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1. Introduction

Allergic rhinitis (AR) is the most common respiratory allergic disorder which results from an IgE-mediated inflammation in the nasal mucosa. The first strategies for the controlling of AR are the avoidance of allergen exposure and using pharmacotherapeutic agents to relief the

acute symptom (Bacharier et al. 2008). However, allergen-specific immunotherapy has been introduced as the best disease-modifying treatment for pollen allergy with a long-term effect (Malling 1998; Wahn 2016; Wahn et al. 2009b). Allergen-specific immunotherapy (SIT) involves immunological pathways to modify allergenic responses by reorienting of a Th2-like allergic response toward a Th1-like non-allergic response and as well as TCD4⁺ regulatory cells, that is associated with IL-4, IL-5 and IL-13 reduction, IFN- γ and IL-10 increasment, and subsequently effector cell-induced allergic inflammation suppression (Akdis and Akdis 2011; Jutel and Akdis 2011; Larche et al. 2006; Moingeon et al. 2006; Suarez-Fueyo et al. 2014). SIT is usually applied as subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT) (Schmitt et al. 2015; Wahn et al.). Some problems associated with SCIT including high risk of side effects and anaphylaxis reactions (Durham 2006; Lockey et al. 2001). However, SLIT requires at least 50–100 times more allergen than SCIT to achieve a similar level of efficacy (Seitzer et al. 2003), but patients as well as companies interested to SLIT are increasing due to needle-free administration and reduced side effects (Joshi et al. 2014). During SLIT, the allergen is captured locally (i.e. within the oral mucosa) by Langerhans-like DCs following phagocytosis, macropinocytosis or receptor-mediated endocytosis. After that, DCs mature and migrate to proximal draining lymph nodes, involved in their adhesion and trafficking to target tissue (Hubbell et al. 2009; Larche et al. 2006). Eventually, as a consequence of the circulation of allergen-specific activated effector T cells throughout the body and the persistence of memory cells, a local (i.e. sublingual) administration of the allergen during desensitization results in both systemic and mucosal protective immune responses (Larche et al. 2006).

Many studies have been showed that the optimal size of vaccine delivery systems could improve the safety and stability of the vaccine and facilitate antigen presentation to the T cells by targeting of professional antigen presenting cells (APCs) especially Dendritic cells

(DCs)(Clawson et al. 2010; Combadiere and Mahe 2008; Hubbell et al. 2009; Igartua et al. 2001; Scholl et al. 2006). Moreover, the size of NPs dramatically affects their migration to the secondary lymph organs and therefore, the immune response (Brozek et al. 2010; Nakajima and Takatsu 2007; Stemeseder et al. 2017). In salari et al study, PLGA NPs containing rChe a 3 allergen used sublingually and showed impact on Th2 shifted to Th1 and Treg responses that indicated the use of sublingual dendritic cell(Salari et al. 2015). Poly (D, L-lactic-co-glycolic acid) (PLGA) NPs is a biocompatible as well as biodegradable copolymer which approved by the US Food and Drug Administration (FDA). In different studies, the efficiency of nanopatform based on PLGA NPs has been evaluated for anticancer drug delivery and overcoming multidrug resistance (Nie et al. 2017; Peng et al. 2018; Zeng et al. 2013). Nowadays, PLGA is assayed for allergen-specific immunotherapy of allergic diseases especially in human (Klose et al. 2008; Scholl et al. 2004). PLGA is appropriate for the formulation of peptides or proteins due to the protection them from the degradation, high loading efficiency, release drugs slowly, allowing it to be applied as an immunostimulatory adjuvants in allergic vaccines (Ulery et al. 2009; Xiao et al. 2013).

Different size of PLGA NPs can be obtained by altering chemical changes as well the synthesis method (Elamanchili et al. 2007; Lu et al. 2009). In our previous study, we have developed the size control methods by changing the preparation condition during formation of PLGA NPs containing rChe a 3 allergen (Hajavi et al. 2017).Chenopodium album (Lambs quarter), the important allergenic weed in the Chenopodiaceae family, is one of the most reasons of allergic diseases, with the prevalence of %62.9 in Iran(Ezeamuzie et al. 2000; Fereidouni et al. 2009). Until now via molecules analysis, three allergens have been identified from Chenopodium album pollen, Che a 1, Che a 2 and Che a 3(Barderas et al. 2004). Che a 3 belong to Polcalcin families that are small acidic protein with a molecular weight of 9k Da and involved in messaging and budding of pollen. Che a 3 cause IgE–mediate histamine

release from basophils of allergic people which cause type I allergy symptoms (Barderas et al. 2005; Ledesma et al. 1998; Martinez et al. 2002). This allergen has been purified, cloned and produced as recombinant protein in the *E.coli* as a host (Nouri et al. 2012a). The purpose of the current study was to load of recombinant rChe a 3 allergen into PLGA NPs with different size of (about 200, 400 and 800 nm), ii) and evaluation of size and dose effects of delivery system on the Th2 immune response (IgE, IgG1 and IgG2a and some cytokine) after SLIT in a mouse model of experimental allergic rhinitis.

2. Materials and methods

2.1 Materials

PLGA 50:50 with a molecular weight of 30000-60000 Da and free carboxyl end groups was obtaining from Sigma-Aldrich (Sigma Chemical Co, St. Louis, MO, USA). Two analytical grades of Polyvinyl alcohol, as a stabilizers, with different degrees of hydrolyzation and polymerization (PVA, 87–89% hydrolyzed, MW: 31,000-50000 and MW: 30,000-70,000 Da, 87–90% hydrolyzed, were also supplied by Sigma-Aldrich (Sigma Chemical Co, St. Louis, MO, USA). The organic solvent, Dichloromethane (DCM) (MW=84.93 g/mol) was obtained from Merck, Germany. Micro BCA protein assay kit was provided by Parstous, IRAN. Al (OH)₃ was also purchased from Sigma-Aldrich. Biotin-conjugated anti-mouse IgE and horseradish peroxidase (HPR)-conjugated anti-mouse IgG were from eBioscience (San Diego, CA, USA). Also, Mouse interleukin (IL)-2, 4, 5, IL-5, IL-10, TGF- β and interferon gamma (IFN- γ) enzyme-linked immunosorbent assay (ELISA) kits were obtained from eBioscience (San Diego, CA, USA). All other chemical reagents and solvents were commercial products of analytical or reagent grade. Phytohaemagglutinin (PHA), Culture medium RPMI 1640 and their supplementation were obtained by Gibco (Gibco™, UK).

2.2 Animals

Female, BALB/c mice (specific pathogen-free grade, aged 6–8 weeks, weight 16–20g) were obtained from the Medical School of Mashhad University of Medical Sciences, Mashhad, Iran. Animals were maintained at the Animal Care Services of the Nanotechnology Research Center (Mashhad University of Medical Sciences, Mashhad, Iran), according to the local guidelines for animal care. All the animal experiments and handling procedures were approved by the Institutional Animal Ethics Committee (No. 930731) of the Mashhad University of Medical Sciences.

2.3 Methods

2.4 Expression and purification of recombinant Che a 3 antigen

According to our previous study (Nouri et al. 2012b; Salari et al. 2015), the recombinant Che a 3 antigen was produced in *Escherichia coli* BL21 Codon Plus (DE3) RIL cells (Stratagene, USA), purified by Ni-NTA affinity chromatography column (Parstous Co., IRAN) and finally the IgE reactivity of rChe a 3 to *C. album* allergic patients' sera was confirmed. The elution, which including resultant protein was dialyzed and freeze for using in different part of project.

2.5 Synthesis of different sizes of PLGA-rChe a 3 allergen NPs

Encapsulation of rChe a 3 allergen into PLGA NPs was performed by W/O/W Double-emulsion solvent evaporation method with some modifications (Feczko et al. 2008). Briefly, 0.3 ml aqueous phase containing 1mg of rChe a 3 in PBS was added to 10 mg of PLGA in 1.2 ml of DCM as an organic phase and then the mixture was emulsified by using a probe sonication (Hielscher Ultrasonic, Germany) for 1 minute (70W electric power input) in ice bath. The resulting primary water-in-oil (W/O) emulsion was added to 5 ml ice-cold aqueous PVA solution (2%) with different MW and concentration and further emulsified using a probe sonicator to prepare water-in-oil-in-water (W1/O/W2) double emulsion. This emulsion was under stirring at room temperature 800rpm for 2 h to evaporate the organic solvent. NPs

were then collected by centrifugation (Sigma 3-30ks, Germany) at 14,000(20400 g) rpm/min for 30 min at 4°C. Pellets washed three times with distilled water to completely remove PVA and then the purified nanoparticle suspension was stored at 4°C. Also, empty NP was prepared for control immunization.

2.6 Particle size and quantifying of rChe a 3 encapsulation and loading efficiencies of NPs

Mean particle size, size distribution, polydispersity index (PDI) of nanoparticles were determined by Zeta sizer (Nano-ZS, Malvern Instruments Ltd, Malvern, UK) based on dynamic light scattering at 25°C. The encapsulation and loading efficiencies of rChe a 3 loading into PLGA NPs was determined by indirect method. After collection of NPs by centrifugation, the amount of unencapsulated allergen in supernatants was measured by a micro BCA (bicinchoninic acid) protein assay Kit. The encapsulation efficiency (EE) of the rChe a 3-loaded nanoparticles and the protein loading capacity (Moral et al.) of the fabrication process were calculated using the following equations:

$$\text{Entrapment efficiency (EE\%)} = \frac{[\text{initial amount of allergen} - \text{amount of allergen in the supernatant}]}{[\text{initial amount of allergen}]} \times 100$$

$$\text{Loading capacity (LC } \frac{\mu\text{g}}{\text{mg}} \%) = \frac{[\text{initial amount of allergen} - \text{amount of allergen in the supernatant}]}{[\text{initial amount of polymer}]} \times 100$$

2.7 Evaluation of structural integrity of the rChe a 3 allergen by SDS-PAGE

The integrity and stability of rChe a 3 allergen before and after encapsulation in PLGA NPs was investigated using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) assays according to Laemmli with some modifications (Laemmli 1970). Briefly, rChe a 3 allergen, empty PLGA and PLGA-rChe a 3 NPs in different sizes were analyzed using 15% acrylamide separation gels with a 5% stacking gel under reducing conditions. Samples

were diluted at a 1:1 ratio with sample buffer and boiled at 95°C along with low molecular weight marker (Parstous, Iran), five min for rChe a 3, but 30 min for PLGA-rChe a 3 and empty PLGA(Silva et al. 2015). After loading of samples and electrophoresis, gels were stained with Coomassie brilliant blue 0.1%, w/v.

2.8 Animal sensitization and sublingual immunotherapy

The animal sensitization model and specific immunotherapy were designed by combining the previous literature (Lu et al. 2011; Marazuela et al. 2007; Yu et al. 2010). The mice were randomly divided into thirteen groups (n=6), as a show in Fig.1. Mice model were sensitized to rChe a 3 by intraperitoneal (i.p.) injection with 10 µg rChe a 3 in PBS adsorbed on Al(OH)₃ at days 0 and 14. Negative control animals were sensitized with equivalent amounts of phosphate-buffered saline (PBS)/Alum. From days 21 to 24, 1% of rChe a 3 in PBS was aerosolized for 20min per day, using similar doses as above. To assess immune humoral responses, blood samples were taken at day 25(end of sensitization phase) through the retro-orbital sinus. The blood samples were centrifuged for 10 min at 4000 rpm 4 °C, and the serum was collected and stored at -70 °C until analysis for IgE, IgG1 and IgG2a titers. The treatment was initiated on day 28. The PLGA-rChe a 3 groups (9 groups) were sublingually treated with NPs containing 12.5, 25 and 50 µg of rChe a 3 with different sizes (200,400 and 800). Other groups included normal group (negative control, not-sensitized, not-treated), PBS group (positive group, sensitized, PBS-treated), PLGA and rChe a 3 groups that were sensitized but treated by PLGA and rChe a 3 in equivalent amount, respectively. All groups received a total of 9 doses of the vaccines (or control), one time per week for 2 months. Mice were held on their back for 2 minute to prevent immediate swallowing of the allergen. On days 90 and 91 (end of treatment), all groups were intranasally challenged by rChe a 3(1%) for 20 min to per group as described in sensitization phase. Twenty-four hours after the last aerosolize challenge, mice were euthanized, blood was obtained by cardiac puncture, and

serum was recovered. In addition, spleen cells were harvested and Bronchoalveolar lavage (BAL) and Nasal airway lavage (NAL) fluid was collected. Finally, lung and nasal removed for histopathological analysis.

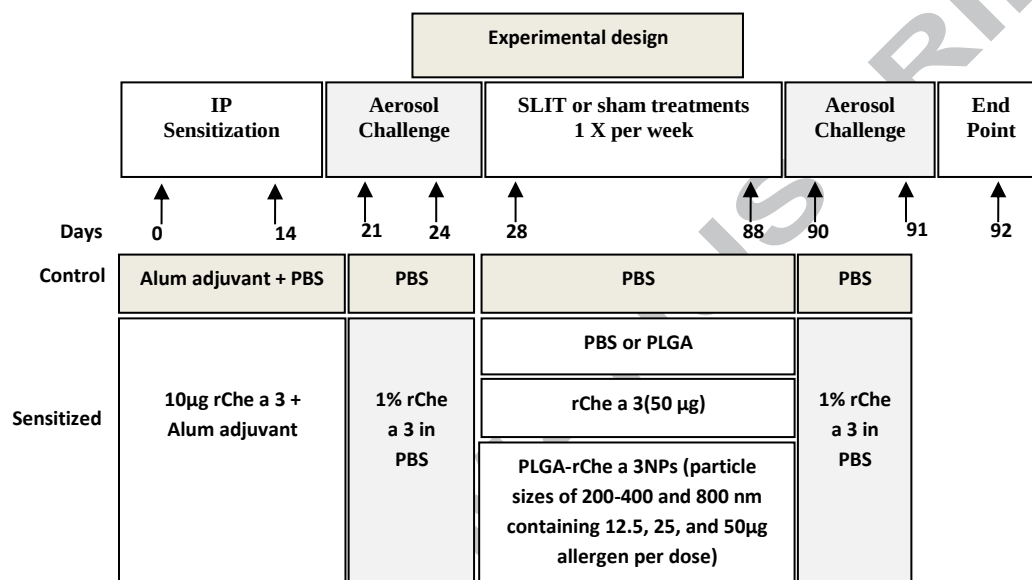


Fig.1. Experimental design. Thirteen groups of mice (n=6 per group) were used in the experiment. Abbreviations: PLGA, poly (lactic-co-glycolic acid); rChe a 3, recombinant *chenopodium album*; SIT, specific immunotherapy; ip, intraperitoneal injection; sc, sublingual injection.

2.9 Determination of total IgG1, IgG2a and specific-allergen IgE in serum against rChe a 3 allergen

Serum rChe a 3-specific IgE antibody was measured in an established indirect enzyme-linked immunosorbent assay (ELISA) as described previously (Nouri et al. 2013). Serum IgG1 and IgG2a allergen antibodies were also measured using ELISA kit according to the manufacturer's protocol (Zymed Laboratories Inc., USA and BD Biosciences, San Diego, CA, USA). Briefly, the anti-mouse IgG1 and IgG2a capture antibodies were coated with PBS and incubated overnight at 4°C. The plates, which were washed with Phosphate buffered saline with Tween-20 (PBST) and blocked with 2% bovine serum albumin (BSA)/PBST (250 µL/well), were incubated at 37°C for one hour. Serum samples were diluted by 1% BSA-PBST and added to the test wells. After washing the plates with PBST, the antibodies were detected with HRP-

conjugated anti-mouse IgG antibodies, finally, the samples were treated with TMB substrate buffer, and the reaction was stopped after 15 min by 1N HCl. The absorbance of the samples were measured at 450 nm using a Stat fax[®] 2100 microplate reader (Awareness Technology, Palm City, Fla., USA).

2.10 Cytokine assays in secretion of spleen cells after SLIT

To measure the cytokine production, 24 hours after the last immunization, spleens from individual mice were removed aseptically, minced, filtered through a sterile filters, washed with PBS, and resuspended in RPMI 1640 medium (Gibco Ltd., Paisley, Scotland) supplemented with 10% inactivated fetal calf serum (FCS) and 100 U/ml of penicillin and 100 µg/ml of streptomycin. Erythrocyte lysate were collected by centrifugation. Cells were cultured in 24-well flat-bottomed culture plates (Nunc, Denmark) in duplicate at a density of 2×10^6 cells/ml, and stimulated with rChE a 3 (10 µg/ml), PHA (1 µg/ml), or media at 37 °C. The culture medium was then collected and centrifuged at 4°C and 1,500 rpm (Salari et al. 2015). Supernatants were harvested after 72 h and analyzed for IL-2, IL-4, IL-10, IFN- γ , and TGF- β using mouse ELISA kits (eBioscience, San Diego, California) according to the manufacturer's instructions.

2.11 MTT cell Proliferation assay

For T cell proliferation assays, spleen cells of each group were pooled and seeded at 2×10^6 cells per well in 96-well round-bottomed culture plates in triplicate (Nunc, Denmark). For stimulation, the cells were cultured for three days with rChE a 3 (10 µg/ml), PHA (1 µg/ml) as a positive control, or medium alone at 37°C. Cell proliferation in response to the rChE a 3 was determined using a colorimetric immunoassay for quantification of cell proliferation based on the MTT proliferation assay kit (Roche Diagnostics, Germany) following the manufacturer's instructions. Cell proliferation calculated at the mean OD at 570 nm of stimulated cultures (minus unstimulated cultures) to that of unstimulated cultures.

2.12 Bronchoalveolar Lavage Fluid analysis

To determine the number and type of cells recruited to the airways, Bronchoalveolar lavage (BAL) fluid was collected and processed as described by George et al (George et al. 2006). After being mice euthanized, the lung was immediately lavaged via the trachea cannula and by using three doses of 1 mL of PBS. BAL fluid was centrifuged at 2000rpm for 10 min at 4°C and the supernatant was stored at -70°C for determination of IL-5 cytokine level. The cells in precipitate were resuspended with 500 µL of PBS, which was utilized for cell counting (10µL) using a hemocytometer and, because of better harvest cells, then suspension centrifuge as say above and pellet used for cell differential counting(490µL). Cells were fixed in methanol for 2-3 min and then stained with May-Grunwald Giemsa(Reactifs RAL, Martillac, France). At least 200 cells were counted for eosinophils according to their morphologic characteristics, under an optical microscope (Olympus, Center Valley, PA).

2.13 Nasal lavage fluid analysis

Nasal lavage fluid (NLF) was collected 24 hours after the last intranasal provocation with rChe a 3. When mice were anesthetized, thoracotomy was performed in each group 24 hours after the final allergen challenge. NAL by 22-gauge catheters was performed after partial tracheal resection(Shin and Ye 2012). The catheter was inserted into the tracheal opening in the direction of the upper airway and into the nasopharynx. The nasal passages were perfused gently with 1 mL PBS and NAL fluid was collected from the nares. NAL fluid was centrifuged at 2000 rpm for 7 minutes at 4°C, and the precipitate was evaluated for cell count with a hemocytometer. The slides were prepared, stained with Diff-Quick (Dade Behring AG, Dudingen, Switzerland), and evaluated for counts of eosinophils by observing at least 300 cells per slide at 400 magnification.

2.14 Lung and Nasal mucosa histology

For Histopathology studies, Lungs were removed, fixed in 10% formaldehyde, cut into 5 mm sections and stained with hematoxylin and eosin(H&E). Lung sections were observed under light microscopy (Axiophot 2 model, Zeiss, Jena, Germany) and photographed with a digital camera (Spot Insight Color, Diagnostic Instruments, MI, and USA). Additionally quantitative analysis in each slide, five airway sections were randomly selected and the degree of peribronchiolar and perivascular inflammation was evaluated (Khakzad et al. 2012). The number of eosinophils per unit airway area was determined by morphological criteria and enumerated per high-powered field. For histological examination of the nasal mucosa, the head was fixed in 10% buffered formalin solution 24 h after the last intranasal antigen challenge(Tsunematsu et al. 2007). The Frontal sections of the nasal tissues were stained with H&E. The stained slides were dehydrated, mounted, and observed under a light microscope (Axiophot 2 model, Zeiss, Jena, Germany) and photographed with a digital camera (Spot Insight Color, Diagnostic Instruments, MI, USA).For quantitative of lung sections analysis, in each slide, five airway sections were randomly selected and the degree of peribronchiolar and perivascular inflammation was evaluated as follows: 0: no cells; 1: a few cells; 2: a ring of cells, 1 cell layer deep; 3: a ring of cells, 2: 4 cells deep; 4: a ring of cells, more than 4 cell layers deep.

2.15 Statistical Analysis

Statistical and graphing software GraphPad Prism version 6.0(GraphPad, San Diego, CA) was used for all statistical analyses. All assays were compared using one-way analysis of variance followed by Tukey's post-test to compare all pairs of treatments. Differences were considered significant at p values that were less than or equal to 0.05. Experiments were performed three times.

3. Results

3.1 Expression and purification rChe a 3 antigen

The rChe a 3 protein was produced in BL21 cells at 16°C in a shaker with a shaking speed of 200 rpm. SDS-PAGE of rChe a 3 protein after purification by Ni-NTA affinity chromatography column were performed using 15% acrylamide separation gels under reducing conditions and Coomassie blue staining of gel indicated distinct allergen band (Fig. 2A).

3.2 Physicochemical characterizations of nanoparticles in different sizes

PLGA NPs containing rChe a 3 allergen were prepared by double emulsion solvent evaporation method and formulation conditions were optimized to obtain different sizes of NPs (about 200, 400 and 800 nm). As shown in table 1, different concentrations and Mol. Wt. of PVA were used to obtain different sizes (MW: 31,000-50000, 2% and 1% for 200 and 400 nm, respectively and MW: 30,000-70,000 Da, 1% for 800 nm). Also, sonication time for these NPs were 1 min in first sonication for all, but 4, 1 and 4 min for 200, 400 and 800 nm, respectively, in second sonication time. The particle size (nm), polydispersity (PDI), encapsulation efficacy (%) and loading efficiency (%) of these NPs were evaluated and presented in table 1. the maximum encapsulation of rChe a 3-encapsulated in PLGA was obtained with the 400 nm NPs. On the other hand, it was found NPs with size 400 nm had higher percent of loading efficacy (4.44%) compared to 200 nm and 800 nm NPs.

Table1: characterization of nanoparticle in different sizes (Mean±SD)

Formulations	Mean size(nm)	PDI	PVA Con (%)	PVA Mw(Da)	Type of solvent	Second sonication time	Mean EE (%)	Mean (µg/mg)
F1	232±35	0.126±0.045	2	31000-50000	DCM	4	43.6±1.55	3.14±0
F2	430±40	0.375±0.105	1	31000-50000	DCM	1	60.4±0.55	4.44±0

F3	727±65	0.132±0.064	1	30000-70000	DCM	4	51.3±1.75	3.77±0
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3.3 Structural integrity of the rChe a 3 loaded PLGA by SDS-PAGE

During rChe a 3 encapsulation, the allergen is exposed to mechanical and chemical treatments which could contribute to a loss of structural and antigenic integrity. We used SDS-PAGE to detect structural alters in our allergen after encapsulating in NPs. The bands of 13 kDa, PLGA- rChe a 3 and rChe a 3 are almost similar, establishing that rChe a 3 integrity after encapsulation in PLGA was without alterations and was not affected by the entrapment procedure (Fig.2).

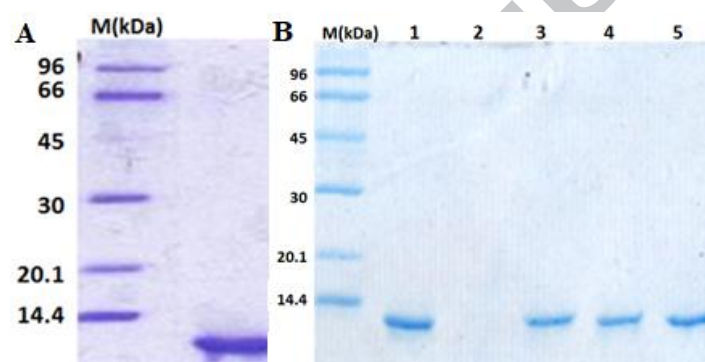


Fig. 2. A; SDS-PAGE analysis of rChe a 3, after expression and after purification. B; SDS-PAGE stability analysis of rChe a 3 loaded of PLGA nanoparticles by SDS-PAGE and Coomassie blue staining of gel. Notes: M: Molecular mass markers, 1: rChe a 3, 2: empty PLGA, 3, 4 and 5: rChe a 3 loaded from PLGA nanoparticles 200, 400 and 800 nm, respectively.

3.4 Th2 responses in rChe a 3 sensitized mice

BALB/c mice sensitized by rChe a 3 allergen as described in the Methods section. On day 25, serum samples were collected and analyzed for the induction of humoral immune responses by measurement of the level of IgG1, IgG2a and specific-allergen IgE against rChe a 3 allergen with ELISA kit. As shown in Fig.3, sensitized mice altered Th2 antibody responses to Th1 with elevated titers of rChe a 3-specific IgE and IgG1 antibodies. In contrast, no specific IgE or IgG1 production was observed in control mice receiving PBS in alum gel suspension.

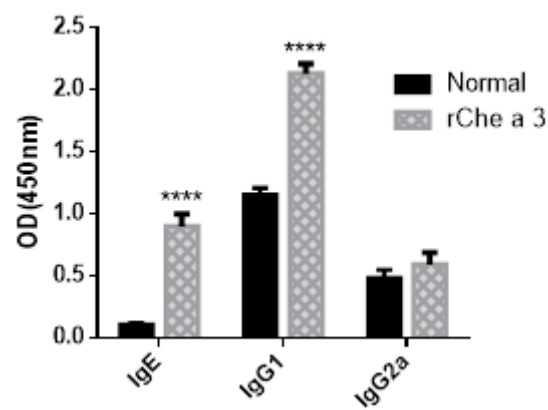
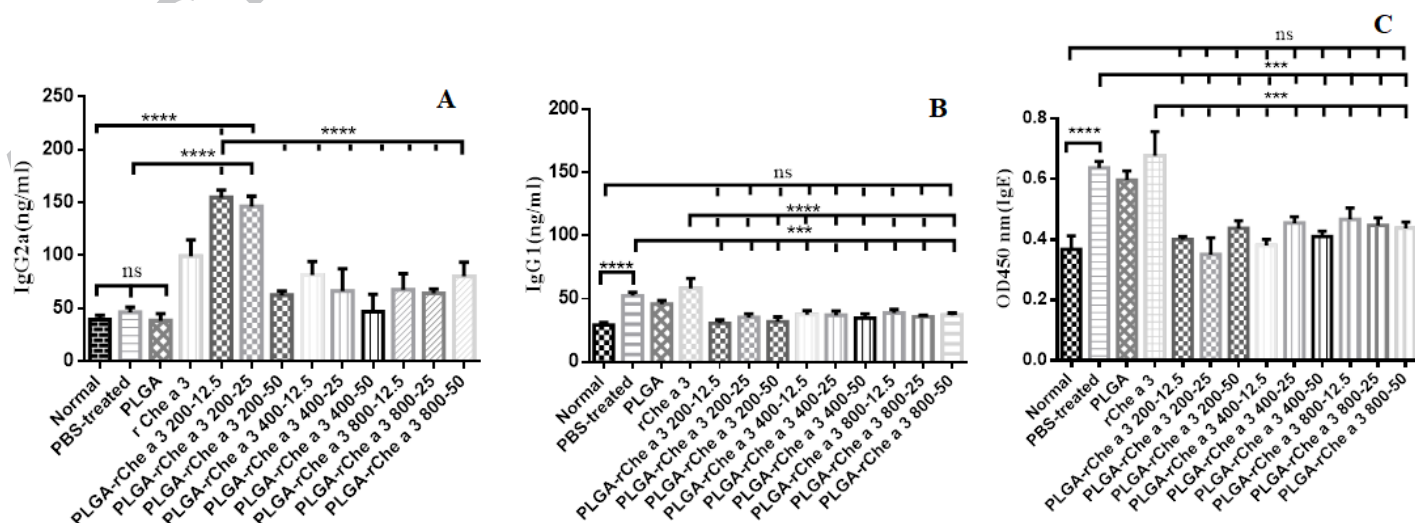


Fig.3. Antibodies (IgG2a, IgG1 and IgE) level in serum after sensitization of mice on day 25. Normal: not sensitized; rChe a 3: sensitized by rChe a 3 as allergen.

3.5 Protective Antibodies after SLIT

To determine the efficacy of the sublingual immunotherapy, serum IgE, IgG1 and IgG2a levels were measured by ELISA. Both rChe a 3 and PLGA-rChe a 3-treated groups showed higher serum levels of allergen-specific IgG2a than the normal, PBS and PLGA-treated groups (Fig. 4A). Furthermore The highest IgG2a level was observed by PLGA NPs with a mean diameter of 200 nm containing 12.5 and 25 μ g/ml allergen. Serum IgG1 level in treated mice with PLGA-rChe a 3 NPs were significantly lower than the PBS-treated group ($p < 0.0001$) and similar to normal group (Fig. 4B). According to results, the serum IgE levels in the PBS-treated group were significantly higher than those in the normal



group($P<0.0001$). The results showed that serum IgE level decreased by encapsulation of allergen into PLGA NPs at different size and dose (Fig. 4C).

Fig. 4. Antibodies (IgG2a, IgG1 and IgE) levels in serum after sublingual immunotherapy. A: IgG2a, B: IgG1 and C: IgE. ns, nonspecific. * $P<0.05$, ** $P<0.01$, *** $p<0.001$, **** $p<0.0001$ versus untreated rhinitis model control group. Normal, healthy control group; untreated, untreated asthma model group; PLGA- rChe a 3, rChe a 3-loaded PLGA nanoparticle group in three size and concentration; PLGA, blank PLGA nanoparticle control group; rChe a 3, rChe a 3/Al (OH)₃ vaccine group.

3.6 Cytokine secretion of spleen cells after SLIT

The analysis of rChe a 3-specific IL-2, IL-4, IL-10, IFN- γ and TGF- β cytokine concentrations in supernatants of spleen cells cultures following re-stimulation by rChe a 3 was shown in Fig. 5 A, B, C, D and E, respectively. After SLIT, TGF- β secretion was significantly increased in all treatment groups by PLGA-rChe a 3 NPs compared to normal, PBS and PLGA-treated groups. Group treated by PLGA-rChe a 3 200 nm (12.5 μ g) produced the highest amount of TGF- β secretion ($p<0.0001$). (Fig. 5A). All PLGA-rChe a 3 treatment groups was induced the production of IFN- γ more than control groups and allergen-treated group. This significant increasment was observed in treatment groups with PLGA-rChe a 3 NPs of 200 nm f (12.5 and 25) ($p<0.0001$). (Fig. 5B). Following SLIT, rChe a 3-specific IL-2 production in PLGA-rChe a 3 NPs treatment groups increased compared to the control groups and allergen-treated group. PLGA-rChe a 3 with a size of 200 nm (12.5, 25 and 50 μ g) indicated an significantly enhancement in IL-2 secretion ($p<0.0001$) (Fig. 5C). Although the results showed a significantly difference in the reduction of the IL-4 in all treatment groups with PLGA-rChe a 3 in compare to allergen-treated group ($p<0.0001$). The lowest IL-4 production were seen in mice treated with PLGA-rChe a 3 200 nm (Fig. 5D). In case of IL-10, there was no significant difference between all receiving groups of PLGA-rChe a 3 and normal, PBS, PLGA and rChe a 3 treated groups (Fig. 5E).

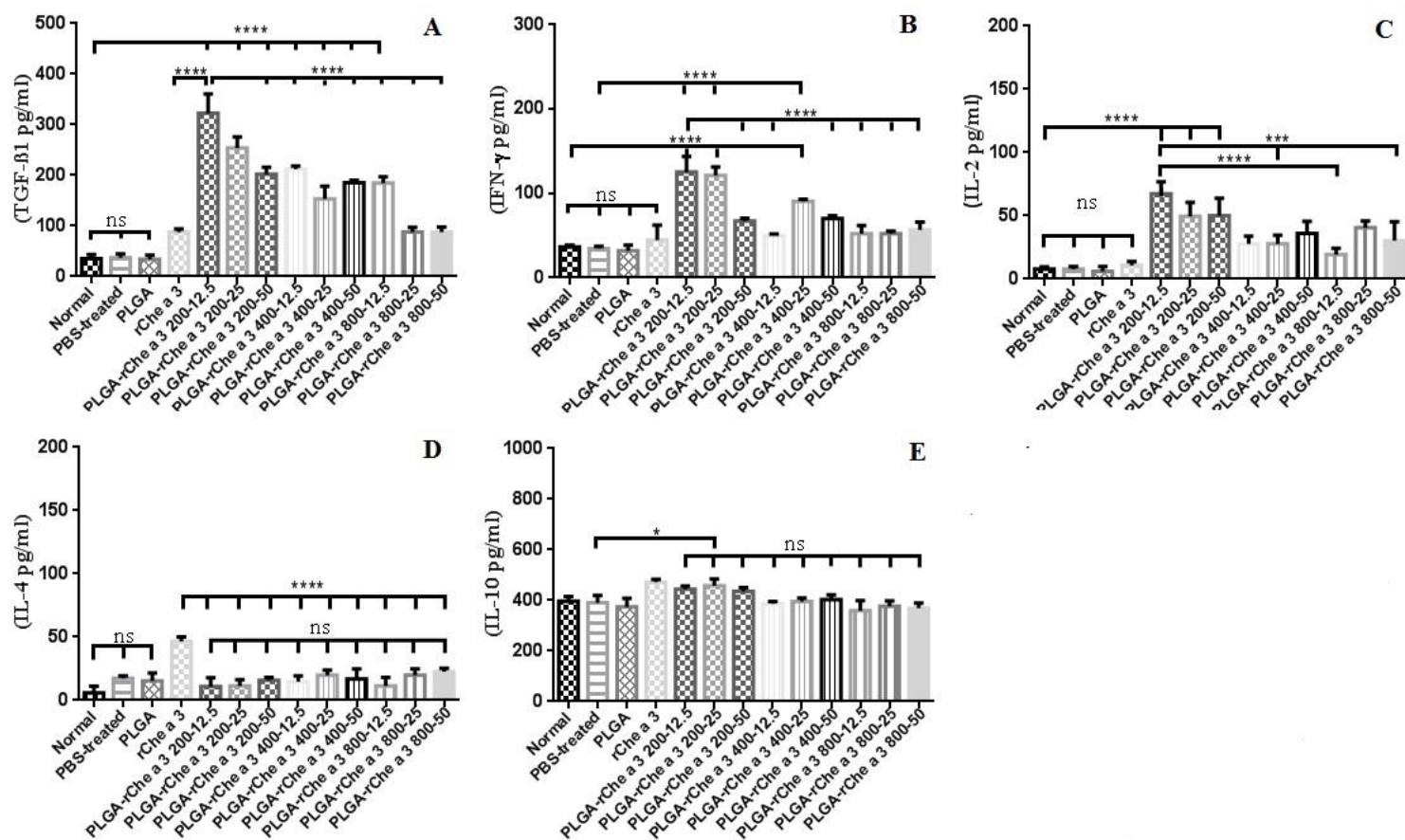


Fig.5. Cytokine levels in supernatant of spleen cell culture after re-stimulation with rChE a 3. A: TGF- β , B: IFN- γ , C: IL-2, D: IL-4 and E: IL-10. ns, nonspecific. * P <0.05, ** P <0.01, *** p < 0.001, **** p < 0.0001 versus untreated rhinitis model control group. Normal, healthy control group; untreated, untreated rhinitis model group; PLGA- rChE a 3, rChE a 3-loaded PLGA NP group in three size and concentration; PLGA, blank PLGA nanoparticle control group; rChE a 3, rChE a 3/Al (OH)₃ vaccine group.

3.7 IL-5 production in Broncho alveolar lavage fluid after SLIT

As showed in Fig.6, IL-5 level increased significantly with the sensitization of animals in contrast to normal group (p < 0.0001). There is a significant decrease in IL-5 level between PLGA-rChE a 3 NPs receiving grouped in compare to PBS and PLGA-treated (p <0.05). In all PLGA-rChE a 3 treated groups this cytokine level were showed statistically significant decrease in compare to rChE a 3 treated group (p < 0.0001). Analysis between different sizes and doses of PLGA-rChE a 3 treatment groups were not shows statistically significant difference.

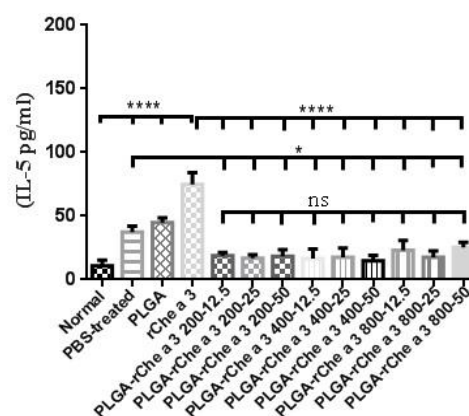


Fig.6. IL-5 levels in Broncho alveolar lavage fluid after SLIT. ns, nonspecific. * $P < 0.05$, ** $P < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus untreated rhinitis model control group. Normal, healthy control group; untreated, untreated rhinitis model group; PLGA- rChe a 3, rChe a 3-loaded PLGA nanoparticle group in three size and concentration; PLGA, blank PLGA nanoparticle control group; rChe a 3, rChe a 3/Al (OH)₃ vaccine group.

3.8 Proliferative response

In vitro proliferation response of re-stimulation of splenocyte with rChe a 3 is presented in Fig.7. The results showed a heterogeneous pattern of proliferative responses. The stimulation with all PLGA-rChe a 3 groups were less than normal and PBS-treated groups, but difference was not statistically significant. The decrease of proliferation in PLGA-rChe a 3 200 nm was greater than that of other PLGA-rChe a 3 treated groups in comparison to rChe a 3 group ($p < 0.05$).

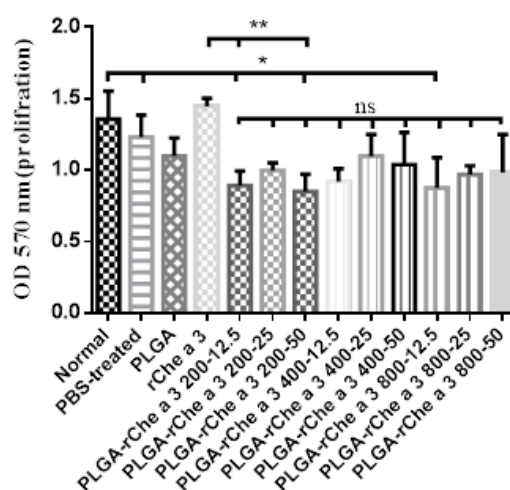


Fig. 7. In vitro proliferation assay. ns, nonspecific. * $P < 0.05$, versus normal and untreated rhinitis model group. Normal, healthy control group; untreated, untreated rhinitis model group; PLGA- rChe a 3, rChe a 3-loaded PLGA nanoparticle group in three size and concentration; PLGA, blank PLGA nanoparticle control group; rChe a 3, rChe a 3/Al (OH)₃ vaccine group.

3.9 Bronchoalveolar and Nasal lavage fluid analysis cell counting

BALF analysis showed the total counts of cells were significantly reduced after treatment of mice with PLGA-rChe a 3 NPs compared with the PBS, PLGA and allergen treated groups. This reduction was more prominent in nanoparticle 200 nm (12.5 μ g) ($p < 0.001$). Importantly, there was no significant correlation between nanoparticle 200 nm (12.5 μ g) and normal group, which showed indicative of a good therapeutic effect of this treatment group. ($p < 0.0001$) (Fig.8A). Nasal lavage total cell counts showed higher reduction in PLGA-rChe a 3 NP 200 nm (12.5, 25 and 50 μ g) compared to normal group. On the other hands, there was no difference between all PLGA-rChe a 3 treatment groups. Furthermore, there was a statistically significant difference between normal and rChe a 3 group ($p < 0.0001$) and also between the all PLGA-rChe a 3 treatment groups and rChe a 3-treated group with a decrease in total cell counts ($p < 0.001$) (Fig.8B). Cell differing in BALF showed that significantly decreased in eosinophil by rChe a 3-PLGA group in comparison to PBS-treated group. The highest decrease was observed with NPs having 200 nm sizes. In contrast, there was no statically difference between different rChe a 3-PLGA group (ns) (Fig.8C). On the other hand, eosinophil counting in NALF indicated significantly difference between rChe a 3-PLGA groups in compare to PBS-treated group. In contrast, we not found difference between rChe a 3-PLGA receiving groups (Fig.8D).

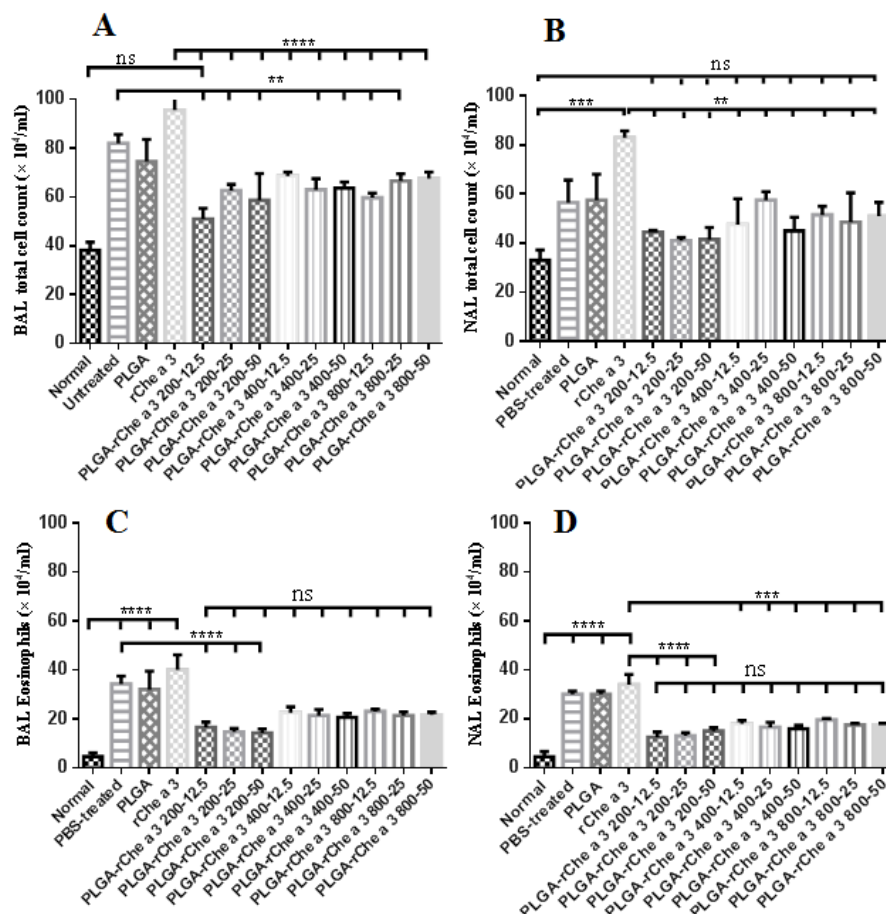


Fig.8. Total cells and eosinophils cell counts. A: total cell in BALF, B: total cell in NALF, C: Eosinophil count in BALF and D: Eosinophil count in NALF. ns, nonspecific. * $P < 0.05$, ** $P < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus untreated rhinitis model control group.

3.9 Histological analysis lung and nasal mucosa

Lung histological examination is one of way to the determination of the severity of allergic airways inflammation. As shown in Fig. 9A, the analysis of lung tissue in the normal group did not show injuries or edema, as well as infiltration of inflammatory cells in the trachea and blood vessels. Accordingly, lung sections analysis of PBS-treated group indicated squamous metaplasia of airways epithelium and also perivascular and peribronchiolar inflammation (Fig. 10), chronic interstitial pneumonia, furthermore, eosinophil accumulations inside of the airways (Fig. 9B). Lung tissue examination in rChe a 3 treated group showed hyperemia and also perivascular and peribronchiolar inflammation around of most of the vessels and bronchioles, respectively (Fig. 9C). Lung tissue in the PLGA-rChe a 3 treated groups showed

rare accumulation of mononuclear cells around of a bronchiole, other parts of the lung were normal similar to that seen in the normal group (Fig. 9D). The histological studies showed peribronchial and perivascular cellular infiltrates in lung tissues in rChe a 3 and PBS-treated groups in compare to normal group (Fig. 10). Normal group nasal histology did not show pathologic alterations (Fig. 9E). However nasal sections of PBS-treated group display Eosinophils infiltration to nasal mucosa, that proven sensitization but without treatment group (Fig. 9F). In rChe a 3 sensitize and treated group were observed Eosinophils accumulation in nasal cavity and nasal mucosa ((Fig. 9G). The nasal histology in PLGA-rChe a 3treated groups, Presence of a few eosinophils in nasal mucosa was seen, similar to normal group that proven treatment impact in allergic mouse (Fig. 9H). Histopathological studies showed treatment with rChe a 3-PLGA resulted to decreased total and eosinophil cell infiltration into BALF and NALF in contrast to the sensitized PBS-treated group.

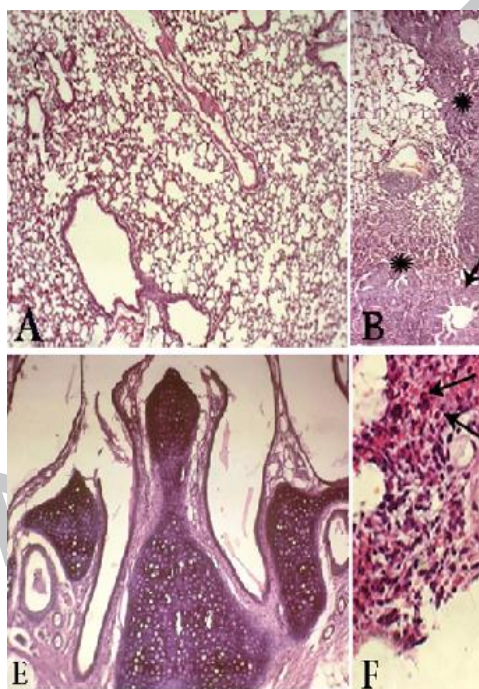
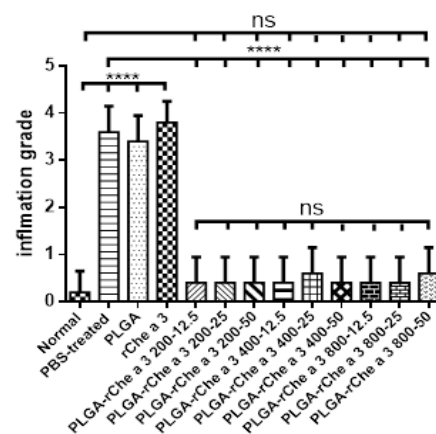


Fig.9. Histological analysis of lung and upper panel, Histopathological findings of mice in different groups (A; Normal, B;



nasal mucosa.
lung in BALB-c
PBS

(Peribronchiolar inflammation (black arrows), chronic interstitial pneumonia (asterisks), C; rChe a 3 (Peribronchiolar inflammation (thick black arrows), perivascular inflammation (thin black arrows), hyperemia (asterisks)) and D; PLGA-rChe a 3 (Local accumulation of mononuclear cells around of a bronchiole (arrow)) (staining with hematoxylin and eosin (H&E, 100 X). lower panel, Histopathological findings of nasal mucosa in BALB-c mice in different groups (A; Normal, B; PBS (Presence of infiltration eosinophils in nasal mucosa (arrows), C; rChe a 3 (Eosinophils accumulation in nasal cavity and nasal mucosa (arrows) and D; PLGA-rChe a 3 (Presence of a few eosinophils in nasal mucosa(arrows)) (staining with hematoxylin and eosin (H&E, 400 X except normal group that is 100 X).

Fig. 10. Quantitative analysis of lung, degree of peribronchiolar and perivascular inflammation in all groups. ns, nonspecific. * $P < 0.05$, ** $P < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus untreated rhinitis model control group.

4. Discussion

Allergic airways disease, as a frequent chronic multifactorial disorder, mainly results from airborne pollens (Stemeseder et al. 2017). Allergen specific immunotherapy conducted via the sublingual route is being successfully used to treat patients with type I respiratory allergies (Brozek et al. 2010; Canonica et al. 2009; Canonica et al. 2014; Wahn et al. 2009a). In the current study, we investigated the influence of rChe a 3 allergen encapsulated in PLGA NPs with different size and various doses in the immunologic effects after sublingual administration to previously rChe a 3-sensitized BALB/c mice. For example, B-lactoglobulin (BLG) loaded in PLGA by single dose of oral immunization resulted to decrease IgE and also induction of tolerance in mice, furthermore, remarkably reduce allergen amount 10,000 times, in compare to allergen using soluble and orally (Fattal et al. 2002; Pecquet et al. 2000). Other studies showed using PLGA as encapsulating vector of rChe a 3 in compare to soluble form of rChe a 3 had better induction of tolerance [14, 75]. In this research, three sizes of

PLGA NPs containing rChe a 3 allergen was synthesized by manipulation of PVA MW and concentration, also, solvent type and sonication time, the most EE and LC was observed in 400 nm of NPs. A possible explanation for our results about the effect of particle size on drug loading is allergen loss during emulsification mostly due to partitioning of rChe a 3 from the dispersed oil droplets into the external water phase. Also, The relative higher surface: volume ratio of smaller droplets will result in relative higher drug loss and hence a lower higher EE for smaller microparticles (Chen et al. 2016). During encapsulation of allergen into nanoparticle, the maintenance of the proteins integrity is necessary for its immunological activity (Kammona and Kiparissides 2012). In this study, SDS-PAGE analyses showed the structural integrity of rChe a 3 loaded to PLGA NPs is similar to unloaded allergen. In other studies, similar results were also obtained about the maintenance of protein integrity after encapsulation into PLGA NPs. For example, in the study by Sharif et al., dust mite allergen, Der p 1, entrapped into PLGA microparticles. The results indicated that protein integrity and ability of the protein to bind to IgG and IgE was not significantly affected during encapsulation process (Sharif et al. 1995). Different studies demonstrated that DCs are critically involved in the induction of tolerance against allergens, which is a major mode of action of immunotherapy (Novak 2006). On the other hand, antigen uptake by DCs resulted to Th1 response induction and therefore tolerance (Hamdy et al. 2011; Rebouças et al. 2012). The antigens encapsulated into PLGA NPs could efficiently deliver to these cells. However, the size of PLGA NPs can affect the induction either cellular or a humoral immune responses. In a research by Joshi et al, Der p2 entrapment in smaller size of PLGA NPs revealed more decrease the allergic inflammatory response and also more increment the IgG2a level resulting in shift of the immune response to Th1 [12]. It was shown that nanoparticle size is a key factor in particle absorption on mucosal surfaces, so that more antigens could present after using of smaller nanoparticles following to better

stimulation of immune responses in systemic lymphoid tissues [65]. In study by pelkmans, it was revealed that particulate vaccines from 20 to 200 nm generally captured by receptor-dependent endocytosis mechanism that led to stimulation of CD4, CD8, and Th1 type immune responses(Pelkmans 2005), Larger sized particles (0.5–5 μm) are taken up by macropinocytosis, while particles greater than 0.5 μm are predominantly taken up by phagocytosis, and primarily ingested by macrophages and resulted in activation of humoral immunity(Fifis et al. 2004). In addition, particles with smaller size act as adjuvants which are more effectively in maturation and activity of DCs (Xiang et al. 2006). In another study, it was demonstrated that DCs preferably uptake nanoparticles with a size of 20-200 nm, while macrophages internalizes particle the 0.5 to 5 μm (Fifis et al. 2004). It was proven while larger particles (500–2000 nm) were mostly associated with DCs at the in injection site, small particles (20–200 nm) were found in LN-resident DC and macrophages, suggesting their free drainage to the lymph node (LN) (Manolova et al. 2008).

Our results indicated rChe a 3-loaded PLGA nanoparticles having size in the range of 200-800, efficiently inhibited production of serum IgE, IgG1 and also stimulated serum IgG2a compared to free allergen. Scholl et al proved that the allergen (Bet v 1) entrapped in PLGA microcapsules slowly released and effectively stimulated production of antibody IgG2a (Scholl et al. 2004).These results demonstrated that smaller size and lower dose of PLGA NPs employed for sublingual immunotherapy could decrease the induction of Th2-polarized antibody responses and increaseTh1 type immune responses.

In in vivo studies, mice treated with smaller nanoparticles (200 nm) showed significantly higher levels of IgG2a antibody than other sizes of PLGA-rChe a 3 NPs. Furthermore, this treated group of mice had somewhat similar levels of IgG1 and IgE antibodies compared to larger nanoparticles, but lower levels in compared to untreated and PLGA groups. After

sublingual immunotherapy IgG2/IgG1 ratio showed a statistically significant between PLGA-rChe a 3 treated with 200 nm (12.5 and 25 μ g/ml) and PBS-treated groups. However, there were no significant difference between the normal group with PBS, PLGA and rChe a 3 treated groups (Fig. 4E). This obviously indicates that smaller size of PLGA nanoparticles employed for sublingual immunotherapy decreases the induction of Th2-polarized antibody responses. Gutierro et al. findings showed no difference in IgG2a stimulation by PLGA NPs containing BSA from 200 to 1000 nm size after administration with subcutaneous, oral and intragastric pathways. The administration of 1000 nm particles generally stimulated a higher serum IgG response than that gained with the administration of 500 or 200 nm sized nanospheres, the immune response for 500 nm particles being similar than that obtained with 200 nm by the subcutaneous and the oral route, and higher by the intranasal route. However, NPs could produce stronger response compared to free antigens with adjuvants (Gutierro et al. 2002). Antigen-specific T cell proliferation may be change after immunotherapy. In this study, it was shown that PLGA-rChe a 3 NPs with 200 nm size containing 50 μ g allergen could effectively decrease proliferation response of re-stimulation of Splenocyte with rChe a 3. In other studies, which used sublingual route for induction of tolerance, no alteration in T cell proliferation in spleen cells was observed (Brimnes et al. 2007; Kildsgaard et al. 2007). It seems SLIT resulted of suppressive activity of induced Treg cells through secretion of suppressive cytokines such as IL-10 and TGF- β or by cell-cell contact (Fujita et al. 2012; James et al. 2011; Klimek and Pfaar 2013). In our study, in treated groups with PLGA-rChe a 3 NPs with 200 nm size containing 12.5 μ g allergen, the lowest IL-4 level and the highest IFN- γ , TGF- β , and IL-10 levels was observed. Xiao et al. showed treatment of asthmatic murine with rCmP-loaded PLGA NPs resulted to intensely decrease and increase IL-4 and IFN- γ levels, respectively compared to and rCmP as allergen proposing that nanoparticle treatment enhanced the Th1 immune response and repressed the Th2 immune

response (Xiao et al. 2013). In addition, IL-10 levels increased in the PLGA-rCmP and rCmP groups in compare to the model group, which confirmed anti-inflammatory and immunosuppressive function more effectively in these groups (Xiao et al. 2013). Salari et al. showed restimulated splenocytes result in IL-4 levels significantly decreased, but IFN- γ , TGF- β and IL-10 levels, as well as Foxp3 expression, were meaningfully higher than control groups (Salari et al. 2015). Bet v 1 allergen encapsulated into PLGA NPs could change the current Th2 response, leading to a reduction in IgG1 antibody production and a growth in IgG2a antibody titer. Subcutaneously (SC) Demonstrated to be positive in changing meanwhile IFN- γ and IL-10 were produced after the allergen-specific stimulation of splenocytes (Scholl et al. 2004). Major allergen of birch pollen, Bet v1, encapsulated in PLGA nanoparticles orientate the Th2 response to Th1 response, by increasing the Bet v1 IgG2a and as well as IFN- γ and IL-10 levels (Scholl et al. 2006; Scholl et al. 2004). One of the problems associated with sublingual immunotherapy is using at least 50–100 times more allergen than SCIT to achieve a similar level of efficacy. We found that sublingual immunotherapy by 12.5 μ g/dose of rChe a 3 encapsulated in PLGA was effective than other dose (25, 50 μ g) in reducing the eosinophilic infiltration and Th2 response (specific IgE, serum IL-4 and IL-5 in BAL). It seems the use of nanoparticles in our study and the gradually release of allergen from nanoparticles resulted effectiveness lower dose in treatment of allergic rhinitis. On the other hand, this dose is less than the dose used for SCIT. Tourdot et al. also showed more SLIT efficacy with a sub-optimal dose (5 μ g) of rBet v 1a, once formulated within MPA particles with greatest mainly a decrease in Th2 responses (Tourdot et al. 2013).

BALF is an appreciated tool to evaluate cellular and non-cellular parts related with inflammation in the respiratory tract (Kim et al. 2003). Data obtained in groups received PLGA-rChe a 3 nanoparticle confirmed decrease of IL-5 Level and eosinophil infiltrates in

BAL, that proven selective treatment of allergic rhinitis. In Similar studies, it was shown, the levels of IL-5 in BAL fluid in allergen loaded PLGA treatment group were decreasing that proving the absence of inflammatory Th2 responses (Joshi et al. 2014; Tourdot et al. 2013). The total counts for cells and eosinophils were significantly lower in groups treated with selective formulation. After analysis of lung and nasal mucosa histopathology, NALF and BALF cell infiltration of PLGA-rChe a 3- mouse in smaller size and dose proved allergen-specific sublingual immunity production with banned pulmonary infiltration of leukocytes. In study, lung tissue in the PLGA-rChe a 3 treated groups showed histologic modifications like to that in the normal group without apparent damage or edema, The trachea and blood vessels were not infiltrated peripherally by inflammatory cells, similar to Xiao et al, (Xiao et al. 2013). Also we saw nanoparticle size impact on eosinophilia, which was significantly, decreased in compared to medium and large-sized PLGA-rChe a 3 nanoparticle. According to these results, PLGA nanoparticles containing rChe a 3 with a size of 200 nm could effectively enhance Treg and Th1 immunomodulatory response at low dose of allergen (12.5 and 25 $\mu\text{g}/\text{dose}$).

5. Conclusion

In conclusion, the rChe a 3-loaded PLGA nanoparticles with different sizes and doses were capable to inhibit and treat rhinitis allergic mouse model successfully by modifiable Th1/Th2 balance via downregulation of Th2 cytokine expression, inhibition of eosinophil differentiation, and stimulation of Th1 cytokine expression. This study firstly evaluates the effect of size of PLGA particles in producing a vaccine against rChe a 3 allergen. In this study, although 400 and 800 nm nanoparticles also play an effective role in converting Th2 to Th1 responses, however, rChe a 3-PLGA in smaller size(200 nm) of nanoparticle as well as lower dose of allergen (12.5 μg) resulted in highest efficiency of sublingual immunotherapy.

Histological analysis also showed smaller size of nanoparticle decrease airway inflammation and eosinophilia accumulation in lungs and nasal tissue.

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Conflict of interests

No potential conflict of interest was reported by the authors

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Conflict of interests

No potential conflict of interest was reported by the authors

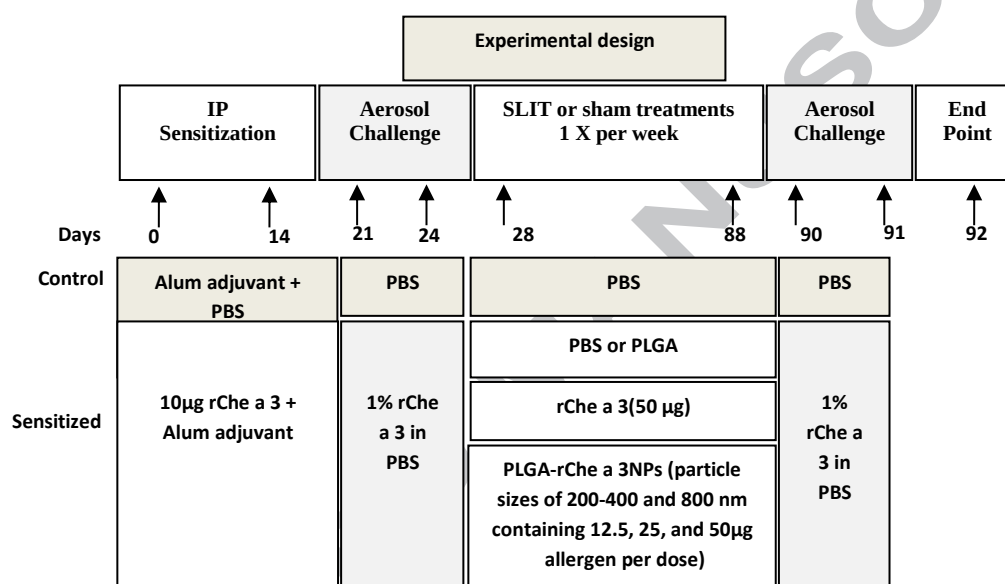


Fig.1. Experimental design. Thirteen groups of mice (n=6 per group) were used in the experiment. Abbreviations: PLGA, poly (lactic-co-glycolic acid); rChe a 3, recombinant *chenopodium album*; SIT, specific immunotherapy; ip, intraperitoneal injection; sc, sublingual injection.

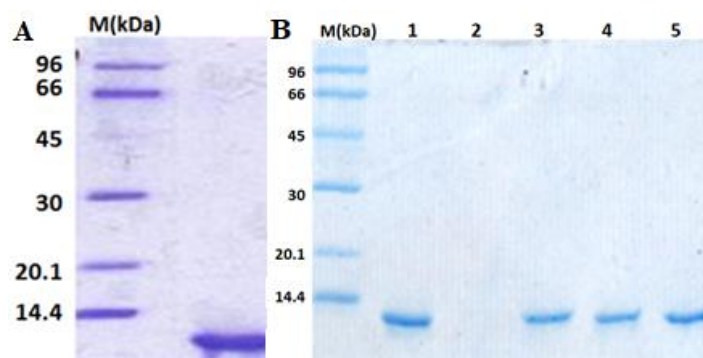


Fig. 2.A;SDS-PAGE analysis of rChe a 3, After expression and After purification. B;SDS-PAGE stability analysis of rChe a 3loaded of PLGA nanoparticles by SDS-PAGE and Coomassie blue staining of gel. Notes: M: Molecular mass markers, 1: rChe a 3, 2: empty PLGA, 3,4 and 5: rChe a 3 loaded from PLGA nanoparticles 200, 400 and 800 nm, respectively.

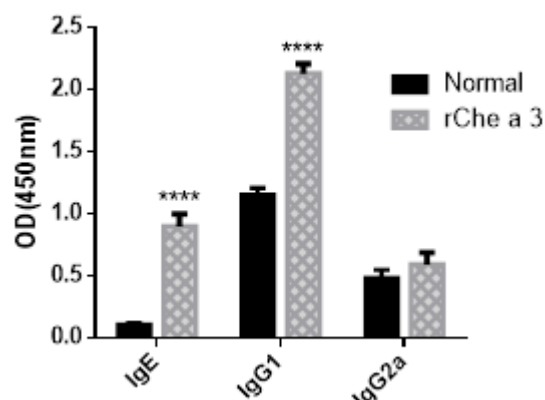


Fig.3.Antibodies (IgG2a, IgG1 and IgE) level in serum after sensitization of mice on day 25. Normal: not sensitized; rChe a 3: sensitized by rChe a 3 as allergen.

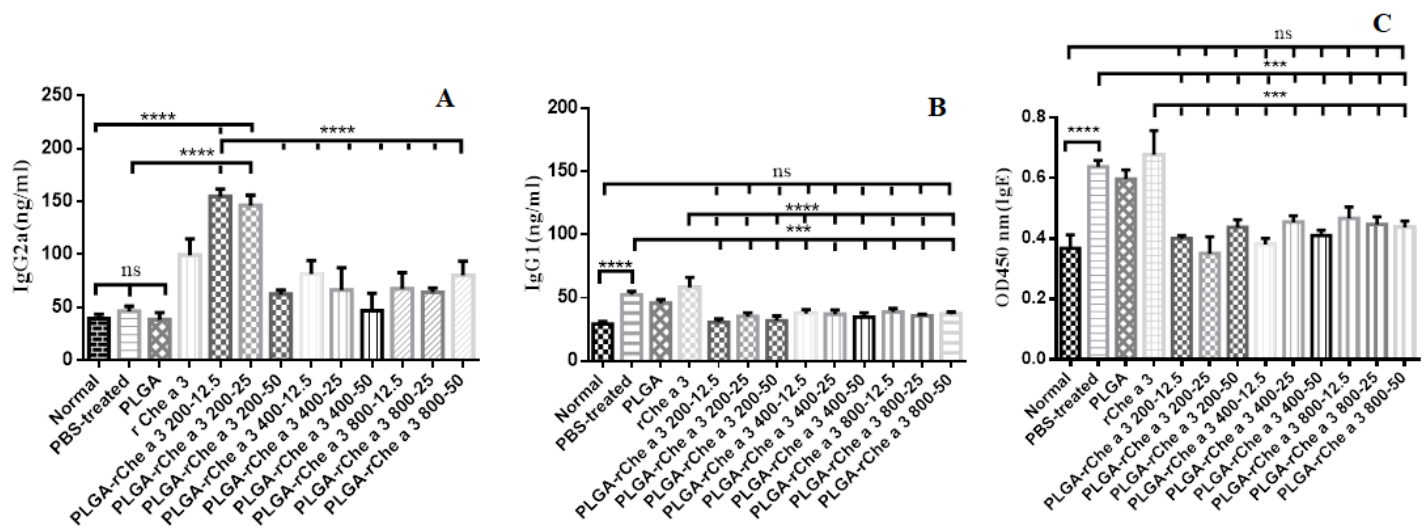


Fig. 4. Antibodies (IgG2a, IgG1 and IgE) levels in serum after sublingual immunotherapy. A: IgG2a, B: IgG1 and C: IgE. ns, nonspecific. * $P < 0.05$, ** $P < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus untreated rhinitis model control group. Normal, healthy control group; untreated, untreated asthma model group; PLGA- rChe a 3, rChe a 3-loaded PLGA nanoparticle group in three size and concentration; PLGA, blank PLGA nanoparticle control group; rChe a 3, rChe a 3/Al (OH)₃ vaccine group

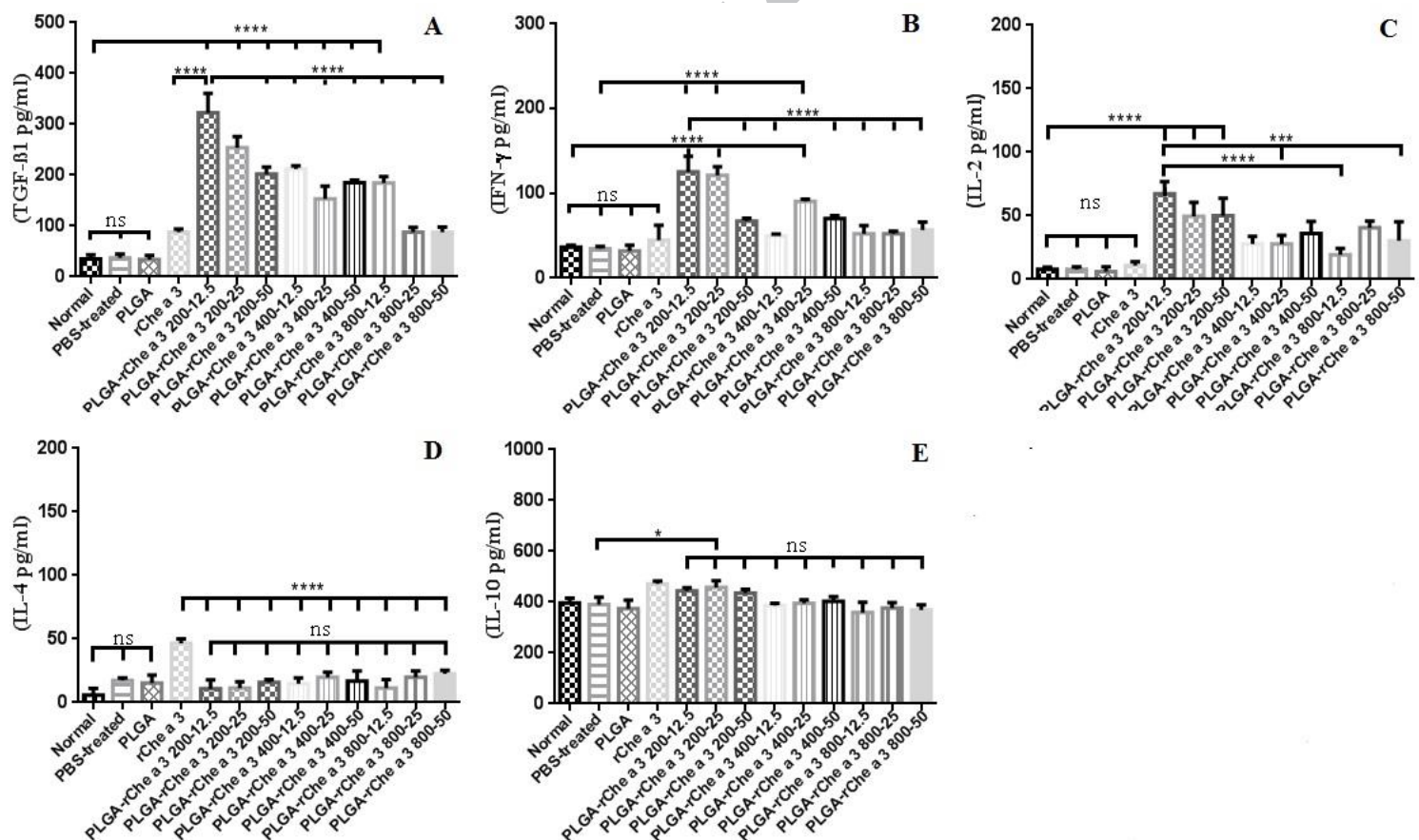


Fig.5. Cytokine levels in supernatant of spleen cell culture after re-stimulation with rChe a 3. A: TGF-β, B: IFN-γ, C: IL-2, D: IL-4 and E: IL-10. ns, nonspecific. * $P < 0.05$, ** $P < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus

untreated rhinitis model control group. Normal, healthy control group; untreated, untreated rhinitis model group; PLGA- rChe a 3, rChe a 3-loaded PLGA NP group in three size and concentration; PLGA, blank PLGA nanoparticle control group; rChe a 3, rChe a 3/Al (OH)3 vaccine group.

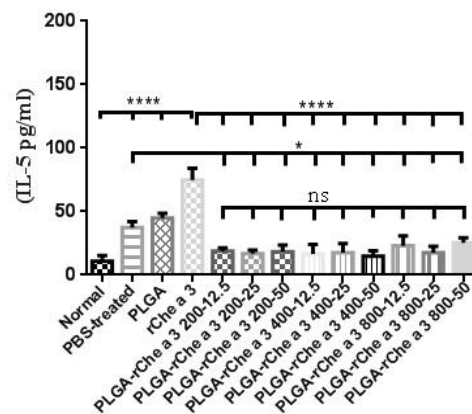


Fig.6. IL-5 levels in Broncho alveolar lavage fluid after SLIT. ns, nonspecific. * $P < 0.05$, ** $P < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus untreated rhinitis model control group. Normal, healthy control group; untreated, untreated rhinitis model group; PLGA- rChe a 3, rChe a 3-loaded PLGA nanoparticle group in three size and concentration; PLGA, blank PLGA nanoparticle control group; rChe a 3, rChe a 3/Al (OH)3 vaccine group.

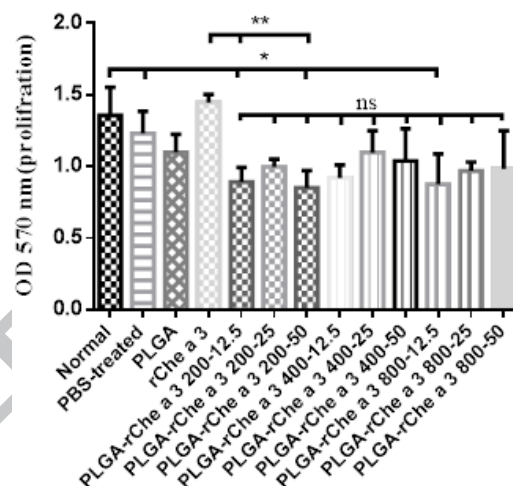


Fig. 7. In vitro proliferation assay. ns, nonspecific. * $P < 0.05$, versus normal and untreated rhinitis model group. Normal, healthy control group; untreated, untreated rhinitis model group; PLGA- rChe a 3, rChe a 3-loaded PLGA nanoparticle group in three size and concentration; PLGA, blank PLGA nanoparticle control group; rChe a 3, rChe a 3/Al (OH)3 vaccine group.

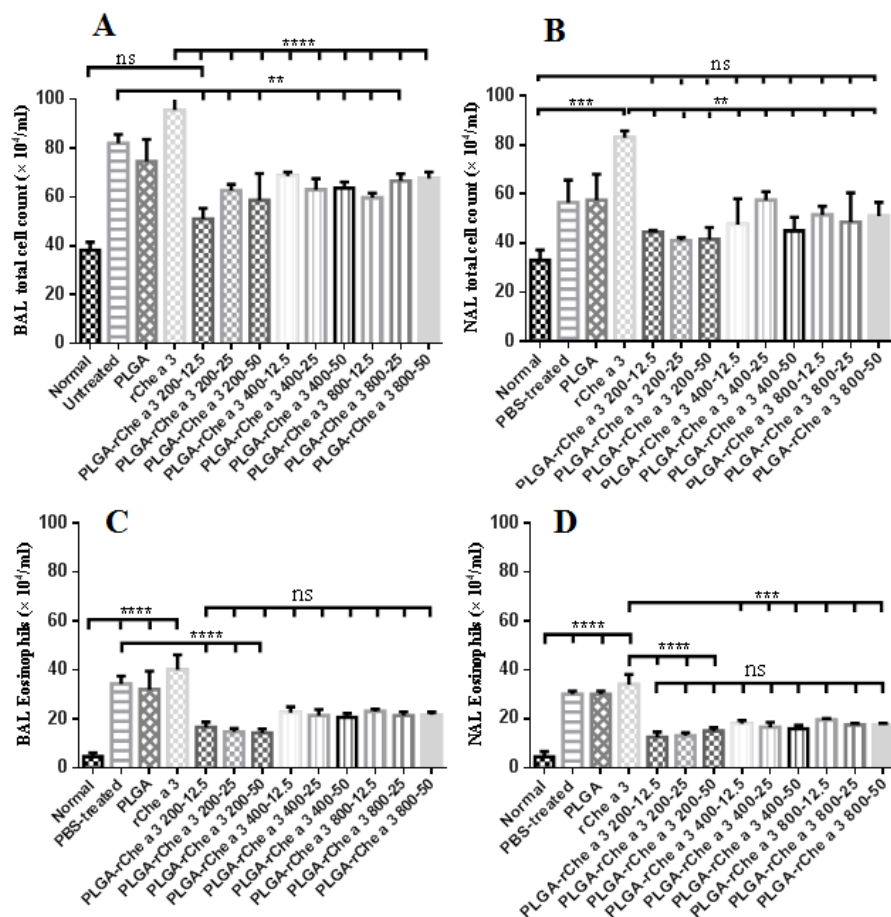


Fig.8. Total cells and eosinophils cell counts. A: total cell in BALF, B: total cell in NALF, C: Eosinophil count in BALF and D: Eosinophil count in NALF. ns, nonspecific. * $P < 0.05$, ** $P < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus untreated rhinitis model control group.

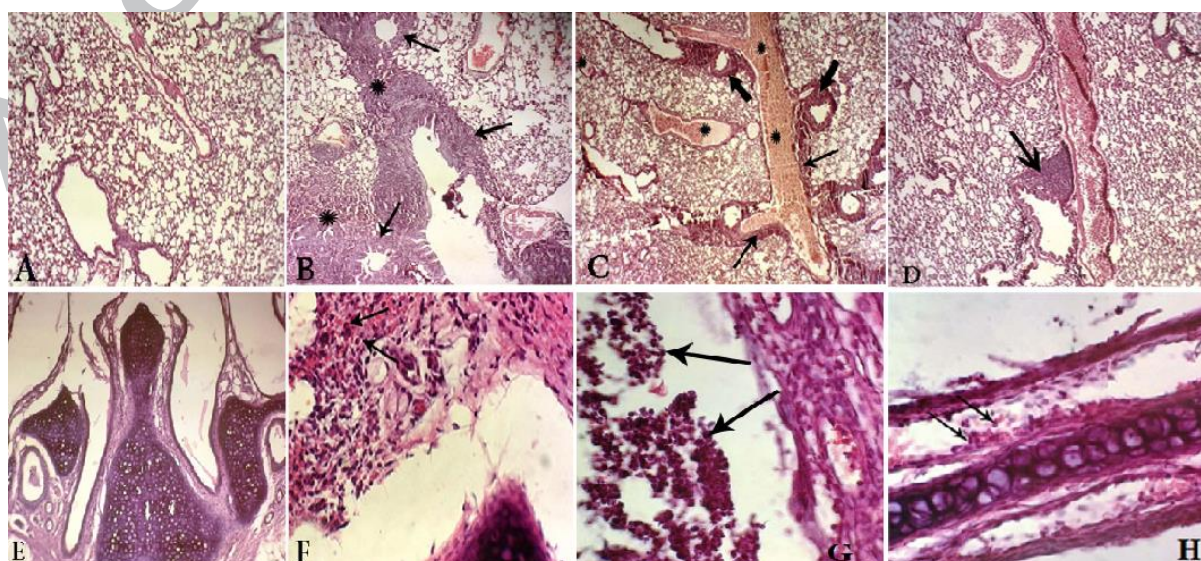


Fig.9. Histological analysis of lung and nasal mucosa. upper panel, Histopathological findings of lung in BALB-c mice in different groups (A; Normal, B; PBS (Peribronchiolar inflammation (black arrows), chronic interstitial pneumonia (asterisks), C; rChe a 3 (Peribronchiolar inflammation (thick black arrows), perivascular inflammation (thin black arrows), hyperemia (asterisks)) and D; PLGA-rChe a 3 (Local accumulation of mononuclear cells around of a bronchiole (arrow)) (staining with hematoxylin and eosin (H&E, 100 X). lower panel, Histopathological findings of nasal mucosa in BALB-c mice in different groups (A; Normal, B; PBS (Presence of infiltration eosinophils in nasal mucosa (arrows), C; rChe a 3 (Eosinophils accumulation in nasal cavity and nasal mucosa (arrows) and D; PLGA-rChe a 3 (Presence of a few eosinophils in nasal mucosa(arrows)) (staining with hematoxylin and eosin (H&E, 400 X except normal group that is 100 X).

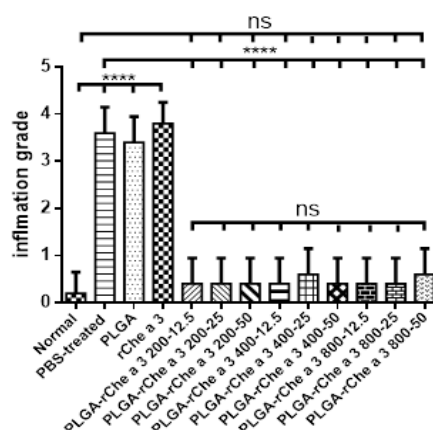


Fig. 10. Quantitative analysis of lung, degree of peribronchiolar and perivascular inflammation in all groups. ns, nonspecific. * $P < 0.05$, ** $P < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus untreated rhinitis model control group.

Table1: characterization of nanoparticle in different sizes (Mean $\pm$ SD)

Formulations	Mean size(nm)	PDI	PVA Con (%)	PVA Mw(Da)	Type of solvent	Second sonication time	Mean EE (%)	Mean LC(μ g/mg) (%)
F1	232 $\pm$ 35	0.126 $\pm$ 0.045	2	31000-50000	DCM	4	43.6 $\pm$ 1.55	3.14 $\pm$ 0.255
F2	430 $\pm$ 40	0.375 $\pm$ 0.105	1	31000-50000	DCM	1	60.4 $\pm$ 0.55	4.44 $\pm$ 0.545
F3	727 $\pm$ 65	0.132 $\pm$ 0.064	1	30000-70000	DCM	4	51.3 $\pm$ 1.75	3.77 $\pm$ 0.351

