ORIGINAL ARTICLE

Evaluation of Pro-inflammatory and Anti-inflammatory Cytokine Levels in Rats Treated with *Hottentotta Saulcyi* Scorpion Venom

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Abstract

Introduction: Scorpion venom contains various biological compounds. Clinical symptoms in individuals and laboratory animals exposed to scorpion venom depend on the response of the host immune system. The secretion of inflammatory cytokines is one of the most critical factors involved in the pathogenesis of scorpion venom.
Methods: This comparative study aimed to evaluate the expression of inflammatory cytokine IL-6 and anti-inflammatory cytokine IL-10 in rats treated with *Hottentotta saulsyi* scorpion venom. The venom was obtained from the Razi Vaccine and Serum Research Institute of Ahvaz branch. After determining the *H. saulsyi* venom LD_{50}, the rats were divided in two groups of test and control (n-12). The test group received 1/3 LD_{50} dose in 0.5 ml of physiological serum by subcutaneous injection per rat. The exact amount of physiological serum was injected into the control group. After that, cardiac blood samples were taken from rats at 0, 4, 24, and 72 hours after anesthesia. After serum preparation, the levels of IL-10 and IL-6 cytokines were measured in both groups using ELISA assays.
Results: The obtained LD_{50} equaled 1.01 mg/kg of the rat's body weight. Four hours after experimentally envenomation, the serum levels of IL-6 and IL-10 were significantly increased compared to the control group ($P < 0.05$); but in the taken samples 24 hours after the treatment, there was no significant difference compared to the control group. During 72 hours, the level of these cytokines decreased in the treatment group compared to the control group.
Conclusion: Changes in inflammatory and anti-inflammatory cytokines levels during scorpion stings can be used as a novel clinical finding to assess patients' status and perform appropriate therapeutic interventions to reduce scorpion sting complications.

Keywords: Hottentotta saulsyi, Pro-inflammatory, Anti-inflammatory, Cytokine, Rat

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INTRODUCTION

Scorpions are venomous animals of the genus Phylum *Arthropoda*, Order *Scorpionida*, Class *Arachnida*, and the spiders order. The bodies of these creatures are hard and rough. Iranian scorpions are reported to belong to the two families *Buthidae* and *Scorpionidae* and include 12 genera and 24 species [1]. These creatures inhabit in all continents except *Antarctica*. The medically main scorpion species belong to the *Buthidae* family, including the genera of *Androctonus*, *Buthus*, *Mesobuthus*, *Buthotus* (*Hottentotta*), *Parabuthus*, and *Leirus* [2). Scorpion sting is one of the most critical health and medical issues in the world's underdeveloped tropical and subtropical countries, which endangers thousands of people's lives yearly. Scorpion stings are currently a health problem in South Africa, the Middle East, the southern United States (Mexico), and the Indian subcontinent. They are a vital concern due to illness and mortality, especially in children in these countries [3].

An investigation estimated 2.3 billion people would be victims of scorpion stings in high-risk areas. About one million people get scorpion stings each year, leading to a mortality of about 0.27% [4]. The global distribution of *Hottentotta saulsyi* scorpions is in Syria, Afghanistan, Iran, Turkey, and Iraq. This scorpion distribution is very high and is found among the boulders in most mountainous parts of the country. In Iran, this scorpion species is most abundant in the Khuzestan province.

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The *Hottentotta saulsyi* scorpion is one of Iran's sixth medically significant scorpions, which is present in almost all parts of the country [5]. The venom of this scorpion species from the *Butida* family is such that the injured person should receive medical care. Reports of injured persons indicate the effect of the poison on the respiratory and cardiovascular systems. Scorpion stings and the resulting damages in Khuzestan province threaten society's health. It is noteworthy that scorpion venom contains many toxins that show a wide range of biological properties and activities [6].

A cascade of systems, cellular elements, and the release of immune system mediators trigger the inflammatory response. Vertebrates respond to antigen exposure by producing antibodies through a series of events involving multiple cell interactions. Initially, T cell antigen receptors identify antigens on antigen-presenting cells surface. Finally, B cells produce antibodies that detect the venom antigens. Two subpopulations of Th cells, Th1, and Th2, are mainly involved in cytokine secretion in response to antigens.

Th1-type cells mainly secrete interleukin-2 (IL-2), tumor necrosis factor (TNF-α), and interferon-γ (IFN-γ), which are responsible for activating macrophages and triggering cellular immune responses to endogenous pathogens. They strengthen the invading cell or the presence of toxins. Th1 cells also produce opsonizing IgG2a antibodies. Th2 cells secrete IL-4 and IL-5 cytokines in allergic conditions and worm infections; also, these cells are involved in the production of antibodies by B cells [7].

Cytokines are compounds secreted by most immune system cells and exhibit different functions. Immunologists classify Cytokines as inflammatory or anti-inflammatory depending on their roles or properties [8]. Inflammatory cytokines such as IL-1, IL-6, and TNF-α are primarily responsible for creating an effective defense against exogenous pathogens. However, overproduction of these intermediates can be harmful and may eventually lead to shock, multiple organ failure, and death. Conversely, anti-inflammatory cytokines, including IL-4, IL-5, and IL-10, are essential for reducing the regulation of the intensified inflammatory process and maintaining homeostasis for the proper functioning of vital organs. However, excessive anti-inflammatory responses may also lead to suppressing safety performance. The balance between inflammatory and anti-inflammatory activities determines the degree and extent of inflammation that leads to different clinical effects [9].

The effects of inflammatory cytokines neutralize anti-inflammatory cytokines. Therefore, the relative concentration of the cytokine on its inhibitor or antagonist determines its outcome. The imbalance of cytokines leads to damage and destruction of organs during severe sepsis and scorpion stings. There is ample evidence for the role of cytokines in scorpion stings. It appears that scorpion stings alter both levels of inflammatory and anti-inflammatory cytokines.

IL-6 is a cytokine with different functions that has inflammatory effects. Various cells produce it, including B and T lymphocytes, monocytes, fibroblasts, keratinocytes, endothelial cells, mesenchymal cells, and particular tumor cells [10]. Researchers observed increased levels of IL-6 in rats which exposed to *Centruroides noxius* and *T. serrulatus* scorpion venom. IL-6 is often used as a marker inflammatory response and has inflammatory and anti-inflammatory effects [11]. This interleukin regulates the synthesis of IL-1 and TNF-α and inhibits the production of GM-CSF, IFN-γ, and MIP-2 [12]. IL-10 is the most important anti-inflammatory cytokine found in the human immune response and is a potent inhibitor of Th1 cytokines production, including IFN-γ, TNF-α, IL-1β, IL-2, IL-6, IL-8, and IL-12. In addition, inflammation is the original stimulus for its production, and IL-1β and TNF-α can directly stimulate IL-10 production. Another property of IL-10 is suppressing oxygen-free radical scavenging and nitric oxide activity by macrophages and prostaglandin production [13]. The secretion of IL-10 is altered in the serum of patients exposed to the scorpion venom *Tityus serrulatus* and in laboratory animals exposed to the scorpion venom of *Androctonus australis hector, Centruroides noxius and T. serrulatus*.

The pathophysiology of the poison entering the body is complex, but there is no doubt that it often triggers a systemic inflammatory response to scorpion stings. The discovery that cytokines can cause disease has opened up new lines of research. Systemic effects of cytokines cause fever and increased symptoms. In scorpion sting, the balance between inflammatory and anti-inflammatory cytokines determines the degree and extent of the inflammation and can lead to various clinical effects. A severe inflammatory reaction is responsible for the pathogenesis of septic shock following scorpion stings. In contrast, an intense anti-inflammatory reaction can lead to the failure of scorpion venom function. According to the above facts, *H. saulcyi* scorpion is one of the most critical scorpion species in Khuzestan province. Venom in the victims' bodies probably changes the balance and amount of inflammatory and anti-inflammatory cytokines. Due to the lack of sufficient information about the action mechanisms of this scorpion venom in the body and target organs, we performed the present study to investigate the inflammatory and anti-inflammatory cytokines released in laboratory rats treated with *Hottentotta saulsyi* scorpion venom.

METHODS

Experimental Animals

This study was an experimental (laboratory) study in which we investigated the inflammatory response and secretion of inflammatory and anti-inflammatory cytokines in laboratory Wistar rats in weight of 250 gr±20 after subcutaneous injection of *H. saulcyi* scorpion venom. The animal handling procedures were carried out under the project license issued by ethical approval of animal welfare, in Razi vaccine and serum research institute of Ahvaz branch, Ahvaz, Iran with ethical code of IR.AJUMS.ABHC.REC.1400.118 Rats were at appropriate light, humidity, and temperature conditions during storage, and we regularly checked their nutrition and water quality. Blood sampling was done with great care and following the

ethical standards of working with animals.

A total of 24 rats were divided into two groups; one as a healthy control group and the other as experimentally scorpion envenomation. Razi Vaccine and Serum Research Institute, Ahvaz branch, prepared the required venom for this study. The lethal dose was determined by intraperitoneally (I.P) injection of increasing concentrations of venom to adequate numbers of the rats. After treatment, the animals were monitored for 24 h and the number of deaths was recorded; Lethal dose (LD) 50% (LD50) was determined using the Spearman–Kaerber method (Hamilton et al., 1977). After determining LD_{50}, 1/3 LD_{50} dose equal to 0.336 mg /kg was injected subcutaneously into 12 rats in the treatment group. In addition, the same volume of physiological saline was injected subcutaneously into 12 rats in the control group.

The rats were anesthetized at blood sampling times using an intraperitoneal injection of 60 mg /kg pentobarbital sodium. The heparinized blood samples were collected from the animal's hearts at 0, 4, 24, and 72 hours after venom injection. Consequently, The ZellBio ELISA diagnostic kit were used to determine the plasma levels of cytokines IL-6 and IL-10.

Date Analysis

Descriptive and inferential statistical methods were applied to data analysis and the SPSS software version 18 was used. Paired sample t-tests were used to compare groups. Data on the assumption of non-normality was restored with the Wilcoxon rank test. One sample t-test was employed to evaluate the confidence intervals. Data were considered statistically significant if P-values were less than 0.05.

RESULTS

By comparing serum IL-10 levels in the control and treatment groups, there was no statistically significant difference between the mean serum IL-10 levels at 0 and 24h in the control and treatment groups (Figure 1). The serum level of IL-10 at 4 h in the treatment group was significantly increased compared to the control group ($p < 0.05$) (Figure 1). Controversially, Serum level of IL-10 at 72 h in the control group compared to the treatment group decreased significantly ($p < 0.05$) (Figure 1).

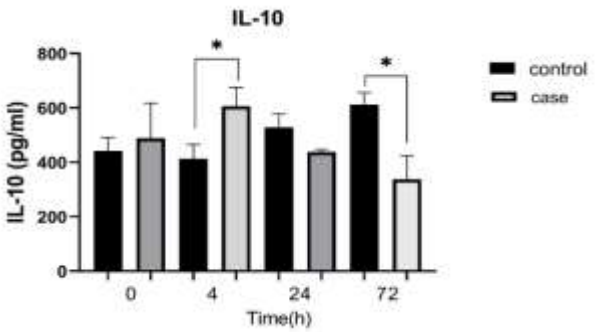

Figure 1. Comparison of the level of rats' serum IL-10 in control and treatment groups at different times.

There was no significant difference between serum IL-10 levels at 0 and 4h. However, serum level of IL-10 were significantly higher at 4h than at 24h and 72h ($p < 0.05$).

Serum levels of IL-6 in rats in the treatment group were significantly increased at 4 h compared to the control group (Figure 3) ($p < 0.05$). Serum levels of IL-6 at 24 h did not show a significant difference between the two groups, but this level at 72 h was significantly higher in control rats' than in the treatment group ($p < 0.05$).

Serum IL-6 levels were significantly increased at 4 h compared to 0 h. ($p<0.05$) But there was no significant difference between 4h and 24h times. At 72 h, serum IL-6 levels were significantly reduced compared to 4 h and 24 h ($p < 0.05$).

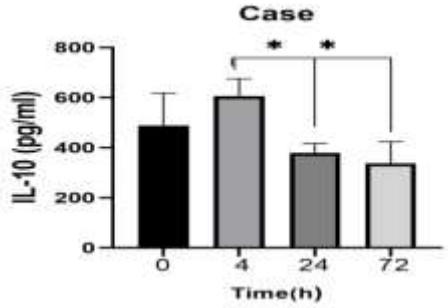

Figure 2. Comparison of the level of rats' serum IL-10 at different times in the treatment group (* $p < 0.05$).

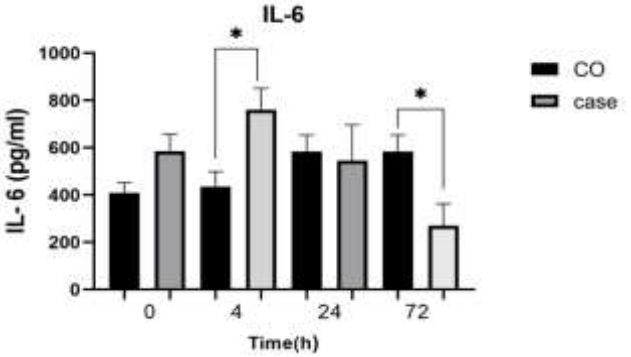

Figure 3. Comparison of the level of rats' serum IL-6 in control and treatment groups (p* <0.05)

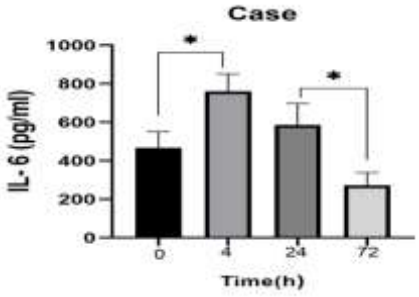

Figure 4. Comparison of the level of rats' serum IL-6 at different times in the treatment group.

DISCUSSION

In this study, we investigated the effect of *Hottentotta saulsyi* venom on the expression of IL-10 and IL-6 cytokines in rats. At 4 h after injection, the level of IL-10 cytokine in the treatment group was significantly increased compared to the control group. In a study conducted by Fukuhara et al., it was observed that following a scorpion sting, the serum level of IL-10 in severe and moderate scorpion stings increases in patients [16], which is in line with the results of the present study .

Tissue damage that occurs during the inflammatory process is an advanced process that may lead to organ dysfunction. Together, pro-inflammatory and anti-inflammatory cytokines play an important role in the regeneration of damaged tissues, both functionally and structurally. One of the critical roles of interleukin 10 is to inhibit the effects of interleukin 6 and other pro-inflammatory factors such as interleukins 2, 8, 12 as well as TNFα and IL-1B [17, 18]. Another cytokine we evaluated in this study was IL-6.4 h; the level of IL-6 was significantly increased compared to time zero and the control group.

In a study conducted by Costal-oliviera et al., it was contended that after injection of *H. lunatus* scorpion venom in murine and field mouse models, the levels of cytokines IL-6, IL-10, TNF-α, IFN- γ and IL-4 increased significantly at intervals of 3 and 6 hours. Still, the increase in IL-6 levels at these intervals was much more significant in other cytokines [19].

IL-6 is one of the essential inflammatory cytokines in the body, which is secreted from immune system cells such as macrophages in various conditions, including inflammation, infectious diseases, and plays a significant defensive role. Uncontrolled response to IL-6, and with other inflammatory cytokines such as IL-1 can cause pathological damage, heart disorders, and shock in individuals [20]. Regulatory agents such as IL-10 are essential to prevent excessive and damaging immune responses. IL-10 prevents the damage caused by these mediators by regulating the immune system responses and modulating the production and secretion of inflammatory mediators, such as shock, and acting [21].

Scorpion venom contains various toxic and immunogenic compounds that stimulate the cells of the immune system and cause the release of inflammatory mediators. If these inflammatory mediators remain uncontrolled, the patient may develop cytokine storm and septic shock. In this study, we observed a simultaneous increase in serum levels of IL-6 and IL-10 in the treated mouse model. There are good reasons to hypothesize that the simultaneous secretion of inflammatory and anti-inflammatory mediators such as IL-10 by immunoregulatory cells adjust immune responses that prevent uncontrolled and harmful reactions against the host despite fighting pathogens Within 24 hours after injection of the scorpion poison. Additionally, we observed that the serum levels of IL-10 and IL-6 cytokines decreased compared to 4 hours and did not show a significant difference from the control group. This decreasing slope became much more noticeable in the period of 72h. Thus, the serum levels of IL-10 and IL-6 in the treated rat model were significantly reduced compared to the initial hours in the control group. In a study by Razi-Jalali et al., they reported that IL-6 and IL-1α cytokines levels peaked between 3 and 6 hours after injection of scorpion venom *mesobuthus eupeus* in rats. However, its amount is gradually reduced so that it returns to its base value in the time periods of 24 and 72h [22].

Cytokines are relatively short-lived mediators that are secreted by the body depending on the conditions and needs, and after performing their function, their amount decreases after a while. Cytokine reducing process is also logical sometimes after a scorpion sting and is an immunological process. Cytokines are secreted 4 to 6 hours after the scorpion sting, due to the body's inflammatory response to scorpion venom, and after the operation, due to their limited half-lives, their levels in the blood begin to decrease. Obviously, when the amount of poison that enters the body and the subsequent stimulation is high, the immune system response will be more intense, and the period of cytokine production will be longer

In this study, like other research that uses similar doses of scorpion venom, the results almost followed a pattern; the increased cytokine production persisted for 3 to 6 hours and then gradually began to decrease. This issue can be due to the perseverance of the venom in the body and the immune response regulation. The immune system begins to secrete inflammatory and regulatory mediators in times of crisis and threat. Still, after neutralizing the threatening factors and preventing harmful responses, it reduces the production level of its mediators and limits the reactions. The current study could be replicated on the envenomed human samples to determine the treatment strategies in each period after envenomation according to the indexing cytokines of the inflammatory or regulatory elements.

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