



Safety and efficiency of active immunization with detoxified antigen against scorpion venom: side effect evaluation

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Abstract

Objective The efficiency and safety of vaccine are the most important properties, however, as any medication, it can induce side effects. This prophylactic therapy could be used to prevent the lethal and pathophysiological effects induced after scorpion envenomation.

Methods In this study, detoxified venom associated to alum adjuvant (V*alum) is used as a vaccine against scorpion venom for immunization of mice. We evaluate the safety and the inflammatory response of this vaccine. We also investigated the protective effect of this formulation against the toxicity of native *Androctonus australis hector* venom.

Results Results showed no adverse events occurred after immunization of animals. This active immunization of animals did not cause change in vascular permeability, no edema formation in the studied organs. Furthermore, there are no IgE production in sera, nor change in the morphology of the mast cells in skin tissues. However, low inflammatory response triggered by activating the recruitment of eosinophils associated to IL-4 and IL-5 release was observed. All immunized animals are protected from the toxic effects of native venom until 6 LD50 and to 7 LD50 after the second challenge.

Conclusion This safe vaccine preparation seems to induce a long-term protection without any risk of deleterious inflammatory response.

Keywords Active immunization · Safety · Inflammatory response · Immunoprotection

Introduction

Scorpion envenomation is a severe syndrome which can lead to multifactorial pathogenesis causing important morbidity and mortality in Maghreb regions [1, 2]. *Androctonus australis hector* (Aah) is the most dangerous scorpion in these regions. Lethality caused by Aah venom is mainly due to its neurotoxins that bind to the sodium voltage-gated channels causing multi-organ failure [3]. Immunotherapy associated with symptomatic therapy remains the currently used approach to treat the stung patients. The efficiency of the immunotherapy is limited by the delay between the sting and the administration of immune sera in at-risk regions. Vaccine approach using detoxified Aah venom by gamma irradiation seems to be a good alternative to the usual therapy [4–7].

Vaccine aimed to produce strong immune response to the administered antigen and to provide long-term protection.

The most commonly used adjuvant in human vaccines is aluminum adjuvant which prolonged the retention of adsorbed antigens at the injection site [8–11]. Injection of alum-adsorbed vaccines can cause the release of cellular contents and inflammation. Moderate inflammatory reaction is one of the potential concerns that can be evaluated in the framework of vaccine safety testing [12]. All effective vaccines activate innate immunity with inflammatory responses that are essential for the induction of adaptive immunity [13–16].

The induced reactions by vaccine may be systemic or localized to the vaccination site. The local reactions are

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commonly characterized by erythema and swelling as the result of a localized inflammatory response to the vaccine solution. Alum-adsorbed vaccines induce local inflammatory nodules at injection sites accompanied with systemic and local production of inflammatory cytokines [13].

The aim of this study was to evaluate the safety and efficiency of the vaccine preparation against the lethal effects of *Aah* venom. The long-term immunoprotective effect provided by this preparation against the lethal effects of *Aah* venom was also evaluated.

Materials and methods

Venom

Lyophilized *Androctonus australis hector* (*Aah*) venom was provided from Pasteur Institute of Algeria. It was irradiated by the Cobalt-60 (2 kGy dose and 765 Gy/h rate) at Algerian Center of Nuclear Research (CNRA) [17]. The toxicity of irradiated antigen was abolished until 20 times of LD₅₀ of native venom. The venom solution was kept at -20°C until use time.

Chemical reagents

Chemical reagents used in this study were of analytic grade. Aluminum hydroxide adjuvant (alum), Ortho-dianisidine hydrochloride, orthophenyldiamine (OPD), May–Grunwald–Giemsa, hydrogen peroxide (H_2O_2), hematoxylin and eosin were obtained from Sigma-Aldrich (St Louis MO, USA). Evans Blue and formamide were from Merck (Darmstadt, FRG).

Animals

Animals were obtained from the animal breeding of Biological Sciences Faculty of the University of Sciences and Technology Houari Boumediene (Algeria) and kept under controlled conditions of humidity, lighting, and temperature throughout the experiments with free access to standard rodent diet and tap water.

This study was conducted with 112 adult female NMRI mice weighing 18–22 g (6–8 weeks-old). All animal experiments were in accordance with the actual guidelines for the care of animal's laboratory.

Immunization schedule

Two groups of mice received three subcutaneous (s.c.) boosts of 100 μg of irradiated venoms associated with alum adjuvant (2%) or alum adjuvant alone at 1 month intervals. Control groups received, respectively, physiological saline

solution (0.9% NaCl) or ovalbumin (OVA) associated with alum. Blood samples and organs (skin, lung, intestine) were collected at various times (30 min, 1, 2, 4 and 24 h) after the last immunization.

Evaluation of adverse reactions

After injection, mice were monitored for symptoms of adverse reactions as described in previous study [18]. Body weight was measured each week for all groups.

Measurement of vascular permeability changes

The extravasation of Evans blue dye from blood vessels to injection site (skin), lungs and intestine was used as an indicator of organ vascular permeability increase due to its high affinity for plasma proteins with which it binds in the first few seconds after its injection [19].

Evans blue dye (EBD) was administered intravenously into the mice at a dose of 20 mg/kg few seconds before the last immunization. Mice receiving the Evans blue were killed after 30 min, 1, 2, 4 and 24 h. Organs (skin, lungs, and intestine) were recovered, weighed, and placed in 2 ml of dimethylformamide. They were incubated at 37°C for 48 h. Absorbance was read at a wavelength of 620 nm. The concentration of Evans blue was determined after extrapolation on a standard curve conducted with various concentrations of Evans blue. The results were expressed in micrograms of dye per microgram of tissue.

Determination of organ water content

The organs (skin, lungs, and intestine) were collected and weighed at several time intervals (30 min, 1, 2, 4 and 24 h). The samples were dried at 60°C for 24 h, and the water content was determined by the following formula: water content = wet weight – dry weight/dry weight [20].

Eosinophil count

To count the eosinophil cells among leukocytes, peripheral blood smears from each mouse were prepared, after fixation in methanol for 10 min; the leukocytes were stained with May–Grunwald–Giemsa. Eosinophil cells were counted in a double-blind manner, using a light microscope at 400-fold magnification, with the total count being 100 leukocytes per slide.

Evaluation of eosinophil peroxidase (EPO) activity

Eosinophil peroxidase is an enzyme contained in the granules of eosinophil cells; it is released after their activation. EPO activity was evaluated in the sera according to

Kroegel et al. [21]. Briefly, 100 µl of reagent solution (10 mg of ortho-Phenylenediamine dihydrochloride, H₂O₂ 0.03%, 10 ml of phosphate buffer, pH 8) was added to 50 µl of diluted serum 1/10 in microplate. Plate was incubated in the dark for 1 h at 37 °C and then read at 490 nm. Enzyme activity was expressed in milli-mole of reduced H₂O₂ per minute and per milliliter of serum (mM H₂O₂/min/ml).

Evaluation of specific IgE levels in sera

Levels of specific IgE were measured in duplicate by ELISA as described previously [22]. Immulon II 96-well plates (Nunc Immuno-Maxisorp, Thermo Scientific) were coated with 100 µl of antigens (5 µg/ml) in 0.1 M carbonate buffer pH 9.6. After overnight incubation at 4 °C, plates were washed 3 times with PBS/0.05% Tween 20 and blocked with 100 µl of PBS (0.1 M; pH 7.4) containing 1% skim milk for 3 h at 37 °C. After three washes, serum samples (1:10 dilutions) were added to the plates and incubated overnight at 4 °C. Plates were then washed, and 100 µl of rat anti-mouse IgE (HRP) was added to each well. The plates were incubated for 90 min at room temperature. After a final wash, 100 µl of the peroxidase substrate O-phenylenediamine was added to each well; the reaction was subsequently stopped with 50 µl of 30% sulfuric acid and the absorbance read at 492 nm.

Evaluation of cytokines levels

The levels of IL-4 and IL-5 cytokines were assayed in sera by ELISA, using Sigma-Aldrich Kit (USA). All cytokine assays were performed according to the manufacturer's instructions. Detection limits were 0.6 pg/ml for IL-4 and 12 pg/ml for IL-5.

Histological analysis of mast cell degranulation

In all animal groups, skin injection site was removed, immediately after death by cervical dislocation and fixed immediately in 10% formaldehyde solution for 48 h at room temperature. The fixed tissues were subsequently dehydrated and embedded in paraffin, according to the routine procedures and 5 µm paraffin sections were stained using toluidine blue to detect mast cells. After staining, sections were observed under oil immersion for counting and morphological features of mast cells.

In vivo protective effect of vaccinated mice against challenged doses of native venom

At 3 and 4 months of the immunization after the administration of the last dose, immunized mice with irradiated

venom associated with alum adjuvant were individually challenged with lethal doses of venom. The LD₅₀ of native *A. australis hector* used throughout this study was 17 µg/20 g body mass by i.p. injection [23]. Mortality was recorded 24 h later [17]. A second challenge was undertaken 6 and 26 months to survived animals. The IgG antibody titers raised against *Aah* venom were evaluated in sera of envenomed mice by ELISA.

Statistical analysis

The data were expressed as mean ± standard deviation and examined for their statistic using one-way ANOVA. P values of less than 0.05 were considered statistically significant.

Results

Assessment of adverse reactions in sensitized mice

All groups of immunized mice were survived during immunization schedule. The body weights gained continuously and consistently throughout the experimental period approximately of 15 g in both groups (Data not shown).

Three months after initial immunization, mice were monitored for adverse reactions. Animal control injected with saline solution or alum adjuvant and immunized animals with irradiated venom associated with alum adjuvant did not show any symptoms of adverse reactions. However, in immunized animals with ovalbumin several symptoms are observed within 10–20 min after the last immunization, including scratching around the nose and head; a change in respiratory rate; tremor and convulsions (Table 1).

Granuloma formation was monitored in all immunized animals. In contrast to group immunized with NaCl, immunized mice with alum adjuvant or adjuvanted irradiated venom developed small subcutaneous granulomas at injection sites which disappear 2 weeks after the last immunization. However, for the immunized animals with ovalbumin, these granulomas persist few months later.

Vascular permeability changes after the immunization schedule

To assess changes in the level of vascular permeability after immunization, Evan's blue dye was injected through the tail veins of mice from the different experimental groups before the last immunizations.

Two hours after the last subcutaneous injection of alum adjuvant, adjuvanted ovalbumin or irradiated venom associated with alum, a significant Evans blue dye extravasation was obtained in skin, lung and intestine when

Table 1 Evaluation of adverse reactions in immunized animals with NaCl, alum, OVA-alum and V*alum

Symptoms of adverse reactions	Immunized animals			
	Control	alum	OVA-alum	V*alum
Scratching and rubbing around the nose and the head	—	—	+	—
Puffiness around the eyes and mouth, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate	—	—	—	—
Wheezing, labored respiration, and cyanosis around the mouth and the tail	—	—	+	—
No activity after prodding or tremor and convulsions	—	—	+	—
Death	—	—	—	—
Number of mice with adverse effects	0/10	0/10	10/10	0/10

Symptoms of adverse reactions were identified for all groups, (+) Appearance of symptoms, (—) absence of symptoms ($n = 10$)

compared with control; the concentration of extravasated Evans blue dye increased significantly in skin, lung and intestine compared to the control (Fig. 1a–c).

Immunized mice with alum adjuvant alone or irradiated venom associated with alum present much less leakage than mice from the immunized animals with ovalbumin (Fig. 1a–c).

Assessment of edema in skin, lung and intestine of immunized mice

The evaluation of the wet weight/dry weight ratios of the skin, lung and intestine, is undertaken to estimate the extent of the edema forming in these organs.

Subcutaneous injection of adjuvanted ovalbumin used as an allergen model induced marked edema in skin, lung and intestine 2 h after the last immunization (Fig. 1d–f). However, there are no significant differences between immunized mice with alum and irradiated venom associated with alum and the control, indicating no edema forming in skin, lung and intestine of immunized animals with the formulation using irradiated venom.

The treated animals with alum and irradiated venom associated with alum showed a significant difference when compared to those immunized with adjuvanted ovalbumin (Fig. 1).

Evaluation of eosinophil recruitment and EPO activity in sera of immunized mice

The evaluation of eosinophil levels in the four animal groups revealed that immunization was able to increase the eosinophil infiltration in blood of immunized mice with alum adjuvant, adjuvanted ovalbumin or adjuvanted-irradiated venom and seems to be more efficient at 24 h post-immunization when compared to those immunized with saline solution. The levels of these inflammatory cells are more important when the immunization was undertaken with adjuvanted ovalbumin compared to that using alum or

adjuvanted irradiated venom (Fig. 2a). These results are in correlation with the eosinophil peroxidase activity, a marker of the recruitment of eosinophil cells, after the immunization (Fig. 2b). These results indicate that adjuvanted irradiated venom induces a lowest inflammatory response which may be necessary for the induction of the specific immune response.

Evaluation of specific IgE antibody levels in sera of immunized animals

The titers of specific IgE to the injected antigens were evaluated by ELISA in sera of immunized mice (Fig. 3a). The IgE titers of immunized animals with ovalbumin were significantly higher than those observed in immunized mice with saline solution. This increase was observed at 30 min, it peaked at 2 h after the last injection of ovalbumin. However, no significant difference was observed in IgE titers of immunized animals with irradiated venom associated with alum and those injected with alum alone compared to the control, indicating that the formulation of irradiated venom associated with alum did not induce IgE production in sera of immunized mice. Significant differences were observed in sera of immunized animals with alum and irradiated venom associated with alum compared to immunized animals with ovalbumin ($P < 0.001$).

Evaluation of cytokine levels of type-2 T-helper cells in sera of immunized mice

Subcutaneous immunization with adjuvanted ovalbumin, alum or adjuvanted irradiated venom resulted in a significant increase of IL-4 and IL-5 levels 2 and 24 h after the last immunization compared to the control (Fig. 3b, c). The concentrations of IL-4 and IL-5 were significantly important in sera of immunized mice with adjuvanted ovalbumin compared to those of immunized animals with alum or adjuvanted irradiated venom (Fig. 3b, c). These results

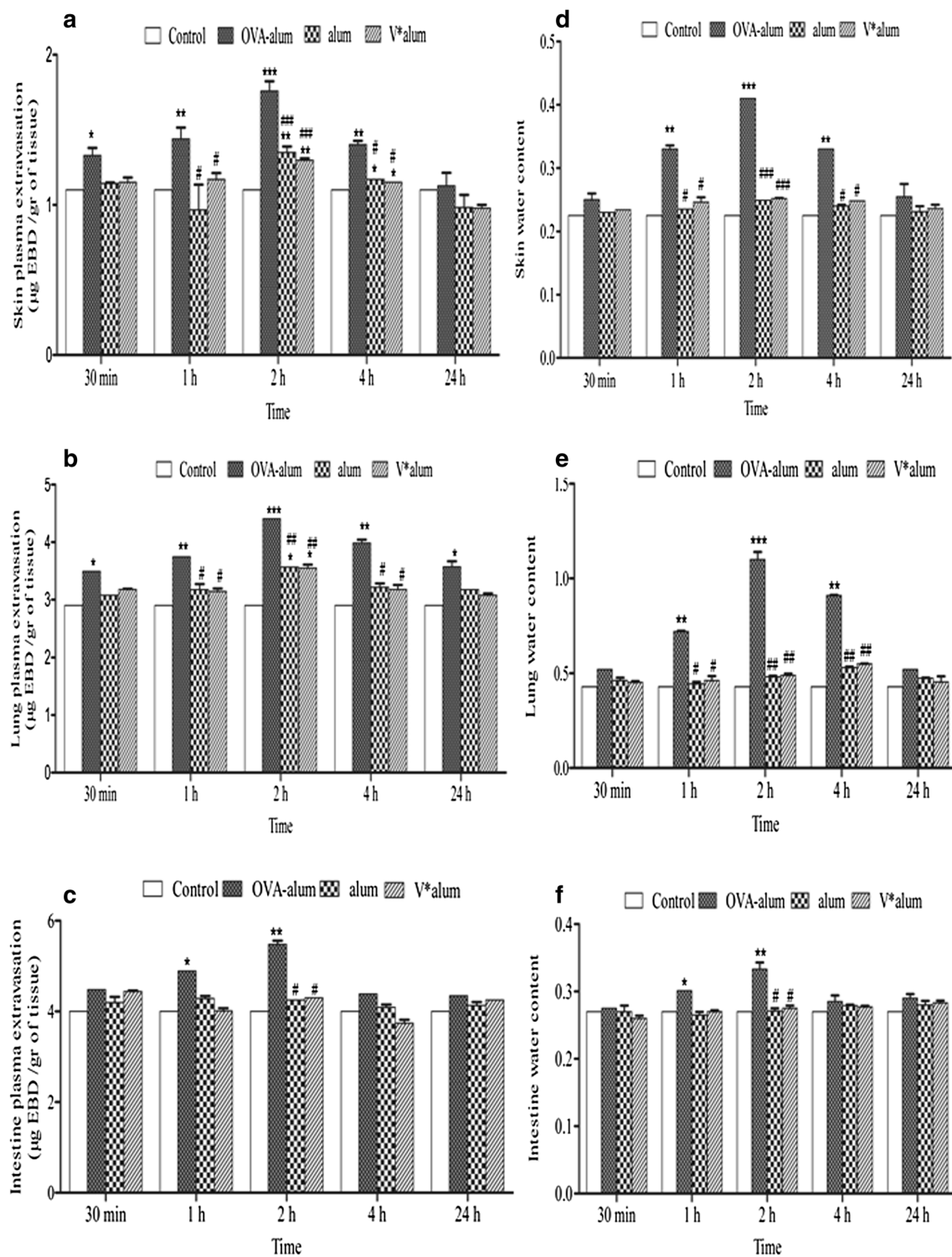


Fig. 1 Effects of previous immunization of mice by NaCl, alum, OVA-alum or V*alum on the extravasation of Evans blue dye (20 mg/kg) in skin (a), lung (b) and intestine (c) at several time intervals. Evans blue levels in the skin, lung and intestine were taken as an index of plasma extravasation and quantified at 620 nm. Assessment of edema forming was performed in skin (d), lung (e) and

intestine (f) of immunized mice. All data were presented as mean $\pm$ SEM ($n = 4$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; immunized animals with adjuvanted OVA versus animal control (vehicle NaCl). # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$; immunized animals with alum and V*alum versus immunized animals with adjuvanted OVA

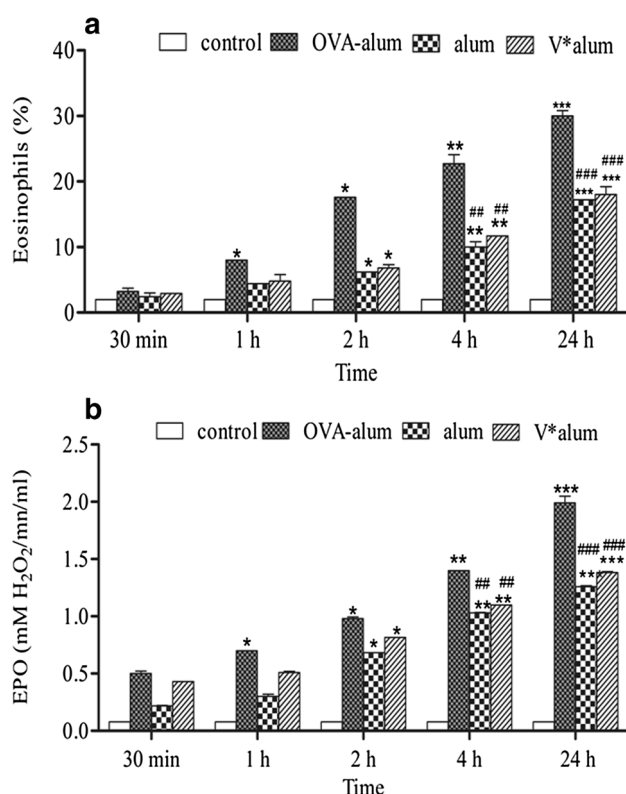


Fig. 2 Peripheral blood eosinophil count expressed as a percentage of eosinophils in the total number of leukocytes (a) and eosinophil peroxidase activity (b) in sera of immunized animals with NaCl, alum, OVA-alum or V*alum. All data were presented as mean $\pm$ SEM ($n = 4$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; immunized animals with adjuvanted OVA versus animal control (vehicle NaCl). ## $P < 0.01$; ### $P < 0.001$; immunized animals with alum and V*alum versus immunized animals with adjuvanted OVA

indicate that the humoral immune Th2 pathway was activated by the adjuvanted irradiated venom.

Mast cell degranulation in skin of immunized mice

The morphology of the mast cells in skin tissues of mice was stained with Toluidine Blue. Histologic analysis of skin tissues showed the absence of degranulated mast cells in skin injection sites of the fourth groups of mice, 30 min post-immunization, all mast cells are full of granules (Fig. 4a, b, d, f). However, degranulation of skin mast cells was detected only 24 h after the last immunization with ovalbumin associated with alum (Fig. 4c). Fully granulated mast cells are evident 24 h post-immunization in skin immunized animals with NaCl, alum or adjuvanted irradiated venom (Fig. 4e, g). These results correlated with the levels of degranulated mast cells 24 h post-immunization in skin of immunized animals with NaCl, alum and adjuvanted irradiated venom which showed an absence of degranulated mast cells (Fig. 4h).

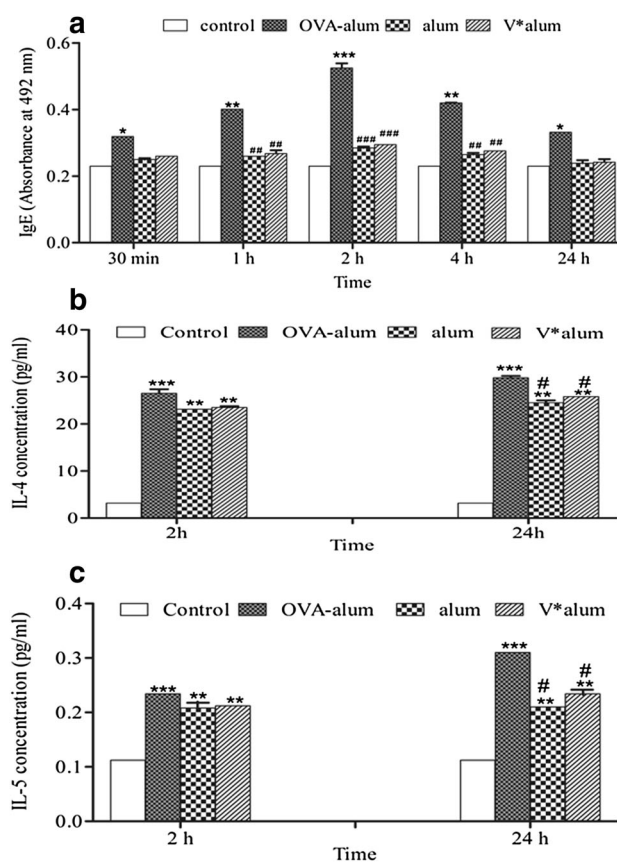
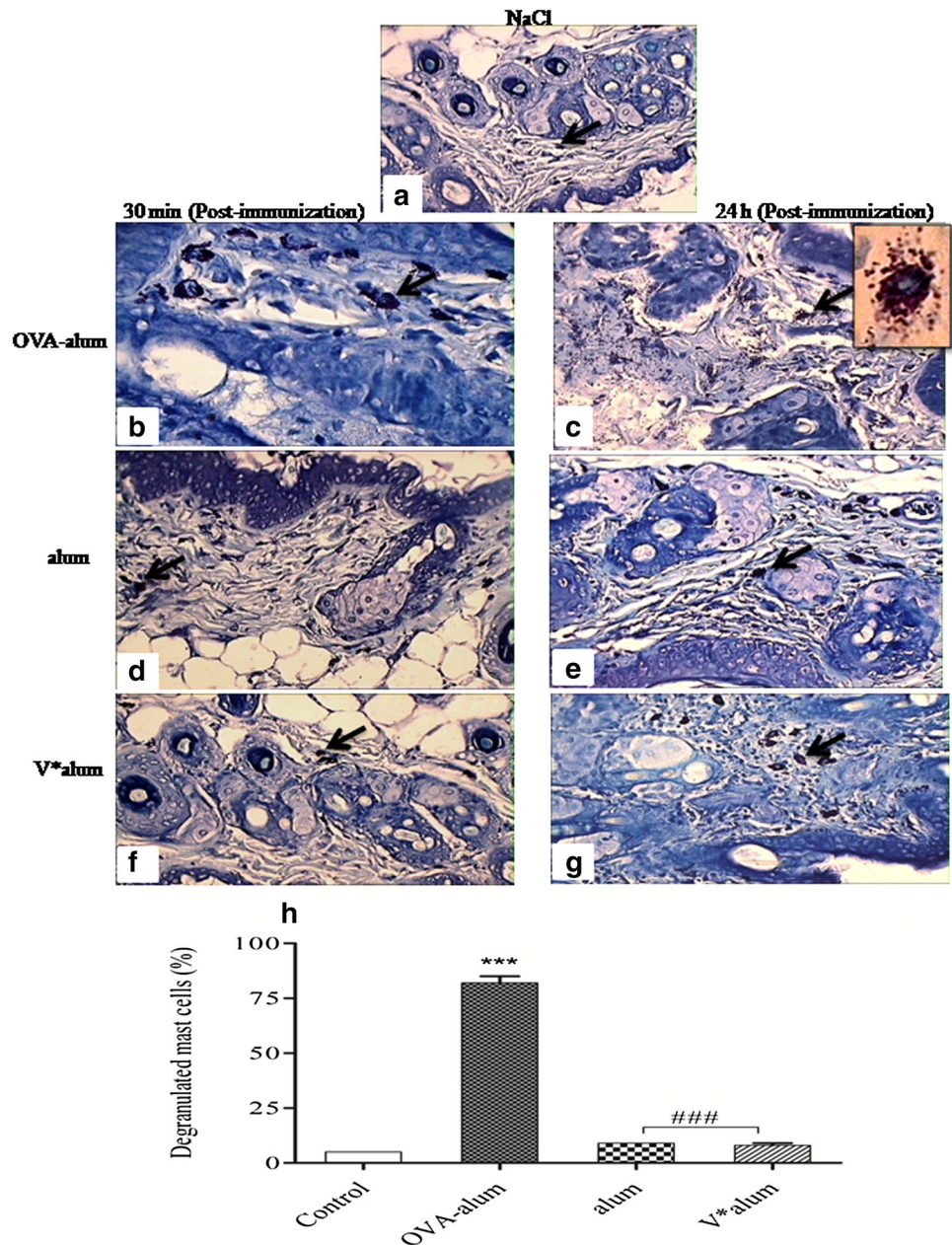


Fig. 3 Quantification by ELISA of the levels of antigens-specific IgE (a), IL-4 (b) and IL-5 (c) in sera after immunization of mice with NaCl, alum, adjuvanted OVA or adjuvanted irradiated venom. All data were presented as mean $\pm$ SEM * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; immunized animals with OVA versus control (vehicle NaCl). # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$; immunized animals with alum and V*alum versus immunized animals with adjuvanted OVA

Protective effect of mice against native venom conferred after immunization with irradiated venom associated with alum

The ability of irradiated venom associated with alum adjuvant to confer an in vivo protective immune response against the toxicity of *Aah* venom was evaluated in mice. Vaccinated mice were challenged with lethal doses of venom by subcutaneous route; the number of survived mice was revealed 24 h after challenge. At 3 and 4 months after the third injection, the immunized mice received, respectively, 4 LD₅₀ and 6 LD₅₀ to observe their protection against the native venom of *Aah* (Table 2). The results showed that immunized mice were totally protected against the challenge with 4 and 6 LD₅₀ of the native venom; these doses did not generate any toxic symptom (Table 2). The second challenge was undertaken with 7 and 5 LD₅₀ after 6 and 26 months, respectively, after immunization

Fig. 4 Histological analysis of mast cell degranulation in skin following subcutaneous immunization. The mast cells in skin were identified 30 min by Toluidine Blue Stain (**b, d, f**) and 24 h (**c, e, g**) after the last immunization. Magnification $\times 400$, $\times 1000$. Arrows indicate mast cells. **a, d–g**: Non degranulated mast cells in skin of immunized animals with NaCl, alum or V*alum (arrows). **b, c** Degranulated mast cells in skin of mice sensitized with OVA-alum (arrows). **h** Percentage of degranulated mast cells in skin sample 24 h post-immunization of animals. One-hundred mast cells were analyzed as described in “Materials and methods”. Values are expressed as mean $\pm$ SEM of 3 mice per group. *** $P < 0.001$; immunized animals with adjuvanted OVA versus animal control. ### $P < 0.001$; immunized animals with alum or with adjuvanted irradiated venom versus immunized animals with adjuvanted OVA



schedule to the survived mice after the first challenge, all vaccinated mice survived to these doses.

Specific antibody titer in sera of survived animals

Sera from survived animals after challenge with lethal doses of *Aah* venom were analyzed to evaluate the levels of IgG antibodies. A significant increase in the specific IgG titers was observed after the challenge with lethal doses of *Aah* venom. The IgG titers were much more important after the second challenge (twofold) compared to those obtained after the first challenge (Fig. 5).

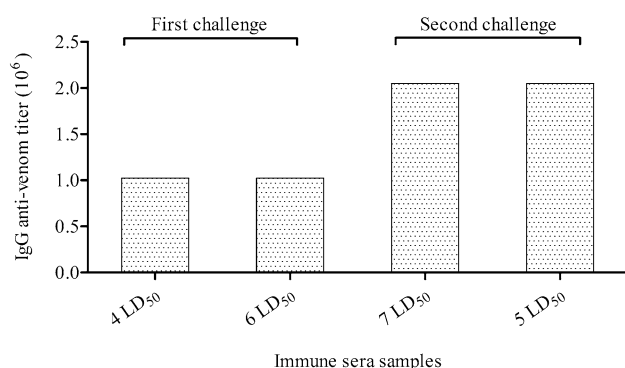
Discussion

Immunotherapy is currently the only specific treatment used for scorpion envenomation; however, this treatment presents some limits. An alternative to this treatment could be a vaccine approach which must stimulate immune response without adverse effects and deleterious inflammatory response. In this study, the safety and efficiency of vaccine approach against *Aah* venom were assessed by the evaluation of the long-term protective effect, adverse effects and the inflammatory response in immunized animals with adjuvanted irradiated venom.

Table 2 Long-term immunoprotective effect of immunized mice with adjuvanted irradiated venom against the toxicity of native *Aah* venom

Challenge	Post-immunization period (months)	Amount of challenge dose (LD ₅₀)	Survived mice
First	3	4	4/4
	4	6	4/4
Second	6	7	4/4
	26	5	4/4

Immunized animals were challenged with lethal doses of native venom ($n = 4$)

**Fig. 5** Antibody titers of immune sera from survived mice to the first and second challenge with lethal doses of native venom

No apparent adverse effects or deaths were observed in all animals immunized with irradiated venom compared to those immunized with ovalbumin which present adverse effects and appeared to be less active.

The results showed also small granulomas occurring at the site of injection of immunized animals with the adjuvant alone or the adjuvanted irradiated venom, which disappear after a few weeks. These granulomas are defined histologically as immunogenic granulomas surrounded by lymphocyte and eosinophil infiltration. Although in some cases, persistent subcutaneous granulomas have been already observed after injection of diphtheria–tetanus–pertussis, hepatitis B vaccines and allergen extracts [15, 24–27].

The observed increase of eosinophils in the blood of immunized animals could be related to the inflammatory reaction, since eosinophils take part in inflammatory processes [28–30].

Furthermore, a significant increase of IL-5 and IL-4 levels in sera of immunized animals was observed. Indeed, the development of eosinophil infiltration may be mediated by inflammatory cytokines IL-5 and IL-4 [31].

The level of IgE can be affected by the secreted cytokine IL-4 by auxiliary T lymphocyte [32–36]. Despite the high

level of the IL-4, no production of IgE in sera of immunized animals with the adjuvanted irradiated venom was observed indicating that this formulation prevents the IgE synthesis while enhances the IgG production by upregulating Id2; a specific inhibitor of class switching recombination (CSR) to IgE, which may be able to modulate B cells to undergo isotype switching and/or memory B cells [37, 38].

The skin tissue analysis showed fully granulated mast cells in skin of immunized mice with adjuvanted irradiated venom, indicating the absence of IgE in their sera, which is responsible for mast cell degranulation [39]. Mast cell degranulation occurred after IgE fixation on specific membrane receptors, releasing series of pre-formed inflammatory mediators such as histamine and proteases [40, 41], which lead to an increase of vascular permeability, causing inflammatory edema [42].

Neither IgE production, nor mast cell degranulation, nor change in vascular permeability and nor edema-forming were observed in the studied organs of immunized animals with adjuvanted irradiated venom. However, a mild inflammation was observed; it might be necessary for the induction of the specific immune response.

The challenge with increased doses of native *Aah* venom to immunized animals with adjuvanted irradiated venom revealed a high level of immunoprotection. Effective protection is achieved after the second challenge at 6 and 26 months after the first envenomation. The long-lasting protective effect could be explained by the high titers of antibodies induced by adjuvanted irradiated venom [9, 10, 17]. The antibody production is carried out by long-lived plasma cells which reach the survival niches in the bone marrow under the effect of the chemokines of stromal cells [43, 44] and by the memory B cells developed during the immunization of mice. Previous studies have reported that memory B cells persist in Germinal Center during several months after immunization [45].

Conclusion

In the present study, it appears that the immunization with irradiated *Aah* venom associated with alum induces low inflammatory response without any adverse effect. This safe formulation presents also an immunoprotective effect at long-term against scorpion envenomation. This safe and non-toxic vaccine formulation could be a good candidate to prevent the severe cases of scorpion envenomation without risk of developing a deleterious inflammatory response.

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