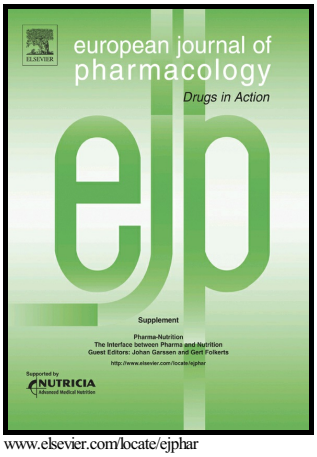

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Conjugated Alpha-Alumina nanoparticle with vasoactive intestinal peptide as a Nano-drug in treatment of allergic asthma in mice

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Abstract

Asthma is a chronic respiratory disease characterized by airway inflammation, bronchoconstriction, airway hyperresponsiveness and recurring attacks of impaired breathing. Vasoactive intestinal peptide (VIP) has been proposed as a novel anti-asthma drug due to its effects on airway smooth muscle relaxation, bronchodilation and vasodilation along with its immunomodulatory and anti-inflammatory properties. In the current study, we investigated the therapeutic effects of VIP when conjugated with α-alumina nanoparticle (α-AN) to prevent enzymatic degradation of VIP in the respiratory tract. VIP was conjugated with α-AN. Balb/c mice were sensitized and challenges with ovalbumin (OVA) or PBS and were divided in four groups; VIP-treated, α-AN-treated, α-AN-VIP-treated and beclomethasone-treated as a positive control group. Specific and total IgE level, airway hyperresponsiveness (AHR), bronchial cytokine expression and lung histology were measured. α-AN-VIP significantly reduced the number of

eosinophils (Eos), serum IgE level, Th2 cytokines and AHR. These effects of α-AN-VIP were more pronounced than that seen with beclomethasone or VIP alone (P<0.05). The current data indicate that α-AN-VIP can be considered as an effective nano-drug for the treatment of asthma.

Keywords

Alpha-Alumina nanoparticle, vasoactive intestinal peptide, allergic asthma

1. Introduction

Asthma is an important and complex, chronic multi-factorial respiratory disease with a higher prevalence in the industrial world (Sharifi et al., 2013). Asthma is characterized by inflammation of the airway, bronchoconstriction, airway hyperresponsiveness (AHR), with symptoms of cough, dyspnea, wheezing and chest pain, and recurrent attacks of breathlessness (Refaat and Aref, 2014; Sharifi et al., 2013). Asthma is found at all age groups but is more prevalent in children. Allergic asthma mostly occurs in children and younger adults whilst non-allergic asthma is more prevalent in older adult-onset asthmatics (Papiris et al., 2001; Zolnoori et al., 2012). Allergic asthma is an important public health problem and a major cause of respiratory morbidity and mortality afflicting more than 300 million individuals globally (Moin et al., 2014; Papiris et al., 2001). A heightened allergic immune response to environmental allergens is the dominant feature in subjects with a genetic predisposition to asthma. In allergic patients, a specific response of Th2 lymphocytes to the specific allergen leads to increased levels of type-2 cytokines and of total and specific IgEs (Bidad et al., 2010; Moin et al., 2014) resulting in eosinophilia (Bidad et al. 2010; Yavari et al. 2016; Athari and Athari 2014).

Asthma has been reported since ancient times and various attempts over the millennia have been made using traditional and complementary medicine to treat this disease. Allergic asthma treatment strategies are classified as: a) bronchodilator drugs (e.g. β2 adrenergic receptor agonists, cholinergic receptor antagonists and inhibitors of phosphodiesterases) and b) anti-inflammatory drugs (e.g., corticosteroids and inhibitors of leukotriene receptors). Unfortunately none of these drugs cures asthma or completely prevents asthma attacks particularly in patients with severe disease. The major aims of

allergic asthma treatment are focused on the prevention of eosinophilic bronchial inflammation, bronchial mucosal hypersecretion, hyperplasia of smooth muscle cells around the airways and airway remodeling (Bel et al., 2014; Ortega et al., 2014; Wechsler et al., 2011).

Vasoactive intestinal peptide is a 28-amino acid peptide (molecular weight: 3326 Da) that was first isolated from the gastrointestinal system. VIP is a powerful airway smooth muscle cell relaxant that provides a longer lasting bronchodilation in comparison to many other drugs (Igarashi et al., 2011; Misaka et al., 2011). VIP also has immunomodulatory and anti-inflammatory effects and therefore may be useful as a candidate drug for allergic asthma treatment (Igarashi et al., 2011; Linden et al., 2003; Misaka et al., 2011; Prasse et al., 2010; Samarasinghe et al., 2010). Systemic administration of VIP has adverse effects such as hypotension, decreases heart rate and increased cardiovascular disorders. These effects are attenuated by administrating VIP via the inhaled route but efficacy is reduced due to enzymatic degradation of VIP in the airways and this loss of potency is the main limitation to its clinical application (Hajos et al., 2008; Misaka et al., 2010; Onoue et al., 2012). Therefore, the aim of this study was to investigate the effect of the conjugated form of this peptide with α-alumina nanoparticle (α-AN), which is more stable due to the protection of enzymatic degradation.

2. Material and methods

2.1. Nanoparticle preparation

Alpha-Alumina nanoparticle (a-Al2O3 80 nm) was purchased from US Research Nanomaterials, Inc., USA. VIP (Abbiotec, USA) solution and α-AN suspension in 0.1 M sodium phosphate and 0.1 M NaCl (pH 7.4) were prepared separately (Hermanson, 2013). Succinimidyl 4-hydrazino nicotinate acetone hydrazone (SANH) (bio-world, USA) and succinimidyl 4-formyl benzoate (SFB) (bio-world, USA) were dissolved in dimethylformamide (DMF) (bio-world, USA) separately at 20 g/l. SFB/DMF solution was added to VIP and SANH/DMF was added to the α-AN suspension. These were then incubated for 2-3 h at room temperature before being desalted using dialysis [dialysis membrane with a molecular weight cut off (MWCO) of 3/5 kDa] against PBS at 4 °C.

During these steps, aldehyde groups blocked the peptide and hydrazine groups blocked the nanoparticles. VIP (containing aldehyde groups) and nanoparticles (containing hydrazine) were placed in citrate buffer (containing sodium citrate 100 mM, hydrochloric acid 150 mM, pH 6) and incubated for 2 h at room temperature. Finally, the non-conjugated VIP was separated from the conjugated VIP using gel chromatography (Hermanson, 2013). The peptide binding to α-AN was measured using a spectrophotometer and measurement of free peptide of supernatant was performed by bicinchoninic acid assay (BCA assay).

For determination of peptide release rate from nanoparticles at different pH (pH 8, 7.4, 7.0, 6.5 and 6.0), 200 μl of peptide-nanoparticle solution was placed separately in the five different pH solutions (phosphate buffered for physiological and alkaline pH, citrate buffered for acidic pH) and the absorbance of the supernatant measured hourly for 10 h, using spectrophotometry at 250 nm and the percentage release calculated. The structural characteristics of the nanoparticles were assessed by transmission electron microscopy. The average size of nanoparticles and the distribution and amount of electric charge were determined by Zeta Plus Zeta Particle size Analyzer. Fourier transform infrared (FTIR) spectroscopy was used following the conjugation.

2.2. Animal model of asthma

Balb/c female mice (6-8 weeks old) were purchased from Pasteur Institute of Iran (Karaj-Iran) and mice were acclimatized under standard laboratory conditions (24±2 °C, 50±5% humidity, and 12 h light-dark cycle and pathogen free condition) for 1 week. All of protocols were approved by the Ethics Committees (Immunology, Asthma and Allergy Research Institute NO: 412/b/236 and Faculty of Medical Sciences, Tarbiat Modares University NO: 52d/6564).

2.3. Animal treatment schedule

Eighty four mice were divided into six groups. Each group contained 14 mice [7 mice for histopathological analysis and 7 mice for bronchoalveolar lavage (BAL)]. Airway inflammation was induced in 5 of the groups using ovalbumin (OVA, Sigma-Aldrich, Netherlands), according to a standard protocol (Szema et al., 2006). Briefly, the mice

were sensitized by intraperitoneal injection of 20 μg of chicken OVA with 50 μl aluminum hydroxide (Sigma-Aldrich, Netherlands) on day 1 and 14. The mice were then challenged by inhalation of 1% OVA solution aerosolized by an ultrasonic nebulizer (NE-U07, Omrom, Japan) for 30 min per day on days 24, 26, 28 and 30 (Fig. 1).

Four of the 5 sensitized and challenged groups were also treated with α-AN (10 mg/ml), α-AN-VIP (10 mg/ml), VIP (10 mg/ml) and beclomethasone (10 mg/ml) on days 25, 27 and 29 (1% solution aerosolized by ultrasonic nebulizer for 30 min). Beclomethasone, a topical corticosteroid was used as a positive anti-inflammatory control for comparison with the anti-inflammatory effect of VIP. The 5th sensitized and challenged groups were used as the placebo control. The mice in negative control group were sensitized and challenged with PBS. Blood, BAL and lung tissue were collected from 7 mice at 48 h after the last challenge on day 30. The remaining 7 mice in each group were used for methacholine challenge and the airway hyperresponsiveness (AHR) measurements.

2.4. Measurement of airway responsiveness

Methacholine challenge test was performed done in all groups of mice two days after the last OVA or PBS challenge (Szema et al., 2006). AHR to methacholine challenge was assessed by determining enhanced pause (Penh). Each mouse was exposed to PBS aerosol to obtain a baseline Penh value and then exposed to doubling concentrations of aerosolized methacholine (MCh, 0.5, 1, 2, 4 and 8 mg/ml based on American Thoracic Society, the ATS board of directors, July 1999). The relative Penh values were determined as a percentage of the PBS Penh value.

2.5. Collection of BAL fluid

The remaining 7 mice in each group were anesthetized by urethane (Sigma-Aldrich, USA) and then tracheotomized. The catheter was placed in trachea and connected to a mechanical ventilator (Inspira ASV; Harvard Apparatus). BAL fluid was collected by washing the lung via the trachea with 1 ml of PBS. BAL cells were collected onto cytospin slides, stained with Wright's stain solution and differential cell counts performed. The BAL supernatant was stored at -70 °C for later analysis of cytokines.

2.6. Measurement of cytokines levels in BAL fluid

IL-2, IL-5, IL-6, IL-13, INF-γ and TNF-α levels in BAL fluid were measured using Bio-Plex Pro™ Mouse Cytokine, Chemokine, and Growth Factor Assays (Bio-Rad, Nederland) as described before after centrifugation of the stored BAL fluid (Mortaz et al., 2011).

2.7. RNA extraction and quantitative real time PCR

Total RNA was isolated from BAL cells using TRIzol (Invitrogen life technologies, NY, USA) according to manufacturer's instructions. Extracted RNA samples were reverse transcribed to first strand cDNA using a cDNA synthesis kit (Maxima First Strand cDNA Synthesis Kit, Thermo Scientific, Rockford, IL, USA) which includes a double-strand specific DNase (dsDNase) that could specifically remove contaminating genomic DNA from RNA samples. Quantitative PCR analysis was performed using a Rotor-Gene SYBR Green PCR Kit and a Rotor-Gene Q thermal cycler (Qiagen, Hilden, Germany). The Rotor-Gene SYBR Green PCR Kit requires minimum optimization procedure and is already enhanced to work with Qiagen cyclers. Primers for the six target genes (IL-2, IL-5, IL-13, IFN-γ and MUC5ac) and one primer-pair for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene (endogenous control) are shown in Table 1.

2.8. IgE levels in serum

Blood samples were obtained from all mice two days after the last challenge and the sera stored. Total IgE (BD Biosciences, USA) and OVA-specific IgE levels (Cusabio Biotech, USA) were measured in sera samples using the appropriate ELISAs.

2.9. Histological analysis

Mouse lungs were isolated and fixed by immersion in 10% neutral buffered formalin then trimmed and embedded in paraffin. Tissue sections were prepared and stained with Hematoxylin and Eosin in (H&E) solution and periodic acid Schiff (PAS). The ratio of mucus production was determined in two separate examinations by visually scoring the intensity of PAS stain in 10 randomly selected microscopy fields on histological

sections at 400x magnification by two pathologists. The absence of any observable PAS stain was scored 0 and low level of PAS stain in negative control histological sections was scored between 0.0 to 0.5 by two pathologists. Moreover, goblet cells (PAS-positive cells) number was quantified per 100 epithelial cells at several randomly selected microscopy fields on histological sections at 400x magnification by two pathologists. The calculated number was Goblet Cell Index (GCI). The GCI was classified to 4 levels/scores as follows; score 0: GCI < 5%, score 1: 5% ≤ GCI < 25%, score 2: 25% ≤ GCI < 50%, score 3: 50% ≤ GCI < 75%, score 4: 75% ≤ GCI ≤ 100%. Eosinophils (Eos) population was examined on histological sections of lung tissue at 1000x magnification by two pathologists in five repeats by each pathologist. Absence of Eos or presence few Eos was scored 0, Incomplete layer was scored between 0.1 to 1, one complete layer of peribronchial/perivascular Eos was scored 1, two complete layers was scored 2, three complete layers was scored 3 and more than three complete layers of Eos was scored 4. Photomicrographs were taken with an Olympus B × 50 microscope equipped with a Leica DFC 320 digital Camera.

2.10. Statistical analysis

Experimental results are expressed as means±S.E.M. Results were tested statistically by a one-way ANOVA followed by Newman-Keuls test for comparing all pairs of groups or two-tailed, non-paired, student's t-test. Analyses were performed using GraphPad Prism (version 5.0). Results were considered statistically significant when $P<0.05$.

3. Results

3.1. Analysis of VIP-conjugated nanoparticles

Measurement of free peptides by the BCA assay indicated that the degree of peptide conjugation to α-AN was approximately 85% (total used peptide: 100 μg/ml, free peptide of supernatant: 15 μg/ml). The structure of the conjugated VIP-nanoparticle was studied by transmission electron microscopy (Fig. 2) which demonstrated the average size of the VIP-nanoparticles at 80±5 nm. FTIR spectroscopy and Zeta potential curve changing showed that the peptide was conjugated with α-AN (Figs. 3A-C) with a poly

disparity index (PDI) for α-AN-VIP of 0.98. The degree of peptide release from the α-AN-VIP nanoparticle at different pH's was analysed over time. The BAL pH is approximately 6.5 and at this pH, 38% (32 μg/ml) of the total conjugated peptide (85 μg/ml) is released after 10 h (Fig. 4).

3.2. Effects of α-AN-VIP on airway hyperresponsiveness

Airway responsiveness was significantly increased (P<0.05) in the OVA-sensitized and challenged mice (8.4±0.15) compared with negative control (2.1±0.10) (Fig. 5). α-AN alone did not affect airway function. In contrast, α-AN-VIP (2.5±0.08) completely prevented the development of AHR in the OVA-sensitized and challenged mice and was more efficacious than beclomethasone (4.2±0.09) or VIP (5.7±0.15) treatment alone (Fig. 5).

3.3. Effects of α-AN-VIP on blood and BAL eosinophilia

The percentage of eosinophils in the blood (Fig. 6A) and BAL (Fig. 6B) cytospins were counted after staining. OVA sensitization and challenge increased the number of blood (2.9±0.3 versus 0.75±0.09%, P<0.05) and BAL eosinophils (1.0±0.1 versus 63.0±5.0%, P<0.05) compared to PBS-challenged negative controls. There was no effect of treatment with α-AN on the number of number of eosinophils in blood (2.9±0.3 versus 3±0.5%) or in BAL (63±5 versus 59±3%). In contrast, the blood eosinophil counts were significantly reduced by treatment with VIP (1.0±0.09%, P<0.05), beclomethasone (1.2±0.09%, P<0.05) and by α-AN-VIP (1.0±0.09%, P<0.05). Furthermore, OVA-induced BAL eosinophil counts were also suppressed by treatment with VIP (20.0±7.0%, P<0.05), α-AN-VIP (8.0±0.1%, P<0.05) and beclomethasone (21.0±3.0%, P<0.05). We were unable to detect neutrophils within the BAL cytospins.

3.4. Effects of α-AN-VIP on Lung histology

Mucus hyper-secretion (3.6±0.3 fold) (Fig. 7B and C) and goblet cell hyperplasia (score: 3.5) (Fig. 7A) were significantly increased in the airway of OVA-challenged mice, compared with negative control (mucus secretion: 0.2±0.1, Fig. 7E and F) (goblet cell score: 0, Fig. 7D). Mucus hyper-secretion in the lung tissue of α-AN-VIP group (0.7±0.1 fold) (Fig. 7K, M and O) was decreased in comparison with VIP (1.6±0.1 fold) (Fig. 7G, H and J) and beclomethasone- treated (1.3±0.2 fold) groups (P<0.05). Histological analyses revealed pathologic features of asthma in the OVA-challenged mice compared with the negative control group. The score of infiltrated eosinophils was significantly decreased in α-AN-VIP treatment group (perivascular: 0.7±0.1, peribronchial: 0.5±0.09) compared to beclomethasone (perivascular: 1.1±0.2, peribronchial: 1±0.4) treated group. Also the beclomethasone treated group showed lower number of eosinophils compared to group that received VIP (perivascular: 1.5±0.1, peribronchial: 1.9±0.25) (P<0.05).

3.5. Effects of the α-AN- VIP on serum levels of IgE

OVA sensitization and challenge significantly enhance serum levels of OVA-specific IgE (240±8 versus 0.0 ng/ml, P<0.05) (Fig. 8A) and total IgE (2150±47 versus 50±2 ng/ml, P<0.05) (Fig. 8B) compared to PBS-control animals. The OVA-induced elevated levels were not affected by α-AN treatment (specific: 230±31 ng/ml, total: 2100±125 ng/ml). However, α-AN-VIP treatment significantly reduced specific IgE (3±0.1 ng/ml) and total IgE levels (110±11 ng/ml) to almost baseline (Fig. 8A, B). The serum levels of total IgE and OVA-specific IgE were also significantly reduced by VIP (35±11 and 380±4 ng/ml respectively) and beclomethasone (35±3 and 240±54 ng/ml respectively) but the effect of α-AN-VIP was significantly more pronounced (P<0.05) (Fig. 8 A, B).

3.6. Effects of α-AN- VIP on cytokines levels of BAL

The levels of IL-2 (117±7 versus 63±6 pg/ml, P<0.05), IL-5 (84±9 versus 45±7 pg/ml, P<0.05), IL-6 (56±11 versus 13±2 pg/ml, P<0.05), IL-13 (121±12 versus 68±4 pg/ml, P<0.05), and TNF-α (190±4 versus 110±9 pg/ml, P<0.05) were increased in the OVA sensitized and challenged positive control animals compared with those seen in the

negative control PBS-treated animals (Fig. 9). Treatment with α-AN did not affect OVA-induced cytokine levels (117±9, 81±14, 54±2, 114±21, 185±11 pg/ml respectively). In comparison, OVA reduced BAL IFN-γ levels and this was also not affected by α-AN exposure (positive control: 21±2, α-AN: 24±8, negative control: 49±4 pg/ml) (P<0.05) (Fig. 9).

In contrast, α-AN-VIP, VIP and beclomethasone all significantly attenuatedthe levels of IL-2 (α-AN-VIP: 65±14, VIP: 110±2, beclomethasone: 80±18 pg/ml), IL-5 (α-AN-VIP: 38±7, VIP: 60±13, beclomethasone: 59±16 pg/ml), IL-6 (α-AN-VIP: 18±3, VIP: 39±2, beclomethasone: 34±1 pg/ml), and IL-13 (α-AN-VIP: 70±3, VIP: 88±9, beclomethasone: 85±18 pg/ml) but restored the level of IFN-γ (α-AN-VIP: 44±1, VIP: 29±3, beclomethasone: 30±9 pg/ml) (all P<0.05) (Fig. 9). The level of TNF-α was only significantly reduced by α-AN-VIP (115±11 pg/ml, P<0.05) in comparison with the OVA positive control group (190±4 pg/ml) 0.05.

3.7. Effects of α-AN- VIP on mRNA levels of cytokines and mucin

In the OVA group, the mRNA expression of IL-2 (4±0.54), IL-4 (8±1.87), IL-5 (2.82±0.46) and IL-13 (2±0.3) mRNA was increased compared to that seen in the PBS-treated negative control group (1.00±0.14, 1.00±0.11, 1.00±0.13 and 1.00±0.09, respectively) (P<0.05). The opposite effect was found with respect to IFN-γ mRNA expression (OVA: 0.5±0.04, negative control: 1±0.16) (P<0.05). In the α-AN-VIP treated group, there was a significant reduction in IL-2 (1.51±0.26), IL-4 (1.41±0.24), IL-5 (1.31±0.24), and IL-13 (1.14±0.17) mRNA expression and a restoration in the amount of IFN-γ mRNA expression (0.81±0.12) compared to OVA group (Fig. 10) (all P<0.05). The expression of MUC5AC mRNA in BAL cells was increased in OVA-challenged animal (11.31±1.2, P<0.05) in comparison to PBS-treated group (1.00±0.18) (Fig. 10F). This induction was significantly reversed in the α-AN-VIP treated group (2.14±0.36, P<0.05) compared to VIP group.

4. Discussion

Conjugated α-AN-VIP was an effective nano-drug in this animal model for asthma by possessing both immunomodulatory and anti-allergic activity. Although α-AN alone was ineffective, the conjugation of VIP with α-AN was more effective that VIP alone due to the protection against VIP enzymatic degradation in the airways. α-AN-VIP attenuated the AHR to methacholine and using histochemical analysis we demonstrated that α-AN-VIP markedly attenuated OVA-induced BAL, blood, peribronchial and lung perivascular eosinophilia. α-AN-VIP treatment also suppressed OVA-induced mucus hyper-secretion in the lung tissue and goblet cell hyperplasia in the airway. Furthermore, α-AN-VIP was more effective than α-AN or VIP alone in suppressing OVA-induced specific and total IgE levels and of asthma-associated cytokine expression. In addition, treatment of OVA-sensitized and challenged mice with α-AN-VIP was at least as effective as beclomethasone, and often more effective, against all parameters measured.

VIP receptors are present on nerves and on smooth muscle cells within the airway and receptor activation results in a rapid smooth muscle relaxation (Evans et al., 2009). Conversely, a reduction in the levels of VIP within the lung is associated with a rise in AHR, airway eosinophilia and allergic asthma (Maruno et al., 1995). In addition, acute administration of VIP to the airways can decrease acute and chronic inflammation in the lung and can control airway inflammation (Maruno et al., 1995; Said, 1989). Moreover, VIP knockout animals show peribronchial inflammation and increase in pro-inflammatory cytokines levels in BAL (Burian et al., 2010; Szema et al., 2011).

Previous studies have also reported a protective effect of VIP on airway remodeling and mucus production. Samarasinghe et al., reported that VIP increased mucociliary clearance and decreased the mucus accumulation in an animal model of asthma (Samarasinghe et al., 2010). Furthermore, Guan et al., found that VIP was able to mediate the repair of damaged bronchial epithelial cell processes (Guan et al., 2009). Therefore, VIP plays a protective role in the lung and can prevent airway remodeling, but it needs to be protected from degradation in the airway (Gonzalez-Rey and Delgado, 2005; Said, 1991; Szema et al., 2011; Yavari et al., 2016).

VIP deficiency, therefore, is related to asthma pathophysiology and the actions of VIP as a strong bronchodilator and as an anti-inflammatory agent have suggested its potential use in the treatment of asthma.

Cytokines play a major role in airway inflammation (Delgado et al., 2002; Sarir et al., 2008) and inhalation of VIP has been shown to be an effective treatment of murine asthma through modulation of cytokine release (Gomariz et al., 2007; Linden et al., 2003; Petkov et al., 2003). More specifically, VIP modulates the Th1/Th2 balance and is a strong immunomodulator (Gonzalez-Rey et al., 2007; Tan et al., 2015). In the present study, we showed that α-AN-VIP treatment inhibits BAL Th2 cytokines production and leads to the production of IFN-γ. The measured immunomodulatory effect of α-AN-VIP was greater than that seen with VIP alone. Indeed, the level of OVA-induced TNF-α significantly decreased only in the α-AN-VIP treated group. Our results are in agreement with the experiments indicating the suppressive effect of VIP on TNF-α production by monocytes and macrophages (Foey et al., 2003). Also VIP decreased the IL-10 serum level in rats administered by LPS (Bik et al., 2004). In a sarcoid model of airway inflammation model, the VIP treatment significantly reduced the TNF-α production by BAL cells. IL-13 production by BAL cells was also attenuated by VIP-treatment (Prasse et al., 2010). Our observations were consistent with these results.

However, a major drawback to its therapeutic utility is that it is very sensitive to enzymatic degradation in the airway. This limits the duration of its effectiveness and would necessitate frequent use as a maintenance therapy thereby prohibiting its development as a therapeutic agent (Delgado et al., 1999; Harmar et al., 2012; Onoue et al., 2007). The efficiency of VIP treatment, therefore, could be optimized by protecting it from enzymatic degradation in airways possibly by using liposomal formulations of VIP by inhalation (Hajos et al., 2008). However in studies using liposomes, although the stability of VIP in the lung was increased, the release of the VIP peptide was slow which decreased the effect of liposome-VIP compared to free VIP (Onoue et al., 2008; Sethi et al., 2005; Stark et al., 2008; Stark et al., 2007).

As indicated earlier, the most important defect of VIP is its instability in the presence of tissue enzymes. VIP in conjugated form showed stability and strong anti-asthma effect. As an alternative approach to liposomes, we conjugated VIP with α-AN which enabled

the peptide to be deposited in the airway at the epithelial surface. Therefore, VIP can be effectively delivered to key target cells within the airway cells with reduced degradation. In the current study, α-AN-VIP demonstrated more pronounced effects compared to VIP particularly on the reduction in OVA-induced eosinophilic inflammation and on the production of type 2 inflammatory cytokines.

According to Inoue et al., the deposition of nanomaterials in the lung does not exacerbate eosinophilic inflammation in murine models (Inoue et al., 2009). Indeed, in our study α-AN had no effect on any of the inflammatory and AHR parameters investigated. One of the concerns with using a nano-delivery system for VIP is the elimination of the compound by mucociliary clearance and alveolar macrophage uptake (Lee et al., 2013; Schürch et al., 1990). α-AN-VIP is a small and high density particle and in inhaled formulation, it could overcome the mucociliary clearance by the rapid penetration to mucus, where it reaches the cells and binds to its receptor (Tan et al., 2015). Therefore, using of an inhaled α-AN-VIP could be an effective and safe application in treatment of asthma.

Limitations of the current study include the use of an acute model of asthma, no investigation of the duration of the α-AN-VIP effects compared to its constituent parts and the use of single doses in a prophylactic manner. Moreover, the use of Penh method for measuring the airway resistance is still a matter of debate but this method got a clear association with the lungs mechanics (airway response). However, some groups indicated good correlation between Penh results and resistance in particular conditions such as those in allergen-sensitized mice challenged with methacholine but in some other experimental situations such as in experiments with anesthetized mice, resistance increased but Penh did not (Tepper and Costa, 2015). It was also shown that the Penh method in severe airway inflammation models gives the reliable results which are consistent with other accepted methods for measuring the airway resistance (Verheijden et al., 2014). Future studies are planned to examine the duration of the effect of α-AN-VIP in a chronic house dust mite model of allergic asthma when given after the last challenge. In this study, we used female mice because female mice are more susceptible to development of allergic asthma than male mice (Melgert et al., 2005). Severe asthma is more common in females (Chung and Wenzel, 2014) and it

may be of use to examine the effect of α-AN-VIP in a model of severe asthma which has a relative steroid insensitivity. It is also unclear whether α-AN-VIP will be of clinical use in patients with non-allergic asthma and this should be studied in models of non-Th2 asthma.

5. Conclusion

In summary, the conjugation of VIP to the α-AN nanoparticle was more effective in reversing characteristics of asthma in a mouse model than either components alone. This indicates that α-AN-VIP could be considered as an effective nano-drug for the treatment of asthma. However, further precise studies should be conducted to monitor the VIP concentration in lungs after release by nanocarrier and to reveal the mechanism of epithelial and other respiratory cells action on released VIP. Since the production of α-AN-VIP nanoparticles is feasible and easy to administer to the airways more preclinical, clinical studies should be performed to help translate these findings to patients. Also toxicology, bioavailability and dose determining studies should be considered to establish the VIP-nanocarrier conjugates as a safe and low side-effect therapy.

Conflicts of interest

There are no conflicts of interest.

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Figures legends

Fig. 1. Diagram of experimental for animal sensitization and treatment. Mice were sensitized by intraperitoneal (IP) injection of 20 µg of ovalbumin along with 50 µl aluminum hydroxide on day 1. The same procedure was repeated at day 14. Five sensitized groups of mice were subsequently challenged with inhaled (IT) 5% ovalbumin solution aerosolized by ultrasonic nebulizer for 30 min per day on days: 24, 26, 28 and 30. Animals were treated with inhaled alpha-alumina nanoparticles (α-AN), vasoactive intestinal peptide (VIP), α-AN-VIP conjugate or beclomethasone aerosolized by ultrasonic nebulizer for 30 min per day on days: 25, 27 and 29.

Fig. 2. Structural characteristics of nanoparticles. The structure of the alpha-alumina nanoparticles (α-AN-VIP) used was assessed by transmission electron microscopy. The figure shows the α-AN-VIP particles at a magnification of x40000. Image is representative of those obtained from 3 independent experiments.

Fig. 3. Conjugation of vasoactive intestinal peptide (VIP) to alpha-alumina nanoparticles (α-AN). The zeta potential curves of (A) α-AN and (B) α-AN-VIP particles was assessed. The shift of the α-AN zeta potential to a more positive charge after conjugation indicates the presence of VIP. (C) Fourier transform infrared (FTIR)

spectroscopy of α-AN-VIP. The wave numbers show the presence of peptide active groups within the nanoparticle structures.

Fig. 4. Effect of pH and time on vasoactive intestinal peptide (VIP) release from alpha-alumina nanoparticles (α-AN) conjugated α-AN-VIP nanoparticles. The α-AN-VIP nanoparticles were incubated at different pH over time and the release of VIP into the supernatant measured. Results are presented as the mean of 3 replicates representative of 3 independent experiments.

Fig. 5. Effect of drugs on airway hyperresponsiveness measured by enhanced pause (Penh) in response to increasing doses of methacholine (Mch). Penh values were increased in the ovalbumin (OVA)-sensitized and challenged mice (OVA, +ve control) compared with negative control mice (PBS, -ve control) for all concentrations of Mch. The effect of vasoactive intestinal peptide (VIP)(OVA-VIP), alpha-alumina nanoparticles (α-AN)(OVA α-AN), beclomethasone (OVA-beclomethasone) and VIP conjugated α-AN nanoparticles (α-AN-VIP)(OVA-α-AN-VIP) on airway responsiveness in sensitized and challenged mice is shown. Sensitized and challenged groups were treated with α-AN (10 mg/ml), α-AN-VIP (10 mg/ml), VIP (10 mg/ml) and beclomethasone (10 mg/ml) on days 25, 27 and 29 by nebulizer for 30 min. The results are presented as the means±S.E.M. $^{*}P<0.05$ compared with OVA +ve control, $^{**}P<0.01$ compared with OVA +ve control, $^{***}P<0.001$ compared with OVA +ve control using a student's t-test, n = 7–9 mice/group.

Fig. 6. Effect of drug treatment of ovalbumin-induced blood and bronchoalveolar lavage (BAL) eosinophils. Mice were sensitized and challenged with ovalbumin (OVA, +ve control) or sensitized and challenged with PBS (-ve control). The effect of vasoactive intestinal peptide (VIP)(OVA-VIP), alpha-alumina nanoparticles (α-AN)(OVA α-AN), beclomethasone (OVA-beclomethasone) and VIP conjugated α-AN nanoparticles (α-AN-VIP)(OVA-α-AN-VIP) on (A) peripheral blood eosinophil and (B) BAL eosinophil percentage are shown as means±S.D. of n=7 animals. Sensitized and challenged groups were treated with α-AN (10 mg/ml), α-AN-VIP (10 mg/ml), VIP (10 mg/ml) and beclomethasone (10 mg/ml) on days 25, 27 and 29 by nebulizer for 30 min. $^{+}P<0.05$ and $^{*}P<0.01$ compared with PBS –ve control, $^{\#}P<0.01$ compared with OVA +ve control.

Fig. 7. Histology of lungs section in treated and untreated animals. In the histological examination tissues stained with Hematoxilin-Eosine peribronchial inflammation, goblet cell hyperplasia within the airway epithelium and mucus hyper-secretion in lung sections were observed. PAS staining was used to show mucus and Goblet cells (which containing mucus in cytoplasm). Goblet cell was showed with yellow arrow and Mucus was showed blue arrow in sections. Lungs sections were prepared and stained with PAS (a, d, g and k) or H&E (b, e, h and m). (a and b): OVA-challenged group shows hyperplasia of goblet cells and mucus hyper secretion. In this group, airway was obstructed by mucus and epithelium of airway had hyperplasia of the goblet cells. (d and e): PBS-challenged group as negative control group which did not receive OVA and treatment. This group had no mucus hypersecretion and obstructed in airway and epithelium of airway had one layer of goblet cells (there is not goblet cells hyperplasia). (g and h): OVA-challenged group that were treated with VIP (10 mg/ml) only. In this group, mucus hypersecretion was decreased in compare of positive control group but had increasing in compare of negative control group. Goblet cells in epithelium of airway were 2-3 layers (goblet cells had hyperplasia in compare of negative control group). (k and m): OVA-challenged group that were treated with α-AN-VIP (10 mg/ml). In OVA, α-AN-VIP group, mucus hypersecretion and goblet cells hyperplasia were controlled and they were similar to negative control group. This group had the lowest mucus hypersecretion (similar normal group) and goblet cells hyperplasia (no hyperplasia) in all asthmatic groups.

Fig. 8. Effect of drug treatment on ovalbumin (OVA)-specific IgE and total IgE in OVA-sensitized and challenged animals. Mice were sensitized and challenged with OVA (+ve control) or PBS (-ve control) and OVA-specific (A) and total (B) IgE measured in peripheral blood using ELISA. The effect of vasoactive intestinal peptide (VIP)(OVA-VIP), alpha-alumina nanoparticles (α-AN)(OVA α-AN), beclomethasone (OVA-beclomethasone) and VIP conjugated α-AN nanoparticles (α-AN-VIP)(OVA-α-AN-VIP) on IgE levels was determined. The results are expressed as means±S.D. of n=7. Sensitized and challenged groups were treated with α-AN (10 mg/ml), α-AN-VIP (10 mg/ml), VIP (10 mg/ml) and beclomethasone (10 mg/ml) on days 25, 27 and 29 by

nebulizer for 30 min. $^{+}P<0.05$ and $^{*}P<0.01$ compared with PBS –ve control, $^{\#}P<0.01$ compared with OVA +ve control.

Fig. 9. Effect of drugs on cytokine production level in bronchoalveolar lavage (BAL) fluid of ovalbumin (OVA) sensitized and challenged animals. Mice were sensitized and challenged with OVA (+ve control) or PBS (-ve control) and the released levels of IL-2 (A), IL-5 (B), IL-6 (C), IL-13 (D), IFN-γ (E) and TNF-α (F) in BAL fluid determined by ELISA. The effect of vasoactive intestinal peptide (VIP)(OVA-VIP), alpha-alumina nanoparticles (α-AN)(OVA α-AN), beclomethasone (OVA-beclomethasone) and VIP conjugated α-AN nanoparticles (α-AN-VIP)(OVA-α-AN-VIP) on cytokine level was determined. IL-2, IL-5, IL-6, IL-13, and TNF-α levels were increased in OVA treated animals compared to –ve controls and this was not affected by α-AN treatment. In contrast, OVA animals had reduced levels of IFN-γ. Treatment with α-AN-VIP, VIP and beclomethasone decreased IL-2, IL-5, IL-6, and IL-13 levels and increased that of IFN-γ. The level of TNF-α was reduced only in α-AN-VIP group. The results are expressed as means±S.D. of n=7. Sensitized and challenged groups were treated with α-AN (10 mg/ml), α-AN-VIP (10 mg/ml), VIP (10 mg/ml) and beclomethasone (10 mg/ml) on days 25, 27 and 29 by nebulizer for 30 min. $^{+}P<0.05$ and $^{*}P<0.01$ compared with PBS –ve control, $^{\dagger}P<0.05$ and $^{\#}P<0.01$ compared with OVA +ve control.

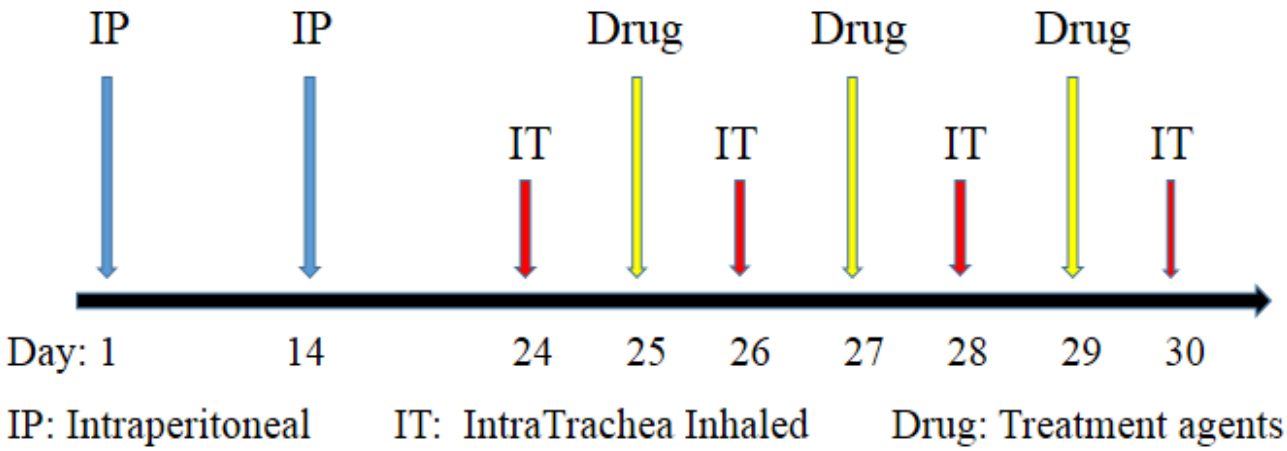
Fig. 10. Effect of drugs on the mRNA expression of cytokines and mucin in bronchoalveolar (BAL) cells sensitized using ovalbumin (OVA) and in challenged animals. Mice were sensitized and challenged with OVA (+ve control) or PBS (-ve control) as described at material and methods section. Then, the expression of IL-2 (A), IL-4 (B), IL-5 (C), IL-13 (D), IFN-γ (E) and MUC5AC (F) at the mRNA level in BAL cells determined using quantitative RT-PCR. The effect of vasoactive intestinal peptide (VIP) (OVA-VIP), alpha-alumina nanoparticles (Alumina) (OVA, Alumina), beclomethasone (OVA, beclomethasone) and VIP conjugated α-AN nanoparticles (Alumina-VIP) (OVA, Alumina-VIP) on mRNA expression was determined. The results are expressed as means±S.E.M. of n=7. Sensitized and challenged groups were treated with α-AN (10 mg/ml), α-AN-VIP (10 mg/ml), VIP (10 mg/ml) and beclomethasone (10 mg/ml) on days 25, 27 and 29 by nebulizer for 30 min. $^{+}P<0.05$ and $^{*}P<0.01$ compared with PBS –ve control, $^{\dagger}P<0.05$ and $^{\#}P<0.01$ compared with OVA +ve control.

Table 1. Primer sequences.

Gene Symbol	5'-3'	Sequence (5'-3')	Length (bp)	Amplicon Size (bp)	Reference
GAPDH	Sense	TGTTCCTACCCCCAATGTGT	20	138	(Lukacs et al. 2010)
	Antisense	GGTCCTCAGTGTAGCCCAAG	20		
IL-2	Sense	TCCAGAACATGCCGCAGAG	19	141	(Overbergh et al. 1999)
	Antisense	CCTGAGCAGGATGGAGAATTACA	23		
IFN-γ	Sense	CAGAGCCAGATTATCTCTTTCTACCTCAGAC	31	132	(McClellan et al. 2008)
	Antisense	CTTTTTCGCCTTGCTGTTGCTGAAG	25		
IL-4	Sense	AGATCATCGGCATTTTGAACG	21	67	(Amsen, de Visser and Town 2009)
	Antisense	TTTGGCACATCCATCTCCG	19		
IL-5	Sense	ACATTGACCGCCAAAAAGAG	21	62	(Seki et al. 2012)
	Antisense	ATCCAGGAACTGCCTCGTC	19		
IL-13	Sense	GGTCCACACAGGGCAACT	18	43	(Seki et al. 2012)
	Antisense	AATAAGATCAAGAAGAAATGTGCTCAA	27		
Muc5ac	Sense	CAGGACTCTCTGAAATCGTACCA	23	128	(Wilson et al. 2007)
	Antisense	AAGGCTCGTACCACAGGGA	19		

The primer sequences were obtained from previous studies and blasted using the BLAST and IDT's Oligo Analyzer (version 3.1) to confirm specificity.

Figure
Fig. 1



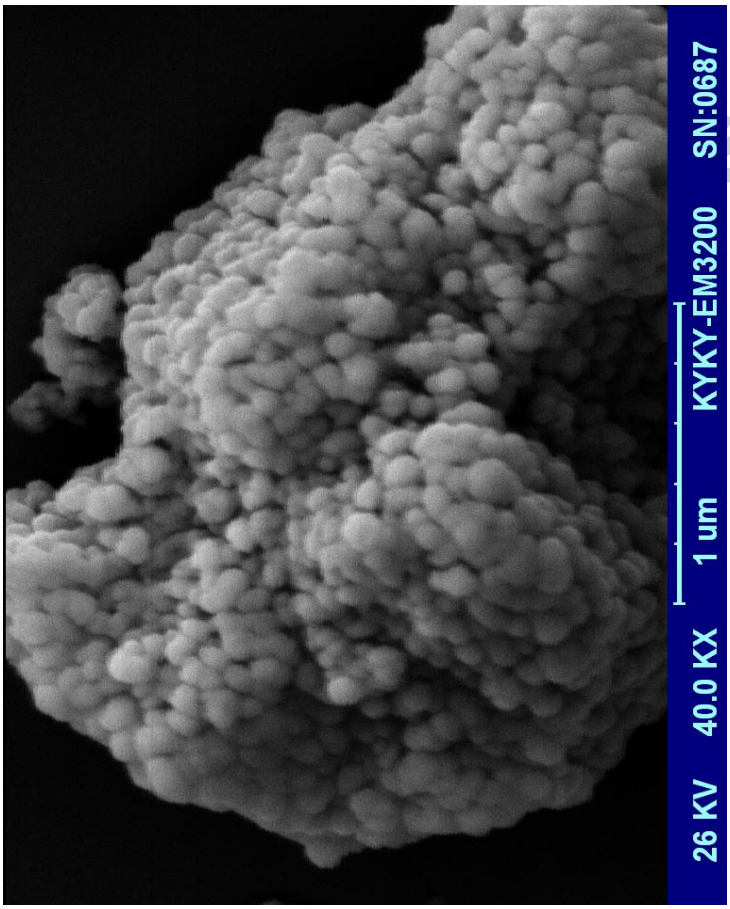
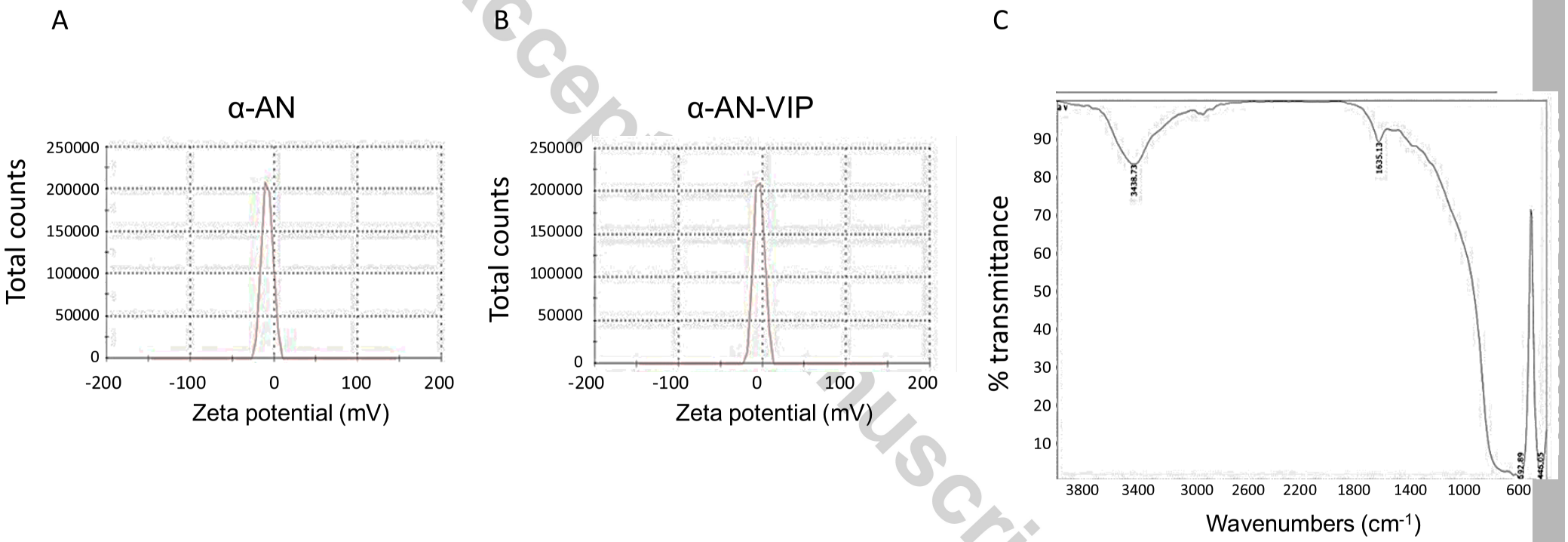


Fig. 2

Fig. 3



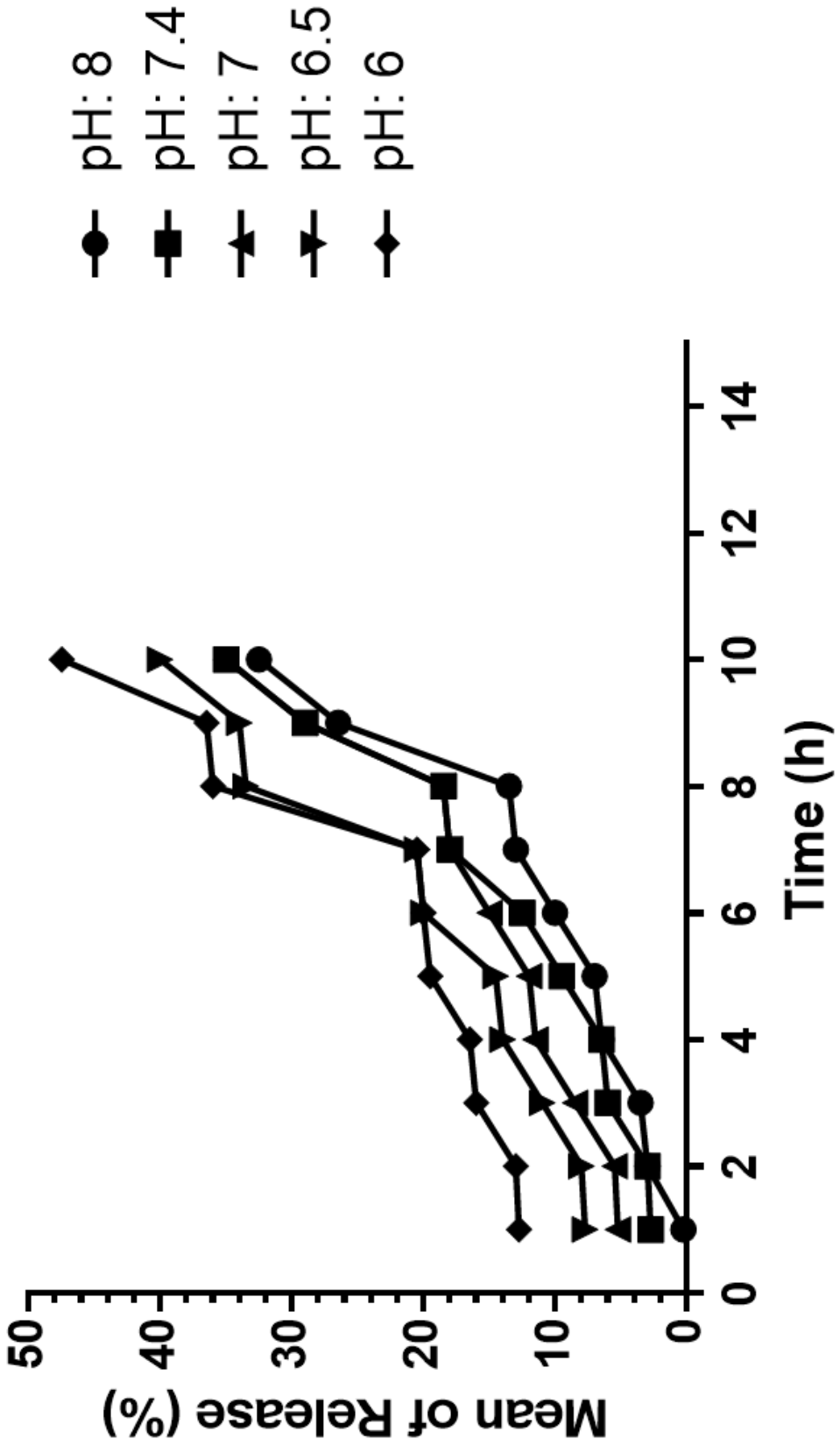
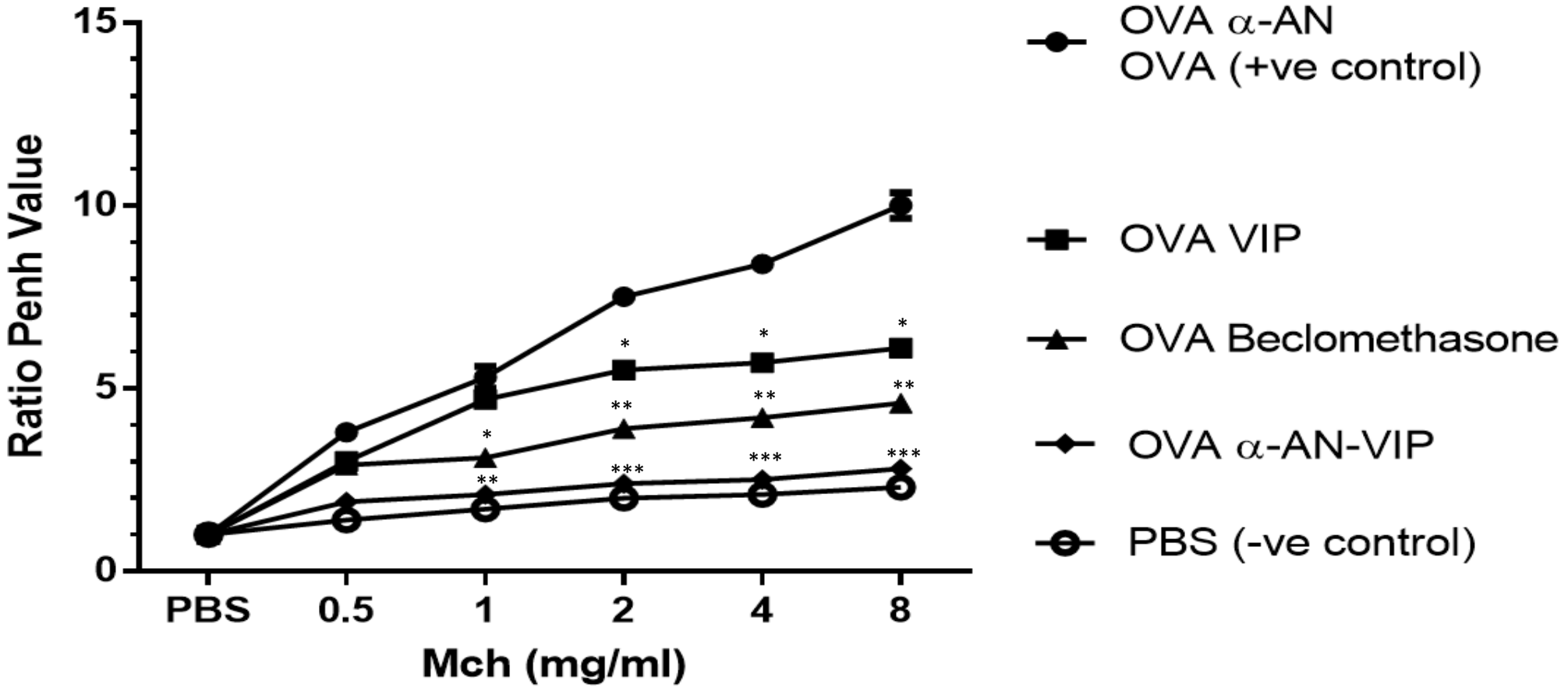

α-Alumina Nanoparticles
pH: 8
pH: 7.4
pH: 7
pH: 6.5
pH: 6
Mean of Release (%)
0
10
20
30
40
50
0
2
4
6
8
10
12
14
Time (h)

Fig. 4

Fig. 5



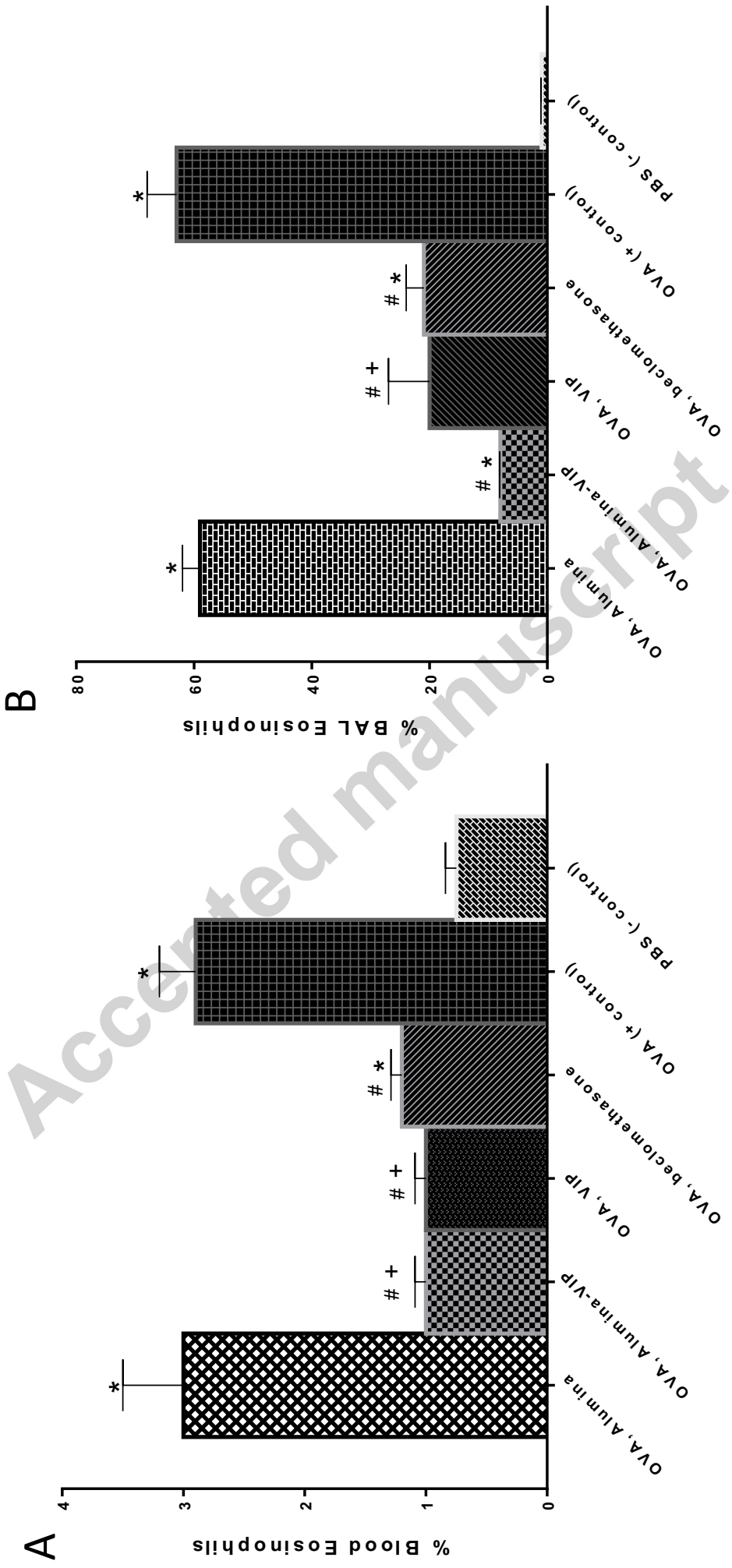


Fig. 6

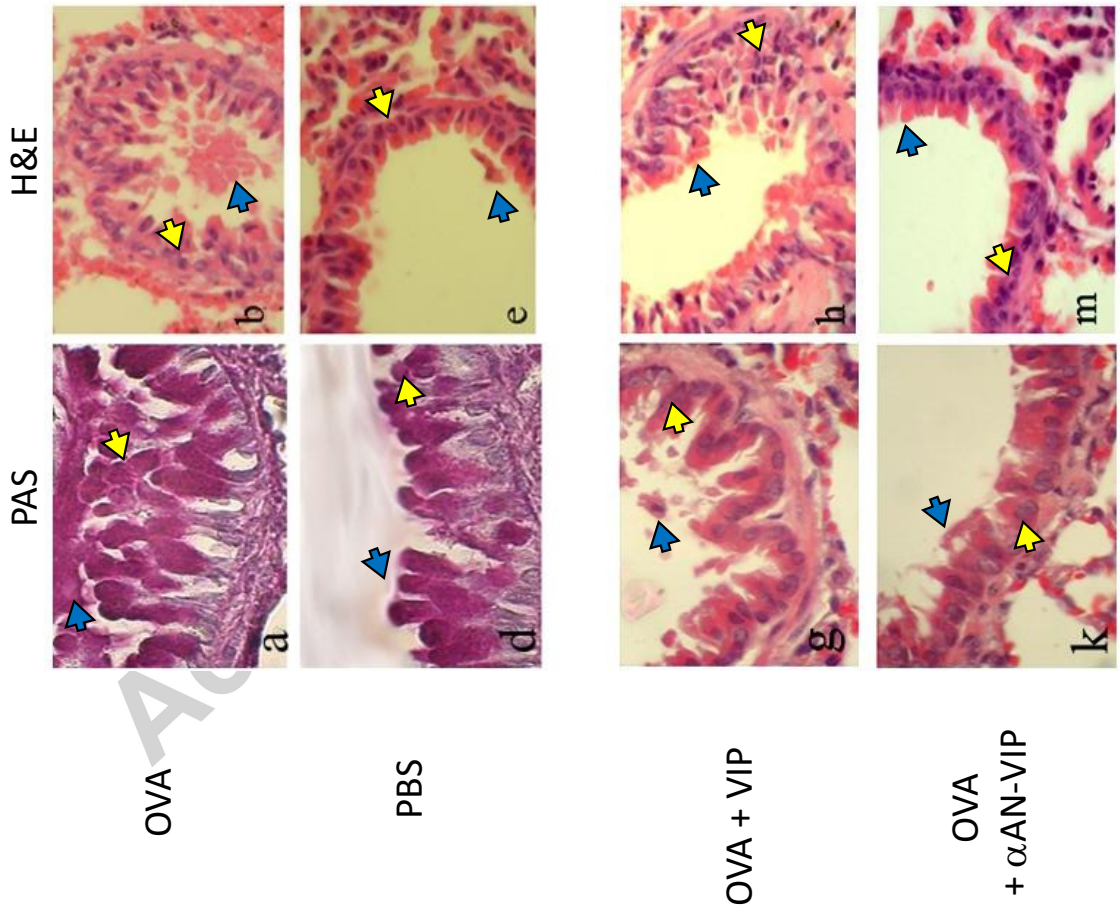


Fig. 7

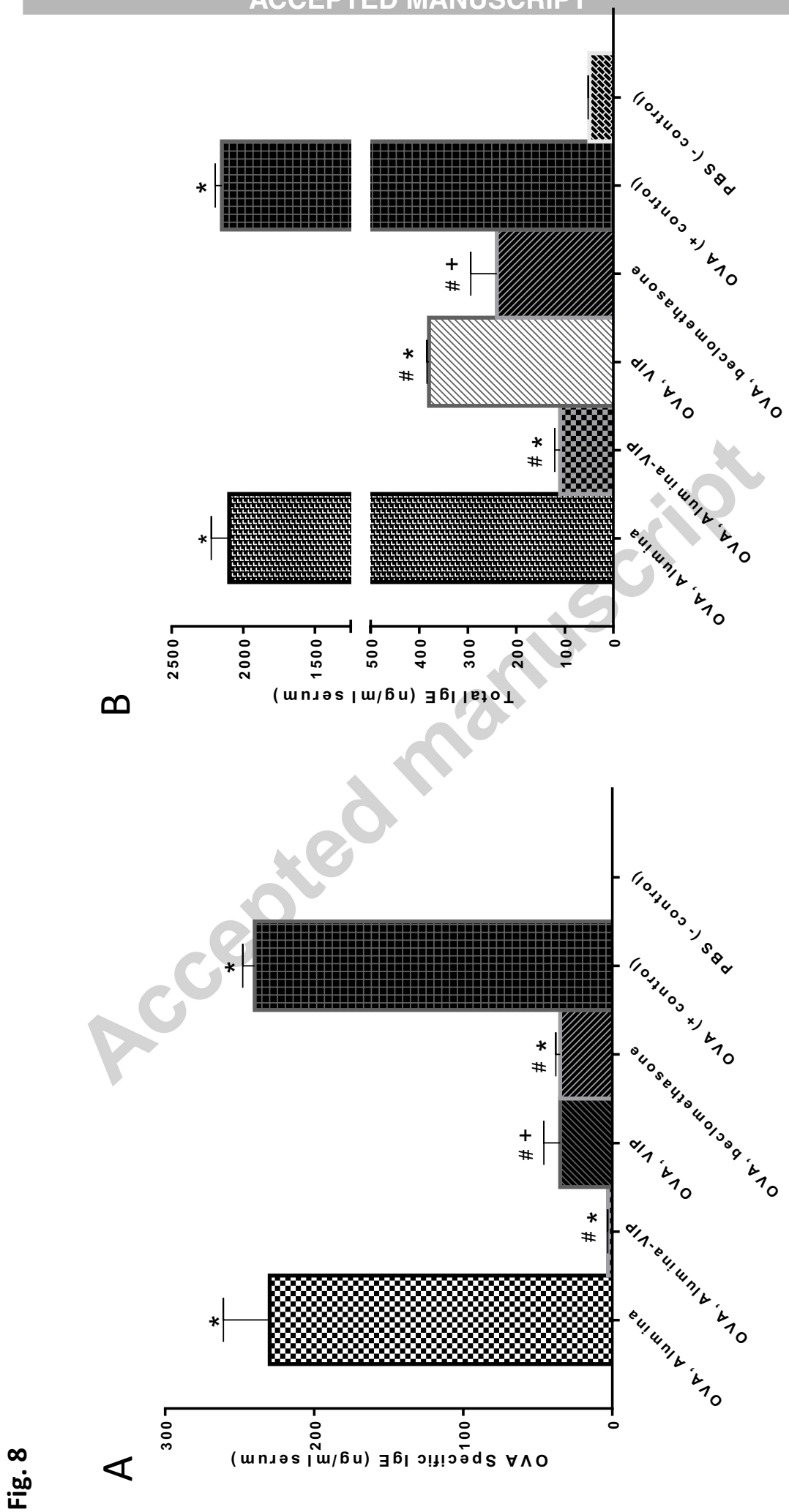


Fig. 8

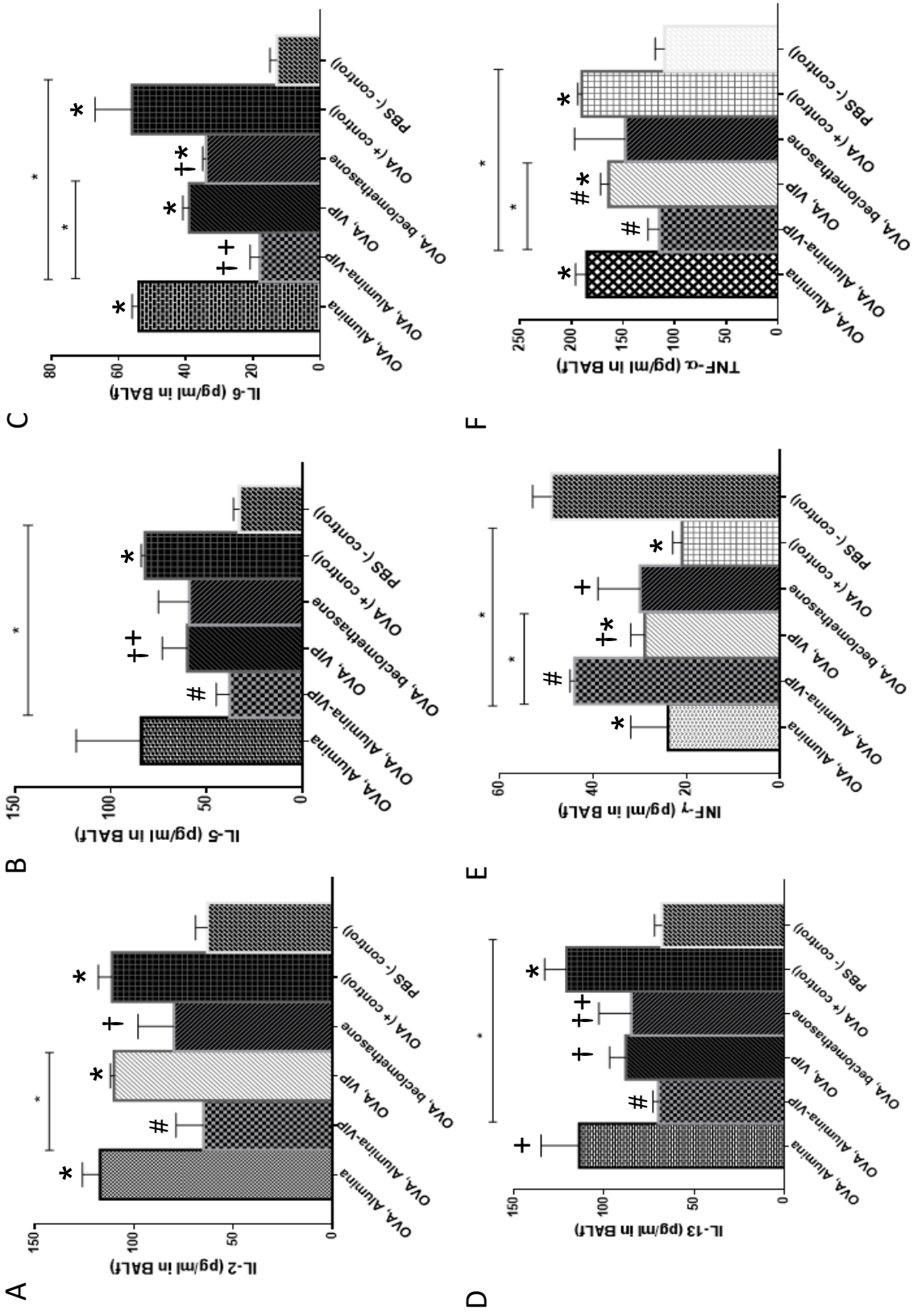


Fig. 9

Fig. 10