

# Lung Immunoreactivity and Airway Inflammation: Their Assessment After Scorpion Envenomation

Sonia Adi-Bessalem,<sup>1,2</sup> Amina Mendil,<sup>1,2</sup> Djelila Hammoudi-Triki,<sup>1,2</sup> and Fatima Laraba-Djebbari<sup>1,2,3</sup>

**Abstract**—Release and activation of pro-inflammatory mediators are among the most important induced factors that are involved in the scorpion envenomation pathogenesis. Inflammatory response and lung reactivity were studied in mice following subcutaneous injection with *Androctonus australis hector* (Aah) venom. Venom immunodetection in lungs and sequestered cell population in the airways were determined. Cytokines, cellular peroxidase activities (eosinophil peroxidase, myeloperoxidase), and IgE antibodies were also assessed. Immunohistochemical study revealed a positive detection of the Aah venom in the alveolar wall, venule lumens, and inside inflammatory cells. Severe lung edema associated with rapid inflammatory response was observed after animal envenomation. Lung neutrophilia and eosinophilia were accompanied with IL-4, IL-5 release, and IgE synthesis. In conclusion, high cytokine levels, recruitment of inflammatory cells (eosinophils and neutrophils), and increased IgE concentration may contribute to the exacerbation and maintenance of the induced inflammatory response in lungs by scorpion venom. These results lead to the better understanding of this induced pathogenesis and could help the physicians to take care of envenomed patients.

**KEY WORDS:** scorpion venom; immunodetection; hyperleukocytosis; eosinophilia; neutrophilia; cytokines; intercellular adhesion molecule; IgE antibodies.

## INTRODUCTION

Voltage-gated Na<sup>+</sup> (Na<sub>v</sub>) channel neurotoxins are major components of scorpion venom and the main agents responsible for the toxic effects of scorpion envenomation [1]. Neurotoxins lead to release massive neurotransmitter and inflammatory modulators. These mediators induce various pathological perturbations including acute respiratory distress syndrome, systemic

inflammatory response syndrome, and multiple organ failure [2–8].

Acute respiratory distress syndrome and airway inflammation are related to massive production of cytokines and other inflammatory mediators by activated macrophages, neutrophils, lymphocytes, and tissue resident cells [2–6, 9–14]. The release of high cytokine level (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, IL-10, and IFN- $\gamma$ ) is usually correlated with severity of envenomation [8, 14–19]. Recruitment of inflammatory cells from blood to the tissue is also a critical event in the development and persistence of airway inflammation [8, 16].

*Androctonus australis hector* venom, Algerian scorpion cause acute lung injury characterized by altered lung function and increase in pulmonary vascular permeability; these alterations are accompanied by protein edema of the alveoli, high level of pro-inflammatory cytokines (IL-1, IL-6, and TNF $\alpha$ ), and infiltration of inflammatory cells in peripheral blood [19–21]. In this study, lung inflammatory response was

<sup>1</sup>Laboratoire de Biologie Cellulaire et Moléculaire, Faculté des Sciences Biologiques, Université des Sciences et de la Technologie Houari Boumédiène, BP 32El-Alia Bab Ezzouar, 16111, Algiers, Algeria

<sup>2</sup>Laboratoire de Recherche et de Développement sur les Venins, Institut Pasteur d'Algérie, Route du Petit Staouéli, Dely Brahim, Algiers, Algeria

<sup>3</sup>To whom correspondence should be addressed at Laboratoire de Biologie Cellulaire et Moléculaire, Faculté des Sciences Biologiques, Université des Sciences et de la Technologie Houari Boumédiène, BP 32El-Alia Bab Ezzouar, 16111, Algiers, Algeria. E-mail: flaraba@hotmail.com

investigated after envenomation. Immune responses and lung reactivity were analyzed by counting and identifying cell populations in the respiratory airway of mice. Cytokine (IL-5 and IL-4), intercellular adhesion molecule (ICAM-1), and IgE levels were also evaluated. This response was compared to the detected biodistribution of the venom by immunostaining. Lung reactivity was also assessed by specific measurement of eosinophil peroxidase (EPO) and myeloperoxidase (MPO) activities as biomarkers of the influx of eosinophils and neutrophils into lungs, respectively.

## MATERIALS AND METHODS

### Nonbiological Materials

The chemicals and reagents used were purchased from Sigma (St. Louis, USA) or Merck (Darmstadt, F.R.G) and were of analytical grade.

### Biological Samples

#### *Venom*

*A. australis hector* (Aah) venom was obtained from the Pasteur Institute of Algeria. It was lyophilized and stored at 4°C. The lethal dose (LD50) of Aah venom is estimated to be 0.85 mg/kg by the i.p. route [22].

#### *Animals*

NMRI mice with an average weight of  $20 \pm 2$  g obtained from the Pasteur Institute of Algeria were kept in controlled temperature and humidity rooms and given free access to food and water. The experiments were carried out in accordance with the current guidelines for the care of laboratory animals.

#### *Specific Antibodies IgG for Aah FtoxG-50*

IgG with high specific affinity for Aah FtoxG-50 were used for immunohistochemistry and were obtained by affinity chromatography on CNBr-activated Sepharose 4B coupled to the toxic venom fraction FtoxG-50 [23].

## Methods

### *Animals and Experimental Protocol*

Animals were divided into two groups of 20 mice per group. The first group, served as control, was injected by s. c. with 100  $\mu$ l of physiological saline solution (0.9% NaCl).

The second group received a s.c. injection of sublethal dose (10  $\mu$ g/20 g body weight) of Aah venom. Blood samples, lungs, and bronchoalveolar lavage (BAL) fluid were collected at various times following the injection.

### *Immunohistochemical Analysis of Aah Venom in Lungs*

Lung tissue samples were embedded in paraffin and sectioned (5  $\mu$ m thick) according to standard histological procedures. Sections were processed for immunohistochemical detection of Aah venom as described by D'Suze *et al.* [24] with a slight modification.

Sections were completely deparaffinized, rehydrated, and washed in phosphate-buffered saline (PBS). They were incubated with 3% hydrogen peroxide ( $H_2O_2$ ) in methanol for 30 min at room temperature to block endogenous peroxidase activity. Antigen was reactivated by incubation in 0.5% SDS for 10 min at room temperature [25], and nonspecific binding sites were blocked by incubation with PBS containing 5% skim milk, 0.1% Triton X-100 for 1 h at room temperature. The slides were incubated overnight at 4°C with a horse polyclonal anti-FtoxG-50 Aah IgG (0.5 mg/ml) diluted in PBS containing 2.5% skim milk and 0.1% Triton X-100. Sections were then incubated with peroxidase-labeled anti-horse IgG (Sigma) for 1 h at room temperature. All incubation steps were followed by extensive washing in PBS. Sections were incubated with substrate solution containing 8%  $NiCl_2$ , 0.05% 3,3'-diaminobenzidine, and 0.02%  $H_2O_2$  in 50 mM Tris-HCl buffer, pH 7.6. Finally, sections were washed with 50 mM Tris-HCl buffer, pH 7.6.

The sections were then counterstained with hematoxylin and examined with a Motic 2000 camera microscope. The images were analyzed with Motic Q Win Plus v.3.2.0 software. Antigen-antibody complexes were visualized as brown deposits. Controls included incubating tissue sections from non-envenomed mice with normal horse serum under the same conditions as described above: no positive reactions were observed in these sections.

### *Lung-to-Body Weight Ratio*

The lung/body weight index (Pulmonary index) indicative of lung water content was calculated as lung weight/total body weight  $\times 100$  [14, 26].

### *Differential Leukocyte Count in Bronchoalveolar Lavage*

To evaluate airway inflammation, the accumulation of inflammatory cells in the BAL was examined at

various times. Mice were killed with an overdose of ether, the chest wall was opened, and the tracheas were cannulated. The airway lumina were washed and the resulting BAL was immediately centrifuged at 500×g for 10 min. Cells pelleted from the BAL were resuspended and supernatants collected and both were stored for further analysis. A hemocytometer (ADVIA, Hematology system) was used for cell counting, and leukocyte populations were stained with Giemsa for identification and counting.

### *Measurement of Eosinophil Peroxidase Activity*

The extent of eosinophil accumulation in the lung tissue was measured by assaying eosinophil peroxidase activity as previously described by Van Oosterhout *et al.* [27]. The lungs recovered from mice after sacrifice were weighed and homogenized with a manual grinder in 0.05 M Tris–HCl buffer pH 8 containing 0.1% Triton X-100. The tissue homogenates were centrifuged at 500×g for 15 min, and the supernatant was collected and kept at 4°C. Aliquots (50 µl) were placed in the wells of an ELISA plate and 100 µl of 0.05 M Tris–HCl buffer pH 8–0.1% Triton X-100 containing 4 mM of H<sub>2</sub>O<sub>2</sub> and a tablet of 10 mM *o*-phenylenediamine were added. The plates were incubated for 1 h at room temperature in the dark, and the absorbance of each sample at 490 nm was determined with an ELISA reader. Results are expressed as changes in absorbance per 100 mg of lung tissue.

### *Measurement of Myeloperoxidase Activity*

The extent of neutrophil accumulation in the lung tissue was measured by assaying myeloperoxidase activity. The lungs of animals that had received vehicle or Aah venom were removed and snap frozen. Upon thawing, the lung tissue was homogenized in pH 7.4 buffer 50 mM potassium phosphate and centrifuged at 10,000×g for 30 min. The pellet was reconstituting in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. After further centrifugation, the pellet was then resuspended in 50 mM potassium phosphate buffer (pH 6.0). One milliliter aliquots of the suspension were transferred into 1.5-ml Eppendorf tubes followed by three freeze–thaw cycles. These were then centrifuged for 15 min at 10,000×g and myeloperoxidase activity in the resuspended pellet was assayed by measuring the change in optical density OD at 460 nm using *o*-dianisidine 1.6 mM and H<sub>2</sub>O<sub>2</sub> 0.4 mM in phosphate buffer 50 mM, pH 6.6. Results were expressed as changes in absorbance per 100 mg of lung tissue.

### *Cytokine and Intercellular Adhesion Molecule Assays*

Cytokines (IL-4 and IL-5) and ICAM-1 were assayed in sera and lung tissue homogenates by a specific two-site sandwich ELISA, using an Amersham Bioscience Kit (USA). All cytokine assays were performed according manufacturers' instructions. Detection limits were 5 pg/mL for IL-4, 12 pg/mL for IL-5, and 5 pg/mL for ICAM-1. ELISA plates were analyzed in a microplate ELISA reader (Sanofi).

### *Measurement of Serum IgE*

An enzyme-linked immunosorbent assay (sandwich ELISA) in 96-well microtiter plates was used to assay IgE. The wells were coated with 100 µL of anti-IgE antibody (5 µg/ml) in 0.1 M sodium carbonate buffer pH 9.6, then serum samples were added with parallel murine IgE standard controls, followed by a biotinylated polyclonal rabbit anti-mouse IgE (1/1,000). The binding of antibody was detected with streptavidine-coupled to peroxidase (1/4,000).

After a final wash, 100 µl of the peroxidase substrate *o*-phenylenediamine was added to each well, and the reaction was subsequently stopped with 50 µl of 30% sulfuric acid. Plates were read at 490 nm using an ELISA reader and the quantity of IgE in the test sample was interpolated from the calibration curve constructed from the standard IgE.

### *Statistical Analysis*

All results are expressed as means±SD. The statistical significance of differences between groups was analyzed by a Student *t* test. Differences were considered significant if *p* values were <0.05

## RESULTS

### **Immuno-Detection of Aah Venom in Lung Tissue**

Immunohistochemistry was used to identify the cellular targets of venom in lung tissue after experimental envenomation. Staining of lung tissue for Aah venom was diffuse and intense with no nuclear involvement in envenomed animals. Venom was immunodetected throughout the lung interstitium and in blood vessels (Fig. 1b). The venom was abundant inside neutrophils and macrophages (Fig. 1c). A similar distribution has been reported in rams envenomed with *Tityus discrepans* venom [24].

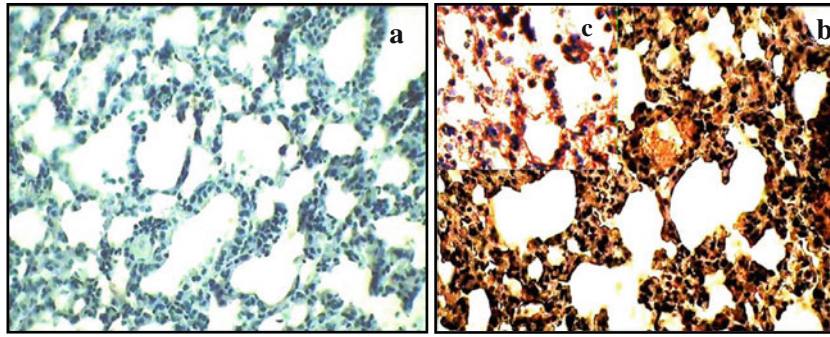


**Fig. 1.** Immunohistochemical detection of *Androctonus australis hector* venom in lung tissue following envenomation (10  $\mu$ g/20 g body weight). Zones in brown are positive for Aah venom. Nuclei are counterstained with hematoxylin. No staining of lung structure for venom in non-envenomed mice (control).  $\times 400$  magnification.

The alveolar septa of envenomed mice were thicker than normal; this may have been a consequence of edema formation. Indeed, the lung-to-body weight ratio was  $1.8 \pm 0.03$  for envenomed animals, significantly higher than the  $1 \pm 0.01$  for saline-injected control mice ( $p < 0.01$ ). We previously showed that Aah venom causes the formation of inter- and intra-alveolar edema in lung tissue [19, 21].

#### Bronchoalveolar Lavage Cell Counts

Cellular infiltration in the lung was estimated by counting the various leukocyte populations in bronchoalveolar lavage. The neutrophil, lymphocyte, and monocyte counts in BAL fluid were higher in the treatment group 45 min after venom challenge than in controls (Fig. 2). Monocytes ( $24 \pm 2.4\%$ ) and neutrophilic granulocytes ( $13 \pm 0.9\%$ ) were significantly more numerous, with maximal values after 2 and 4 h, respectively. Eosinophil cells ( $6 \pm 0.5\%$ ) were also significantly more numerous than in controls 45 min after the venom inoculation. The accumulation of eosinophils in lung fluid is often associated with an allergic response, but also, with parasitic infiltration and in some autoimmune disorders [28, 29]. Lymphocyte counts declined significantly 4 h after envenomation. Twenty-four hours after envenomation, there was no significant difference in the cell composition of the BAL liquid from venom-treated and control groups.

#### Quantification of intercellular adhesion molecule (ICAM-1), cytokines (IL-4 and IL-5), cellular peroxidase activities (EPO and MPO), and IgE antibodies

To investigate the migration of leukocytes mobilized by Aah venom injection, we analyzed the expression of

cell-adhesion molecules at various times. The accumulation of cells and ICAM expression in lung were higher in envenomed animals than in controls. The injection of venom led to a substantial increase in the ICAM-1 concentration in serum to a maximum of  $75.5 \pm 6.5$  pg/ml after 3 h, followed by a decline to  $54.75 \pm 4.8$  pg/ml at 24 h. The increase of ICAM-1 in lung was important and more rapid, peaking at  $204.5 \pm 18.5$  pg/ml after 30 min, then declining and increasing again to  $177 \pm 15.17$  pg/ml after 2,880 min (Fig. 3).

The concentration of IL-4 increased significantly in serum ( $231 \pm 11.5$  pg/ml) and lungs ( $63.5 \pm 3.17$  pg/ml), reaching maximum values at 60 min and dropping to baseline values 180 min after venom injection (Fig. 4a). There was also a large increase in the serum concen-

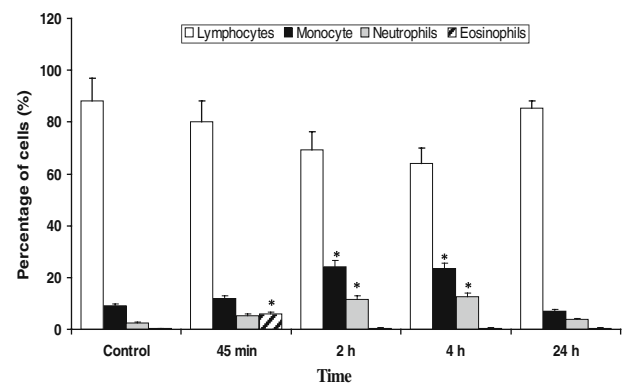


**Fig. 2.** Time course of leukocyte counts in bronchoalveolar lavage fluid after injection of a sublethal dose of *Androctonus australis hector* venom (10  $\mu$ g/20 g body weight) or sterile saline (control) in mice. Bars represent means  $\pm$  SD ( $n=4$ ); \* $p < 0.05$  compared with the control group.

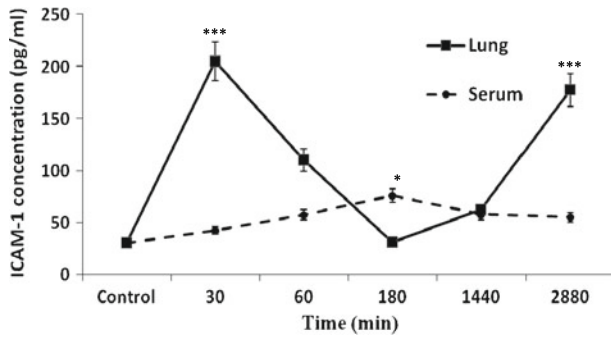


**Fig. 3.** Time course of ICAM-1 concentration in mouse serum and lung after injection of a sublethal dose of *Androctonus australis Hector* venom (10 µg/20 g body weight) or sterile saline (control). Means±SD are shown (n=4); \**p*<0.05, \*\*\**p*<0.001 compared with the control group.

tration of IL-5 at 60 min and a gradual decrease again to baseline after 180 min. The time course of IL-5 release in the lungs showed a biphasic response with peaks at 1 h (47.5±4.5 pg/ml) and 24 h (50±4.8 pg/ml; Fig. 4b).

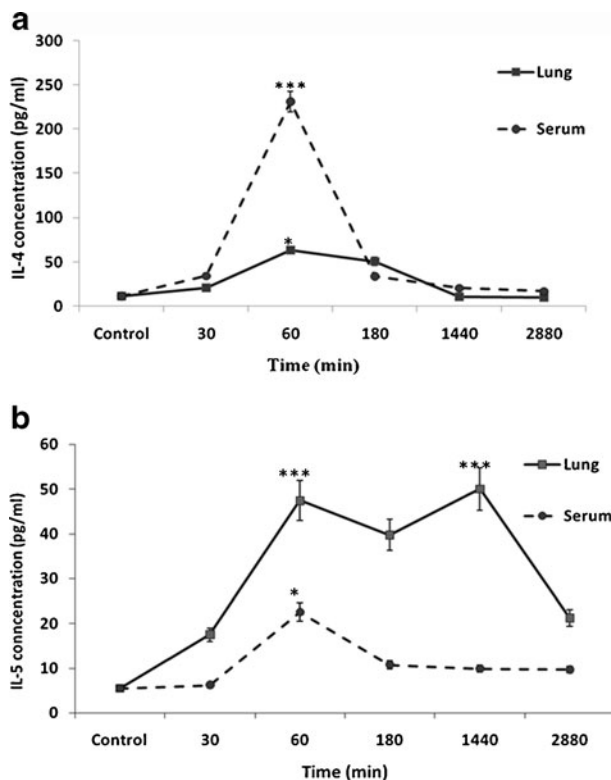


**Fig. 4.** Serum and lung concentrations of IL-4 (a) and IL-5 (b) after injection of a sublethal dose of *Androctonus australis Hector* venom (10 µg/20 g weight) or sterile saline (control) in mice. Bars represent mean±SD (n=4); \**p*<0.05, \*\*\**p*<0.001 compared with the control group.

Cytokines, IL-4 and IL-5, play important roles in the increase of IgE and in the recruitment of eosinophils especially in lungs [28]. We studied eosinophil influx by assaying eosinophil peroxidase in lung tissue. In control mice, EPO activity was 0.09±0.016 absorbance/100 mg tissue whereas following envenomation, EPO activity increased to a value of 0.65±0.034 absorbance/100 mg tissue (Fig. 5). The pulmonary levels of myeloperoxidase, an index of the number of infiltrating neutrophils, in venom-treated animals (0.313±0.061) were more significant to those of control animals (0.089±0.011; Fig. 5).

Aah venom injection was followed by a substantial increase in IgE production at all time points studied (from 30 min to 21 days), peaking at 45 min (5.29±0.45 ng/ml) after venom injection (Fig. 6).

## DISCUSSION

Scorpion venoms caused associated upper airway obstruction with severe damage in lung tissue such as diffuse injury in the alveolar capillary barrier and interstitial edema with marked leukocyte infiltration [19–21, 24, 30].

Release and activation of pro-inflammatory mediators are among the factors which appear to be important in the pathogenesis of many of the deleterious effects of scorpion envenomation [15, 17, 31–34].

The obtained results state that Aah venom was responsible of lung immunoreactivity (increase of IgE and cytokine levels, hyperneutrophilia, hypereosinophilia....) and airway inflammation. These results are in correlation with previous data which demonstrates an increase in the serum levels of pro-inflammatory cytokines (IL-1, IL-6, and TNF-α), airway resistance, and significant accumulation of polymorphonuclear cells into peripheral blood [19–21].

We report also that Aah venom immunostaining was intense in pulmonary parenchyma. Furthermore, edema characterized by significant increase of lung to body weight ratio was observed in lungs. There was a rapid inflammatory response (within 120 min) in the lungs of envenomed mice, resulting in a significant infiltration of leukocytes (neutrophils, eosinophils, and lymphocytes) and Th2 cytokine production (IL-4 and IL-5).

Th2 cells were dominant in the airway responsiveness and Th2 cytokines, especially IL-6, IL-4, and IL-5 play a pivotal role in the pathophysiology of bronchopulmonary distress [28, 35, 36]. Activated mast cells

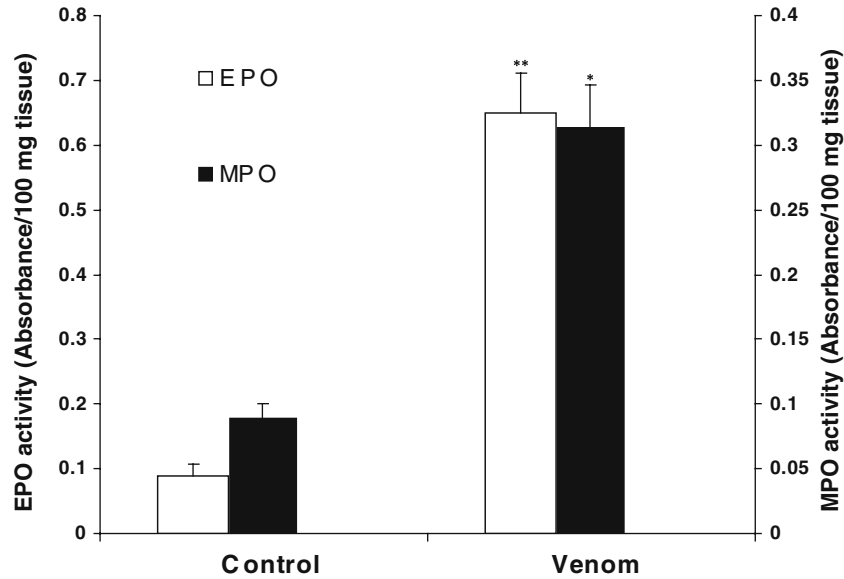


**Fig. 5.** Effects of a sublethal dose of *Androctonus australis hector* venom (10  $\mu$ g/20 g body weight) on EPO and MPO activities in the lung. Controls were injected with sterile saline. Bars represent mean $\pm$ SD ( $n=4$ ); \* $p<0.05$ ; \*\* $p<0.01$  compared with the control group.

produce also IL-4 and IL-5 which may be essential for IgE isotype switching [28, 36].

Increased release of IL-5 is accompanied by a marked accumulation of eosinophils in lung fluid and increase of EPO activity followed by the IgE production.

Shah and collaborators [37] reported pulmonary infiltration with blood eosinophilia after scorpion sting. Immediate hypersensitivity reaction to *Centruroides vittatus* scorpion venom has been also observed in envenomed patients [38].

Eosinophilia and IgE production could be related with hypersensitivity of the animals to components of the Aah venom.

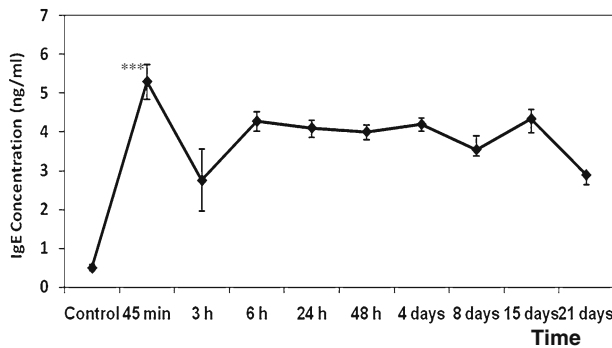


**Fig. 6.** Kinetics of IgE production in mice after injection of a sublethal dose of *Androctonus australis hector* venom (10  $\mu$ g/20 g body weight) or sterile saline (control). Bars represent means $\pm$ SD ( $n=4$ ); \*\*\* $p<0.001$  compared with the control group.

Envenoming with Aah venom leads to the increased levels of pulmonary neutrophils, as determined by both, direct BAL cell counting and measurement of MPO activity in lung homogenates. Neutrophils infiltrate lungs and migrate into the airways lead to the release of oxygen free radicals and enzymes (proteases and myeloperoxidase) exacerbating damage to endothelial cells and to subendothelial matrix proteins, and inducing tissue injury [39–41]. The activation of platelet-activating factor receptors, production of chemokines, and adhesion molecules (ICAM-1) were responsible of neutrophil recruitment in lungs after scorpion envenomation [40–42].

Furthermore, many studies suggest that the release of inflammatory factors (eicosanoids, kinin, and reactive oxygen species) and cytotoxic leukocyte-derived products such as EPO from activated eosinophils could be involved in severe lung injury [8, 11, 43–46].

Our findings suggest that the increased cytokine levels (IL-4 and IL-5), the recruitment of inflammatory cells (eosinophils and neutrophils), and the increased concentration of IgE antibodies contribute to the onset and maintenance of the pulmonary edema caused by *A. australis hector* scorpion venom. The involvement of these inflammatory markers may explain in part the induced reactivity of lung tissue in envenomed mice. The use of inhibitors, and in particular anti-inflammatory drugs, may be useful to elucidate the mechanism of bronchopulmonary reactivity after scorpion envenomation.

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