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Evaluation of the efficacy of isopathic immunotherapy in the treatment of allergic asthma in BALB/C mice

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ABSTRACT

Introduction: Homeopathy is a therapeutic method based on the fundamental principle of “like cures like.” Homeopathic remedies are extremely dilute but involve vigorous shaking at each dilution. Isopathy is one approach of homeopathy, in which the causative agents or products of a disease are used to treat the same disease. Allergen immunotherapy is the only potential disease-modifying treatment for allergic patients. Subcutaneous immunotherapy is more effective than sublingual immunotherapy. However, subcutaneous immunotherapy is ineffective at a low dose, whereas at high doses it can result in an unacceptably high frequency of systemic reactions. In the current study, we evaluated the efficacy of isopathic immunotherapy with highly diluted ovalbumin (HD OVA) in the treatment of OVA-induced allergic asthma in BALB/c mice.

Methods: BALB/c mice were sensitized with OVA and alum. Two weeks later, the mice received HD OVA on days 21, 22, 32 and 41 (8 h after the last challenge) of the treatment. The mice were challenged with OVA (5%) aerosols on days 35, 38 and 41 for 20 minutes using an ultrasonic nebulizer and sacrificed the next day.

Results: Isopathic immunotherapy significantly reduced lung tissue inflammation, the number of eosinophils in bronchoalveolar fluid, allergen-specific IgE and interleukin-4 production. It also insignificantly increased the production of transforming growth factor-beta and proliferation of regulatory T cells against the allergen.

Conclusion: Isopathic immunotherapy may be a good candidate treatment for allergic asthma.

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Asthma; inflammation; allergy; immunotherapy; isopathic immunotherapy; homeopathy

Introduction

Allergen immunotherapy (AIT) is the only potential disease-modifying treatment for allergic patients [1]. There are two routes of administration for AIT: subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT). SCIT is more efficacious and presents fewer safety concerns compared to SLIT [2,3]. However, there are serious issues with SCIT. It is ineffective at a low dose, while high doses can result in an unacceptably high frequency of systemic reactions [4]. Thus, the indication for SCIT is patients that, despite a supervised trial of medical treatment, continue to display uncontrolled symptoms [3]. Therefore, it is essential to improve the efficacy and decrease the side effects of SCIT.

Homeopathy is a therapeutic method based on the fundamental principle of “like cures like.”

Homeopathic remedies are extremely dilute and are prepared using vigorous shaking at each dilution [5]. According to homeopathic rules, repeated serial dilution and vigorously shaking (succussing) the medicinal substances does not diminish their therapeutic power but is expected to enhance their therapeutic power. The main criticism of this concept is when the dilutions exceed Avogadro's constant (6.022×10^{23} /mol), the probability of the existence of even a single molecule of the original substance approaches zero [6].

Isopathy is a homeopathy approach in which the causative agents or products of a disease are used to treat the same disease [7]. Some studies have used isopathic immunotherapy to treat allergic diseases. The method of using isopathic immunotherapy for the treatment of allergic diseases is comparable to the immunotherapy represented in the allergy medicine

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literature [8]. Classical homeopathy is a highly individualized therapeutic system [9]. This individuality makes the right diagnosis by the homeopathic practitioner challenging and time consuming and has complicated clinical studies of the efficacy of classical homeopathic remedies, compared to conventional medicine. Furthermore, because the mechanism of action of homeopathic remedies is unknown, studying the effects of homeopathic remedies on cells or animal model of diseases is also difficult. In isopathic immunotherapy, remedies are prepared in the same manner as classical homeopathic remedies [7] and the method of diagnosis is the same as conventional allergen immunotherapy [8]. Thus, isopathic immunotherapy might be the most suitable candidate to address the main issue that currently limits homeopathy, namely the possibility of therapeutic effects in a highly diluted remedy.

Some studies have evaluated the efficacy of isopathic immunotherapy in the treatment of patients with respiratory allergic diseases. Most, but not all, the studies concluded that isopathic immunotherapy was effective [4,6,8,10–14].

Animal models are useful tools to investigate the mechanisms of diseases. Mouse models of allergic asthma share many clinical features of the disease with those in humans. For example, mice can develop a Th2 immune response against the allergen, produce antigen-specific IgE and develop airway inflammation due to allergic response [15]. Studying the pharmaceutical activity or efficacy of homeopathic remedies in animal models could be very useful in clarifying the mechanism of action of homeopathic remedies [16,17].

In the current study, we evaluated the efficacy of isopathic immunotherapy with highly diluted ovalbumin (OVA) in the treatment of OVA-induced allergic asthma in BALB/c mice.

Methods

Animals

Six-to-eight-week-old healthy male BALB/c mice were used for the experiments. The mice were purchased from Razi Vaccine and Serum Research Institute (Karaj, Iran). They were housed in polycarbonate cages (20.7 × 26.5 × 14 cm) one week prior to the experiments with free access to standard food and water, and a 12:12 light/dark cycle. The bedding material used for cages was wood chips. All the experiments were conducted in accordance with the

Animal Care and Use Protocol of Urmia University of Medical Sciences, Urmia, Iran.

Preparation of highly diluted OVA

OVA (Grade V, Sigma-Aldrich, USA) were prepared in 6C homeopathic dilutions and succussed according to Homeopathic Pharmacopeia of the United States specifications for centesimal scale potencies [18]. A 6C dilution is 1:1,000,000,000,000 (10^{-12}) w/v of the original substance. Here, it was equal to 10–12 mg/ml of OVA, which was designated highly diluted (HD) OVA.

Allergy induction, treatment and challenge protocol

The mice were sensitized on days 0 and 7 using 10 µg OVA in 100 µl alum that was delivered intraperitoneally. Before starting the treatments, the mice were randomly divided into three groups. The HD group received 20 µl HD OVA subcutaneously on days 21, 22, 32 and 41 (eight hours after the last challenge) as the treatment. The untreated group received 20 µl normal saline subcutaneously. Healthy mice were considered as the normal group. All mice were challenged with OVA (5% in pyrogen-free saline) aerosols on days 35, 38 and 41 for 20 min using a model NE-U17 ultrasonic nebulizer (Omron, Japan). Twenty-four hours after the last challenge, the mice were sacrificed to perform different experiments [19–21]. Five mice per group were used to perform different experiments. A flow diagram of the protocol is provided in Figure 1.

Pathological evaluation of lung tissue

Twenty-four hours after the last challenge, the mice were anesthetized deeply using a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) [22]. The trachea of each mouse was exposed by removing the tissues above the trachea and carefully puncturing the trachea using a tracheal cannula. The trachea and tracheal cannula were tied by a surgical suture. Blood samples were collected for allergen-specific IgE measurement and then 1 ml of 10% formalin was slowly injected into the lung through the tracheal cannula. The lung was harvested and fixed in 50 ml of 10% formalin for at least 24 h, embedded in paraffin and used for sectioning. The sections were stained with hematoxylin and eosin (H&E) stain. Determination and analysis of tissue inflammation and its severity were performed

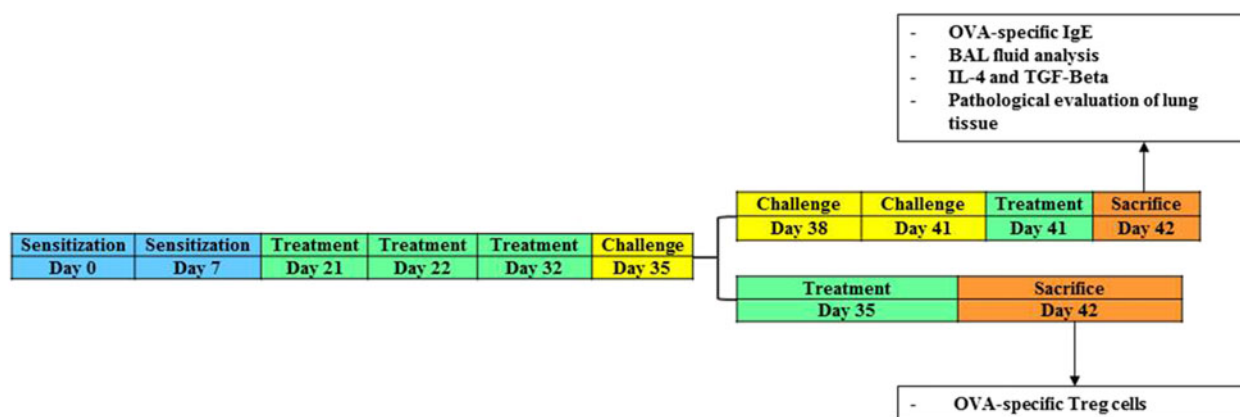


Figure 1. Flow diagram of the experimental protocols.

by a pathologist who was blinded to the group assignments. In addition, digital images were obtained from the lung sections after the analysis. A semi-quantitative scoring system was used to evaluate and grade the lung inflammation, where +5 was considered as the highest inflammation degree (more than three cells deep) and accumulation of inflammatory cells around the majority of vessels and bronchioles and +1 reflected the lowest inflammation rate with a small number of inflammatory foci [23].

Bronchoalveolar lavage (BAL) fluid analysis

The mice were sacrificed on day 42. Their tracheas were punctured using a tracheal cannula and fixed by a surgical suture. The airways of the mice were lavaged twice with 0.9 ml cold phosphate buffered saline (PBS) containing 5% bovine serum albumin (BSA; Merck, Germany), transferred into 15-ml polystyrene tubes and kept on ice. The BAL fluid was centrifuged at 2000 rpm and 4°C for 5 min, and the cell pellet re-suspended in 100 µl of PBS. The volume was spread on the surface of glass slides and dried at laboratory temperature. The dried film on the slide was fixed in methanol for 5 min. Methanol-fixed slides were stained with Wright-Giemsa stain. Eosinophil counting was performed by considering 20 random fields per slide (at least 200 cells per slide) [24,25].

Measurements of interleukin (IL)-4 and transforming growth factor-beta (TGF-β)

Splenocytes were cultured to compare the production of cytokines. The spleen was removed aseptically and single-cell suspensions were prepared in RPMI 1640 medium (Gibco, UK). Red blood cells were lysed osmotically using ammonium chloride buffer (0.9%). The cells were washed twice in RPMI 1640. After the

final wash, the cell pellet was re-suspended in RPMI containing 10% fetal bovine serum (FBS, Gibco). The viable cells were counted using Trypan blue (0.4% w/v). Cells (1×10^5) suspended in RPMI 1640 supplemented with 10% FBS, 100 IU/ml penicillin and 100 mg/ml streptomycin were added to each well of a 96-well V-Bottom Microplate. The cells were stimulated with 40 µg/ml OVA and incubated at 37°C in an atmosphere of 5% carbon dioxide. Seventy-two hours later, the plates were centrifuged and the supernatants were collected and refrigerated at -70°C. The concentration of IL-4 and TGF-β in the supernatants was estimated using a commercial enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, USA). All the samples including standards, blank and tests were prepared in duplicate [26,27].

Determination of OVA-specific IgE levels in serum

The serum of blood samples collected on day 42 were used to determine OVA-specific IgE by ELISA using 96-well microtiter plates. The blood samples were centrifuged at 2000 rpm for 10 min at 4°C, and the separated sera were collected and stored at -80°C. The optimum dilution of the sera and the optimum dose of OVA to be used in the ELISA were determined using the checkerboard assay. First, 200 µl of allergen (0.5 µg/ml OVA) in coating buffer (0.1 M carbonate, pH 9.5) was added to each well. The coated plates were incubated at 4°C overnight in a humid chamber. The microplate washed with PBS containing 0.05% Tween 20 (PBST) three times to remove unattached allergens and then blocked with 250 µl PBST containing 5% BSA and incubated for 2 h at 37°C. After washing the plates with PBST, 200 µl of diluted serum was added to each wells and incubated at 37°C for 2 h. After washing three times with PBST, the plates were incubated with 200 µl horseradish peroxidase conjugated goat anti-

mouse IgE (Abnova, Taiwan) and incubated for 1 h at room temperature. The microplate was washed three times with PBST and the reaction was developed by adding 200 μ l of 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide substrate and incubated for 30 min in the dark. The reaction was terminated by the addition of 50 μ l of 2 N H₂SO₄ and the absorbance was read at 450 nm [26,28]. The obtained optical densities were used for comparison between the groups, since no OVA-specific standards were available.

Evaluation of T regulatory (Treg) cell population

The mice were treated on days 21, 22, 32 and 35 (eight hours after the challenge). The mice were challenged once on day 35 for 20 min. Seven days later (day 42) they were sacrificed, and spleens were removed aseptically. The spleens were homogenized by 1 ml cold RPMI 1640 (Gibco) and the erythrocytes were lysed using 0.9% cold ammonium chloride for 5 min and the cells were washed twice with RPMI 1640. After the final wash, the cell pellet was re-suspended in RPMI containing 10% FBS (Gibco). The viable cells were counted using Trypan blue (0.4% w/v). The viability was never less than 98%. The cells (1×10^7) were suspended in 1 ml of culture medium and cultured in 24-well microplates using 1 ml of RPMI 1640 containing 200 μ g/ml OVA, 10% FBS, 100 IU/ml penicillin and 100 mg/ml streptomycin (total volume of 2 ml per well). Each microplate was incubated at 37°C in an atmosphere of 5% carbon dioxide for 72 h. The cells were centrifuged for 5 min at 2000 rpm and 4°C. The pellet was re-suspended in flow cytometry staining buffer and viability was measured using Trypan blue (viability was never less than 70%). A suspension containing 1×10^6 cells/ml was prepared. The cells were stained by a mouse Treg staining kit according to the manufacturer's instructions (eBioscience, USA). The samples were analyzed by flow cytometry (Sysmex-Partec, Germany) and the results were analyzed using FlowJo software. CD4 and FOXP3 positive cells were considered as Treg cells [29] and the percentages obtained were compared between the groups [30].

Statistical analyses

One-way analysis of variance (ANOVA) was followed by Tukey's *post hoc* test. A *p* values < 0.05 was considered significant.

Results

Pathological evaluation of lung tissue

Recruitment of inflammatory cells into the lung tissue was evaluated in a single blind manner by a pathologist. Accumulation of inflammatory cells around the majority of vessels and bronchioles was evident and was also evaluated. As shown in Figure 2(A), administration of HD OVA led to a significant (*p* = 0.009) reduction of the inflammation score compared to the no-treatment group. Five mice per group were used to evaluate inflammation in the lung tissue.

BALF fluid analysis

As shown in Figure 3(A), administration of HD OVA reduced the number of eosinophils in BAL fluid compared to the no-treatment group. The highest number of eosinophils was observed in the no-treatment group and the treatment significantly reduced eosinophil numbers in BAL fluid (*p* = 0.001).

IL-4 and TGF- β measurement

IL-4 production by splenocytes was determined upon exposure to the allergen (OVA, 40 μ g/ml). Treating of mice with 20 μ l of HD OVA reduced IL-4 production significantly compared to the no-treatment group (*p* = 0.001) (Figure 4(A)). An increase in TGF- β production was evident in the HD group, but was not statistically significant compared to the no-treatment group (Figure 4(B)). By contrast, treating mice with HD OVA significantly increased TGF- β /IL-4 ratio compared to the no-treatment group (*p* = 0.002) (Figure 4(C)).

Determination of OVA-specific IgE levels in serum

An indirect ELISA technique was used to evaluate OVA-specific IgE levels in sera. Administration of HD OVA significantly (*p* = 0.025) reduced the allergen-specific IgE production and its concentration in the serum compared to the no-treatment group (Figure 5).

Evaluation of Treg cell population

An *in vitro* assay was used to evaluate the Treg cell population by culturing of splenocytes in the presence of OVA (200 μ g/ml). To reduce spillover between phycoerythrin (PE) and PE-cyanine 5 (CY5) fluorochromes to the minimum amount, we considered CD4⁺ and FOXP3⁺ (fluorescein isothiocyanate

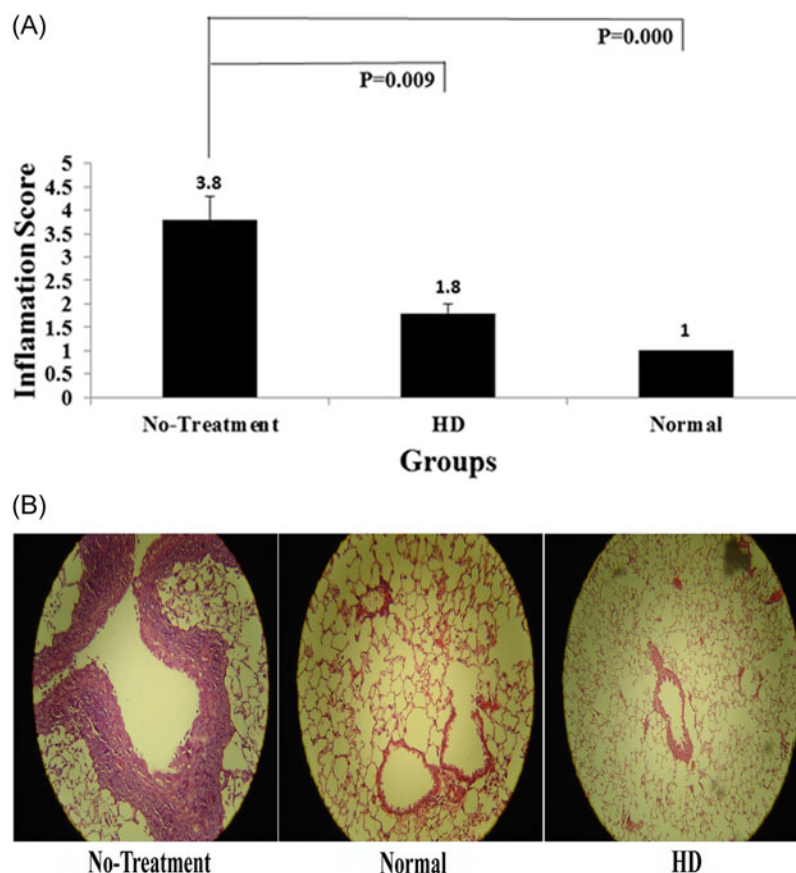


Figure 2. (A) Effects of administration of highly diluted (HD) OVA on the inflammation score in the lung tissue. Administration of 20 μ l HD OVA significantly reduced lung tissue inflammation compared to the no-treatment group. (B) Digital images of lung tissue. The highest inflammation score is seen in the no-treatment group and the lowest inflammation score is observed in the normal group. Treatment with HD OVA significantly reduced lung tissue inflammation compared to the no-treatment group.

[FITC]+ and PE-CY5+) cells as Treg cells [22]. The population in the HD group was higher than the no-treatment group, but this difference was not statistically significant (Figure 6(A)).

Discussion

Isopathic immunotherapy of OVA-induced allergic asthmatic mice significantly reduced inflammation of lung tissue ($p=0.009$), eosinophil number in BAL fluid ($p=0.001$), IL-4 production by splenocytes ($p=0.000$) and OVA-specific IgE levels in sera ($p=0.025$). This immunotherapy also significantly increased the ratio of TGF- β /IL-4 produced by the splenocytes ($p=0.002$) and increased (albeit insignificantly) TGF- β production and the proliferation rate of FOXP3 + CD4+ Treg cells.

To the best of our knowledge, this is the first study on the efficacy of isopathic immunotherapy in a laboratory animal model. The results support those of most of the other studies that have explored the efficacy of this kind of immunotherapy on allergic diseases in humans and have reported beneficial effects

[8,11–14]. However, another study reported that isopathic immunotherapy was not effective in the treatment of allergic diseases [31].

Inflammation plays an important role in the pathophysiology of allergic asthma. One of the most important steps in the treatment of asthma is the tight regulation of inflammation [32]. Presently, isopathic immunotherapy significantly ($p=0.009$) reduced the inflammation of the lung tissue. This is consistent with the finding that the therapeutic effects of SCIT on allergic asthma are accompanied by reduction of airway inflammation [33].

Eosinophils play a key role in the pathogenesis of asthma [34]. We showed that isopathic immunotherapy resulted in diminished numbers of eosinophils in BAL fluid. This is in agreement with the finding that SCIT decreases the number of eosinophils in BAL fluid of allergic asthma in mice [33].

The most important players in the inflammation of asthma are Th2 cells. IL-4 is the most important cytokine produced by Th2 cells [35]. We measured the production of IL-4 by cultured splenocytes exposed to

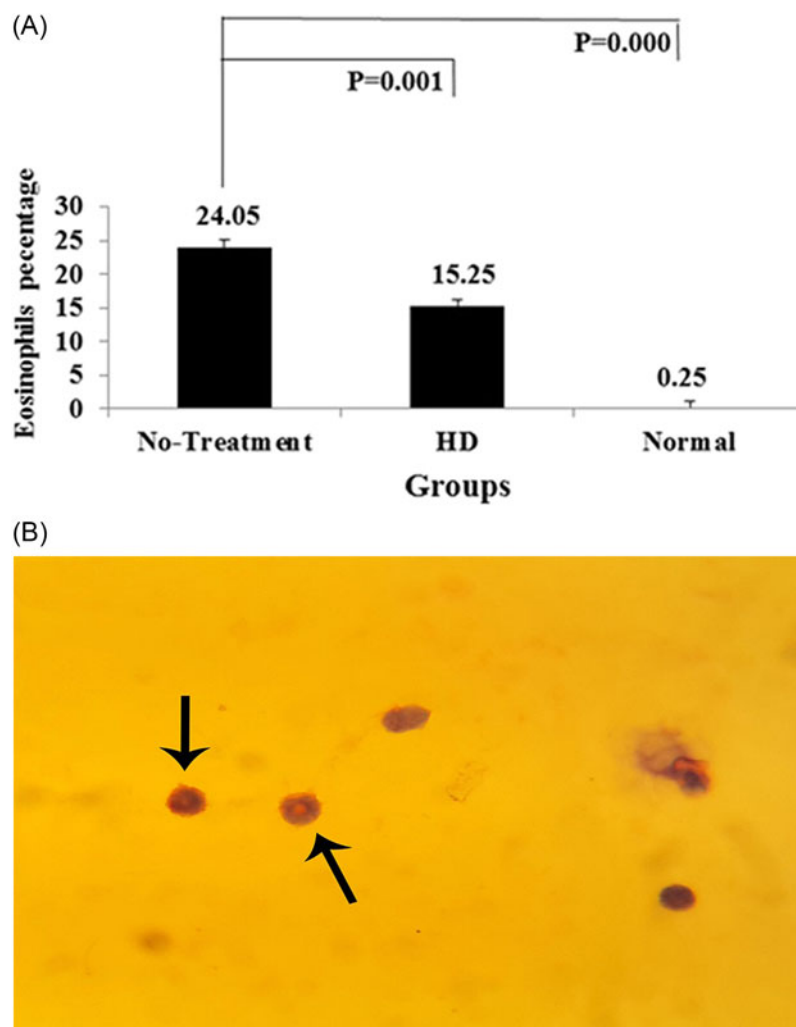


Figure 3. (A) Effects of HD OVA administration on the percentage of eosinophils in BAL fluid. Administration of high-diluted OVA significantly reduced the eosinophil percentage compared to the no-treatment group. (B) Digital image of eosinophils. Peripheral smear of BAL fluid stained with Wright-Giemsa stain. Eosinophils are indicated by the black arrows.

the OVA allergen. Isopathic immunotherapy significantly ($p=0.000$) reduced the production of IL-4 by the splenocytes. This finding indicated that isopathic immunotherapy reduced the deviation of immune responses toward Th2. This is consistent with the finding that SCIT decreases the serum level of IL-4 in a mouse model of asthma [36].

IgE-mediated hypersensitivity to antigen(s) is the most important mechanism underlying the development of allergic asthma [37]. Presently, isopathic immunotherapy significantly ($p=0.025$) decreased the serum levels of OVA-specific IgE. As the reduction of serum levels of allergen-specific IgE is always due to the reduction of deviation of immune responses toward Th2 [38], this result is consistent with our finding that isopathic immunotherapy decreased Th2 immune deviation. Furthermore, this result agrees with the finding that the therapeutic effects of SCIT

on allergic asthma are accompanied by the reduction of serum allergen-specific IgE levels [33,36].

Isopathic immunotherapy increased the production of TGF- β , but this increase was not significant. However, the TGF- β /IL-4 ratio was significantly ($p=0.002$) increased. TGF- β plays a vital role in generation, function and stabilization of CD4 + Foxp3 + Treg lymphocytes [39,40]. It has also been shown that there is an increased ratio of IL-4/TGF- β and Th2/Treg cells in patients with moderate to severe asthma [41]. Thus, increasing the TGF- β /IL-4 ratio by isopathic immunotherapy is consistent with our finding that isopathic immunotherapy increased the proliferation of OVA-specific FOXP3 + CD4+ Treg cells. However, this increase was not statistically significant. The therapeutic effects of SCIT on allergic asthma are accompanied with significant increases of TGF- β production and Treg cell proliferation [33,41]. Such findings have led to the claim that the immunoregulatory activity of Treg

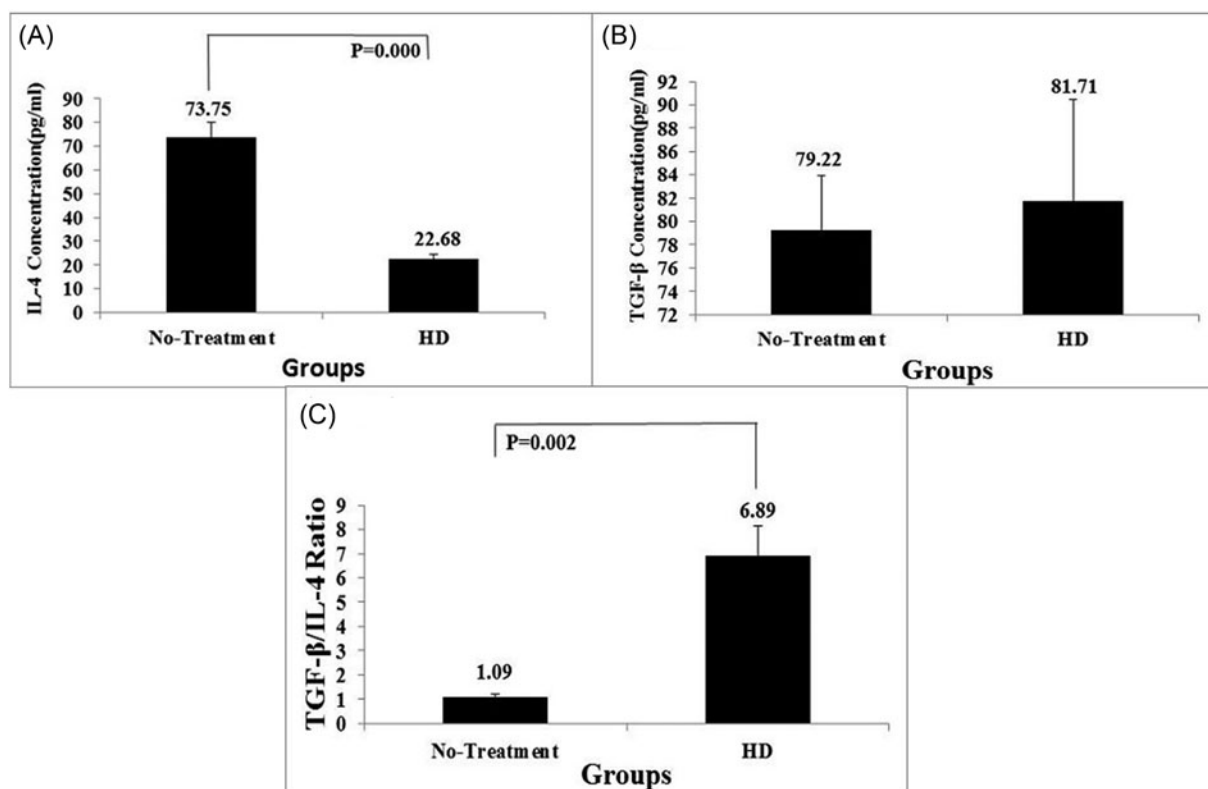


Figure 4. Effect of treatment of mice with HD OVA on the cytokines production by splenocytes. (A) Splenocytes were cultured in RPMI and stimulated by 40 μ g/ml OVA for 72 h. IL-4 concentration was measured in the supernatant. Treatment of mice with HD OVA significantly reduced IL-4 production by splenocytes. (B) Administration of HD OVA increased TGF- β production by splenocytes when exposed to OVA but the increase was not significant statistically. (C) Treatment of mice with HD OVA resulted in an incremental change of the TGF- β /IL-4 ratio compared to the no-treatment group.

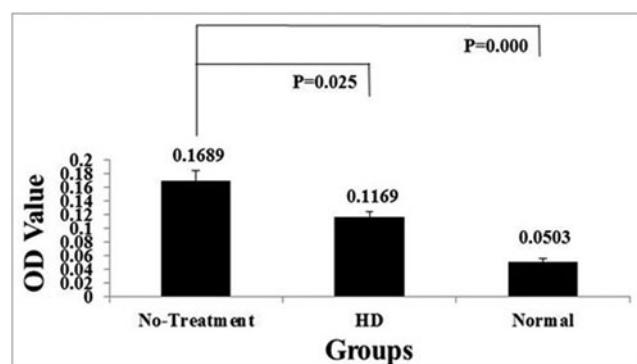


Figure 5. Effect of HD OVA administration on OVA-specific IgE production. Treating the mice with HD OVA reduced OVA-specific IgE concentration in the serum.

cells is the main mechanism for the clinical efficacy of SCIT [37,42]. Therefore, it is conceivable that, in addition to induction of antigen-specific FOXP3 + CD4+ Treg cell proliferation, there might be other important mechanisms underlying the efficacy of isopathic immunotherapy. This remains to be explored. Furthermore, our finding that isopathic immunotherapy increased antigen-specific FOXP3 + CD4+ Treg cell proliferation and production of TGF- β , albeit in a

statistically nonsignificant manner, is consistent with our previous suggestion that the induction of regulatory anti-inflammatory pathways might be one mechanism for the biological effects of homeopathic remedies [43–46]. As we previously explained in our suggested mechanistic model, based on our and other experimental research studies, when a correct homeopathic remedy is administered to a patient, sensory neurons are stimulated and sensitized due to inflammatory or nociceptive stimuli of any origin. Hence, different regulatory mechanisms, including the activation of descending antinociceptive and/or cholinergic anti-inflammatory pathways, are probably triggered. This leads to the restoration of homeostasis [43,46]. Our current findings suggest that Treg cells may play such a role for isopathic immunotherapy. However, further studies are necessary to determine the exact role of Treg cells in isopathic immunotherapy-induced tolerance.

Conclusion

To our knowledge, this is the first study examining the efficacy of isopathic immunotherapy in an animal model. The administration of HD OVA efficiently

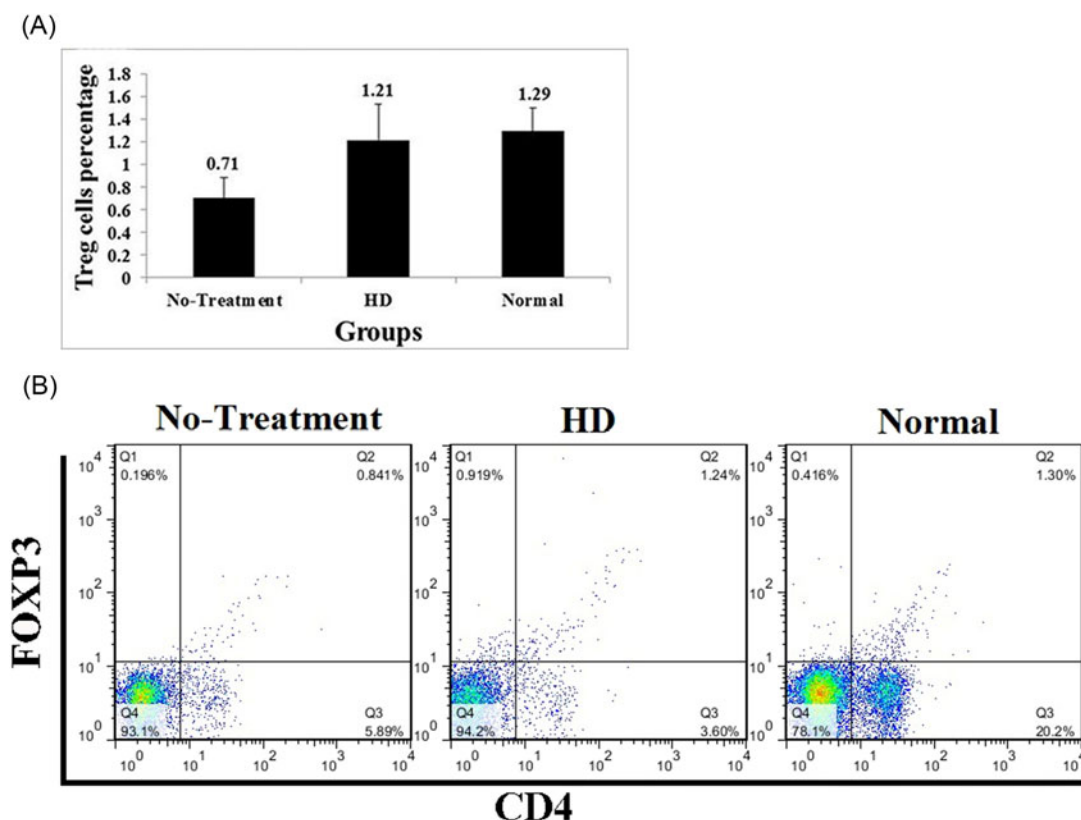


Figure 6. (A) Effect of HD OVA treatment on FOXP3 + CD4 + Treg cell population. Administration of HD OVA increased the rate of Treg cell proliferation compared with the no-treatment group. The difference was not statistically significant. (B) Expression of CD4 and FOXP3 in splenocytes from BALB/c mice. FL3 indicates FOXP3 (PE-Cy5) and FL1 specifies CD4 (FITC).

diminished the severity of OVA-induced allergic asthma in BALB/c mice. Thus, isopathic immunotherapy may potentially be considered as a therapeutic method for the treatment of allergic asthma. The findings are a positive step toward determining the mechanism underlying the biological effects of isopathic immunotherapy on allergies. This knowledge will be valuable in revealing the mechanism of therapeutic effects of homeopathic remedies in the future. Further studies are required to confirm our findings.

Disclosure statement

No potential conflict of interest was reported by the authors.

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