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Impact of scorpion venom as an acute stressor on the neuroendocrine-immunological network

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ABSTRACT

Although immunomodulatory property and many other pharmaceutical applications of scorpion venom have been addressed before, no studies were reported about its application as a neuroimmunomodulator at therapeutic dose. In this study, we conceptualized the property of scorpion venom, capable of inducing the acute pain and neurotoxicity can cause acute stress resulting in the modulation of immune cells through HPA axis. The whole venom from *Hottentotta rugiscutis*, a widely seen scorpion in the region of eastern Karnataka, was extracted and injected a single dose of 1 mg/kg b.w. to Swiss albino mice and then erythrocytes and leukogram were measured. Whole brain AChE activity, corticosterone, cytokines and NO levels in plasma were also evaluated at various time points. Hrv didn't show any histopathological changes in the lymphoid organs and at the site of injection. However, lymphocytes and neutrophils did get altered at 2 h post-injection. Plasma corticosterone, cytokine levels such as IL-1β, IL-6, TNF-α and IL-10 and the AChE activity were significantly increased in a time-dependent manner. Based on these results, it may be predicted, Hrv's ability to cause acute stress resulted in the activation of HPA axis, which stimulates the release of glucocorticoid hormones which in turn elicits the immunomodulation of leukocytes by altering the levels of pro and anti-inflammatory cytokines. Thus, we can conclude, the impact of acute stress induced by Hrv can intercommunicate the signals between neuroendocrine-immune systems.

1. Introduction

Stress is interpreted as a real or perceived danger alert which triggers an individual response. Many reports emphasize the effect of stress on immunity. Stress and stress hormones are shown to regulate the quantity and proportions of blood leukocytes, thus regulating the non-specific immunity. Besides that, acute stress was known to enhance the cell-mediated immunity (Dhabhar and McEwen, 1996) and immune cells are known to be regulated by central nervous system during stress, through Hypothalamic-Pituitary-Adrenal (HPA) axis mediated hormones such as adrenocorticotrophin (ACTH), glucocorticoids, and nor-epinephrine (Shepard et al., 2005).

Scorpion venom is a complex mixture of several proteins, peptides, enzymes, and neurotoxins, having pharmacological and therapeutic properties. Immunomodulatory role of venom from few scorpion species have been analyzed and found to trigger the production of cytokines, nitric oxide, reactive oxygen species and other immune mediators (Petricevich, 2006; Petricevich et al., 2007; Abdoon and Fatani, 2009).

The ability of a stressor to instigate the physiological response depends on the perception of the individual. Opioids administration in the absence of acute pain was observed to result in high infectious disease prevalence. Contradict to that, opioid administration along with acute pain was shown to be protective (Page, 2005). Such acute pain can be conceptualized as a physical and psychological stressor. Scorpion venom with highly toxic proteins induces severe acute pain, a form of acute stress, by which it may send a stress signal to central nervous system (CNS). Moreover, scorpion venom may trigger the neuroendocrine-immunological axis with its ability to release catecholamines, bradykinins, and corticosterone, which may induce the release of inflammatory messengers such as cytokines and nitric oxide. Thus, further studies are needed to understand the role of scorpion venom as an acute stressor and its role in neuroimmunomodulation.

Furthermore, *Hottentotta rugiscutis* (Pocock, 1897), a Buthidae

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scorpion, is prevalent in Chirathagundu and Hindaskatte regions of Karnataka causing considerable public health problems (Nagaraj et al., 2015). However, there are no further reports on the biochemical and immunological properties of Hrv.

Taken together, the present study was designed to understand the effect of scorpion venom as an acute stressor on neuroendocrinological messengers and its influence on inflammatory mediators. Hence, the kinetics of acetylcholine esterase and corticosterone including selective cytokines and nitric oxide responses were investigated in the presence of *Hottentotta rugiscutis* whole venom. In addition, hematological and pathological changes in lymphoid organs were also studied in the mouse model system.

2. Materials and methods

2.1. Scorpion and scorpion venom

Hottentotta rugiscutis scorpions were collected from Chirathagundu and Hindaskatte regions of Bellary district, Karnataka, India. They were maintained in plastic containers and fed weekly once with insects. Venom was obtained by mild electrical stimulation (8 V) of telson for 2–3 s. Venom was pooled, diluted with 10 vol of phosphate buffered saline (PBS) and centrifuged at 15,000 rpm for 20 min to remove the mucus. The supernatant was collected, lyophilized and stored at −20 °C and dissolved in PBS for further use (Nagaraj et al., 2015).

2.2. Animals

Female Swiss albino mice of 6–8 weeks old (20–25 g) were used in all the experiments conducted. They were kept at room temperature (RT) and fed with rodent chow and tap water *ad libitum*. All procedures performed in studies involving animals were in accordance with the guidelines approved by the institutional animal ethical committee (National College of Pharmacy, Shivamogga).

For each experiment, four groups of mice (n = 5) were injected with *Hottentotta rugiscutis* venom (Hrv) subcutaneously, a single dose of 1.0 mg/kg body weight and sacrificed at four different time points (0.5, 2, 6 and 24 h) for all the experiments. The control group was injected with the PBS alone.

2.3. Histopathology of lymphoid organs and skin histology

Lymphoid organs such as the spleen, thymus, and inguinal lymph nodes were harvested from Hrv treated and control mice and fixed in 10% buffered formalin (pH 7.4). 5 μm thin sections of paraffin-embedded tissues were prepared, stained with Hematoxylin-Eosin and observed under a light microscope for any pathological changes (Kiernan, 1999).

A trichotomy was conducted on the back of the animal at the site of venom injection and evaluated in a random and blind mode for any abnormal signs such as edema formation, hemorrhage, redness etc (Fialho et al., 2011).

2.4. Total blood cell count and differential blood cell count

Blood samples were collected from Hrv treated and control mice at different time points in K_2-EDTA containing tubes and made two aliquots. One aliquot was used for the separation of plasma by centrifugation at 2000 rpm for 15 min, to quantify the levels of corticosterone, nitric oxide, and selected cytokines. Another aliquot was used for blood cell counts. Red blood cell (RBC) count and differential Leukocyte counts (Lymphocytes, Monocytes, Eosinophils, Basophils and Neutrophils) were determined using hematological auto analyzer (Sysmex, USA).

2.5. Quantification of acetylcholine esterase (AChE)

Acetylcholinesterase activity of the whole brain was determined by modified Ellman's method (Schwartz et al., 2007). Briefly, brain tissue was homogenized (0.04 g/ml) in 20 mM Tris buffer (pH 7.4) containing 50 mM NaCl, 10 mM EDTA, 0.5% Tween 20 and 40 U/ml aprotinin (Sigma), centrifuged at 15,000 rpm/4 °C for 20 min and supernatant was collected. 100 μl of the supernatant was loaded to each well of 96 well microtiter plate and 50 μl of 0.49 mg/ml 5, 5′-dithiobis-2-nitrobenzoic acid (Sigma) in PBS containing sodium bicarbonate (0.1875 mg/ml) was added. After 5 min incubation at RT, 50 μl of 0.542 mg/ml acetylthiocholine iodide (Sigma) solution in 0.1 M PBS was added and optical density was measured at 405 nm in a Multiskan microplate reader (Thermo scientific, Finland) for every 2 min intervals up to 12th min.

2.6. Estimation of corticosterone

Corticosterone concentration was quantified by competitive ELISA as per manufacturer's protocol (Arbor assays, USA). Briefly, 50 μl of plasma sample or standard was added to each well of microtiter plate. 50 and 75 μl of assay buffer was added to wells which served as maximum binding well and nonspecific binding (NSB) well respectively. Then, 25 μl of corticosterone conjugate was added to all the wells followed by 25 μl of corticosterone antibody except for NSB wells. The plate was incubated for 1 h at RT with continuous shaking. After washing with PBS, 100 μl of TMB solution was added to each well and incubated for 30 min without shaking. 50 μl of stop solution was added and optical density was measured at 450 nm in a microplate reader. The detection limit of the assay is 16.9 pg/ml.

2.7. Estimation of pro and anti-inflammatory cytokines

IL-1β, IL-6, IL-10, and TNF-α level in plasma were determined using double-ligand cytokine ELISA kit (Biolegend, USA). In brief, flat-bottomed 96-well Nunc Maxisorp microtiter plates were coated with 100 μl/well of antibody specific for a particular cytokine prepared in coating buffer (pH 9.5) and incubated overnight at 4 °C. Wells were washed with PBS (pH 7.4) and incubated for 1 h at RT with buffer containing 10% fetal calf serum to avoid non-specific binding. Standard curves were constructed using recombinant IL-1β, IL-6, IL-10, and TNF-α. Plasma and standard samples were loaded into separate wells, incubated for 1 h at RT and washed. The appropriate biotinylated anti-cytokine antibody was added and incubated for 1 h. After washing, 100 μl/well of Avidin-HRP (1:1000) was added and incubated for 45 min at RT and washed thoroughly. Finally, 100 μl of TMB (Sigma) was added, and the reaction was arrested after 15 min with 100 μl of 1 M H_2SO_4. The intensity of the color developed was measured at 450 nm with a microplate reader. The results were expressed as ng cytokine/ml plasma. The sensitivity of the assay is approximately 2 pg/ml for IL-6; 4 pg/ml for TNF-α; 16 pg/ml for IL-10 and IL-1β.

2.8. Estimation of total nitric oxide

The total nitric oxide (NO) level in plasma was estimated as the total nitrate/nitrite concentration after the reduction of nitrate to nitrite using colorimetric assay kit (Cayman chemicals, US). Briefly, Nitrate standard was added to uncoated polystyrene plates to get a concentration ranging from 5 to 35 μM and the final volume was made up to 80 μl with assay buffer. Then, plasma samples were added to wells with a maximum of 40 μl. 10 μl of Nitrate reductase and 10 μl of nitrate reductase cofactor were added to each sample and standard wells. One well was added with 200 μl of assay buffer