Toxicon 100 (2015) 13–19

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

A forward to optimization of antivenom therapy: An *in vivo* study upon the effectiveness of the antivenom against early and delayed nephrotoxicity induced by the venom of the Iranian scorpion *Hemiscorpius lepturus* in rat

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ARTICLE INFO

Article history:
Received 17 October 2014
Received in revised form
7 February 2015
Accepted 31 March 2015
Available online 3 April 2015

Keywords:
Nephrotoxicity
In-vivo
Hemiscorpius lepturus
Alanin Amino Peptidase (AAP)
N-Acetyl-β-D-Glucosaminidase (NAG)
Polyvalent antivenom

ABSTRACT

The aim of the present *in vivo* study was to identify the optimal effective dose, the most favorable time and the route of administration of the available polyvalent scorpion antivenom against the toxic effects induced by *Hemiscorpius lepturus* (*H. lepturus*) venom in rat. The end point for assessment included measurement of alanin-amino-peptidase (AAP) and N-acetyl-b-D-glucosaminidase (NAG), biochemical urine analysis and histopathological assessment. The results showed that a single subcutaneous 50 μg of the venom produced significant increase in the AAP and NAG enzyme activity, urinary biochemical parameters and induced histopathological structural abnormalities in the renal system. The optimal effective co-administered dose of the antivenom was 0.5 ml, which when administered 1 and 2 h of envenomation by intravenous (IV) and subcutaneous (SC) routes respectively produced significant protection against these toxic effects. Prudently, the significance of these findings need to be assessed in further clinical studies.

1. Introduction

Hemiscorpius lepturus is the most dangerous scorpion in Iran (Jalali et al., 2010). It is commonly found in south and south-western regions of Iran and in other parts of the world such as Iraq and Yemen (Pipelzadeh et al., 2007). Unlike other scorpion species in this area and many other regions of the world, which mainly produce autonomic system over stimulation, the sting of this scorpion causes severe dermal necrosis and renal damage. These effects may be attributed to its cytotoxic properties (Pipelzadeh et al., 2006; Jalali et al., 2011a; Isbister and Bawaskar, 2014). Previous epidemiological studies reported that *H. lepturus* venom causes pathological changes such as congestion, bleeding and tissue necrosis in different organs particularly in kidneys (Pipelzadeh et al., 2007).

Preparation of antivenom has been hampered by the fact that venoms have low antigenicity and preparation of specific antibodies has been difficult. In fact, the production of the available polyvalent antivenom that contains horse $F(ab')_2$ antibodies against *H. lepturus* venom has been made possible only a decade ago. Despite this, the effectiveness of the serotherapy using the available polyvalent is still a disputable subject. Furthermore, despite the large amount of research on scorpion envenoming, there is as yet no clear dose/effectiveness nor time/efficacy relationship related antivenom therapy (Ismail and Abd-El Salam, 1988). This perhaps is due to the inherent variations of scorpion species, subjects under investigation, the conditions of envenoming, the dose of injected venom and the time at which the antivenom was administrated. Furthermore, treatment outcomes using antivenom alone or in combination with various pharmacological interventions vary with

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http://dx.doi.org/10.1016/j.toxicon.2015.03.023
0041-0101/

the type of scorpion species involved (Isbister and Bawaskar, 2014). There is as yet no generally agreed standard treatment protocol among the attending physicians for management of *H. lepturus* envenomed victims other than the use of an IM multivalent antivenom serum manufactured by Razi Institute, Karaj, and close observation of the patients for any other adverse effects, which are treated by symptomatic means (Radmanesh, 1990). Furthermore, there is still some scepticism regarding the effectiveness and safety of the antivenom in clinical practice.

Urine Amino-Alanine Peptidase (AAP) is an important marker early acute renal failure (Burdmann et al., 1994; Gaedeke et al., 1996). This enzyme is mainly found in brush border membranes of renal proximal tubules. Enhancement of AAP enzyme activity in urine samples is considered as an appropriate marker of early renal toxicity. N-acetyl-β-D-glucosaminidase (NAG) is another enzyme biomarker used for assessment renal tubular integrity (Bazzi et al., 2002). This lysosomal enzyme is mainly produced and excreted by proximal renal tubules. The enhancement of activity of this enzyme occurs when there is an increase in protein concentration in the tubular cells and its measurement is considered as an alternative non-invasive method for assessment of ongoing tubular injury (Gadalean et al., 2013).

The exact mechanism of that mediates this venoms' nephrotxicity is not clearly known. It has been speculated that *H. lepturus* scorpion has no direct nephrotoxicty, but is attributed to hemoglobinuria as the main factor for secondary nephrotoxicty. However, it is possible that it has direct nephrotoxic activity (Borchani et al., 2013).

Previous *in vitro* studies demonstrated that the antivenom was effective in neutralizing the haemolytic activity of the venom from this scorpion (Jalali et al., 2011b). It was prudent to assess if the antivenom is also effective under *in vivo* conditions to neutralize early and delayed nephrotoxicty. The aims of the present *in vivo* animal study were to find an answer to three main questions: firstly, by measuring two important nephrotoxic biomarkers namely AAP and NAG, as well as detection of hemoglobin and protein in the urine to establish if the venom from this dangerous scorpion produces early nephrotoxicity in rat? If it does, then at what optimal dose? Secondly to assess the delayed toxic effects induced following administration of the allocated optimal dose of this venom by assessing of renal histopathological changes after 7 days of envenoming. Thirdly, to clarify if the available antivenom is effective in preventing these early and delayed toxicities, and if so, at what antivenom/venom ratio dose, by which route and what is the optimal delay period between envenomation and antivenom administration?

2. Materials and methods

2.1. Animals

Male inbred adult N. Mari rats (200–240 g) were purchased from the animal house of Jundishapur University of Medical Sciences, Ahvaz, Iran. Animals were fed on a standard, commercial, pellet diet (Shushtar Khorakdam Co., Iran), and had free access to tap water. Animal house temperature was adjusted at 21 ± 2 °C and a 12-h light/dark cycle was maintained while the study was carried out. The Ethics committee of the Jundishapur University, Ahvaz approved the design of the experiments.

2.2. Preparation of stock solution of the venom

The venom of *H. lepturus* was collected following milking by electrical stimulation of the telson, freeze-dried and stored under refrigeration conditions. On the day of the experiments, a stock solution of the venom was prepared by dissolving in normal saline at 2 mg/ml concentration.

2.3. Collection of urine samples

Collection of urine samples, after 24 h of envenoming, was made by forced diuresis. For this purpose, each animal was administered 1 ml/100 g body weight tap water by a feeding tube and placed in a metabolic cage. During the next hr, urine samples were collected. Control values for the selected parameters were measured from the same animals a day before envenoming.

2.4. End points for assessment of renal toxicity

2.4.1. Measurement of NAG enzyme assay

The protocol for measurement of NAG enzyme activity was adopted as described by (Hosseini et al., 1997a). In short, the collected urine samples were centrifuged at 8000 rpm for 10 min. Two hundred μl of PNP-NAG fraction (9 mmol/L) prepared in acetate buffer and then transferred into tubes and incubated at 37 °C for 15 min. In the next step, the enzyme activity was inhibited by addition of 525 μl glycin buffer to blank and test sample tubes. Absorptions were determined at 504 nm for each fraction against blank sample. NAG activity was determined by plotting p-nitrophenol standard curve. Each enzyme activity unit is a representative of enzyme content which is able to liberate 1 μmol p-nitrophenol from PNP-NAG per min.

2.4.2. Measurement of AAP enzyme

Three ml of collected urine samples were transferred and centrifuged at 8000 rpm for 15 min. 0.1 ml the supernatant was removed and mixed with 2.2 ml of Tris buffer, and incubated at 30 °C for 5 min. The AAP enzyme activation was initiated by addition of 0.22 ml L-Alanine-P-Nitroaniline solution (75 mmol/L Tris buffer). The released p-nitroaniline, after 30 s, was measured for 3 min by UV spectrophotometer (PC 1650, UK) at 405 nm wavelength. One AAP enzyme activation unit was considered to be equivalent to enzyme content which releases 1 μmol p-nitroaniline from L-alanine-P-nitroaniline/min (Hosseini et al., 1997b). AAP enzyme activity was calculated for each sample by use of the following relationship:

$$AAP\ activity = \frac{Abs \times Vt}{Vu \times \varepsilon}$$

Where, Abs, Vt, Vu and ε, are the mean absorption over 1 min, the total sample volume, is the urine sample volume the cuvette length respectively.

2.4.3. Measurement of urinary biochemical parameters

Standard laboratory reagent strip (Uriyab-8, Bakhtar Bioshimi, Kermanshah, Iran) was used for detection of hemoglobin urine, protein, urobillinogen, and glucose. The results of which were confirmed using chemical methods of analysis. The urine samples from all groups were collected 1 h before envenoming (for control value) and after 30 min, 1, 2 and 3 h of the experimental intervention. Furthermore, the urine samples were observed centrifuged and examined microscopically for presence of intact red blood cells (RBC).

2.4.4. Histological evaluation

After one week the animals from different groups were sacrificed and a kidney from two animals from each group was removed for histological evaluation, using Hematoxylin and Eosin staining technique.