and pH stability of *Hemiscorpius lepturus* venom. The venom samples dissolved in Normal saline preincubated at (a) different temperatures (4, 20, 60 and 100 °C) for 60 minutes or different pH adjusted media (1, 4, 7, 9 and 11) for 60 minutes on ice with stirring(b). After pretreatment the retained hemolytic activity (10 μ g/ml) were evaluated. The results are expressed as mean \pm SD(n=3).P<0.5 versus control value.

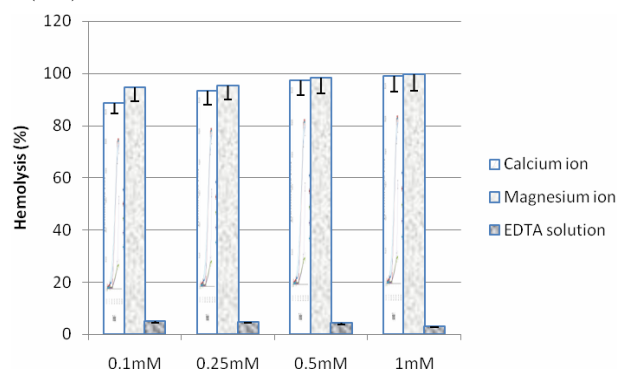


Figure 4. The hemolytic activity of *Hemiscorpius lepturus* treated with different doses of Ca²⁺, Mg²⁺ and EDTA solution in distilled water (0.1, 0.25, 0.5 and 1 Mm) at 37 °C after 12 hours. All results are expressed as mean \pm SD (n=3).

Hemolytic changes caused by proteases. After treatment with proteases, the hemolytic activity of *H. lepturus* was altered. Trypsin and papain had opposite effects on this purpose and Papain reduced the hemolytic potency to 38% versus complete hemolysis. The hemolytic activity (%) decreased down to 46.3 \pm 2.3 and 38.4 \pm 4.6 by preincubation with 5 and 10U/ml papain respectively (Figure 5). Full hemolytic effects were occurred by preincubation with trypsin.

Effects of *H. lepturus* on platelet lysis. According to Figure 6, Incubation of platelets with escalating doses of venom (2.5, 5 and 10 μg) had no lytic effect since LDH levels in milieu were not different with treated samples with Normal saline as control after 5 minutes.

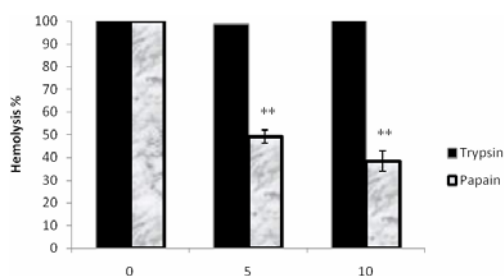


Figure 5. The hemolytic activity of *Hemiscorpius lepturus* treatment with trypsin and papain. ** $P < 0.01$ vs 0; Results are expressed as mean \pm SD (n=3).

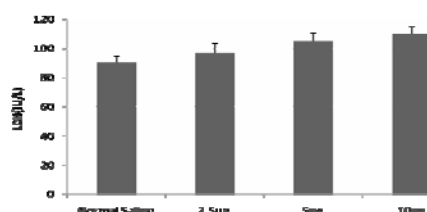


Figure 6. Effect of the incubation of human rich plasma with different doses of *H.lepturus* venom on Lactate dehydrogenase (LDH) leakage. Data are expressed as mean \pm SD(n=3).

In vitro determination of hemostatic parameters.

Based on our study ,incubation of escalating doses of *H. lepturus* venom with human plasma neither caused clotting nor lysis of the clot formed. The coagulation factors in this study showed significant differences between separate groups (Table 1). The PT and APTT were increased from 13 to 35 ± 2 and >60 seconds and 45 ± 2 to >120 seconds following incubation with 10 and 50 μg of venom respectively showing hemorrhagic nature of this remedy.

DISCUSSION

Medical envenomations by *H. lepturus* are characterised by hemoglobinuria leading to hematuria. Cytotoxic effects on all tissues including the skin (edema, cellulitis, bullae and necrotic lesion spreading

gravitationally), cardiovascular system and central nervous system are other pathologic findings. These are very similar to those brought about by *Loxosceles* envenomation (Dyachenko *et al* 2006, Málague *et al* 2002).

Table 1. In vitro *H. lepturus* venom effects on coagulation parameters. Data are shown as mean \pm standard deviation (n=3). PT,

Venom concentration ($\mu\text{g/ml}$)	PT(S)	APTT(S)
0	13 ± 1	45 ± 2
10	$35 \pm 2^{**}$	$>120^{**}$
50	$>60^{**}$	$>120^{**}$

T, activated partial thromboplastin time.

** $P < 0.01$ vs 0; Results are expressed as mean \pm SD (n=3).

Hemoglobinuria is seen in majority of patients probably due to hemitoxin (Srairi-Abid *et al* 2008), while systemic clinical manifestations may be seen in a small group of patients especially children (Dehghani & Fathi 2012, Mashak *et al* 2000). Our results showed that the reconstituted *H. lepturus* venom with normal saline had strong toxin or toxins with delayed hemolytic properties. This is shown by sigmoidal curve of hemolysis upon time with peak effects occurring between 12 and 18 hours similar to other venomous creatures specially *Loxosceles intermedia* (Chaves Moreira *et al* 2009).This is unlike some jelly fish venoms (Uechi *et al* 2005) and other creatures (Rekow *et al* 1983). The present study shows that this venom is

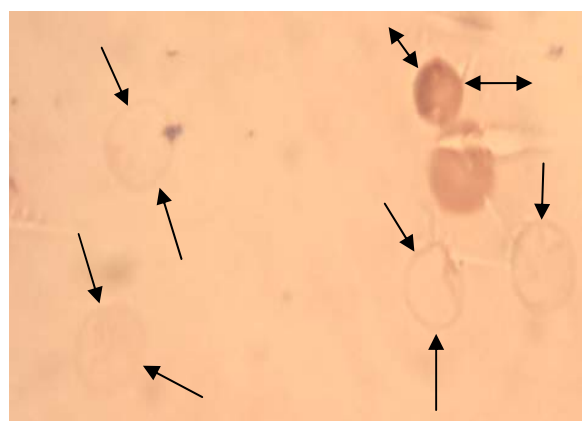


Figure 7. Hemolytic cells characterized by *H. lepturus* venom (10 $\mu\text{g/ml}$) containing erythrocyte ghosts (arrows) and one spherocyte (double arrow), one day after envenomation. Magnification is 1000 \times .

changed significantly in different temperatures but diminished abruptly following boiling an hour at 100 °C predicting its proteinaceous structure (Chung *et al* 2001b).

The hemolytic activity of *H. lepturus* was preserved at pH 3-13 but diminished significantly at pH 1 (Feng *et al* 2010). The hemolytic activity persisted upon wide variation in temperature and pH, making it preserved in different laboratory conditions. Calcium and Magnesium ions could enhance the hemolytic activity of this venom in different concentrations representing the metalloproteinase nature of *H. lepturus* venom (Chaves Moreira *et al* 2009) producing spherocytes and predominantly erythrocyte ghosts (Figure 7) in our supernatant probably due to pore formation in cell membranes and hemoglobin efflux (Ingraham *et al* 1981, Don *et al* 2004). EDTA solution (1mM) significantly reduced the hemolytic properties of this scorpion venom even greater than its effect on *Chironex fleckeri* (Baxter & Marr 1969). This is shown by chelating metals like calcium and magnesium that are necessary for hemolysis. Hemolysis is not dependent on blood group and red cell disorders. Blood groups A, B and O lysed in the same manner like red cells obtained from thalassemic, glucose 6 phosphate dehydrogenase deficient and sickle cell patients following toxin treatment (data not shown). Hemolytic activity by *H. lepturus* venom showed unpredictable results on erythrocytes of four different species (cats, horses, sheep and humans) suggesting that in vitro sensitivity to hemolysis is highly variable probably due to different lecithin to sphingomyelin ratios and sphingomyelin content in red cell membrane. In our study, time interval of hemolysis was similar in all animals and horse erythrocytes was the least sensitive among the species analyzed due to low sphingomyelin (14%) content in its erythrocytes membranes making it susceptible to lysis with phospholipase dependent enzymes (Ochi *et al* 2003). *Hemiscorpius lepturus* venom had no effect on blood clotting since in vitro study on human blood plasma showed no clotting

capacity when incubated with escalating doses of this venom. At the same time coagulation factors were significantly elevated following incubation of *H. lepturus* venom with human rich plasma. This was shown by changes in PT and PTT. This finding was in agreement with one in vivo study on human victims (Khosravi 2008) showing great differences in PT and APTT after envenomation, depicting greater affinity of *H. lepturus* venom for common coagulation factors (factors II, V and X). So evaluation of envenomed patients to prevent hemolytic uremic syndrome and disseminated intravascular coagulation is mandatory. Due to similarities in clinical manifestations by this scorpion and loxosceles, (Hemoglobinuria, hemolysis and necrotic dermal ulcers) (Málaque *et al* 2002, Dyachenko *et al* 2006), we believe that erythrocyte ghost cell formations in our *In vitro* hemolytic process was induced probably by pore forming reactions depending on divalent cations like magnesium and calcium ions. Metalloenzyme(s) like Heminecrosis as the first hemolytic and dermonecrotic toxin with sphingomyelinase activity induced complement dependent hemolytic reactions (Heidarpour *et al* 2012), like other venoms (Bass *et al* 1983). Further studies to elucidate the role of other involving enzymes in this pathway seems justifiable. Papain has been reported to produce inhibitory effects on hemolysis of some cnidarian venoms (Chung *et al* 2001a). Treatment of *H. lepturus* with papain as a cysteine protease enzyme induced a great inhibitory change in hemolytic activity while trypsin treatment had no effect. It seems that the hemolytic protein(s) in this venom was degraded and neutralized via incubation with papain making it suitable as a useful remedy in the treatment of envenomed patients to inhibit its hemolytic potency. Lactate dehydrogenase as a cytosolic enzyme is used to determine platelet lysis induced by incubation with venoms. Increasing doses (2.5, 5 and 10 µg) of *H. lepturus* venom could increase percentage of platelet lysis maximally to 99.7% after 4 hours (not shown) while no increase in LDH enzyme was seen in short time intervals (5 and 120 minutes). This rules out the

platelet lysis as is seen in *Loxosceles* venom (Tavares et al 2004). This necessitates more experiments to be performed to evaluate platelet aggregation potency of this venom. This study provided us the fundamental information about the preserving factors (Temperature and acidity) to stabilize the hemolytic properties in addition to degradation of hemolytic proteins of *H. lepturus* with papain enzyme. According to previous reports and this study it seems that metalloenzymes have a role in pore forming reaction and hemoglobin efflux induced by this venom. Some more in vitro and in vivo experiments must be performed on this venom to analyze its chemical structure and preventing the undesirable effects in stung patients.

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