

Edematogenic, hemorrhagic, and myonecrotic effects of the *Pseudocerastes persicus* venom in mice

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Abstract

Snakebites represent a significant public health concern in Iran, where the Persian horned viper (*Pseudocerastes persicus*) is endemic. This study aimed to evaluate, for the first time, the local pathological effects of its venom in mice. Paw edema was induced in different groups (n = 6 in each group) by intraplantar injection of venom (10, 50, and 100 µg/paw). Increase in paw thickness (%) was measured using a digital caliper over time. Pathological changes (blood cell extravasation, edema, and leukocyte infiltration at the injection site) were assessed via hematoxylin and eosin staining. Pentavalent snake antivenoms (RVSRI and PSA) were tested by preincubation with venom or intraperitoneal administration pre- or post-exposure. Subcutaneous injections of the *Pseudocerastes persicus* crude venom 10-100 µg/paw resulted in marked dose-dependent paw edema in mice. As a whole, both antivenoms diluted in normal saline (100 µl) significantly reduced paw edema, and hemorrhagic and histological properties were reduced when incubated with the challenge dose (50 µg) for 30 minutes. However, when infused intraperitoneally, 10 minutes before or immediately after venom exposure, both failed to neutralize the pathological changes. In parallel settings, intramuscular and subcutaneous injections of the venom (50 µg) into the gastrocnemius muscle and the dorsal areas of anesthetized mice caused severe changes. According to our preliminary study, this venom exhibits extreme edematogenic, hemorrhagic, and myonecrotic effects, necessitating further investigation to develop effective treatment strategies for envenomation. Understanding these dynamics is crucial for introducing effective antivenoms and, if necessary, new therapeutic modalities, given economic constraints and the need for therapeutic interventions in the management of snakebites.

Keywords: Persian horned viper; Paw edema; Venom; Antivenom.

1. Introduction

Snakebite, a neglected tropical disease, is prevalent in tropical and subtropical regions, including Iran, making

it an appropriate focus for herpetological analysis. It is estimated that Iran is home to 45 non-venomous, 27 venomous, and 11 semi-venomous snake species [1].

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Envenomation is common in deserts and warm climates, particularly in hot rural areas with limited access to healthcare centers. Based on previous studies, both *Macrovipera lebetinus* and *Echis carinatus*, belonging to the Viper family, are responsible for the majority of medical envenomations in this region [2, 3].

Pseudocerastes persicus, commonly known as the Persian horned viper, belongs to the Viperidae family and is primarily present in the Middle East, particularly in the Zagros mountains [4]. Despite its widespread distribution, there is only one reported case of biting—a 33-year-old herpetologist from Kerman province—who experienced local pain and significant swelling without systemic or laboratory abnormalities [5]. Meanwhile, this envenomation is associated with several biochemical properties, including caseinase activity, hemolysis, L-amino acid oxidase activity, cytotoxicity, neurotoxicity, coagulant effects, and the activation of factor X and prothrombin in laboratory animals [6–8].

According to current medical knowledge, despite the availability of recommended treatments for viper envenomation, the only approved and effective method for snakebite remains the prompt intravenous administration of commercial mono- or polyvalent antivenom, along with transportation of the patient to the nearest hospital [9]. In Iran, Razi Vaccine and Serum Research Institute (RVSRI) and Padra Serum Alborz (PSA) corporations develop anti-snake serums [10]. Both pentavalent products (pepsin-derived antibodies) could neutralize five endemic snakes (*Echis carinatus*, *Macrovipera lebetina*, *Vipera albicornata*, *Pseudocerastes persicus*, and *Agkistrodon halys*). It should be noted that the production of snake antivenom is a complex process involving venom collection, horse immunization, antibody production, and extensive testing.

As reported in a previous study, this venom contains a high concentration of destructive and proteolytic enzymes, most notably Phospholipase A₂, which is present in greater amounts than in other viper venoms and is primarily responsible for its toxic effects [8]. This study aimed to evaluate experimentally, for the first time, the edematogenic, hemorrhagic, and myonecrotic properties of this venom. For further assessment, pathological samples were prepared to evaluate inflammatory cell infiltration, red blood cell

extravasation, and ultimately, edema induced by this venom. In a parallel study, the neutralization capacity of the edematogenic properties was evaluated using pentavalent RVSRI and PSA antivenoms, with three doses of each product. This study may serve as a primary animal study to define the properties of its instinct and its important destructive enzymes, and paves the way for finding the most suitable treatments for human bites.

2. Materials and Methods

2.1. Animals and Treatments

Male albino mice weighing 20–30 g were provided by Bushehr University of Medical Sciences. The animals were transferred to the animal facility several days prior to the experiment to allow acclimatization. They were maintained under standard laboratory conditions (12:12-hour light/dark cycle, 24–26°C, with free access to food and water). The mice were housed in PVC cages, with three animals per cage.

Pseudocerastes persicus lyophilized venom was obtained from RVSRI. It was stored at -20° C until use. Samples were freshly prepared in 0.9% NaCl solution immediately before each experiment. RVSRI and PSA antivenoms were kindly provided as gifts by their respective manufacturers. All other reagents and chemicals used in this study were obtained from reputable suppliers in Iran.

2.2. Experimental protocol

Initially, animals were divided into three groups (n = 6 in each group). Hind paw edema was measured as described in previous studies [11]. Lyophilized venom (10, 50, and 100 µg/paw) dissolved in 100 microliters of normal saline was injected into the right hind paw intraplantarly (i.pl.) under light anesthesia (ketamine 50 mg/kg) [12]. An equal volume of vehicle (normal saline) was injected into the contralateral hind paw as a control. A response was evaluated by measuring the thickness of the paw edema at regular intervals up to 60 minutes using a digital caliper (Guanglu Co., China). The final result was calculated as the difference in paw thickness between the treated and control paws divided by the control paw thickness. The results were expressed as percentages. In a parallel experiment to evaluate myotoxic activity, *Pseudocerastes persicus* venom

dissolved in normal saline (50 µg/100 µL) was injected intramuscularly into the right gastrocnemius muscle and subcutaneously into the dorsal and right sides of the mice's bodies (n = 6 per group). An equal volume of normal saline was applied to the contralateral organs (the left calf and lumbar region) by the same route. Treated organs were prepared an hour post-treatment for histological examination, following photographic documentation.

2.3. Histological Preparation

At the end of the experiments, all mice were humanely sacrificed. Tissue samples, including the treated tarsal region, skin, and muscle, were excised, photographed, and fixed in 10% formalin for 24 hours. Samples were subsequently dehydrated in ethanol, cleared in xylene, and, finally, embedded in paraffin wax. Paraffin-embedded blocks were sectioned at 5-µm thickness using a rotary microtome. The sections were placed on glass slides, rehydrated, and stained with hematoxylin and eosin (H&E) for histological evaluation. Parameters such as structural interruption, subplantar edema, red blood cell extravasation, and the existence of immune cells were assessed under a standard light microscope at various magnifications (Olympus CX22, USA).

2.4. Polyvalent antivenom neutralization potency

The neutralization properties of three doses (10, 30, and 50 µl) of both antivenoms against the edema-forming potential (50 µg/paw) were assayed following preincubation at room temperature for 30 minutes in parallel experiments. Three incubated mixtures of snake venom and each antivenom, with the same final volume (100 µl), were prepared and injected subcutaneously into the right paw area (n=6 in each group). Injection of normal saline into the contralateral paw was used as a control. After an hour, the mice were humanely sacrificed, and the ability of both antivenoms to prevent edema formation was evaluated by measuring paw thickness at different time points. Pathological changes were microscopically assessed in all samples after obtaining macroscopic images, and the neutralizing properties of our polyclonal antivenoms were assessed in tarsal injections.

2.5. Statistical Analysis

All values were analyzed using SPSS 26 and are presented as mean ± standard error (SE). For intergroup comparisons, a one-way analysis of variance (ANOVA) with Tukey's post hoc test was used. A p-value of less than 0.01 was considered statistically significant.

3. Results and Discussion

3.1. The edematogenic properties of the *Pseudocerastes persicus* venom

As shown in **Figure 1**, 50 µg of this venom induced a statistically significant increase in paw edema ($p < 0.001$), even stronger than 10 µg of the same venom ($p < 0.05$). A clear dose-dependent response was observed, with higher doses leading to greater swelling. Macroscopic and microscopic examinations performed one hour after venom injection revealed diffuse edema and hemorrhage compared to the contralateral paws. This effect was further enhanced at 20 minutes with 50 µg per paw, reaching statistical significance ($p < 0.001$). According to **Figure 2A**, edema formation began at 5 minutes, with a mean increase of $43.21 \pm 3.41\%$, and reached its peak value at 20 minutes ($64.31 \pm 5.22\%$). It is stated that edema progressively declined and completely resolved within two hours, while necrotic appearances of the paws persisted, as shown in **Figure 2B**.

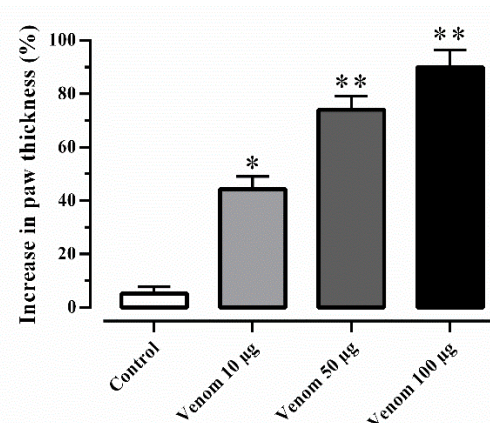


Figure 1. Dose-dependent increase in paw thickness observed 20 minutes after subcutaneous injection of *Pseudocerastes persicus* venom into the right hind paw of mice. The left hind paw served as the control and received 100 µL of normal saline under the same conditions. Each column represents the mean ± SEM of six mice. * $P < 0.05$ and ** $P < 0.001$ compared to control.

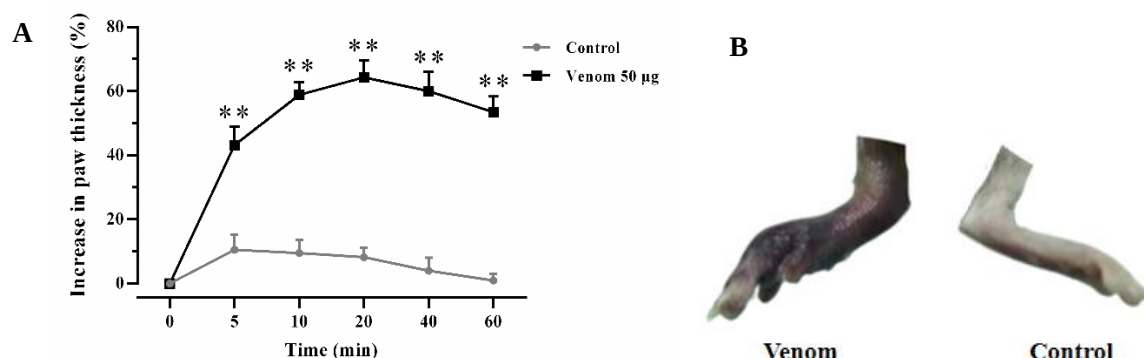


Figure 2. Time-dependent edema from 5 minutes to 1 hour after injection of 50 µg/paw of *Pseudocerastes persicus* venom (A), and its necrotic effects including edema, discoloration, and dysmorphic appearance of the right tarsal area compared to the left (B). ** $P < 0.001$ compared to control.

3.2. Neutralizing effects of incubated venoms and antivenoms

As shown in **Figure 3**, 30-minute incubation of 50 microliters of RVSRI or PSA with *Pseudocerastes persicus* venom (50 µg) decreased paw edema from $64.31 \pm 5.22\%$ to $24.31 \pm 4.08\%$ and $31.54 \pm 5.77\%$, respectively. Low doses of both antivenoms (10 and 30 µl) showed no potency in neutralizing the edematogenic and pathological devastating effects. It is worth noting that neither pre- nor post-intraperitoneal administration of either antivenom (administered 10 minutes before or after envenomation) exhibited neutralizing activity (data not shown).

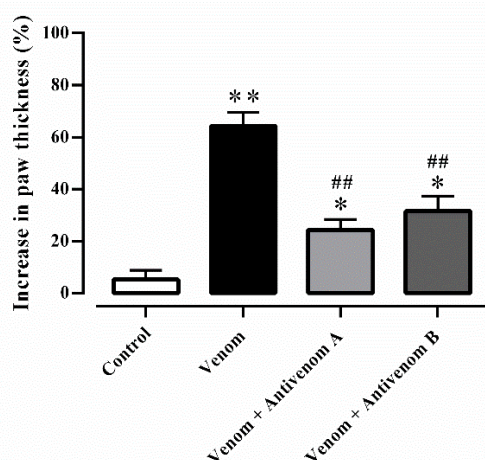


Figure 3. Antivenom efficacy in reducing paw edema induced by *pseudocerastes persicus* venom (Antivenom A: RVSRI, Antivenom B: PSA). Each column represents mean $\pm$ SEM of six mice. * $P < 0.05$ and ** $P < 0.001$ compared to control, ## $P < 0.001$ compared with venom.

3.3. Histopathological studies

Figure 4 illustrates the histopathological alterations in the animal's paw after horned viper venom injection, as well as the mitigating effects of antivenoms on these changes. Pathological analysis of mouse paws treated with normal saline as a control revealed no observable changes at either the macroscopic or microscopic levels after envenomation. Samples showed compact, well-organized collagen fiber bundles with no vascular abnormalities, although minor disruption of fibroblasts was observed. On the contrary, after venom injection, pronounced edema was visualized, leading to the separation of adipose tissues and other substructures, in addition to gangrene and necrosis of the injected paws. Red blood cell extravasation and recruitment of lymphocytes and macrophages were observed beneath the epithelial cell layer. It is noted that 30-minute incubation of *Pseudocerastes persicus* venom with either of the two antivenoms (50 µl) showed no functional destructive effects (**Fig 4**).

3.4. Myotoxic and hemorrhagic activity

An hour post-injection, the animals were sacrificed by cervical dislocation. The right and left gastrocnemius muscles and dorsal skin regions were dissected and photographed macroscopically, then prepared for hematoxylin and eosin (H&E) staining. Based on macroscopic and microscopic observations, injection of the challenge dose (50 µg) induced gross myonecrotic and hemorrhagic effects in the gastrocnemius and dorsal regions of the mice, respectively (**fig 5, 6**).

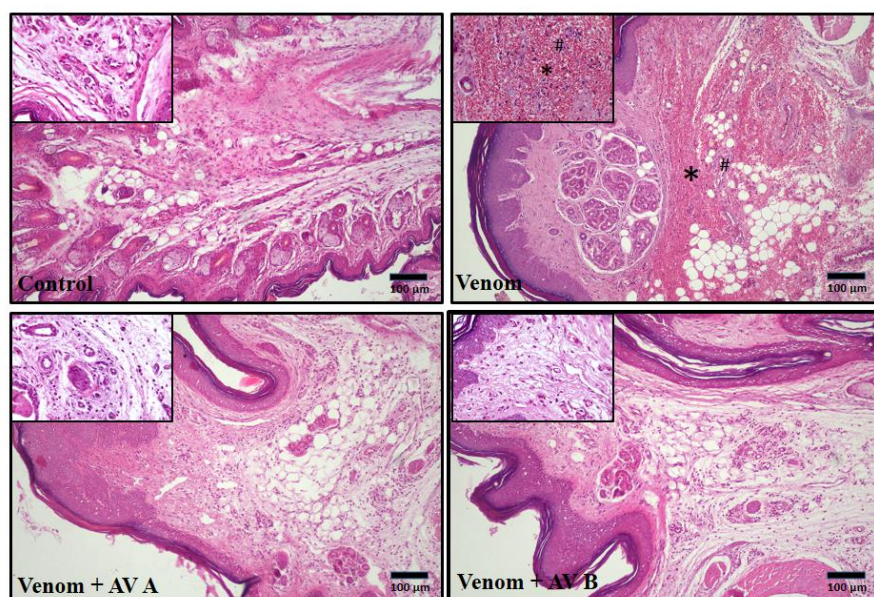


Figure 4. Neutralizing effects of RVSRI (AV A) and PSA (AV B) antivenoms on the pathological changes induced by *Pseudocerastes persicus* venom. Edema, hemorrhage, red blood cell (RBC) extravasation, and immune cells infiltration were observed within a short period, as shown at both low and high magnifications (upper right), compared to normal saline injection (upper left). When the venom (50 μ g) was pre-incubated with equal volumes of RVSRI or PSA antivenom (50 μ l), its hemorrhagic and immunogenic effects were neutralized, while its edematogenic activity was significantly reduced (lower left and right). * Red blood cells extravasation, # White blood cells infiltration.

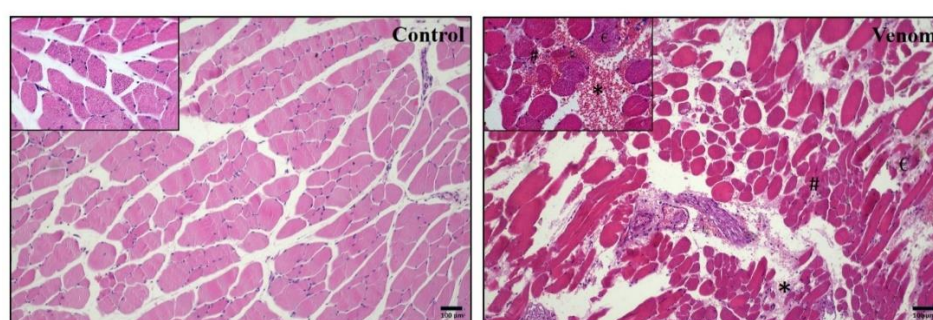


Figure 5. Myonecrotic effects of *Pseudocerastes persicus* venom. Intramuscular injection of *Pseudocerastes persicus* venom induced severe tissue damage, including myocyte bundle disruption, intramuscular hemorrhage, and immune cell infiltration, as shown at both low and high magnifications (right), compared to normal saline administration (left). * Red blood cells extravasation, # White blood cells infiltration, € Myocytes bundle destruction.

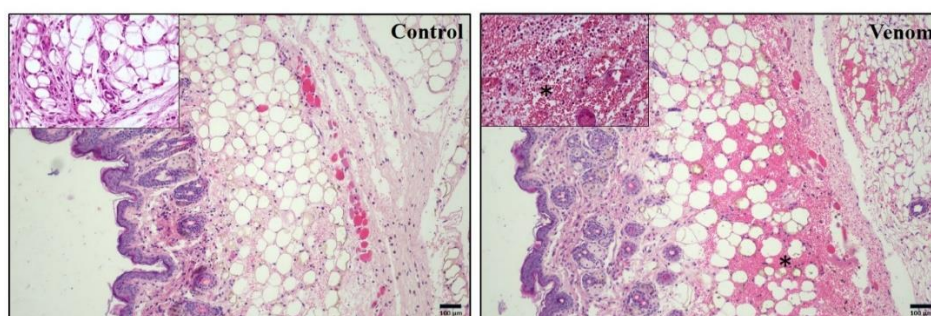


Figure 6. Destructive effects of *Pseudocerastes persicus* venom on the skin. Subcutaneous injection of *Pseudocerastes persicus* venom resulted in extensive hemorrhage, edema, and immune cell infiltration, as shown at both low and high magnifications (upper right), compared to normal saline injection (upper left). * Red blood cells extravasation, # White blood cells infiltration.

Following venom injection, the architecture of the gastrocnemius muscle and skin was severely disrupted compared to the control, with red blood cell extravasation and infiltration of lymphocytes and macrophages observed in both areas (Fig. 5, 6).

3.5. Discussion

Snakebites cause a lot of injuries and mortalities in different parts of the world, particularly in warm climates. In a 7-year epidemiological study (2004-2011), the rate of animal bites was 2.9 per 100,000 in Iran [13]. Fortunately, the mortality rate was low (0.09 per 100,000), likely due to the availability of antivenoms and appropriate medical care [14]. There are few reports of human envenomation by this snake in Iran. On the other hand, there has been no research conducted clinically on the effectiveness of Iranian antivenoms as the initial step in treatment. In this preliminary study, the edematogenic activity of the venom and the neutralizing potential of the corresponding antivenoms were thoroughly evaluated by inducing paw edema in mice through subcutaneous injection of the prepared venom.

Paw edema, hemorrhage, skin necrosis, and myonecrotic effects are the cardinal characteristics of the family Viperidae. According to our experiment (Fig 1), ten micrograms of the *Pseudocerastes persicus* venom induced paw edema ($P < 0.05$), and this capacity was approximately higher than that of *Philodryas nattereri* and *Bothrops* (10 -50 microgram/paw) [15, 16]. Notably, this capacity was lower than that of Egyptian *Cerastes cerastes* and *Echis carinatus* venom, necessitating further investigation [17]. Different snake metalloproteinases and, most importantly, phospholipase A2, are responsible for the rapid onset of edema due to hemorrhage-intensifying activity in snake venom [18, 19]. According to a previous study, the Persian horned viper found in Semnan Province possesses phospholipase A2, as well as serine proteases, metalloproteinases, and other phospholipases [6]. These properties are compatible with the edematogenic and destructive characteristics of this venom in our study. As evident in Fig. 2B, necrosis of the injected paw is observed due to the probable effects of *Pseudocerastes persicus* enzymes, such as metalloproteinases, which are also found in Viperidae [20]. There is no national

research evaluating the full complement of enzymes in this snake, and the effects of temperature, age, location, and gender on these enzymes should be clarified.

Antivenom infusion after envenomation, in addition to medical facilities, is an effective and commonly used remedy for envenomed patients. RVSRI antivenom, as an established Iranian corporation, has a significant responsibility in treating human patients. In our study, the capacity of this pentavalent snake antivenom, in addition to renewed and compatible PSA antivenom, was assessed. They were investigated in three phases (pre-injection, incubation, and post-injection) of the same volume. Based on our study, incubating venom and antivenom together for 30 minutes at room temperature statistically decreased the venom's edematogenic properties, similar to studies on vipers [21].

In contrast, intraperitoneal administration of both antivenoms, either 10 minutes before or after venom injection, failed to prevent swelling. It is evident that, due to its rapid development (10 minutes) compared to other vipers, the edematogenic phase is challenging to neutralize with standard equine pentavalent antivenom both before and after venom injection [22]. Venoms contain low-molecular-weight factors that induce edema, suggesting that the immunogenicity of some components may be insufficient to achieve effective neutralization. However, such components are responsible for only a minor portion of the edema induced by crude venoms [23]. It is worth noting that, given the low incidence of human envenomation by this snake and, from an economic perspective, producing monovalent antivenoms in either company would be impractical. However, our results demonstrate the anti-edematogenic properties of the currently available polyvalent products.

4. Conclusion

Snakebite mortality in Iran is extremely low compared to other countries and regions, such as sub-Saharan Africa, where approximately 20,000 deaths occur annually [24]. This low fatality rate can be attributed to the presence of well-equipped tertiary medical centers and the availability of high-quality antivenoms produced by RVSRI, a well-established and reputable institution and PSA corporation. Due to the lack of human bite studies in Iran, this animal study is the first to experimentally

demonstrate the edematogenic, myonecrotic, and dermonecrotic properties of this venom, as well as the neutralizing effects of PSA and RVSRI polyvalent antivenoms. Finally, continued research into the palliative efficacy of existing pharmacological agents should be conducted in animal studies.

Ethics approval

All experimental procedures were performed according to the EU 2010 declaration rules and institutional guidelines for the care and use of laboratory animals and were subsequently approved by our University (Permission number: IR.BPUMS.AEC.1402.003).

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Conflict of interest

The authors declare no conflicts of interest.

Data availability

Data is available upon request from the corresponding author.

Authors Contributions

RS and HF outlined the study; RS and HF performed experiments and discussed the results; RS managed and directed the study; RS, SEFS and HF wrote the draft of the manuscript. EK and RS finalized the manuscript. All authors reviewed and approved the final version of the manuscript.

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Using artificial intelligence chatbots

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